

1 **First Archaeal rDNA sequences from coastal waters of Argentina:**
2 **unexpected PCR characterization by using eukaryotic primers**

3 *Running title: First Archaea rDNA sequences from the Argentine Sea*

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5 **Primeras secuencias de ADNr de Archaea en aguas costeras de**
6 **Argentina: inesperada caracterización por PCR usando cebadores para**
7 **eucariotas**

8 *Titulo corto: Primeras Secuencias de ADNr de Archaea del Mar Argentino*

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2 **ABSTRACT.** Many members of Archaea, a group of prokaryotes recognized three
3 decades ago, colonize extreme environments. However, new research is showing that
4 Archaeans are also quite abundant in the plankton of the open sea, where are fundamental
5 components that play a key role in the biogeochemical cycles. Although the widespread
6 distribution of Archaea the marine environment is well documented there are no reports
7 on the detection of Archaea in the Southwest Atlantic Ocean. During the search of
8 picophytoplankton sequences using eukaryotic universal primers, we retrieved archaeal
9 rDNA sequences from surface samples collected during Spring at the fixed EPEA Station
10 (38°28'S-57°41'W, Argentine Sea). From environmental DNA and using PCR
11 methodology, two DNA fragments of about 1,700 and 1,450 bp were visualized after
12 electrophoresis in agarose gels, which were separately purified, cloned and sequenced.
13 BLAST analysis showed that sequences of the highest size corresponded to eukaryotic
14 organisms and, unexpectedly, those of about 1,460 bp corresponded to Archaeal
15 organisms. Phylogenetic analysis showed that Archaeal sequences belong to
16 Euryarchaeota of the Marine Group II, which is characterized as a methanogenic lineage.
17 This is the first report on the presence of Euryarchaeota-Group II sequences in
18 environmental water samples of the Argentine Sea. The fact that Archaea sequences were
19 amplified with primers non specific for this group may suggest an unexpected abundance
20 of these organisms in the early spring in the Argentine Sea.

21

22 **Keywords:** Euryarchaeota, Argentine Sea, environmental rDNA, PCR methodology,
23 primer design

1 **Resumen.** Muchos miembros de las Archaea, un grupo de micrororganismos descriptos
2 hace aproximadamente treinta años, colonizan ambientes extremos. Sin embargo, las
3 investigaciones más recientes han demostrado que las Archaeas también son abundantes
4 componentes del plankton marino, siendo algunos grupos de Archaeas componentes
5 fundamentales de los ecosistemas marinos debido a su rol clave en los ciclos
6 biogeoquímicos. Aunque la ubiquidad de las Archaeas ha sido bien documentada, hasta el
7 momento no hay reportes de la presencia de representantes de este grupo en el mar
8 Argentino. En un estudio de biodiversidad orientado a determinar secuencias de
9 picoeucariotas utilizando cebadores universales para eucariotas, encontramos secuencias
10 de ADNr de Archaeas en muestras recolectadas durante la primavera en la estación fija
11 EPEA (38°28'S-57°41'W, Mar Argentino). A partir de ADN ambiental y mediante el uso
12 de la metodología de PCR, obtuvimos dos fragmentos de aproximadamente 1.700 y 1.460
13 bases, los cuales fueron separados y visualizados después de electroforesis en geles de
14 azarosa, y luego purificados, clonados y secuenciados. El análisis del BLAST mostró que
15 las secuencias de tamaño superior correspondían a organismos eucariotas y las secuencias
16 de menor tamaño pertenecían a Archaea. El análisis filogenético mostró que las
17 secuencias de Archaea se agrupan con Euryarchaeota Marina Grupo II, caracterizado
18 como un linaje metanógeno. Éste es el primer reporte de la presencia de secuencias de
19 Euryarchaeota-Grupo II en aguas del mar Argentino. El hecho de que las secuencias de
20 Archaea hayan sido amplificadas con cebadores no específicos para este grupo, sugeriría
21 una inesperada abundancia de estos organismos durante los inicios de primavera en el
22 Mar Argentino.

23

24 **Palabras clave:** Euryarchaeota, Mar Argentino, ADNr ambiental, metodología PCR,
25 diseño de cebadores.

1 INTRODUCTION

2 Microbial diversity is clearly a topic of considerable importance and interest. In the past
3 decades the most surprising discoveries in biodiversity arose from studies on the
4 distribution of microbial communities in the ocean. Marine ecosystems are continually
5 subject to oscillations in environmental conditions. It is now widely recognized that
6 climate change and biodiversity are interconnected (Bowland 2006). Because global
7 warming is expected to have a significant influence on hydrologic cycle over the next
8 several centuries and thus on species composition, the analysis of the current biodiversity
9 is urgent. Increasing amount of knowledge has been reported in the last decades;
10 however, the introduction of molecular methodologies and metagenomic analyses opened
11 new avenues in the understanding of marine microbial diversity. Using these tools, the
12 ubiquitous presence of completely novel lineages, with no representatives in cultures, has
13 been established for the three domains of life: Bacteria (Giovannoni *et al.* 1990), Archaea
14 (DeLong 1992, Fuhrman *et al.* 1992), and more recently Eukaryota (Díez *et al.* 2001,
15 López-García *et al.* 2001a, Massana *et al.* 2002, Romari and Vaulot 2004, Groisillier *et*
16 *al.* 2006, Lovejoy *et al.* 2006).

17 Achaeans are microscopic single-celled organisms that constitute a group of
18 prokaryotes, recognized in 1977 as an independent monophyletic group. Although
19 initially they were believed to be limited to anaerobic, hyperthermal, and highly saline
20 habitats, they were also found in both marine and freshwaters environments (DeLong
21 1992, Fuhrman *et al.* 1992, Massana *et al.* 1997 and 1998, Murray *et al.* 1999, Massana *et*
22 *al.* 2000, Karner *et al.* 2001, Auguet and Casamayor 2008). Thus, it is recognized that
23 marine archaeal populations are diverse, complex and widespread (Danovaro 2010).
24 There is now increasing evidence that marine Archaea make an important contribution to
25 the biogeochemical nitrogen and carbon cycles (Bartossek *et al.* 2010).

1 Based on 16S rDNA phylogeny from cultivated organisms, marine Archaea are
2 phylogenetically distributed through four main taxonomical clusters: one cluster of
3 Crenarchaeota, the Marine group I (MGI), and three clusters of Euryarchaeota, group II,
4 III and IV (Galand *et al.* 2009). Members of the marine Group I have a key role on the
5 biogeochemical cycles, being a fundamental component of the marine ecosystem.
6 Although Crenarchaeota consist mainly of thermophilic species, the genome
7 *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus*, two non-thermophilic strains
8 were completely sequenced (Preston *et al.* 1996, Könneke *et al.* 2005, Bartossek *et al.*
9 2010). Archaea of Group II of planktonic *Euryarchaeota* have more varied metabolisms
10 (hence the name “eury-,” meaning variable), but most biochemical studies have focused
11 on methanogenesis, a unique property of some Archaea (comprising Halobacteriales,
12 Thermoplasmatales, Thermococcales, Sulfolobales, Pyrodictiales, Archaeoglobus,
13 Methanobacteriales). Representatives of Group III are restricted to deep waters, having
14 been found in waters below the photic zone (Galand *et al.* 2009). Group IV was first
15 discovered by Rodriguez-Valera (1979) and sequences of its members were clearly
16 distinct from all known planktonic Archaea (López García *et al.* 2001a).

17 Analyses of rDNA sequences from environmental samples have revealed that
18 Archaea are ubiquitous and far more abundant than previously assumed (Stein and Simon
19 1996, Karner *et al.* 2001, DeLong 2003). Culture-independent techniques based on 16S
20 rDNA analyses showed the existence of Archaea in the open-ocean, marine sediments,
21 soils and freshwater lake sediments (Massana *et al.* 2000, Schleper *et al.* 2005, Galand *et*
22 *al.* 2009, Bartossek *et al.* 2010). Particularly, marine Archaea have been shown to reside
23 in coastal and offshore temperate and cold waters worldwide (DeLong 1992, Fuhrman *et*
24 *al.* 1992, Massana *et al.* 1997, Galand *et al.* 2010). Karner *et al.* (2001) found that pelagic
25 *Crenarchaeota* form North Pacific Ocean Gyre comprising more than 30% of the total

1 microbial cells from 200 m to 5,000 m. Herndl *et al.* (2005) estimated that at depth of 100
2 m *Euryarcheota* contributed about 17% of picoplankton abundance of the North Atlantic
3 Sea while the contribution of *Crenarchaeota* was about 18.5%. Primers to detect archaeal
4 sequences by PCR approach have been designed to amplify all prokaryotic 16S rDNA
5 genes and are referred to as ‘universal’ (DasSarma and Fleischmann 1995, Reysenbach
6 and Pace 1995, Vetriani *et al.* 1999, Baker *et al.* 2003,
7 http://bioinfo.unice.fr/454/VC/archaea_primers_sorted_by_Fsequences.html) or for taxa
8 specific detection (Baker *et al.* 2003). López-García *et al.* (2001 a and b) found
9 Euryarchaeota sequences belonging to Marine Group II and III of the Antarctic Polar
10 Front sea water by using and designed different primer sets for 16S rDNA amplification.

11 To our knowledge there are no reports on the detection of Archaea in the Southwest
12 Atlantic Ocean. In this study, we report the presence of rDNA archaeal sequences in
13 surface water samples of the Argentine Sea that constitute the beginning of more
14 comprehensive studies to understand the contribution of prokaryotes to biogeochemical
15 cycles of marine ecosystems.

16

17 **MATERIAL AND METHODS**

18

19 **Sample collection**

20

21 Water samples were collected by the cruises conducted monthly on-board the RV
22 “Capitan Canepa” (INIDEP). Surface water samples were collected in September,
23 October and November at the fixed EPEA Station in the Argentine Sea (38°28’S -
24 57°41’W at 27 nautical miles south of Mar del Plata, Argentina). Environmental
25 characteristics of sampling site (photosynthetically active radiation, temperature and

1 salinity) were described by Silva *et al.* (2009). Water samples (2.5 liters) were taken
2 using a bucket at the surface, prefiltered through a 25 µm pore size to eliminate
3 microplankton components, passed through a polycarbonate membrane of 3 µm pore size
4 (Nuclepore) to remove nanoplankton components, and finally filtered through a 0.2 µm
5 pore size (Durapore). Filters were transferred into a cryovial tube, immediately frozen in
6 liquid nitrogen, and stored at -80°C until nucleic acid extraction.

7

8 **Nucleic acid extraction**

9

10 Genomic DNA was extracted from marine samples (4 subsamples at each collect)
11 according to standard protocols (Sambrook and Russell 2001). Nucleic acid extraction
12 began with the addition of lysozyme (1 mg mL⁻¹) to the filter unit and incubation at 37°C
13 for 30 min. Then, proteinase K (0.5 mg mL⁻¹) and sodium dodecyl sulfate (SDS, 1%)
14 were added, and the filter was incubated for 2 h at 55°C. The lysate was recovered from
15 the filter, which in turn was rinsed with 1 mL of lysis buffer (Tris-HCl 50 mM, pH 8;
16 EDTA 40 mM, pH 8; sucrose 0.75 M; nuclease-free water) with lysozyme (1 mg/mL).
17 All centrifugations were performed at 13,000 rpm and at 4°C. After centrifugation for 7
18 min, the upper phase was transferred to a clean tube. The pooled lysates were extracted
19 twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) and
20 once with chloroform-isoamyl alcohol (24:1). After removing any residual phenol by
21 centrifugation, the aqueous phase was transferred into a new 1.5 mL tube containing 750
22 µL cold isopropanol and 1/10 volume sodium acetate (0.3 M final concentration, pH 5.2).
23 Tubes were placed in -20°C freezer overnight. After a centrifugation for 30 min the
24 supernatant was decanted into a beaker and the DNA pellet washed with 200 µL 70%
25 ethanol at -20°C. The DNA pellet was dried and re-suspended in 50 µL PCR water and

1 stored at -20°C until use. DNA concentration was determined by measuring absorbance at
2 260 nm.

3

4 **PCR amplification, cloning and sequencing**

5

6 Extracted DNA was used as template in PCR reactions using eukaryotic 18S ribosomal
7 DNA (rDNA)-specific primers to Eukarya EukA 5'-AACCTGGTTGATCCTGCCAGT-
8 3'; EukB 5'-TGATCCTTCTGCAGGTTACCTAC-3' (Medlin *et al.* 1988). The PCR
9 conditions were as follows: initial DNA denaturizing for 3 min at 94°C, followed by 30
10 cycles of denaturizing for 45 s at 94°C, annealing for 1 min at 55°C, extension for 3 min
11 at 72°C, plus one additional cycle with a final 10-min chain elongation at 72°C. The 25-
12 µL reaction volume contained 50 ng of DNA and 5 pmol of each primer. Following
13 amplification, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel
14 and DNA fragments were visualized with ethidium bromide. DNA from the agarose gel
15 was extracted using the QIAGEN MinElute gel extraction kit. The purified PCR products
16 were cloned into the pGemTeasy cloning Vector kit (Promega). The recombinant plasmid
17 was inserted into *Escherichia coli* DH5α competent cells, which were grown in LB
18 medium at 37°C for 20 min. Cultures were sprayed on LB/Ampicillin/IPTG/X-Gal (1 mL
19 100 mg mL⁻¹ ampicillin, 0.12 g isopropyl-β-D-thiogalactopyranoside (IPTG) in 5 mL
20 deionised water; 0.10 g 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) in 2 mL
21 N,N-dimethylformamide). Twenty colonies of each sample (a total of 100 positive white
22 colonies) were separately grown (37°C, over night) in LB medium with ampicillin. The
23 presence of rDNA inserts was confirmed by colony PCR using the same primers and
24 amplification conditions. The PCR products were digested with the restriction
25 endonuclease *Hae*III. All digestions were completed independently and performed in 15

1 μL of volume with 3 μL of PCR product, 10X buffer and 3 units of restriction
2 endonuclease. The solution was incubated at 37°C for one hour. Digested samples were
3 run by electrophoresis (80 V, 3 h) in agarose gels (2.5%) (Meta Phor. Cambrex Bio
4 Science Rockland Inc. Me USA). Agarose gels were stained with ethidium bromide and
5 the restriction fragment length polymorphism (RFLP) products were visualized with UV
6 transillumination. Size of inserts was confirmed by *EcoR1* restriction enzyme (Promega,
7 Madison, USA) by plasmid digestion. The inserts of clones with different RFLP patterns
8 were sequenced (Macrogen, Korea). Sequence was deposited in GenBank
9 (BankIt1405479, uncultured HQ541865).

10

11 **Phylogenetic analysis and rDNA thermodynamic properties prediction**

12

13 Comparisons of rDNA sequences were performed using nucleotide sequences available in the
14 databases at the National Center for Biotechnology Information (NCBI,
15 <http://www.ncbi.nlm.nih.gov/BLAST>). Sequence alignments were generated using the
16 CLUSTAL W software and graphic representations of phylogenetic trees were performed
17 using the MEGA4 software (Tamura *et al.* 2007). The trees were statistically evaluated with
18 non-parametric bootstrap analysis (number of replicates = 1,000). The secondary structures for
19 ribosomal RNA were predicted using RNADRAW (Vienna RNA package;
20 <http://www.rnadraw.com>) and RNAfold program (available at [http://rna.tbi.univie.ac.at/cgi-](http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)
21 [bin/RNAfold.cgi](http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)).

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23

1 **RESULTS AND DISCUSSION**

2

3 In our study of picophytoplankton diversity of the Argentine Sea we used EukA/EukB
4 primers to PCR amplify eukaryotic small subunit ribosomal rDNA genes (Medlin *et al.*
5 1988) from DNA extracted from surface-sea samples collected during spring in the
6 Argentine Sea. Diez *et al.* (2001) had been demonstrated the specificity of EukA/EukB
7 primer pair to construct clone libraries of eukaryotes and the ability of the primers to
8 recover distant phylogenetically-related eukaryotic groups (Stramenopiles, Alveolates,
9 Prasinophytes as well as Chrysomonad, Cercomonads and Fungi) from North Atlantic,
10 Antarctica, and Mediterranean Sea surface waters. Moreover, those primers were
11 successfully used in most recent biodiversity studies of marine picoeukaryotes (Groissillier
12 *et al.* 2006, Guillou *et al.* 2008, Hoppenrath *et al.* 2009, Not *et al.* 2009). The analysis of
13 the amplification products using EukA/EukB primers after separation by electrophoresis
14 on agarose gel, revealed that while a DNA fragment of expected size (1,700 bp) was
15 present in all the samples, an additional band of 1,460 bp was visualized in all samples
16 collected in September and October (fig. 1a). About 66% of the analyzed samples showed
17 the second band. The two DNA amplified fragments were separately purified and cloned
18 in *E. coli* cells (fig. 1b). Further sequencing of the inserts revealed that nucleotide
19 sequences of 1,700-bp bands corresponded to eukaryotic organisms. In the case of
20 samples collected in September, the sequences were ascribed mainly to Stramenopiles
21 (e.g. *Bolidomonas* sp.) and Alveolata (e.g. *Laboea* sp.) whereas those of October matched
22 with sequences belonging to Stramenopiles (e.g. *Pedinella* sp.) and Chlorophyta
23 Prasinophyceae (e.g. *Bathycoccus* sp.). Amplified DNA of samples collected in
24 November produced only one band of about 1,700 bp and sequences matched with
25 sequences belonging to Alveolata (e.g. *Noctiluca* sp.). Surprisingly, nucleotide sequences

1 of the 1,460-bp inserts corresponded to archaeal rDNA, whose RFLP patterns were
2 compared (fig. 2). The sequences of the inserts matched with an uncultured marine
3 Archaea grouped with the Marine Group II of Euryarchaeota (fig. 2).

4 The sequence alignments of Archaea 16S rDNA and 18S rDNA universal primers
5 (EukA/EukB) used for PCR amplification confirmed that the primer pair shares 100 %
6 identity with archaeal rDNA regions. Analyses with the SILVA rRNA database
7 (<http://www.arb-silva.de>) indicated that EukA and EukB primers would amplify a very
8 small number of Archaea sequences (2 out of 11,954 Archaea available sequences).
9 Therefore, the fact that Archaeal sequences could be recovered from the environmental
10 DNA with primers designed for eukaryotes suggests that some scarce Archaea strains of
11 Marine Group II were very abundant at least during September and October, considering
12 the obvious competition in the annealing step between Archaea and Eukaryotic sequences
13 for the primers.

14 The identification of Archaea sequences led us to analyze the primers reported to
15 specifically retrieve sequences of these organisms. We compare EukA-EukB sequences
16 with the Archaea primers and with the more abundant sequence we obtained in this study
17 (HQ541865) (table 1). Whereas some of the reported primers align with the HQ541865
18 sequence in more internal positions than EukA-EukB, others have poor or no
19 complementation with HQ541865. Baker *et al.* (2003) proposed two new primer pairs
20 (A571F 5'-GCYTAAAGSRICCGTAGC-3'/UA1204R 5'-TTMGGGGCATRCIKACCT-
21 3' and A751F 5'-CCGACGGTGAGRGRYGAA-3'/UA1406R 5'-
22 ACGGGCGGTGWGTRCAA-3') to amplify sequences from Crenarchaeote and
23 Euryarchaeota type strains. Also Gantner *et al.* (2011) presented two new archaeal
24 primers (340F 5'-CCCTAYGGGGYGCASCAG -3' and 1000R 5'-
25 GGCCATGCACYWCYTCTC-3') which were designed from 8,500 aligned archaeal

1 sequences by using the SILVA database. They reported that designed primers showed a
2 high archaeal specificity (< 1% bacteria amplification) covering 93 and 97% of available
3 sequences for Crenarchaeota and Euryarchaeota respectively. However, these primers
4 have a high level of degeneracy, which could lead to amplify non-target genes or
5 domains. From non-degenerative primers used to the comparison (table 1), only one
6 aligns with one of the primers used in this study. The specific eukaryotic EukA primer
7 matches with EK4F primer, designed by Robb *et al.* (1995) and later reported as very
8 high specific for methanogen sequences (Baker *et al.* 2003), but which does not match
9 with any other Archaeal group. Remarkably, EukA primer has four additional bases than
10 the EK4F which allowed the specific retrieval of the Marine Group II of Euryarchaeota
11 sequences.

12 It has been shown that the two major groups of planktonic Archaea (Crenarchaeota
13 and Euryarchaeota) might account for about one-third of all prokaryotic cells in the global
14 ocean (Karner *et al.* 2001). Sequences of the Marine Group II of Euryarchaeota have been
15 found in both, the Sta Barbara Channel, California, from 0 to 200 m (Massana *et al.*
16 1997) as well in the Arctic and Antarctic surface waters (Murray *et al.* 1999, Bano *et al.*
17 2004). Although they were reported as more abundant in surface waters in Pacific and
18 Beaufort Sea, their presence was also reported in different oceanic regions sampled at
19 depths between 5 and 200 m (Massana *et al.* 2000, Karner *et al.* 2001, Herndl *et al.* 2005,
20 DeLong *et al.* 2006, Galand *et al.* 2009). In the South Atlantic Sea, however, the presence
21 of Archaea remained undescribed until the present report.

22

23 **Conclusion**

24

1 Euryarchaeota sequences affiliating with the known Group II are recognized to be
2 widespread in the oceans worldwide (Stein and Simon 1996, DeLong 2003) and this
3 study contributes with the identification of the first 16S rDNA sequences belonging to
4 Euryarchaeota-Marine Group II in environmental surface water samples of the Argentine
5 Sea, where could play an important role in biogeochemical cycles. The casual way that
6 led to these results, in turn, gives more weight to the finding, since it highlights the
7 relative abundance of these Archaea in certain months of the year, and how unlikely it
8 would have been to detect them using reported information.

9

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11

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14

15 **References**

16 Auguet JC, Casamayor EO. 2008. A hotspot for cold crenarchaeota in the neuston of high
17 mountain lakes. *Environ. Microbiol.* 10: 1080–1086.

18 Baker GC, Smith JJ, Cowan DA. 2003. Review and re-analysis of domain-specific 16S
19 primers. *J. Microbiol. Meth.* 55: 541-555.

20 Bano N, Ruffin S, Ransom B, JT Hollibaugh. 2004. Phylogenetic Composition of Arctic
21 Ocean Archaeal Assemblages and Comparison with Antarctic Assemblages. *Appl.*
22 *Environm. Microbiol.* 70: 781-789.

23 Bartossek R, Nicol GW, Lanzen A, Klenk HP, Schleper C. 2010. Homologues of nitrite
24 reductases in ammonia-oxidizing archaea: diversity and genomic contexte. *Environ.*
25 *Microbiol.* 12: 1075–1088.

1 Bowland JA. 2006. Climatic Change: Melting away marine biodiversity in the Canadian
2 Arctic. In: Atlantic Canada Marine Biodiversity Essay Content. Available in
3 http://www.marinebiodiversity.ca/en/pdfs/Biodiversity_Bowland.pdf (Accession
4 February 2011).

5 Casamayor, R. Massana, S. Benlloch, L. Overeas, B. Diez and V. Goddard *et al.*, 2002.
6 Changes in archaeal, bacterial, and eukaryal assemblages along a salinity gradient by
7 comparison of genetic fingerprinting methods in a multipond solar saltern scaling
8 plots, *Appl. Environ. Microbiol.* 4: 338–348.

9 Danovaro R, Editor. 2010. Archaeal Diversity Analysis Using 16S rDNA T-RFLP
10 (Terminal-Restriction Fragment Length Polymorphisms) In: *Methods for the Study of*
11 *Deep-Sea Sediments, Their Functioning and Biodiversity*, Chapter 21. Taylor and
12 Francis Group. 415 pp.

13 DasSarma S, Fleischmann EF. 1995. *Archaea: a laboratory manual – Halophiles*. Cold
14 Spring Harbour Laboratory Press, New York, pp. 269-272.

15 DeLong EF . 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA*
16 89: 5685–5689.

17 DeLong EF, Preston CM, Mincer T, Rich V, Hallam SJ, Frigaard NU Martinez A,
18 Sullivan MB, Edwards R, Brito BR, *et al.* 2006. Community genomics among
19 stratified microbial assemblages in the oceans interior. *Science* 311: 496–503.

20 DeLong EF. 2003. Oceans of Archaea: Abundant oceanic Crenarchaeota appear to derive
21 from thermophilic ancestors that invaded low-temperature marine environments.
22 *ASM News* 69: 503-511.

23 Díez B, Pedrós-Alió C, Massana R. 2001. Study of genetic diversity of eukaryotic
24 picoplankton in different oceanic regions by small-subunit rRNA gene cloning and
25 sequencing. *Appl. Environ. Microbiol.* 67: 2932–2941.

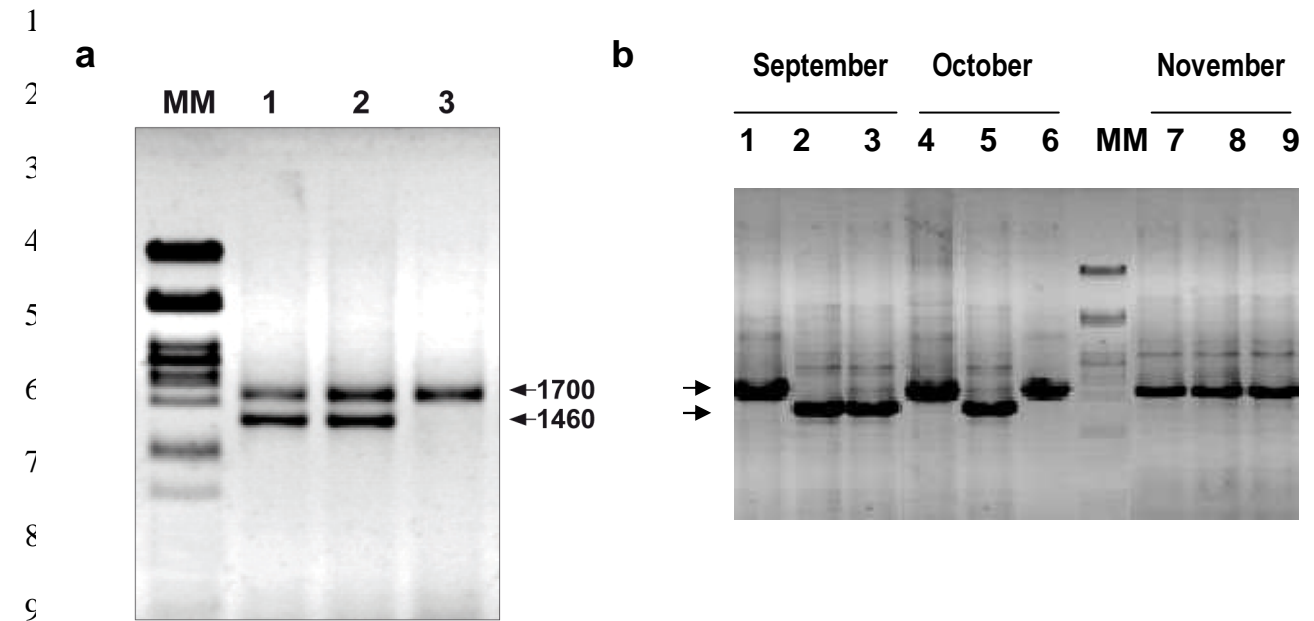
- 1 Embley TM, Finlay BJ, Thomas RH, Dyal PL. 1992. The use of rRNA sequences and
2 fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate
3 *Metopus palaeformis* and its archaeobacterial endosymbiont, J. Gen. Microbiol.
4 138:1479–1487.
- 5 Fuhrman J, McCallum K, Davis AA. 1992. Novel major archaeobacterial group from
6 marine plankton. Nature 356:148-149.
- 7 Galand PE, Casamayor EO, Kirchman DL, Potvin M, Lovejoy C. 2009. Unique archaeal
8 assemblages in the Arctic Ocean unveiled by massively parallel tag Sequencing.
9 ISME J. 3: 860–869.
- 10 Galand PE, Gutierrez-Provecho C, Massana R, Gasol JM, Casamayor EO. 2010. Inter-
11 annual recurrence of archaeal assemblages in the coastal NW Mediterranean Sea
12 (Blanes Bay Microbial Observatory). Limnol. Oceanogr. 55: 2117–2125.
- 13 Gantner S, Andersson AF, Alonso-Sáez L, Bertilsson S. 2011. Novel primers for 16S
14 rRNA-based archaeal community analyses in environmental samples. J. Microbiol.
15 Methods 84: 12-18.
- 16 Giovannoni SJ, Britschgi TB, Moyer CL, Field KG. 1990. Genetic diversity in Sargasso
17 Sea bacterioplankton. Nature 345: 60-63.
- 18 Groisillier A, Massana R, Valentin K, Vaulotl D, Guillou L. 2006. Genetic diversity and
19 habitats of two enigmatic marine alveolate lineages. Aquat. Microb. Ecol. 42: 277–
20 291.
- 21 Guillou L, Viprey M, Chambouvet A, Welsh RM, Kirkham AR, Massana R, Scanlan D J,
22 Worden AZ. 2008. Widespread occurrence and genetic diversity of marine parasitoids
23 belonging to Syndiniales (Alveolata). Environm. Microbiol. 10: 3349–3365.

- 1 Herndl GJ, Reinthaler T, Teira E, van Aken H, Veth C, Pernthaler A, J Pernthaler. 2005.
2 Contribution of Archaea to Total Prokaryotic Production in the Deep Atlantic Ocean.
3 *Appl. Env. Microbiol.* 2303–2309.
- 4 Hoppenrath M, Bachvaroff TR, Handy SM, Delwiche CF and Leander BS. 2009.
5 Molecular phylogeny of ocelloid-bearing dinoflagellates (Warnowiaceae) as inferred
6 from SSU and LSU rDNA sequences. *BMC Evolutionary Biology* 9: 116.
- 7 Huber JA, Mark Welch DB, Morrison HG, Huse SM, Neal PR, Butterfield DA, Sogin
8 ML. 2007. Microbial population structures in the deep marine biosphere. *Science* 318:
9 97–100.
- 10 Karner MB, DeLong EF, Karl DM. 2001. Archaeal dominance in the mesopelagic zone
11 of the Pacific Ocean. *Nature* 409: 507-510.
- 12 Könneke M, Bernhard AE, Torre JR, Walker CB, Waterbury JB, Stahl DA. 2005.
13 Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437: 543–
14 546.
- 15 López-García P, López-López A, Moreira D, Rodríguez-Valera F. 2001b. Diversity of
16 free-living prokaryotes from a deep-sea site at the Antarctic Polar Front. *FEMS Microb.*
17 *Ecol.* 1251: 1-10.
- 18 López-García P, Moreira D, López-López A, Rodríguez-Valera F. 2001a. A novel
19 haloarchaeal-related lineage is widely distributed in deep oceanic regions. *Environ.*
20 *Microbiol.* 3: 72-78.
- 21 Lovejoy C, Massana R, Pedrós-Alió C. 2006. Diversity and Distribution of Marine
22 Microbial Eukaryotes in the Arctic Ocean and Adjacent Seas. *Appl. Environ.*
23 *Microbiol.* 72: 3085-3095.

- 1 Massana R, DeLong EF, Pedros-Alio C. 2000. A few cosmopolitan phylotypes dominate
2 planktonic archaeal assemblages in widely different oceanic provinces. *Appl. Environ.*
3 *Microbiol.* 66: 1777–1787.
- 4 Massana R, Murray A, Preston CM, DeLong EF. 1997. Vertical distribution and
5 phylogenetic characterization of marine planktonic Archaea in the Santa Barbara
6 channel. *Appl. Environ. Microbiol.* 63: 50–56.
- 7 Massana R, Taylor LT, Murray AE, Wu KY, Jeffrey WH, DeLong EF. 1998. Vertical
8 distribution and temporal variation of marine planktonic Archaea in the Gerlache
9 Strait, Antarctica, during early spring. *Limnol. Oceanogr.* 43: 607–617.
- 10 Massana R, Guillou L, Díez B, Pedrós-Alió C. 2002. Unveiling the Organisms behind
11 Novel Eukaryotic Ribosomal DNA Sequences from the Ocean. *Appl. Environ.*
12 *Microbiol.* 68: 4554-4558.
- 13 Medlin L, Elwood HJ, Stickel S, Sogin ML. 1988. The characterization of enzymatically
14 amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71: 491–499.
- 15 Murray AE, Wu KY, Moyer CL, Karl DM, DeLong EF. 1999. Evidence for circumpolar
16 distribution of planktonic Archaea in the Southern Ocean. *Aquat. Microbiol. Ecol.* 18:
17 263–273.
- 18 Not F, del Campo J, Balague V, de Vargas C, Massana R. 2009. New Insights into the
19 Diversity of Marine Picoeukaryotes. *PLoS ONE* (www.plosone.org) 4: e7143.
- 20 Ovreas L, Forney L, Daae FL, Torsvik V. 1997. Distribution of bacterioplankton in
21 meromictic Lake Saelenvannet, as determined by denaturing gradient gel
22 electrophoresis of PCR amplified gene fragments coding for 16S rRNA. *Appl.*
23 *Environ. Microbiol.* 63: 3367–3373.

- 1 Preston CM, Wu KY, Molinski TF, DeLong EF. 1996. A psychrophilic crenarchaeon
2 inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. Proc. Natl.
3 Acad. Sci. USA 93: 6241-6246.
- 4 Reysenbach A-L, Pace NR. 1995. In: Robb FT, Place AR (eds.) Archaea: A Laboratory 6
5 Manual – Thermophiles. Cold Spring Harbour Laboratory Press, New York, pp. 101-
6 107.
- 7 Robb FT, Sowers KR, DasSharma S, Place AR, Schreier. HJ. 1995. Thermophiles, In:
8 Fleischmann EM (ed.), Archaea: a laboratory manual. Cold Spring Harbor Press, Cold
9 Spring Harbor, N.Y. pp. 202-203.
- 10 Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A. 1979. Isolation of
11 extreme halophiles from seawater. Appl. Environ. Microbiol. 38: 164-165.
- 12 Romari K, Vaultot D. 2004. Composition and temporal variability of picoeukaryote
13 communities at a coastal site of the English Channel from 18S rDNA sequences.
14 Limnol. Oceanogr. 49: 784-798.
- 15 Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn, Cold
16 Spring Harbor Laboratory Press, NY.
- 17 Schleper C, Jurgens G, Jonuscheit M. 2005. Genomic studies of uncultivated archaea.
18 Nat. Rev. Microbiol. 3: 479-488.
- 19 Silva RI, Negri RM, Lutz VA. 2009. Summer succession of ultraphytoplankton at the
20 EPEA coastal station (Northern Argentina). J. Plankton Res. 31: 447–458.
- 21 Stahl DA, Amann R. 1991. Development and application of nucleic acid probes in
22 bacterial systematics. In: Stackebrandt E, Goodfellow M (Eds.), Nucleic acid
23 techniques in bacterial systematics, John Wiley and Sons Ltd., Chichester, UK
- 24 Stein JL, Simon MI. 1996. Archaeal ubiquity. Proc Natl Acad Sci U S A. 93: 6228–6230.

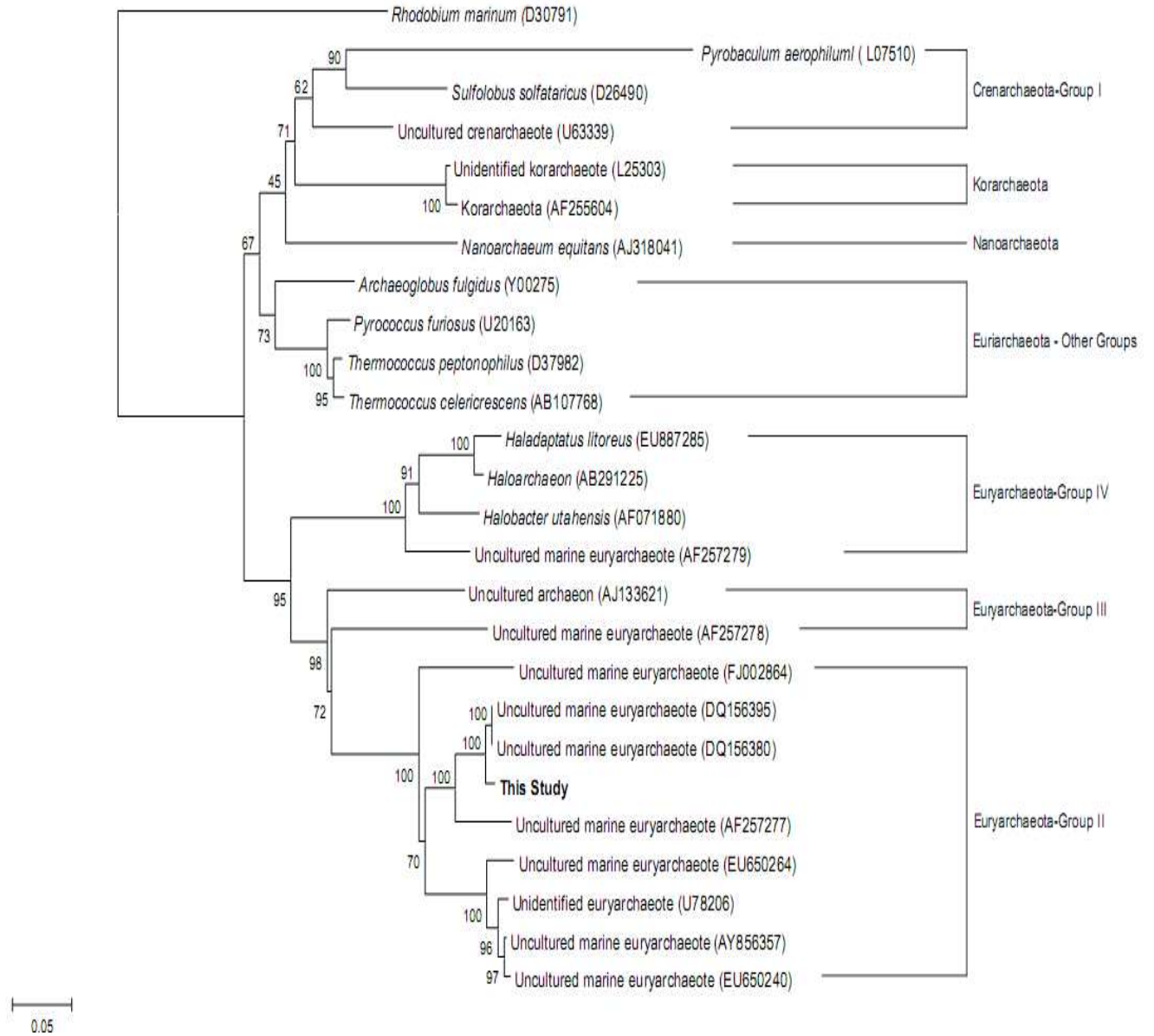
- 1 Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics
- 2 Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599.
- 3 Vetriani C, Jannasch HW, McGregor BJ, Stahl DA, Reysenbach A-L. 1999. Population
- 4 structure and phylogenetic characterisation of marine benthic Archaea in deep-sea
- 5 sediments. *Appl. Environ. Microbiol.* 65: 4375-4384.



11 **Figure 1.** Agarose gel electrophoresis of DNA fragments amplified by PCR: **a)** from
 12 genomic DNA from representative surface samples collected at the Argentine Sea during
 13 spring (lane 1, in September; lane 2, in October; and lane 3, in November) using the
 14 primer pair EukA-EukB; **b)** from colonies of transformed *Escherichia coli* (colony-PCR)
 15 harbouring amplified DNA fragments mentioned in **a)**. Amplification products from
 16 samples collected in September (lanes 1-3), in October (lanes 4-6) and in November
 17 (lanes 7-9). MM, 500-bp DNA ladder (MeBep Bioscience). Arrows indicate the two
 18 amplicons obtained. The 1,460-bp fragment corresponds to archaeal rDNA sequences.
 19 DNA fragments were visualized with ethidium bromide.

20
 21
 22
 23

1 **Figure 2.** RFLP patterns obtained from 1,460-bp fragments. The PCR products were
2 incubated with *Hae*III and the digestion products were separated by electrophoresis in
3 agarose gels (2.5%). DNA fragments were visualized after ethidium bromide staining.



3

4 **Figure 2.** Phylogenetic 16S rDNA-based tree for partial sequences showing the
 5 phylogenetic position of novel archaeal 16S rRNA sequence identified from surface water
 6 samples of the Argentine Sea. All genomic information was downloaded from the
 7 Microbial Genomes resource of the National Center for Biotechnology Information
 8 (NCBI). Crenarchaeota-Group I: *Pyrobaculum aerophilum*l (L07510), *Sulfolobus*
 9 *solfatarius* (D26490), Uncultured crenarchaeote (U63339); Korarchaeota: Unidentified
 10 korarchaeote (L25303), Korarchaeota (AF255604); Nanoarchaeota: *Nanoarchaeum*

1 *equitans* (AJ318041); Euriarchaeota; Other Groups: *Archaeoglobus fulgidus* (Y00275),
2 *Pyrococcus furiosus* (U20163), *Thermococcus peptonophilus* (D37982) *Thermococcus*
3 *celericroscens* (AB107768); Euryarchaeota-Group IV: *Haladaptatus litoreus*
4 (EU887285), Haloarchaeon (AB291225), *Halobacter utahensis* (AF071880), Uncultured
5 marine euryarchaeote (AF257279); Euryarchaeota-Group III: Uncultured archaeon
6 (AJ133621), Uncultured marine euryarchaeote (AF257278); Euryarchaeota-Group II:
7 Uncultured marine euryarchaeote (FJ002864) Uncultured marine euryarchaeote
8 (DQ156395); Uncultured marine euryarchaeote (DQ156380), Uncultured marine
9 euryarchaeote (AF257277), Uncultured marine euryarchaeote (EU650264), Unidentified
10 euryarchaeote (U78206), Uncultured marine euryarchaeote (AY856357), Uncultured
11 marine euryarchaeote (EU650240). Marine alphaproteobacteria *Rhodobium marinum*
12 (D30791) was used as an out group.

Table 1. Comparison of coverage of Archaea retrieved sequence-with commonly used non-degenerate Archaea primers included those for Eukarya (EukA and EukB) used at this study. Positions where each primer matches within the Archaea sequence HQ541865 are indicated.

Primer name	Sequence (5'-3')	Commentary	Position in HQ541865 sequence of this study	Reference
Forward				
EukA	AACCTGGTTGATCCTGCCAGT		1	Medlin <i>et al.</i> (1988)
21F	TTCCGGTTGATCCYGCCGGA		None	DeLong (1992)
958R	YCCGGCGTTGAMTCCAATT		None	DeLong (1992)
1100A	TGGGTCTCGCTCGTTG		None	Embley <i>et al.</i> (1992)
Ab787F	ATTAGATACCCGGGTA		715	DasSarma and Fleischmann (1995)
PARCH 340f	CCCTACGGGG(C/T)GCA(G/C)CAG	T and G to match	307	Ovreas <i>et al.</i> 1997
EK4F	CTGGTTGATCCTGCCAG		3	Robb <i>et al.</i> (1995).
A344F	ACGGGGTGCAGCAGGCGCGA		311	Casamayor <i>et al.</i> (2002)
958arcF	AATTGGANTCAACGCCGG	N=T	893	Huber <i>et al.</i> (2007)
Reverse				
EukB	TGATCCTTCTGCAGGTTACCTAC		1436	Medlin <i>et al.</i> (1988)
ARCH 915R	GTGCTCCCCGCCAATTCCT		849	Stahl and Amann (1991)
PARCH519r	TTACCGCGGC(G/T)GCTG	G to match	448	Ovreas <i>et al.</i> 1997
PREA1100r	(T/C)GGGTCTCGCTCGTT(G/A)CC		None	In Ovreas <i>et al.</i> 1997
1048arcRmajor	CGRCGGCCATGCACCWC	R and W=A	976	Huber <i>et al.</i> (2007)

