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Archaeal Communities in Mangrove Soil Characterized by 16S rRNA Gene Clones

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An archaeal 16S rRNA gene library was constructed from mangrove soil. Phylogenetic analysis revealed archaea in mangrove soil including the *Crenarchaeota* (80.4%) and *Euryarchaeota* (19.6%) phyla. The archaeal community in mangrove soil appears to be a mixture of organisms found in a variety of environments with the majority being of marine origin.

Keywords: mangrove, archaea, phylogeny, 16S rDNA

Mangrove forests are found in the inter-tidal zones along tropical and subtropical coasts and play an important role in the coastal ecosystem. They supply raw materials and help to protect coastlines from erosion, storm damage and wave action. They also help to treat effluents, provide breeding and growth habitats for numerous organisms, along with refuge and feeding zones for marine organisms, and furnish large quantities of organic matter to adjacent coastal waters (Ronnback, 1999). Costanza's research suggests that the service value of mangrove ecosystem is rated fourth among sixteen various ecosystems around the world (Costanza *et al.*, 1997). Mangrove is recognized as a highly productive ecosystem, in which an efficient mechanism of recycling and conserving nutrients is a key element. The diverse bacterial and fungal communities living in the mangrove ecosystem play a major role in nutrient transformation (Holguin *et al.*, 2001). Microflora in mangrove are composed of a combination of terrestrial soil, marine and freshwater microorganisms (Ananda and Sridhar, 2002). While the importance of bacteria and fungi in mangrove biogeochemical cycles is well established, our knowledge of archaea in mangrove habitats remains extremely limited. The *Archaea*, one of the three domains of life, have long been believed to primarily inhabit extreme environments; however, recent studies based on cultivation-independent methods have demonstrated that archaea are much more diverse and widespread than previously suspected (Chaban *et al.*, 2006). The two major phyla, *Crenarchaeota* and *Euryarchaeota*, can account for as much as one-third of all prokaryotic cells in the global oceans (Karner *et al.*, 2001). Many novel archaea groups seem to be confined to specific geographical locations or to ecosystems that have similar geochemistry, but other groups appear to be widely distributed (Schleper *et al.*, 2005).

In order to gain new insight into the archaeal communities of mangrove soil and to build foundational information for future research, we have used the 16S rRNA gene clone library approach to survey the archaeal community found in the soil from a mangrove reserve zone on Hainan Island, China.

Mangrove soil samples were collected from a natural mangrove forest plot (N 19°57′18.0″, E 110° 34′51.7″) that contained only indigenous mangrove species *Kandelia candel* trees in the Dongzhaigang National Nature Reserve, Hainan Island, P.R. China. In October 2004, five soil cores within a 100 m^2 area were collected from depths of 0-30 cm, pooled and homogenized, then stored in sterile polypropylene bags. They were immediately placed on ice and transported to the laboratory within a 4 h period. The samples were then stored at −70°C until analyzed.

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Soil DNA was directly extracted using the FastDNA® SPIN Kit for soil (Q-BIOgene, USA) according to the manufacturer's instructions. Soil sample (0.5 g) was initially bead beaten with the FastPrep® Instrument for 45 s at a speed of 5.5 m/s. Three replicate DNA extracts for each sample were pooled. The clone library construction was carried out as previously described (Munson *et al.*, 1997). Briefly, archaeal 16S rDNA was amplified using the *Archaea*-specific primers 21F: 5′-TTCCGGTTGATCCYGCCGGA-3′ and 958R: 5′-YCCGGCGTTGAMTCCAATT-3′ (De Long, 1992). Three cycles of reconditioning PCR were performed to eliminate "heteroduplexes" (Thompson *et al.*, 2002). Five replicate PCR products were pooled to minimize PCR bias. The amplicon was ligated into the pGEM-T vector (Promega, USA), and the ligation products were transformed into competent *E. coli* DH5α cells. Amplified rDNA restriction analysis (ARDRA) was then performed using T7: 5′-TAATACGACTCACTAT AGGG-3′ and SP6: 5′-ACGATTTAGGTGACACTATA G-3′ primers. The PCR product was digested using the restriction enzymes *Msp*I and *Afa*I (Takara), respectively. One to three clones from each RFLP type were sequenced using an ABI 3730 Automated Sequencer (Applied Biosystems). Each pair of sequences were initially aligned using the 'BLAST 2 Sequences' (Tatiana, 1999) available through the NCBI; sequence similarities of ≥97% were considered the same phylotype and represented by a single type sequence (McCaig *et al.*, 1999). The sequences were examined for possible chimeric artifacts using the programs CHIMERA_CHECK (Cole *et al.*, 2003), and a Bellerophon (Huber *et al.*, 2004). Rarefaction curve was produced with a web-based program written by C.J. Krebs and J. Brzustowski (University of Alberta, Edmonton, Canada) available online at http://www.biology.ualberta.ca/jbrzusto/rarefact.php. A GenBank Blast search was performed for each type sequence in order to sort out the closest relatives. The selected sequences were aligned automatically with the CLUSTAL X program, version 1.83 (Thompson *et al.*, 1997). The phylogenetic tree was constructed by the neighbor-joining method based on a Kimura 2-parameter matrix in the MEGA program, version 3.1 (Kumar *et al.*, 2004). The confidence values of branches of the phylogenetic tree were determined using bootstrap analysis based on 1,000 re-samplings. The sequences obtained in this study were deposited in the GenBank database under accession numbers DQ363752 to DQ363847.

Archaeal DNA was successfully extracted and amplified from mangrove soil samples, and an archaeal 16S rDNA library was constructed. A total of 432 randomly chosen colonies were analyzed, and 426 insert-containing clones were identified. After ARDRA, a total of 149 distinct RFLP types were identified, of which 51 RFLP types represented a single clone; the other 98 RFLP types represented two or more clones, the largest of which was 18 clones. After initial alignment of each pair of sequences of these clones, 107 distinct sequence types were obtained. Through chimeric artifact examination, 11 potential chimeric artifacts were recognized and excluded from further analysis. A total of 96 distinct sequence phylotypes that represented 397 clones were used in diversity analyses. Rarefaction analysis showed the number of clones sequenced in this study covered most of the archaeal diversity of the library (Fig. 1). The coverage C (Good, 1953) of the library was 91.7%, which also indicated that most of the diversity in the library had been detected. GenBank Blast search revealed that all of the sequences retrieved in this study could be grouped into the *Archaea* domain, and most of them were related to uncultured archaeal members. The similarity ranged from 87.2% to 99.3% compared to

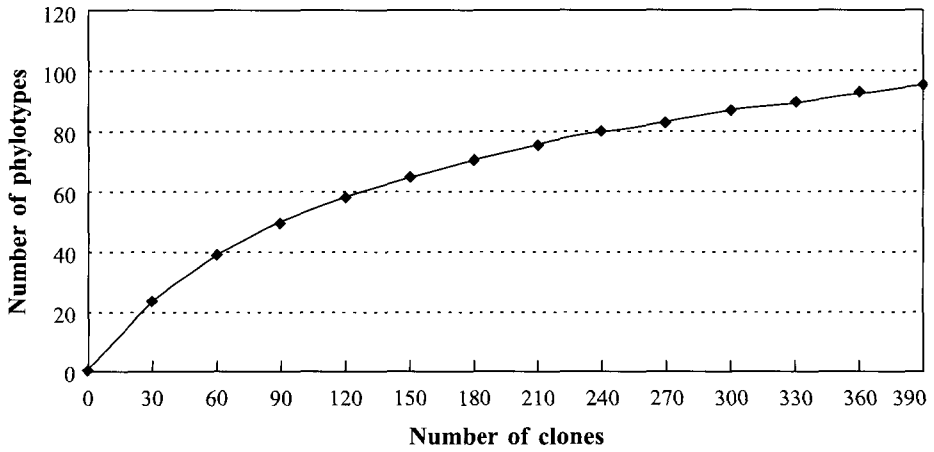

Fig. 1. Rarefaction curves generated for 16S rRNA genes in the clone library from samples collected in the mangrove soil. Clones were grouped into phylotypes based on sequence similarity of ≥97%.

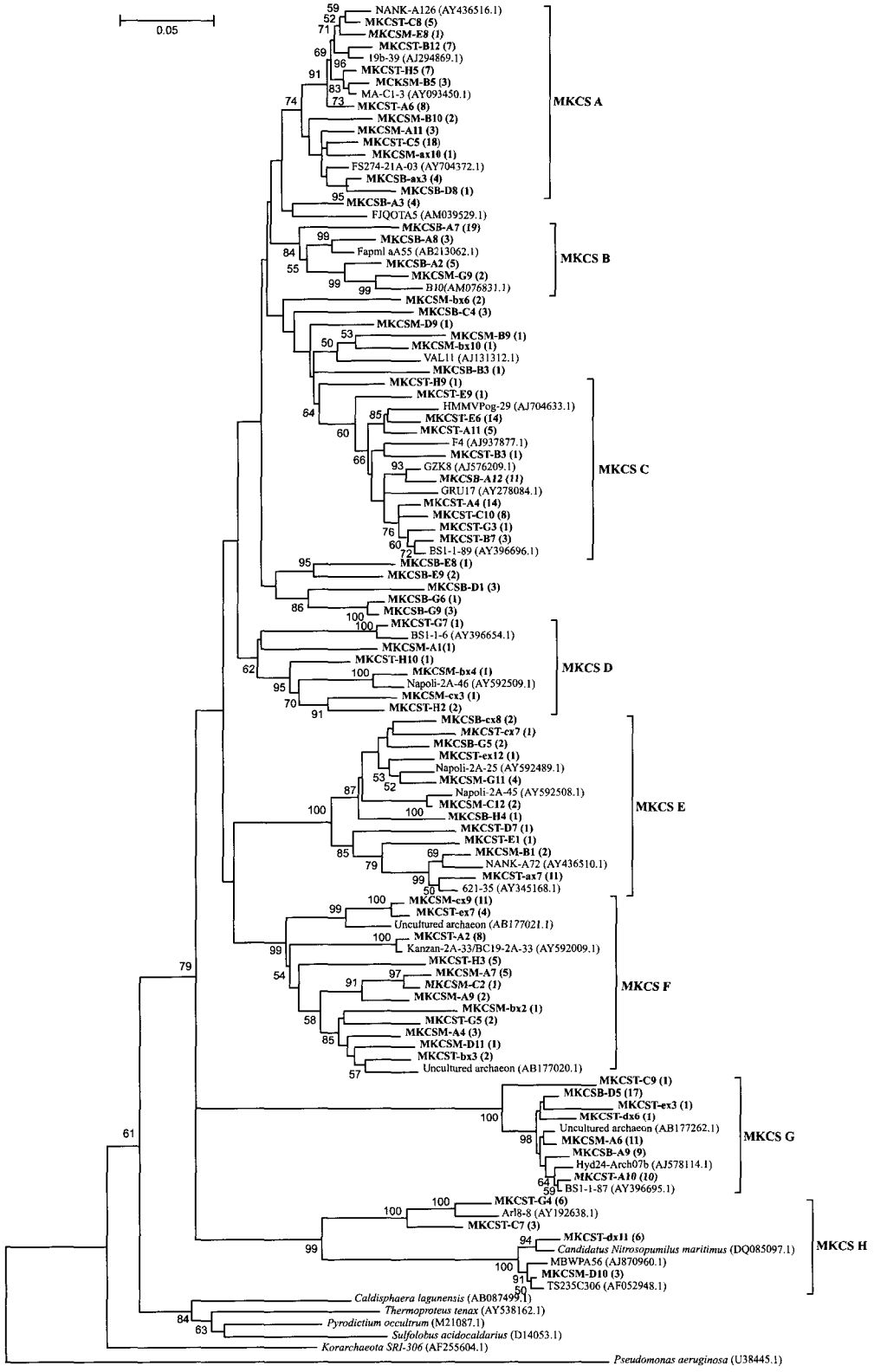

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences of the *Crenarchaeota* clones obtained from mangrove soil. The tree was constructed via the neighbor-joining method. *Korarchaeota SRI-306* (AF255604.1) and *Pseudomonas aeruginosa* (U38445.1) were used as the outgroups. Bootstrap values above 50 are shown as percentage. Bar indicates 5% nucleotide changes per 16S rDNA position. Sequences obtained from this study are given in bold type. The numbers in parentheses indicate the number of times the phylotype was found in the library.

archaeal 16S rDNA sequences available in GenBank as of May 2006. Phylogenetic analysis showed that 319 clones (78 sequences phylotypes) belonged to phylum *Crenarchaeota*, accounting for 80.4% of the clones or 81.3% of the sequence phylotypes in the clone library (Fig. 2); the remainder were euryarchaea. No korarchaea or nanoarchaea were found, which was easily understood, as members of those groups were all isolated from high temperature environments.

None of the retrieved crenarchaeal sequences could be grouped with previously cultured crenarchaea, with the exception of the recently isolated and cultured *Candidatus Nitrosopumilus maritimes* (Konneke *et al.*, 2005); most of these sequences were clustered within eight groups (MKCS-A to MKCS-H) (Fig. 2). Sixty clones (15.1% of the total) were placed into MKCS-A, and they are all closely related to sequences available in the public database (similarity range 93-98%, with most of them above 97%). The closest relatives in the database were isolated from marine sediments, such as clone MA-C1-3 (AY093450.1) isolated from marine deep-subsurface sediments near the Nankai Trough off Japan, clone FS274-21A-03 (AY704372.1) isolated from crustal fluids at Baby Bare Seamount located in the northeast Pacific Ocean, and clone NANK-A126 (AY436516.1) from anaerobic marine sediment core. This implied that crenarchaea in group MKCS-A were organisms of marine origin and distributed in anaerobic marine environments.

MKCS-H was the only group including an already cultured *Crenarchaeote*, *Candidatus Nitrosopumilus maritimes*, which was recently isolated from seawater and known to grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite (Konneke *et al.*, 2005); clone MKCST-dx11 was 98.1% identical to its 16S rDNA sequence. All 18 of the clones in this group were closely related to 16S rDNA sequences in the public database (similarity of 96.4-99.3%). In addition, we noted that the closest relatives were all isolated from marine habitats, represented by clone Ar18-8 (AY192638.1) isolated from marine sponge tissue from Korea, clone TS235C306 (AF052948.1) isolated from seawater particles in the North Sea near Netherlands' Island, and clone MBWPA56 (AJ870960.1) from deep-sea sediment in a tropical west Pacific Warm Pool; this suggested that these clones were of marine origin and widely distributed in seawater and sediments.

MKCS-C accounted for 14.9% of the clones or 10.4% of the sequence phylotypes in the clone library, and many reference sequences were clustered into this group. Those reference sequences were obtained from differ-

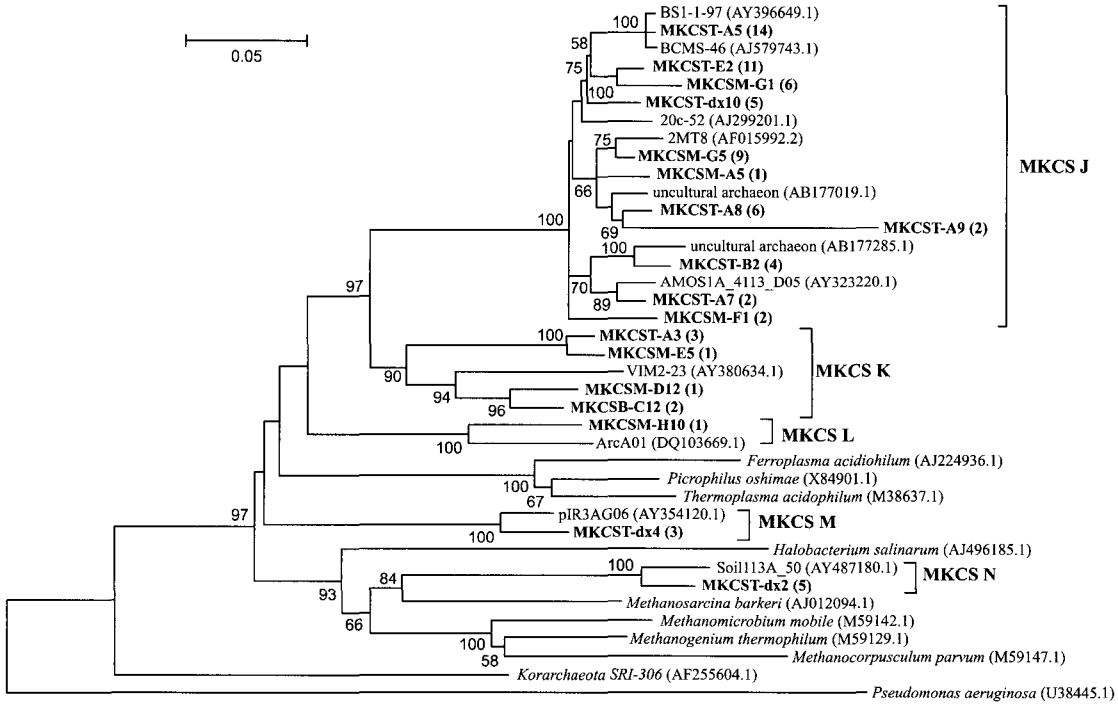

Fig. 3. Phylogenetic tree based on the 16S rDNA sequences of the *Euryarchaeota* clones obtained from mangrove soil. The tree was constructed via the neighbor-joining method. *Korarchaeota SRI-306* (AF255604.1) and *Pseudomonas aeruginosa* (U38445.1) were used as the outgroups. Bootstrap values above 50 are shown as percentage. Bar indicates 5% nucleotide changes per 16S rDNA position. Sequences obtained from this study are given in bold type. The numbers in parentheses indicate the number of times the phylotype was found in the library.

ent environments, such as anaerobic landfill leachate (Huang *et al.*, 2003), freshwater (M.Vich, 2005, GenBank description), marine sediment (Knittel *et al.*, 2004, GenBank description) and tidal flat sediment (Kim *et al.*, 2005). Another noteworthy group was MKCS-G, which included 50 clones that were closely related to sequences in the public database (similarity of 93-98%). Most of the closest relatives came from marine habitats.

Seventy eight clones (18 sequences phylotypes) were determined to belong to *Euryarchaeota* phylum (Fig. 3), accounting for 19.6% of the clones or 18.7% of the sequence phylotypes of the library; all of them clustered within five groups (MKCS-J to MKCS-N). Clone MKCST-dx2 grouped with *Methanosarcina barkeri,* which is a methane-producer and widespread in anaerobic environments. However, clone MKCST-dx2 was closely related (95% similarity) to an uncultured euryarchaea obtained from acid forest soil in southern Cameroon (Donovan *et al.*, 2004). Group MKCS-J contained 62 clones and several reference sequences, 2MT8 from salt marsh sediment (Munson *et al.*, 1997) and AMOS1A_4113_D05 from methane cold seep sediment (Girguis *et al.*, 2003) which were previously assigned to the *Thermoplasmatales* order. Clone MKCST-dx4 and a previously determined sequence belonging to *Thermoplasmatales* clone pIR3AG06 from Rainbow hydrothermal vent sediments (Nercessian *et al.*, 2005) comprised group MKCS-M. Groups MKCS-J to MKCS-M shared common ancestry with the group of cultured members of *Thermoplasmatales*. This suggests that these four groups may be in *Thermoplasmatales*. As euryarchaea in the library were considered, it was surprising to note that 93.6% euryarchaeal clones were affiliated with the *Thermoplasmatales*. This is similar to the archaeal communities found in tidal flat sediment (Kim *et al.*, 2005), but quite different from many other environments previously examined. Mangrove forests and tidal flats, all located within continent and marine contact zones, are periodically flooded and exposed to seawater. Therefore, the presence of numerous *Thermoplasmatales* archaea in mangrove soil may be of ecological significance.

Compared to most of the other archaeal 16S rDNA clone libraries cited in the present paper, the greatest number of sequence phylotypes were found in mangrove soil, although sequence similarities of ≥97% were considered as one phylotype in our study. This suggests the archaeal diversity in mangrove soil is relatively high. In addition, the environments in which the closest-related archaea are found are highly diverse and include marine sediment and water, freshwater, soil and landfill leachate, etc. This suggests that the archaeal communities in mangrove soil are a mix of organisms found in marine, terrestrial and freshwater environments.

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Reference

Ananda, K. and K.R. Sridhar. 2002. Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. *Can. J. Microbiol.* 48, 871-878.

Chaban, B., S.Y.M. Ng, and K.F. Jarrell. 2006. Archaeal habitats from the extreme to the ordinary. *Can. J. Microbiol.* 52, 73-116.

Cole, J.R., B. Chai, T.L. Marsh, R.J. Farris, Q. Wang, S.A. Kulam, S. Chandra, D.M. McGarrell, T.M. Schmidt, G.M. Garrity, and J.M. Tiedje. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res.* 31, 442-443.

Costanza, R., R.d Arge, R. de Groot, S. Farberk, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R.V. O'Neill, J. Paruelo, R.G. Raskin, P. Suttonkk, and M. van den Belt. 1997. The value of the world's ecosystem services and nature capital. *Nature* 387, 253-260.

De Long, E.F. 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci.* 89, 5685-5689.

Donovan, S.E., K.J. Purdy, M.D. Kane, and P. Eggleton. 2004. Comparison of EuryArchaea strains in the guts and food-soil of the soil-feeding termite Cubitermes fungifaber across different soil types. *Appl. Environ. Microbiol.* 70, 3884-3892.

Girguis, P.R., V.J. Orphan, S.J. Hallam, and E.F. De Long. 2003. Growth and methane oxidation rates of anaerobic methanotrophic Archaea in a continuous-flow bioreactor. *Appl. Environ. Microbiol.* 69, 5472-5482.

Good, I.L. 1953. The population frequencies of species and the estimation of population parameters. *Biometrika* 40, 237-264.

Holguin, G., P. Vazquez, and Y. Bashan. 2001. The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. *Biol. Fertil. Soils* 33, 265-278.

Huang, L.N., Y.Q. Chen, H. Zhou, S. Luo, and C.Y. Lan. 2003. Characterization of methanogenic Archaea in the leachate of a closed municipal solid waste landfill. *FEMS Microbiol. Ecol.* 46, 171-177.

Huber, T., G. Faulkner, and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequence in multiple sequence alignments. *Bioinformatics* 20, 2317-2319.

Karner, M.B., E.F. De Long, and D.M. Karl. 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean.

Nature 409, 507-510.

Kim, B.S., H.M. Oh, H. Kang, and J. Chun. 2005. Archaeal diversity in tidal flat sediment as revealed by 16S rDNA analysis. *J. Microbiol.* 43, 144-151.

Konneke, M., A.E. Bernhard, J.R. delaTorre, C.B. Walker, J.B. Waterbury, and D.A. Stahl. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437, 543-546.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 5, 150-163.

McCaig, A.E., L.A. Glover, and J.I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Appl. Environ. Microbiol.* 65, 1721-1730.

Munson, M.A., D.B. Nedwell, and T.M. Embley. 1997. Phylogenetic Diversity of Archaea in Sediment Samples from a Coastal Salt Marsh. *Appl. Environ. Microbiol.* 63, 4729-4733.

Nercessian, O., Y. Fouquet, C. Pierre, D. Prieur, and C. Jeanthon. 2005. Diversity of Bacteria and Archaea associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. *Environ. Microbiol.* 7, 698-714.

Ronnback, P. 1999. The ecological basis for economic value of seafood production supported by mangrove ecosystems. *Ecol. Econ.* 29, 235-252.

Schleper, C., G. Jurgens, and M. Jonuscheit. 2005 Genomic studies of uncultivated Archaea. *Nature Res. Microbiol.* 3, 479-488.

Tatiana, A., T. Thomas, and L. Madden. 1999. Blast 2 sequences – a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* 174, 247-250.

'Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.

Thompson, J.R., L.A. Marcelino, and M.F. Polz. 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res.* 30, 2083-2088.

Vetriani, C., H.W. Jannasch, B.J. Macgregor, D.A. Stahl, and A.L. Reysenbach. 1999. Population structure and Phylogenetic Characterization of Marine Benthic Archaea in Deep-Sea Sediments. *Appl. Environ. Microbiol.* 65, 4375-4384.