Extremophiles (2008) 12:651–656
DOI 10.1007/s00792-008-0170-x

ORIGINAL PAPER

Nanoarchaeal 16S rRNA gene sequences are widely dispersed in hyperthermophilic and mesophilic halophilic environments

Ana Casanueva · Ncebakazi Galada · Gillian C. Baker · William D. Grant · Shaun Heaphy · Brian Jones · Ma Yanhe · Antonio Ventosa · Jenny Blamey · Don A. Cowan

Received: 6 March 2008 / Accepted: 22 April 2008 / Published online: 14 June 2008

Abstract The Nanoarchaeota, proposed as the fourth sub-division of the Archaea in 2002, are known from a single isolate, *Nanoarchaeum equitans*, which exists in a symbiotic association with the hyperthermophilic Crenarchaeote, *Ignicoccus*. *N. equitans* fails to amplify with standard archaeal 16S PCR primers and can only be amplified using specifically designed primers. We have designed a new set of universal archaeal primers that amplify the 16S rRNA gene of all four archaeal sub-divisions, and present two new sets of Nanoarchaeota-specific primers based on all known nanoarchaeal 16S rRNA gene sequences. These primers can be used to detect *N. equitans* and have generated nanoarchaeal amplicons from community DNA extracted from Chinese, New Zealand, Chilean and Tibetan hydrothermal sites. Sequence analysis indicates that these environments harbour novel nanoarchaeal phylotypes, which, however, do not cluster into clear phylogeographical clades. Mesophilic hypersaline environments from Inner Mongolia and South Africa were analysed using the nanoarchaeal-specific primers and found to contain a number of nanoarchaeal phylotypes. These results suggest that nanoarchaeotes are not strictly hyperthermophilic organisms, are not restricted to hyperthermophilic hosts and may be found in a large range of environmental conditions.

Keywords Nanoarchaeaota · 16S rRNA · Phylogeny · Primer · Hyperthermophilic · Hypersaline

Introduction

In the past decade, three new sub-divisions have been described within the Archaea: the Korarchaeota,

Nucleotide sequence data reported are available in the GenBank database under the accession numbers AY727886-AY727891, AY571283, AY572420, EF562614-EF562627 and EU493244-EU493252.

Communicated by T. Matsunaga.

A. Casanueva · N. Galada · D. A. Cowan (✉)
Department of Biotechnology,
Institute for Microbial Biotechnology and Metagenomics,
University of the Western Cape, Private Bag X17,
Bellville 7535, South Africa
e-mail: dcowan@uwc.ac.za

G. C. Baker
Department of Biodiversity and Conservation Biology,
University of the Western Cape, Bellville 7535, South Africa

W. D. Grant · S. Heaphy
Department of Microbiology and Immunology,
University of Leicester, Leicester LE1 7RH, UK

B. Jones
Genencor International, Leiden, The Netherlands

M. Yanhe
Institute of Microbiology,
Chinese Academy of Sciences,
100080 Beijing, China

A. Ventosa
Department of Microbiology and Parasitology,
University of Seville, 41012 Seville, Spain

J. Blamey
Fundación Biociencia, José Domingo Cañas,
2280, Ñuñoa Santiago, Chile

Nanoarchaeota and Thaumarchaeota (Barns et al. 1996; Huber et al. 2002; Brochier-Armanet et al. 2008). The subdivision Nanoarchaeota is known from a single isolate (Huber et al. 2002), two environmental DNA samples (Hohn et al. 2002), and 19 DNA sequences (McCliment et al. 2006), all isolated from hyperthermophilic environments. *Nanoarchaeum equitans*, the type specimen for the Nanoarchaeota, is a 400 nm coccus typically found associated with the chemolithotrophic Crenarchaeote *Ignicoccus hospitalis* (Paper et al. 2007). Studies of the *Nanoarchaeum* genome suggest a parasitic lifestyle, given the absence of a number of core metabolic pathways (Waters et al. 2003). *Ignicoccus* cells grow as well in pure culture as in co-culture with *Nanoarchaeum*, but attachment of *N. equitans* cells to *Ignicoccus* prevents the host cells from dividing, causing a prolonged lag phase (Jahn et al. 2008), suggesting that their relationship is not mutualistic. The true phylogenetic position of *N. equitans* remains a matter of debate; multi-locus phylogenetic studies have placed the clade as a sister-branch of the Crenarchaea (Ciccarelli et al. 2006) rather than a novel phylum, while other studies suggest that the placement of this novel group as a separate phylum is still unresolved (Brochier et al. 2005).

Nanoarchaeum equitans (Huber et al. 2002) failed to amplify using published universal archaeal primers and most nanoarchaeal sequences have been obtained by amplification with nanoarchaea-specific 16S rRNA gene primers (Hohn et al. 2002; McCliment et al. 2006). We have recently designed a new set of archaea-specific primers with 100% similarity to sequences from all four existing archaeal sub-divisions (Baker et al. 2003). These primers have been shown to amplify archaeal type strains and successfully detect archaeal cells in environmental samples that failed to amplify using published universal archaeal primers (Baker and Cowan 2004; Baker et al. 2003). In this study, the acquisition of new nanoarchaeote-like sequences has allowed us to design two new nanoarchaeal-specific PCR primer sets. Here, we report the use of all primer sets to identify novel nanoarchaeote-like taxa from Chinese, New Zealand, Chilean and Tibetan hydrothermal sediments. Using these primer sets we have demonstrated for the first time that nanoarchaeote-like phylotypes are not exclusively restricted to hyperthermophilic environments.

Materials and methods

Sampling

Hydrothermal sediment samples were taken from sites TC11 and TC9 (pH 8.0, average temperature 83°C, N24°57′ E98°26′) in the Rehai thermal region near Tenchong, SW China; from sites TOK17 (pH 6.0, 96°C) and TOK3 (pH 6.5, 86.5°C) in the Tokaanu region, North Island, New Zealand; from sites TA22 (pH 6.3, 80°C), TA101 (pH 7.0, 85°C), TA62 (pH 8.0, 85°C) and TA91 (pH 5.0, 85°C) in the Géiseres del Tatio volcanic region (S22°19′ W68°12′), Chile and from site B1.1 (pH 8.0, 79°C, N 30°00′ E90°28′) in Yangbajing hot spring in Tibet. Hypersaline samples were collected from a saltern pond EN2 (pH 7.5, 18°C, N43°44.426′ E112°02.081′) in Inner Mongolia, China; from Rooipan salt pond sediments (pH 6.9–7.6, 20°C, S33°25.740′ E18°32.541′) in the Western Cape, South Africa.

Growth conditions and DNA extraction

Aliquots of EN2 and Rooipan hypersaline samples were inoculated into 25 ml of media DSM 97, DSM 276 and DSM 372 (http://www.dsmz.de/microorganisms/html/media) and incubated for 2 days at 37°C with shaking. The enrichment cultures were centrifuged at 10,000*g* for 10 min and resuspended in 500 µl TE, 30 µl 10% SDS and proteinase K to a final concentration of 100 µg/ml, and incubated at 37°C for 1 h. The lysate was extracted three times with phenol-chloroform and the resulting aqueous phase was washed and precipitated with ethanol. The DNA solution was dialysed overnight in 50 mM TE buffer at 4°C.

Sediment metagenomic DNA extraction

DNA was extracted from duplicate 0.5 g sediment samples using a modified Zhou method (Stach et al. 2001).

PCR and cloning

Archaeal primer pair A571F/UA1204R, and nanoarchaeal primer pairs A571F/N961R and A571F/N989R (Table 1) were used to amplify genomic DNA extracted from Chinese, New Zealand, Chilean, Tibetan, Inner Mongolian and South African samples. Amplification conditions were as described by Baker et al. (2003). Nanoarchaeal primer pair 9bF/511mcR (Huber et al. 2002) (Table 1) was used for the amplification of metagenomic DNA from Chinese, Inner Mongolian and South African samples. Amplicons were purified using the GFX^{TM} PCR purification kit (Amersham Biosciences), ligated into the pTZ57R vector (InsT/Aclone^{TM}, Fermentas Life Sciences) and transformed into electrocompetent DH5α *E. coli.*

Amplified ribosomal DNA restriction analysis

White colonies from the resulting libraries were amplified by colony PCR using the same PCR primers as used above. The amplicons were digested with *Rsa*I, *Mbo*I, *Hae*III and

Table 1 16S rRNA gene primer sequences used in PCR amplification

Primer	Target site	Sequence 5′–3′	Reference
A571F	571–588	GCYTAAAGSRICCGTAGC	Baker et al. 2003
UA1204R	1187–1204	TTMGGGGCATRCIKACCT	Baker et al. 2003
N961R	944–961	CMATTAAACCGCRCACCC	This study
N989R	972–989	GGTTTCCGGTGTCAGTTC	This study
9bF	9–27	CCCGTTGATCCTGCGGGAG	Huber et al. 2002
511mcR	498–511	CTTGCCCACCGCTT	Huber et al. 2002

*Alu*I at 37°C for 16 h and compared on 2.5% agarose gels by amplified rDNA restriction analysis (ARDRA).

Phylogenetic analysis

Several clones representing each of the ARDRA types were sequenced using M13F and M13R primers. Sequences were checked for similarity to known sequences using BLASTn (http://www.ncbi.nlm.nih.gov) and aligned to closely related representatives from their taxonomic subdivision using ClustalX (Thompson et al. 1997). Tree construction was carried out using the distance based method (neighbour-joining tree using a Kimura-2-parameter correction) and maximum parsimony, each with 1,000 bootstrap replicates, using Mega 4 (Kumar et al. 2004) and Phylo_win 2.0 (Galtier et al. 1996).

Results

Phylogenetic diversity in the Chinese and New Zealand hydrothermal samples

The archaeal primer set A571F/UA1204R (Baker et al. 2003) was used to amplify metagenomic DNA from Chinese samples TC11 and TC9. Inserts from 71 and 36 clones from TC11 and TC9 amplicon libraries, respectively, were amplified using these archaeal primers. Restriction analysis of the 630 bp amplicons using *Rsa*I, *Hae*III and *Mbo*I yielded eight unique ARDRA patterns. Sequencing of the different ARDRA patterns revealed the presence of euryarchaeal, crenarchaeal and novel nanoarchaeote-like sequences. The phylogenetic positions of the novel nanoarchaeote-like phylotypes are shown in Fig. 1. Samples TC9 and TC11 from Rehai, China contained nanoarchaeal-like phylotypes, which clustered into four closely related clades and differed from each other by 1–12%. These new nanoarchaeote-like sequences shared 80–89% base identity with the three previously published sequences from *N. equitans* (Huber et al. 2002) and uncultured nanoarchaeotes CU-1 and OP-9 (Hohn et al. 2002), and consistently grouped together irrespective of the algorithm applied (Fig. 1).

Amplification of metagenomic DNA from sample TC11 using nanoarchaeal primer pair 9bF/511mcR yielded a large amplicon library from which 100 clones were analysed using *Rsa*I, *Hae*III and *Alu*I. A single ARDRA pattern was identified and sequenced. The novel nanoarchaeote-like sequence had 85–88% identity to previously published sequences from *N. equitans* (Huber et al. 2002), uncultured nanoarchaeotes CU-1 and OP-9 (Hohn et al. 2002) and uncultured nanoarchaeotes identified by McCliment et al. (2006). The phylogenetic position of this novel phylotype TC11N10 is shown in Fig. 2.

Metagenomic DNA from New Zealand hydrothermal sites TOK3 and TOK17 was amplified using the archaeal primer pair A571F/UA1204R. The inserts from 281 clones were amplified using the archaeal-specific primers and digested with *Rsa*I, *Hae*III and *Alu*I. Fifteen unique ARDRA patterns were identified in the New Zealand libraries, containing representatives of the Crenarchaeota, Euryarchaeota and Nanoarchaeota. The novel nanoarchaeal-like sequences, TOK173, TOK17C9, TOK353 and TOK315, which differed from each other by 3–5%, were more closely related to *N. equitans* and uncultured nanoarchaeotes CU-1 and OP-9 than to the nanoarchaeote-like sequences from the Chinese samples TC9 and TC11 (Fig. 1). These New Zealand phylotypes shared 85–91% identity with the published nanoarchaeal sequences (Huber et al. 2002; Hohn et al. 2002), as compared to 84–90% with the nanoarchaeal-like sequences from Chinese samples.

New nanoarchaeal-specific primer design

Using the new nanoarchaeal-like 16S rRNA gene sequences from the Chinese and New Zealand samples and the three previously published sequences (Huber et al. 2002; Hohn et al. 2002), two new nanoarchaeal-specific primers were designed by searching for conserved areas within the nanoarchaeal sequences, which were not conserved across the other three archaeal sub-divisions. The region chosen for primer N961R had 100% complementarity to the Chinese and New Zealand nanoarchaeal-like phylotypes, as well as to the published environmental DNA sequences (clones CU1 and OP9), except for *N. equitans* which had two mismatches. Primer N961R was therefore designed

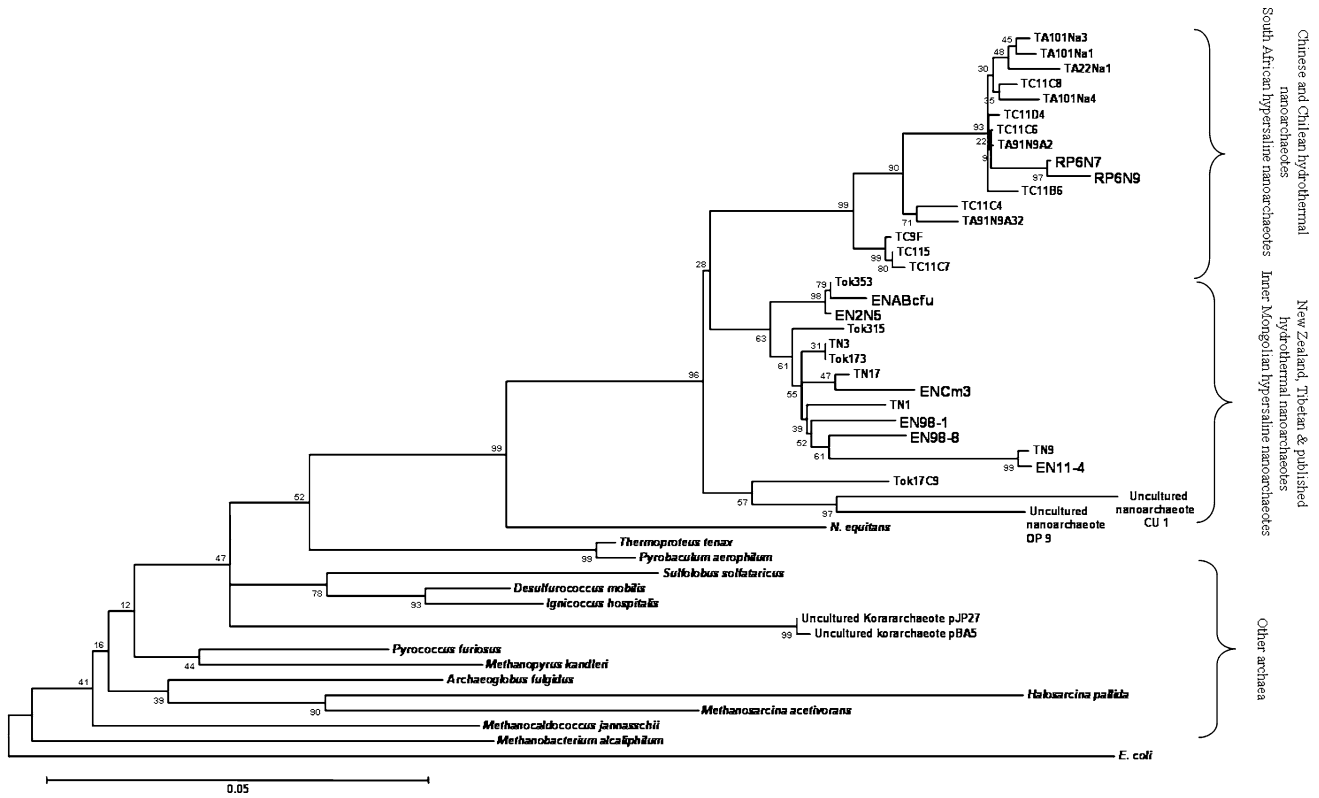

Fig. 1 Phylogenetic tree as determined by neighbour-joining analysis of a 400 bp alignment of 16S rRNA gene sequences. The Chinese (TC9 and TC11) and New Zealand clones were amplified with primers A571F and UA1204R, the Chilean clones were amplified using primer pairs A571F/N961R and A571F/N989R, while the Tibetan, Inner Mongolian and South African clones were amplified using A571F/N989R

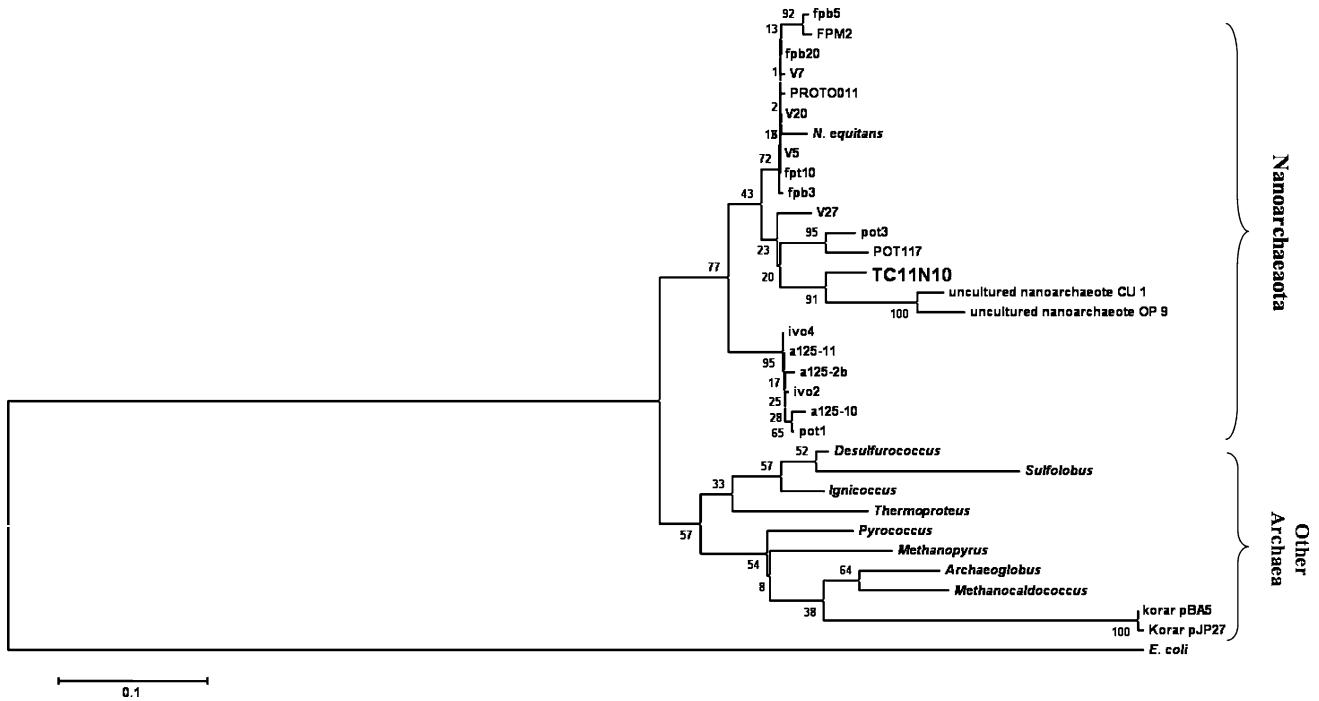

Fig. 2 Phylogenetic tree constructed by neighbour-joining analysis of a 470 bp alignment of 16S rRNA sequences amplified using primers 9bF/511mcR from Chinese sample TC11

with degeneracies at those two positions. Primer N989R, which contained no degeneracies, was designed to have 100% complementarity to the nanoarchaeal-like sequences from Chinese and New Zealand samples, but containing mismatches to the other three published sequences.

The new reverse primers N961R and N989R were used in conjunction with universal archaeal primer A571F, producing amplicons of 400 and 418 bp, respectively. These primer sets were compared to amplification using the universal archaeal primer pair A571F/UA1204R. Both primer sets were tested against crenarchaeal (*Solfolobus solfataricus*), euryarchaeal (*Thermococcus litoralis*), and nanoarchaeal (*N. equitans*) DNA, and a hydrothermal sediment sample (TA101) from the Tatio volcanic region in Chile. The A571F/UA1204R archaeal primers amplified 16S rRNA genes from all four DNA samples (Fig. 3, lanes 1–4). The nanoarchaeal-specific N961R primer set was used successfully to amplify DNA from nanoarchaeal DNA and the Chilean hydrothermal sediment sample and did not amplify crenarchaeal or euryarchaeal DNA (Fig. 3, lanes 5–8). The N989R primer set amplified DNA from the Chilean hydrothermal sediment, but showed no product with either crenarchaeal or euryarchaeal DNA (data not shown). This primer pair did not amplify DNA from *N.* equitans, presumably due to the presence of mismatches in the primer sequence. The PCR product amplified with the nanoarchaeal-specific primers from the Chilean sediment sample was cloned and the resulting recombinants were sequenced using M13 primers. Sequence analysis revealed high similarity to nanoarchaeal phylotypes.

Nanoarchaeal diversity in Chilean and Tibet hydrothermal samples

The nanoarchaeal-specific primer sets were used to amplify 16S rRNA genes from Chilean hydrothermal samples. Inserts from 22 clones from the TA22 library, 43 clones from the TA101 library and 48 clones from the TA91 library were reamplified using the same primer sets. Reamplified inserts were digested with *Rsa*I, *Hae*III and *Alu*I and grouped according to ARDRA pattern (data not shown). Sequences from each group separated into three different clades with high identity (95–99%) to the Chinese nanoarchaeal-like sequences (Fig. 1).

Amplification of 16S rRNA genes from community DNA preparations from Tibetan hydrothermal site DNA using the nanoarchaeal-specific primer set A571F/N989R yielded four sequences with high similarity to phylotypes from New Zealand samples (Fig. 1), but which separated into two different groups.

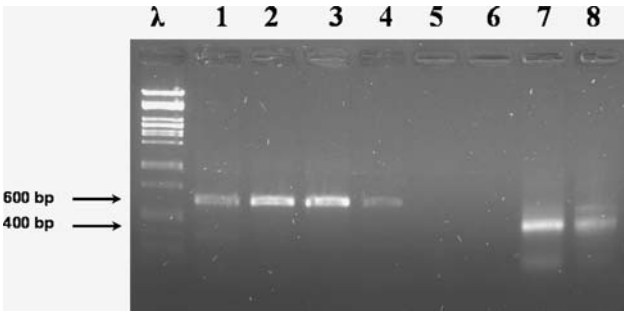

Fig. 3 TBE 1% agarose gel containing PCR products using primers A571F/UA1204R (*lanes 1–4*) and A571F/N961R (*lanes 5–8*) from crenarchaeal (*lanes 1 and 5*), euryarchaeal (*lanes 2 and 6*), nanoarchaeal (*lanes 3 and 7*) and hydrothermal sediment (*lanes 4 and 8*) DNA templates. PstI-digested λ DNA was used as marker

Identification of novel nanoarchaeal phylotypes in mesophilic hypersaline samples from inner Mongolia and South Africa

Metagenomic DNA from Inner Mongolian hypersaline lake sample EN2 was amplified using primer set A571F/N989R. Following ARDRA using *Rsa*I, *Hae*III and *Alu*I, a single phylotype was identified, EN2N5. This sequence had high identity (94–99%) to the New Zealand and Tibetan nanoarchaeote-like sequences (Fig. 1), while sharing 86–88% identity with the *N. equitans* and uncultured CU-1 and OP-9 nanoarchaeote clones. Aliquots from EN2 sample were inoculated into different halophilic media. Nanoarchaeal primers A571F/N989R were used to amplify DNA from mixed enrichment cultures in medium DSM 372. The resulting clones were analysed by ARDRA using *Rsa*I, *Hae*III and *Alu*I. Four clones showing different ARDRA patterns were sequenced and found to have high similarity to nanoarchaeote-like sequences from the New Zealand and Tibetan samples and to EN2N5 (Fig. 1). Clone ENA-Bcfu shared 99% identity to EN2N5, while clones EN98-1, EN98-8 and ENCm3 were 95–96% identical to EN2N5.

Sediment from a South African hypersaline pond (Rooipan, Western Cape) was used to inoculate medium DSM 372. Metagenomic DNA from enrichment cultures was PCR amplified using primer pair N989R/A571F. Clones from the amplicons were analysed and two different patterns were identified. These clones contained sequences with high identity (94–98%) with the Chinese and Chilean nanoarchaeote-like sequences (Fig. 1).

Together, these sequence data provide evidence that nanoarchaeal-like organisms are present in hypersaline systems.

Discussion

All nanoarchaeal-like sequences grouped together with high bootstrap support and were more closely related to

each other than to sequences from other archaeal phyla (Fig. 1). Despite the low bootstrap values obtained for branches containing the different clones within the Nanoarchaeaota group, it is interesting to note that the phylotypes from Chinese and Chilean hydrothermal samples, together with the South African hypersaline samples, form a stable phylogenetic cluster clearly distinguishable from the cluster containing the Tibet and New Zealand hydrothermal phylotypes and Inner Mongolian hypersaline phylotypes.

The lack of a complete 16S rRNA gene sequence for these novel phylotypes makes it impossible to deduce whether they can be classified as different taxa. However, it is clear that if a similar divergence was found over the remainder of the 16S rRNA gene, these phylotypes would be classified as distinct taxa at the species or even genus level, a result which would suggest that multiple nanoarchaeal species co-existed in a single environment. As previously noted (Hohn et al. 2002), there is a wide geographical distribution of members of the Nanoarchaeota, with new members being found in Asia, Oceania, South America and Africa. However, our data do not support the existence of phylogeographic clustering, with phylotypes from widely separate geographical regions having high similarity. We speculate that the geographic distribution of the different nanoarchaeal-like phylotypes is dependent on the host distribution, which is influenced in turn by geochemical parameters, irrespective of geography.

All previous nanoarchaeal signals were derived from hyperthermophilic sites, both terrestrial and marine (Huber et al. 2002; Hohn et al. 2002). Here, we show that phylotypic signals, which show reproducible and reliable nanoarchaeal identity can be recovered from habitats, which differ in key physicochemical properties from those previously known as nanoarchaeal sources. The identification of nanoarchaeal-like signals in mesophilic hypersaline water and sediment samples, which are very unlikely to be contaminated by hydrothermal sources, suggests that nanoarchaea may be much more widely distributed and more physiologically flexible than previously thought. One intriguing consideration from our observation is that hypersaline nanoarchaeal hosts in hypersaline systems are almost certainly extreme halophiles. While we have as yet not identified the putative host(s), and therefore cannot speculate on whether such an organism is a member of domain Bacteria or Archaea, we suggest that the parasitic lifestyle of nanoarchaea is much more widely adaptable than implied by the (hitherto) restricted host range of known nanoarchaea.

Acknowledgments Chinese, Tibet and Inner Mongolian sediment samples were obtained under the auspices of the joint Chinese-EU-SA MGATech project (QLRT-2001-01972). Chilean sediment samples were obtained by Dr. J. Blamey. GCB was supported by the Royal Society/NRF Program. NG and AC were supported by the NRF HBU-RDP Program. DNA sequencing was conducted by Di James at the University of Cape Town Sequencing Facility and Inqaba Biotech, South Africa. We would like to thank H. Huber for supplying "*N. equitans*" genomic DNA.

References

Baker GC, Cowan DA (2004) 16S rDNA primers and the unbiased assessment of thermophile diversity. Biochem Soc Trans 32:218–221

Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55:541–555

Barns SM, Delwiche CF, Palmer JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci USA 93:9188–9193

Brochier C, Forterre P, Gribaldo S (2005) An emerging phylogenetic core of Archaea: phylogenies of transcription and translation machineries converge following addition of new genome sequences. BMC Evol Biol 5:36

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P (2008) Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeaota. Nat Rev Microbiol 6:245–252

Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P (2006) Toward automatic reconstruction of a highly resolved tree of life. Science 311:1283–1287

Galtier N, Gouy M, Gautier C (1996) Seaview and phylo_win: two graphical tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543

Hohn MJ, Hedlund BP, Huber H (2002) Detection of 16S rDNA sequences representing the novel phylum "Nanoarchaeota": Indication for a wide distribution in high temperature biotopes. Syst Appl Microbiol 25:551–554

Huber H, Hohn MJ, Rachel R, Fuchs T, Wimmer VC, Stetter KO (2002) A new phylum of Archaea represented by a nano-sized hyperthermophilic symbiont. Nature 417:63–67

Jahn U, Gallenberger M, Paper W, Junglas B, Eisenreich W, Stteter KO, Rachel R, Huber H (2008) *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: new insights into a unique intimate association of two Archaea. J Bact 190:1743–1750

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

McCliment EA, Voglesonger KM, O'Day PA, Dunn EE, Holloway JR, Cary SC (2006) Colonization of nascent, deep-sea hydrothermal vents by a novel Archaeal and Nanoarchaeal assemblage. Environ Microbiol 8:114–125

Paper W, Jahn U, Hohn MJ, Kronner M, Näther DJ, Burghardt T, Rachel R, Stetter KO, Huber H (2007) *Ignicoccus hospitalis* sp. nov., the host of "*Nanoarchaeum equitans*". Int J Syst Evol Microbiol 57:803–808

Stach JEM, Bathe S, Clapp JP, Burns RG (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil obtained using different isolation and purification methods. FEMS Microbiol Ecol 36:139–151

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustalx windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Waters E, Hohn MJ, Ahel I, Graham DF, Adams MD, Barnstead M et al (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. Proc Natl Acad Sci USA 100:12984–12988