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Distribution and Diversity of Archaeal Ammonia Monooxygenase Genes Associated with Corals▽†

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Corals are known to harbor diverse microbial communities of *Bacteria* and *Archaea*, yet the ecological role of these microorganisms remains largely unknown. Here we report putative ammonia monooxygenase subunit A (*amoA*) genes of archaeal origin associated with corals. Multiple DNA samples drawn from nine coral species and four different reef locations were PCR screened for archaeal and bacterial *amoA* genes, and archaeal *amoA* gene sequences were obtained from five different species of coral collected in Bocas del Toro, Panama. The 210 coral-associated archaeal *amoA* sequences recovered in this study were broadly distributed phylogenetically, with most only distantly related to previously reported sequences from coastal/estuarine sediments and oceanic water columns. In contrast, the bacterial *amoA* gene could not be amplified from any of these samples. These results offer further evidence for the widespread presence of the archaeal *amoA* gene in marine ecosystems, including coral reefs.

The exceptional biological diversity of coral reef ecosystems is relatively well characterized for many eukaryotic species (see, e.g., reference 32). However, diverse assemblages of microorganisms from the domains *Bacteria* and *Archaea* are also found in association with corals (2, 8, 11, 15, 20, 22, 30, 33, 34, 43, 49), yet very little is known about the ecological function of the microorganisms that comprise these communities (34, 35). Among other important roles, it has been hypothesized that coral-associated microorganisms are critical for nutrient cycling (34), and in fact corals have been shown to harbor symbiotic nitrogen (N)-fixing cyanobacteria, which may represent an appreciable source of this essential nutrient (26). In addition, specific bacterial ribotypes appear to be associated with particular coral species (33, 34), with many of these types being most closely related to known nitrogen fixers and antibiotic producers (34).

While these studies have provided some insight into the ecological function of coral-associated *Bacteria*, members of the domain *Archaea* are also known to be associated with corals: both mesophilic *Euryarchaeota* and *Crenarchaeota* are widely distributed across different reef locations and coral species (20, 49), and *Archaea* appear to be abundant on some coral colonies, numbering $>10^8$ cells cm^{-2} (49). Until recently, few insights into the ecology and physiology of these coral-associated *Archaea*—and the mesophilic *Archaea* in general—were available. However, it has become increasingly clear that many mesophilic *Crenarchaeota* may be capable of ammonia oxidation (9), based on the cultivation of an ammonia-oxidizing archaeon (AOA) (21) and accumulating (meta)genomic (12, 13, 21, 45, 46), molecular (1, 6, 10, 24, 25, 29, 31, 50), and geochemical (19, 23) evidence. More specifically, *amoA* genes putatively encoding the α-subunit of the ammonia monooxygenase enzyme appear to be present in many mesophilic *Crenarchaeota* (10, 12, 13, 21, 25, 50), and Lam et al. (24) recently demonstrated that this gene is actively expressed by some *Crenarchaeota* in the ocean.

Ammonia oxidation is the first and rate-limiting step of chemoautotrophic nitrification—the overall oxidation of ammonia (NH_3) to nitrite (NO_2^-) to nitrate (NO_3^-)—and there is clear evidence for nitrification in association with individual corals (47), within coral reefs (48), and within reef cavities (37). Given the association between *Crenarchaeota* and corals (20, 49), we investigated the distribution and diversity of archaeal *amoA* gene fragments in DNA extracts recovered from coral colonies in Panama, Bermuda, Hawaii, and Puerto Rico. Our results indicate that archaeal *amoA* genes are associated with a variety of coral species.

Sample collection, DNA preparation, and amplification and sequencing of archaeal *amoA* genes. Samples were collected from coral colonies at Whale Bone Bay, Bermuda (August 1999), Bocas del Toro, Panama (April 1999, June 2000, February 2005), La Parguerra, Puerto Rico (January 2002), and Kane'ohe Bay, HI (June 2003) by use of previously described methods (49). Briefly, a punch and hammer or bone clippers were used to collect a single sample from each healthy coral colony; samples were then placed in Ziploc bags underwater, washed with 0.2 μM filtered and autoclaved seawater at the surface, placed on ice, returned to the lab, and stored at −80°C until DNA extraction. *Diploria strigosa*, *Montastraea franksi*, and *Porites astreoides* were sampled in both Bermuda and Panama, *Colpophyllia natans* and *Porites furcata* were sampled only

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in Panama, *Acropora cervicornis* and *Acropora prolifera* samples were collected in Puerto Rico, and samples were collected from *Porites compressa* in Hawaii (see Table S1 in the supplemental material). For DNA extraction, each frozen coral sample was airbrushed (<2.7 bar) with 10× TE (100 mM Tris [pH 8.0]-10 mM EDTA) to remove the tissue and associated microbes. Two milliliters of the coral tissue-TE slurry was pelleted for 30 min at 10,000 × *g* at 4°C. Total DNA was extracted from the pellet by use of the Ultra Clean soil DNA kit (MoBio).

Archaeal *amoA* gene fragments were amplified using the PCR primers Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) and conditions as previously described (10). Triplicate PCRs were pooled, gel purified, and cloned using the TOPO-TA cloning kit (Invitrogen). White transformants were transferred to 96-well plates containing LB broth (with 50 μg/ml kanamycin), grown overnight at 37°C, and PCR screened for the presence of inserts by use of T7 and M13R vector primers. Sequencing of T7/M13 PCR products was performed using vector primers on ABI 3730xl capillary sequencers (PE Applied Biosystems). Coral DNA extracts were also PCR screened for the presence of betaproteobacterial *amoA* genes by use of primers (AmoA-1F* and AmoA-2R) and conditions described previously (36, 44).

Richness, phylogenetic, and statistical analyses. Nucleotide sequences were assembled and edited using Sequencher v.4.2 (GeneCodes, Ann Arbor, MI), and nucleotide and amino acid alignments were generated using MacClade (http://macclade.org). Operational taxonomic units (OTUs) were defined as sequence groups in which sequences differed by ≤5%, and all analyses of richness—including rarefaction analysis (14) and both ACE (5) and Chao1 (3) nonparametric richness estimations—were performed using DOTUR (38).

Neighbor-joining phylogenetic trees (based on Jukes-Cantor-corrected distances) and parsimony trees were constructed based on alignments of DNA sequences by use of ARB (http://www.arb-home.de) (27). Nucleic acid sequences (rather than predicted amino acid sequences) were analyzed in order to highlight the genetic (rather than protein-level) heterogeneity among communities. A total of 979 archaeal *amoA* sequences were included in phylogenetic analysis, which was performed on a 585-bp region corresponding to most of the archaeal *amoA* gene. Additional environmental sequences of less than 585 bp in length from previous studies of marine water columns (10, 13, 50), sediments (10), and soils (25) were excluded from this analysis. Distance- and parsimony-based bootstrap analyses were conducted in PAUP *4.0b10 (Sinauer Associates) and used to estimate the reliability of phylogenetic reconstructions with 500 replicates.

To compare compositional overlap between archaeal *amoA* clone libraries, abundance-based Sørensen-type and Jaccard-type similarity indices (4) were calculated using the program SONS (39). Observed differences among libraries were statistically compared using ∫-Libshuff (40), with 10,000 randomizations and a distance interval, *D*, of 0.01 on PAUP-generated Jukes-Cantor pairwise distance matrices. ∫-Libshuff uses Monte Carlo methods to calculate the Cramér-von Mise statistic by constructing random communities from the entire data set and comparing the coverage of random communities to the observed coverage of our libraries. Significant *P* values were evaluated after correcting for multiple pairwise comparisons by use of the Dunn-Sidák method (42), where $P_{corrected} = 1 - (1 - P_{uncorrected})^{1/k}$, where k is the total number of comparisons (for seven archaeal *amoA* libraries, each compared with the corresponding six other libraries, $k = 7 \times 6 = 42$).

TABLE 1. Richness of archaeal *amoA* libraries from corals[a]

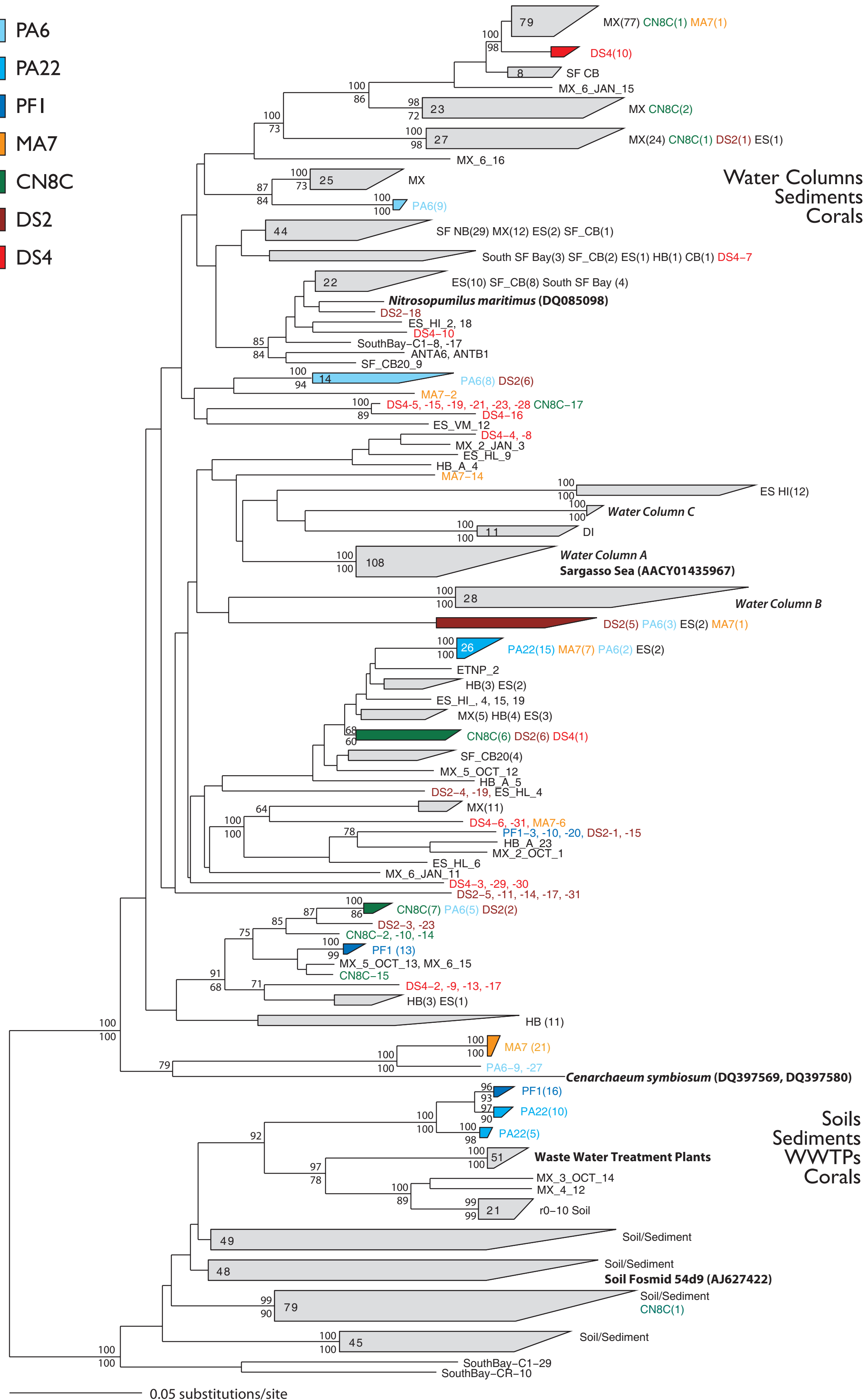
Coral library	No. of clones sequenced	No. of OTUs:			H′ value
		Observed	Estimated by Chao1	Estimated by ACE	
CN8C	23	9	31	27	1.9
DS2	32	12	15	16	2.3
DS4	31	9	10	12	1.9
MA7	33	7	17	31	1.1
PF1	32	3	3	3	0.93
PA6	29	7	7	7	1.8
PA22	30	2	2	2	0.69

[a] All OTUs were defined as having ≤5% difference in nucleic acid sequence alignment, and all indices were calculated using DOTUR.

Distribution and richness of archaeal *amoA* genes associated with corals. DNA was extracted from coral colonies in Bocas del Toro, Panama, Whale Bone Bay, Bermuda, Kane'ohe Bay, HI, and La Parguerra, Puerto Rico and screened for the archaeal *amoA* gene. These DNA samples were drawn from nine different species of coral: *Acropora cervicornis*, *Acropora prolifera*, *Colpophyllia natans*, *Diploria strigosa*, *Montastraea annularis*, *Montastraea franksi*, *Porites astreoides*, *Porites compressa*, and *Porites furcata*. From 40 samples that were screened, the archaeal *amoA* gene was amplified from a total of 12 samples (see Table S1 in the supplemental material). In contrast, in no case could the betaproteobacterial *amoA* gene be PCR amplified.

Of the 12 samples from which the archaeal *amoA* gene was amplified, 10 were previously screened for archaeal 16S rRNA genes, and all contained 16S rRNA genes from *Crenarchaeota* (49). In addition, Wegley et al. (49) quantified *Crenarchaeota* and *Euryarchaeota* for 3 of the 40 coral colonies included in this study via fluorescent in situ hybridization, and *Archaea* numbered 4.1×10^7 to 12×10^7 cells cm^{-2} for these 3 colonies (see Table S1 in the supplemental material). DNA extracts from all three of these coral colonies yielded amplification of the archaeal *amoA* gene, and clone libraries were constructed for two of these (PA6 and PF1). Although previous studies have recovered the *nifH* fragment of the nitrogenase gene in reef sediments and lagoonal waters of the Great Barrier Reef (specifically, Heron Reef) (16, 17), to our knowledge this is the first study in which microbial functional genes involved in nutrient cycling have been directly amplified from coral DNA extracts.

Archaeal *amoA* clone libraries were constructed for a total of seven individual samples drawn from five different species of coral (*C. natans*, *D. strigosa*, *M. annularis*, *P. astreoides*, and *P. furcata*). Libraries were generated from two different samples from *D. strigosa* (DS2 and DS4) and *P. astreoides* (PA6 and PA22) to potentially compare intraspecies variability in archaeal *amoA* diversity. All seven samples were recovered from Bocas del Toro, Panama (see Fig. S2 in the supplemental material). A total of 210 clones were sequenced, ranging from 23 to 33 from each library (Table 1). Richness varied widely



PA6
PA22
PF1
MA7
CN8C
DS2
DS4
MX(77) CN8C(1) MA7(1)
DS4(10)
SF CB
MX_6_JAN_15
MX CN8C(2)
MX(24) CN8C(1) DS2(1) ES(1)
MX_6_16
MX
PA6(9)
SF NB(29) MX(12) ES(2) SF_CB(1)
South SF Bay(3) SF_CB(2) ES(1) HB(1) CB(1) DS4–7
ES(10) SF_CB(8) South SF Bay (4)
Nitrosopumilus maritimus (DQ085098)
DS2–18
ES_HI_2, 18
DS4–10
SouthBay–C1–8, -17
ANTA6, ANTB1
SF_CB20_9
PA6(8) DS2(6)
MA7–2
DS4-5, -15, -19, -21, -23, -28 CN8C–17
DS4–16
ES_VM_12
DS4–4, -8
MX_2_JAN_3
ES_HL_9
HB_A_4
MA7–14
ES HI(12)
Water Column C
DI
Water Column A
Sargasso Sea (AACY01435967)
Water Column B
DS2(5) PA6(3) ES(2) MA7(1)
PA22(15) MA7(7) PA6(2) ES(2)
ETNP_2
HB(3) ES(2)
ES_HI_, 4, 15, 19
MX(5) HB(4) ES(3)
CN8C(6) DS2(6) DS4(1)
SF_CB20(4)
MX_5_OCT_12
HB_A_5
DS2–4, -19, ES_HL_4
MX(11)
DS4–6, -31, MA7-6
PF1–3, -10, -20, DS2-1, -15
HB_A_23
MX_2_OCT_1
ES_HL_6
MX_6_JAN_11
DS4–3, -29, -30
DS2–5, -11, -14, -17, -31
CN8C(7) PA6(5) DS2(2)
DS2–3, -23
CN8C–2, -10, -14
PF1 (13)
MX_5_OCT_13, MX_6_15
CN8C–15
DS4–2, -9, -13, -17
HB(3) ES(1)
HB (11)
MA7 (21)
PA6–9, -27
Cenarchaeum symbiosum (DQ397569, DQ397580)
Water Columns
Sediments
Corals
PF1(16)
PA22(10)
PA22(5)
Waste Water Treatment Plants
MX_3_OCT_14
MX_4_12
r0–10 Soil
Soil/Sediment
Soil/Sediment
Soil Fosmid 54d9 (AJ627422)
Soil/Sediment
CN8C(1)
Soil/Sediment
SouthBay–C1–29
SouthBay–CR–10
Soils
Sediments
WWTPs
Corals
0.05 substitutions/site

among these libraries, with a total of only 2 OTUs recovered in the PA22 library but up to 12 OTUs in the DS2 library (all OTUs defined as ≤5% sequence difference); likewise, the Chao1 and ACE richness estimators ranged from 2 to 31 OTUs, suggesting that some coral colonies have few archaeal *amoA* sequence types associated with them and others have many. The three lowest Chao1, ACE, and observed richness values all corresponded to the three libraries from *Porites* spp. (Table 1). These relatively low levels of richness for *amoA* libraries drawn from *Porites* spp. are quite clear based on rarefaction analysis (see Fig. S1 in the supplemental material), as is the generally higher richness for the two archaeal *amoA* libraries from *D. strigosa*. These findings are analogous to those of Rohwer et al. (34) for bacterial communities, where the Shannon-Wiener diversity index (H′) for *D. strigosa* was 4.2 and H′ for *Porites* spp. was 2.5. For AOA communities, based on archaeal *amoA* libraries, H′ values were 1.9 and 2.3 for the two *D. strigosa* samples and ranged from 0.69 to 1.8 for three samples from *Porites* spp. (Table 1).

Phylogenetic distribution of coral-associated archaeal *amoA* genes. Archaeal *amoA* genes recovered from corals were compared with previously reported environmental sequences from soils, sediments, wastewater treatment plants, and marine water columns and with the *amoA* genes from *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum* (1, 10, 13, 21, 25, 31, 45, 46). Of the 210 coral-associated sequences reported in this study, 178 fell into a large cluster with sequences from water columns and sediments (upper cluster in Fig. 1) and 32 fell into a cluster with soil, sediment, and wastewater treatment plant samples. None of the 210 sequences recovered in this study fell into the two subclusters (A and B) that contain the vast majority of known water column sequences, offering further evidence for the environmental specificity of archaeal *amoA* sequences at the DNA level (1, 9, 10) and supporting the idea that coral-associated *Archaea* are distinct from their planktonic counterparts (20).

With a few important exceptions discussed below, coral-associated archaeal *amoA* sequences were distinct from those currently in the database (Fig. 1). There was little overlap between libraries from different coral colonies, as evidenced by the generally low abundance-based Sørensen's indices of similarity (L_{abd}) among libraries (Table 2). The most similar libraries were PA22 and PF1; however, they shared an L_{abd} of 0.5, which indicates that for a given sequence expected to be found in either library, the probability that it will be found in both libraries is only 50% (4, 39). Along these lines, statistical

TABLE 2. Abundance-based Sørensen- and Jaccard-type similarities among archaeal *amoA* libraries from corals[a]

Coral library	Similarity with indicated library						
	CN8C	DS2	DS4	MA7	PF1	PA6	PA22
CN8C		0.30	0.09*	0.03*	0.04*	0.12	0*
DS2	0.46		0*	0.03*	0.04*	0.26*	0*
DS4	0.16*	0*		0.06*	0*	0*	0*
MA7	0.05*	0.05*	0.11*		0*	0.13*	0.18*
PF1	0.08*	0.08*	0*	0*		0*	0.33*
PA6	0.22	0.41*	0*	0.23*	0*		0.06*
PA22	0*	0*	0*	0.30*	0.50*	0.12*	

[a] Sørensen-type similarities are below the diagonal, and Jaccard-type similarities are above the diagonal. Statistically different ($P < 0.05$ for both) comparisons among libraries in ∫-Libshuff are denoted by a single asterisk.

analysis of libraries by use of ∫-Libshuff found all but two libraries to be significantly ($P < 0.05$) different from one another (Table 2); this indicates that nearly all libraries are representative of significantly different populations of coral-associated AOA (39). The two exceptions to this finding involved the *C. natans* library, which was among the most diverse libraries (Table 1; also see Fig. S1 in the supplemental material) yet shared a comparatively high similarity with the DS2 library (L_{abd} = 0.46) and was not significantly different from the DS2 and PA6 libraries (Table 2).

Although similarities among libraries were generally low, they were in many cases nonzero (Table 2), since there were several instances where an archaeal *amoA* sequence type was found on more than one coral sample or coral species (Fig. 1). For the 16S rRNA gene, Wegley et al. (49) found at least one widely distributed crenarchaeal ribotype present on all three species they studied (*D. strigosa*, *M. franksi*, and *P. astreoides*) in both Bermuda and Panama. Similarly, Kellogg (20) found a euryarchaeal sequence type on all three species sampled (*D. labyrinthiformis*, *D. strigosa*, and *M. annularis*) that constituted 48% of sequenced clones. For the archaeal *amoA* gene, there were multiple cases where a cluster of sequences was found for three species (e.g., for DS2, PA6, and MA7 and for CN8C, PA6, and DS2); however, no single OTU was found in association with all five species included in this study. Where overlaps among coral libraries did occur, they were confined to a few OTUs. This was true even for libraries drawn from the same coral species, as there was no clear trend towards greater similarity in AOA communities drawn from the same species of coral (L_{abd} for DS2 and DS4 = 0; L_{abd} for PA6 and PA22 =

FIG. 1. Phylogenetic relationships among coral-associated archaeal *amoA* sequences and previously reported environmental sequences. This tree is a neighbor-joining tree based on Jukes-Cantor-corrected DNA distances and is midpoint rooted. Sequences are color coded according to coral sample, and each sequence name denotes the coral species, the sample number, and the individual sequence number. For example, DS4-7 represents *Diploria strigosa*, sample number 4, sequence number 7. Previously reported environmental sequences are shown in black, and (meta)genomic sequences are in bold. Clusters are color coded by the most well-represented library within the cluster. A distance scale bar is at the lower left, and bootstrap values (>60%) are indicated at branch points, with distance bootstrap values above the line and parsimony values below. The tree includes 370 environmental sequences from soils, sediments, and the ocean (10) (accession no. DQ14825 to DQ14848 and DQ148573 to DQ148905), 3 sequences from Monterey Bay and Antarctic surface waters (13) (accession no. DQ333419, DQ333421, and DQ433422), 99 sequences from wastewater treatment plants and estuarine sediments (31) (accession no. DQ278494 to DQ278592), 74 soil sequences (25) (accession no. DQ534815 to DQ534888), 218 sequences from estuarine sediments (1) (accession no. DQ500959 to DQ501176), metagenomic sequences from the Sargasso Sea and German soil (accession no. AACY01435967 and AJ627422), the *amoA* gene from *N. maritimus* (accession no. DQ085098), and two *amoA* genes from *C. symbiosum* (accession no. DQ397569 and DQ397580).

0.12). Overall, coral-associated AOA communities appear to be diverse—although different corals commonly share one or more OTUs—with no systematic similarities among species.

Amid this general phylogenetic diversity, there were a few instances where coral-associated archaeal *amoA* sequences were similar to previously reported environmental sequences; for example, a number of coral-associated sequences fell into the upper portion of the tree, which is dominated by sequences from a subtropical estuary located in Mexico's Gulf of California (1). Coral-associated *amoA* sequences were, in several cases, most similar to the only currently known "species" of mesophilic *Crenarchaeota*: DS2-18 shared 95% nucleotide identity to the cultivated AOA *N. maritimus*, while PA6-9, PA6-27, and a group of sequences from *M. annularis* were more distantly related (only 77 to 79% identical) to the *C. symbiosum amoA* gene. In an interesting parallel, Kellogg (20) recovered two crenarchaeal sequences from the surface microlayer of an *M. annularis* coral colony in the Virgin Islands, United States, that were 95% identical to the 16S rRNA sequence(s) from *C. symbiosum*.

Based on differences between the recently published genome of this sponge symbiont and its planktonic relatives (12), as well as specific associations between crenarchaea and their sponge hosts (18, 28), it has been suggested that symbioses between *Crenarchaeota* and other organisms may be common in the marine environment (12). Sharp et al. (41) demonstrated that bacterial and archaeal communities associated with marine sponges are directly transmitted between generations, such that sponges may possess a predictable set of bacterial and archaeal partners that cycle carbon and nutrients within the sponge (41). As putative AOA, one possible role for archaeal symbionts is the removal of nitrogenous host waste products such as ammonia or urea (12). In fact, extremely high rates of ammonia oxidation have been found in association with marine sponges (7). In the case of corals, no study has demonstrated clear symbioses between *Archaea* and coral hosts, although it is clear that *Archaea*—and, based on our results, putative AOA specifically—are occasionally associated with corals.

Of the 210 coral-associated archaeal *amoA* sequences recovered in this study, 32 fell into a large cluster with sequences from soils, sediments, and wastewater treatment plants; of these, a single sequence clustered with soil/sediment sequences. The remaining 31 sequences formed a distinct grouping but were most closely related (83 to 85% nucleotide identity and 96 to 98% amino acid identity) to the dominant sequence type (50 of 75 clones) recently recovered from five different nitrifying wastewater treatment plants (31) as well as to sequences from soil (r0-10 group) (25) and from estuarine sediments (1). Interestingly, half of the sequenced clones (16 of 32) from the PF1 library fell into this cluster, and *Archaea* numbered 4.1×10^7 cells cm^{-2} on this coral colony. Considering the wide range of environmental conditions that might be expected within and between soils, sediments, wastewater treatment plants, and coral reefs—particularly in terms of salinity, oxygen concentrations, and the availability of carbon- and nitrogen-containing compounds—this group should be targeted in subsequent studies of AOA and coral-associated microbial communities.

Archaea can be highly abundant on corals (49); our results indicate that at least some of these organisms possess *amoA* genes and that diverse communities of putative AOA are associated with corals. The general lack of similarity between AOA communities associated with particular coral species or colonies, variability in the richness of these communities, and similarities between coral-associated *amoA* sequences and sequences recovered from other ecosystems all suggest multiple avenues of future research into the microbial ecology and biogeochemistry of coral-associated *Crenarchaeota*.

Nucleotide sequence accession numbers. The archaeal *amoA* sequences reported in this study have been deposited in GenBank under accession numbers EF382414 to EF382623.

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