Archaeal diversity in deep-sea sediments estimated by means of different Terminal-Restriction Fragment Length Polymorphisms (T-RFLP) protocols

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Submitted for consideration to *Current Microbiology*

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Running title: Fingerprinting of deep-sea benthic Archaea

Abstract. Despite the increasing recognition of the quantitative importance of Archaea in all marine systems, the protocols for a rapid estimate of Archaeal diversity patterns in deep-sea sediments have been only poorly tested yet. We collected sediment samples from 11 deep-sea sites covering a wide latitudinal range (from 79°N to 36°N, at depths comprised from 469 to 5500 m) and compared the performance of two different primer sets (ARCH21f/ARCH958r and ARCH109f/ARCH 915r) and three restriction enzymes (AluI, Rsa I and HaeIII) for the fingerprinting analysis (T-RFLP) of Archaeal diversity. *In silico* and experimental analyses consistently indicated that different combinations of primer sets and restriction enzymes can result in different values of benthic Archaeal ribotype richness and different Archaeal assemblage compositions. The use of the ARCH109f/ARCH 915r primer set in combination with AluI provided the best results (a number ribotypes up to 4-folds higher than other combinations), suggesting that this primer set should be used in future studies dealing with the analysis of the patterns of Archaeal diversity in deep-sea sediments. Multivariate, multiple regression analysis revealed that, whatever the T-RFLP protocol utilized, latitude and temperature explained most of the variance in benthic Archaeal ribotype richness, while water depth had a negligible role.

Keywords: Archaea, biodiversity, T-RFLP, deep-sea sediments

Introduction

Archaea are important members within marine ecosystems [3]. While 16S rDNA-based surveys have initially provided evidence for the widespread occurrence of Archaea only in oxygenated coastal waters, more recent studies have indicated that pelagic Archaea are abundant members of meso- and bathypelagic picoplankton [9]. Other studies have revealed that Archaea can also account for a significant fraction of prokaryotic assemblages in both coastal [13] and deep-sea sediments [27]. Molecular studies have consistently revealed how marine Archaeal populations are diverse, complex and widespread, suggesting the need of improving our knowledge on this so-far little studied component of marine biota. In fact, Archaeal diversity and spatial patterns in deep-sea sediments, as well as the environmental factors controlling their distribution, are still very poorly known.

Fingerprinting techniques such as DGGE (Denaturing Gradient Gel Electrophoresis), T-RFLP (Terminal-Restriction Fragment Length Polymorphisms) and ARISA (Automated Ribosomal Intergenic Spacer Analysis) are routinely used in terrestrial and aquatic samples to estimate the number of prokaryotic ribotypes and to investigate spatio-temporal dynamics of prokaryotic assemblages [12, 26]. ARISA is commonly utilized for the study of bacterial assemblages, but cannot be used for Archaea, due to possible lack of a true intergenic spacer region (ITS) in some marine Archaea [10, 15]. The T-RFLP method applied to the 16S rRNA gene has been used for the study of Archaeal diversity in deep-sea sediments [11, 24], but recent studies have stressed the need of improving such molecular methods [3, 8] that, so far have been not yet fully compared and evaluated [26]. A key issue in T-RFLP analyses of benthic Archaeal diversity is the identification of the best primer sets and restriction enzymes [18]. In this regard, the ARCH21f - ARCH958r primer set has been utilized in marine waters and sediments [13, 20, 28], whilst the set ARCH109f - ARCH915r has been mostly utilized in freshwater and terrestrial studies [4, 5, 24]. In fingerprinting analyses, it is also crucial the choice of the most appropriate restriction enzymes [18]. Again, this issue has been so far addressed for Bacteria [7], but not yet investigated for T-RFLP-based studies of benthic Archaeal diversity [4, 24, 26].

We investigated the performance of two primer sets and three restriction enzymes in measuring the deep-benthic Archaeal diversity, in order to evaluate: i) whether different combinations of primer sets and restriction enzymes provide different values of benthic Archaeal diversity, ii) whether

different combinations result in different Archaeal community compositions and iii) whether different combinations lead to different diversity patterns and ecological interpretations on the factors controlling benthic Archaeal diversity in the deep-sea.

Materials and Methods

Sample collection

We collected sediment samples from 11 stations at depths ranging from 450 to 5500 m (from 79°N to 36°N) covering a wide spectrum of deep-sea ecosystems, including open slopes, a trench and a submarine canyon (Table 1). Sediment sampling was carried out using a multiple-corer and/or a NIOZ-type box corer. Once onboard, sub-samples were collected with sterile corers (Ø 3 cm) and the surface sediment (0-1 cm) from each core transferred to sterile test tubes and stored at -20°C until analyses. Replicate sub-aliquots (n=3) of the surface sediment samples were analyzed to determine the concentrations of biopolymeric C, an estimate of the organic C sources available on the sea bottom [7].

Archaeal T-RFLP

We extracted DNA from sediments using the UltraClean Soil DNA Isolation kit (MoBio Laboratoires; [16]). Following spectrophotometric estimates of concentrations, extracted DNA was diluted (5 ng μl^{-1}) and stored at -80°C until analysis. 5 ng of DNA were amplified in 50-µl reactions using the MasterTaq® kit (Eppendorf). We used the following primer sets: ARCH21f (5'-TTCCGGTTGATCCTGCCGGA-3') and ARCH958r (5'- YCCGGCGTTGAMTCCAATT-3') hereafter indicated as set "A21/958", and ARCH109f (5`-ACKGCTCAGTAACACGT-3') and ARCH915r (5`-GTGCTCCCCCGCCAATTCCT-3') hereafter indicated as "A109/915". The reverse primers ARCH958r and ARCH915r had been labeled at the 5' end with the fluorescent dye HEX. Reaction conditions were those indicated by Lanoil et al. [14] for A21/958 and by Chan et al. [4] for A109/915. Negative controls, containing the PCR mixture but no DNA template, were run during each amplification. Positive controls contained 5 ng of genomic DNA of *Methanococcus jannashii* (ATCC 43067D). For each sample, two different PCR were run and subsequently pooled together to minimize

stochastic PCR biases [see 16]. Resulting combined PCR products were purified (Wizard PCR clean-up system, Promega), resuspended in 50 µl of milliQ water and quantified spectrophotometrically. 300-600 ng of purified amplicons were digested in duplicate (37°C, 3 h) in 20-µl reactions containing 10 U of AluI or RsaI or HaeIII. These enzymes were selected after a virtual "*in silico*" T-RFLP using the option "ERPA" on the Mica software (http://mica.ibest.uidaho.edu/enzyme.php). The analysis was carried out by allowing at most 0 mismatches within 0 bases from 5' end of primer. DA RIFARE (att al reverse) This rapid tool, that provides theoretical estimates on optimal primers and restriction enzymes combinations to be used for subsequent T-RFLP determinations, indicated that AluI, RsaI and HaeIII were the three enzymes producing the highest number of possible restriction fragments. Restrictions were stopped by incubating at 65°C for 20 min. Fragment analyses and the interpretation of the T-RFLP electropherograms were carried out as described elsewhere [7, 16]. The amount of DNA analyzed was carefully checked and standardized before capillary electrophoresis (i.e. the same amount of digested products was consistently analyzed for both primer sets).

Data analysis

We calculated Archaeal richness by summing the number of Archaeal ribotypes or Operational Taxonomic Units (OTU) [7]. We explored the presence of significant relationships between richness estimates obtained using the two primer sets using the Spearman-rank correlation. We assessed significant differences in ribotypes number obtained using different primer sets and restriction enzymes by using a 2-way analysis of variance (ANOVA). When significant differences were encountered, we carried out a Student-Newman-Keuls (SNK) post-hoc comparison test (at $p = 0.05$), to ascertain which combination of primers and restriction enzymes gave the highest OTU richness estimate. All ANOVA and SNK tests were conducted using the GMAV 5.0 software (University of Sidney, Australia).

The community composition derived from T-RFLP is best described by means of multivariate statistics [22]. To test the hypothesis that statistical differences exist in Archaeal community composition between sediment samples from different regions, we used the ANOSIM (Analysis of Similarity) tool. The presence of statistical difference between samples is indicated by a significance

level at least $p<0.05$. Archaeal community composition data were then ordinated by means of a multidimensional scaling analysis (MDS), based on a Bray-Curtis similarity matrix. The Bray-Curtis similarity coefficient allows to assess the degree of similarity between T-RFLP profiles obtained from different samples, thus allowing the comparison of Archaeal assemblage structure between samples. For each primer set, a similarity matrix, containing all possible pairwise comparisons, was then generated and used to produce an MDS plot, which results in a visual representation of the Bray Curtis similarity between samples. Both ANOSIM and MDS analyses were performed using the PRIMER v5 software (Plymouth Marine Laboratory).

To assess the extent by which investigated environmental variables (water depth, latitude, longitude, bottom temperature and biopolymeric carbon concentration) explained differences in Archaeal richness, we carried out a non-parametric multivariate multiple regression analysis using the routine "DISTLM forward" [17]. DISTLM forward is a recently-developed computer program, which allows, by performing a multivariate multiple regression on the basis of any distance measure, to identify which environmental variable(s) are responsible for the observed variation in a given biological dataset (in this case, Archaeal OTU richness).

Results and Discussion

The total number of Archaeal ribotypes, obtained by summing up all different ribotypes encountered in sediments samples collected from the 11 stations, ranged from 8 (using the A21/958 primer set in combination with HaeIII) to 81 (using A109/915 and AluI; Table 2). On average, the use of A109/915 primer set resulted in a number of Archaeal ribotypes approximately 4 times higher than the one based on A21/958. The analysis of variance revealed that, independent of the enzyme used, the A109-915 primer set always resulted in the highest number of OTUs. For both primer sets, AluI was the enzyme resulting in the highest number of OTUs as compared to all of the other tested enzymes (Table 3). These results are in agreement with what was predicted by the *in silico* analysis, which indicated that A21/958 set could provide 30 fragments when utilized in combination with AluI, 26 in combination with Rsa and 20 with HaeIII. Conversely, the same *in silico* analysis indicated, for the set 109F/915R,

82 fragments with AluI, 36 with Rsa and 43 with HaeIII. These results provide, for the first time, evidence of the reliability of *in silico* analyses for 16S rDNA Archaeal diversity studies in deep-sea sediments.

It has been recently suggested that, when using the FISH technique, the probe ARCH915 can unspecifically target some bacterial taxa [19]. Thus, the higher ribotype richness observed with A109/915 set could be due to unspecific hybridization of the reverse primer to non-target bacterial 16S rRNA sequences. Conversely, recent studies indicated that all Archaeal primers utilized (including ARCH915) are highly selective for Archaea and do not target any bacterial sequence [1]. Using the RDP tools (http://rdp.cme.msu.edu/), we repeated the *in silico* analysis by Baker et al. [1] on a larger and updated 16S rDNA sequences database. Our analysis indicated that the primers 21F and 958R do not target any of the 418,650 available bacterial 16S rDNA sequences, whereas the primers 109F and 915R target only 8 of them. These results, together with a recent study based on clone libraries [8], suggest that differences in the OTUs number observed with the two primer sets cannot be attributed to a lack of specificity of A109/915.

When the two primer sets were applied to deep sub-surface sediments, the reverse primer A915 matched more sub-surface Archaeal 16S rDNA sequences than the A958 one (i.e. 66 vs 36% of the total) [25]. Since Crenarchaeota are expected to dominate Archaeal assemblages in deep-sea environments [9], the higher performance of the primer set A109/915 compared to A21/958 could be due to better targeting of Crenarchaeota in deep-sea sediments by the former set. This is also supported by the analysis of Baker et al. [1], who reported that the primers A109 and A915 displayed more matches, when compared with the primers A21 and A958, with Thermophilic and Non-Thermophilic Crenarchaeota.

The values of Archaeal richness obtained with the two primer sets in combination with AluI were significantly and positively correlated ($r=0.731$; $P<0.05$ and $r=0.848$; $P<0.01$, respectively), suggesting that different primer sets provide conservative patterns of benthic Archaeal diversity when applied to large sets of samples. Although the use of fingerprinting techniques for obtaining richness estimates can be biased by several factors [2], they are robust enough to allow the comparison of different ecosystems [12].

We also evaluated whether the different primer sets resulted in different Archaeal community composition. The analysis of similarity (ANOSIM) test, conducted to test the presence of statistical difference in assemblage composition, indicated that only the A109/915 primer set revealed significant differences among the different deep-sea sites (ANOSIM, $R = 0.787$, $p<0.01$). Conversely, the use of primer set A21/958 did not result in any statistical difference among the investigated sites (ANOSIM, R = 0,137, n.s.). The analysis of the structure of the Archaeal assemblages, carried out on the results obtained with AluI and based on a MDS ordination, indicated that the two primer sets provided different assemblage structures (Fig. 1), with the A109/915 displaying a higher discriminating power.

To identify the potential drivers of Archaeal diversity in deep-sea sediments, we carried out multivariate multiple regression analyses using the software "DISTLM forward". This analysis revealed that, when using both primer sets, latitude and temperature, together, explained the highest proportion of Archaeal OTU richness variance (ca 55%), whereas longitude, biopolymeric carbon concentrations and water depth explained altogether only 8-10% (Table 4). These preliminary results, despite being based on a relatively limited set of samples and not including other important environmental benthic variables (e.g. pH, redox potential and grain size), suggest that benthic Archaeal biodiversity patterns are potentially influenced by both latitude and deep-sea temperatures.

All fingerprinting techniques have important limitations [2], but can be useful to identify patterns of distribution in space and time or for selecting the samples on which perform further and more sophisticated molecular analyses. The results of our study indicate that the use of the A109/915 primer set in combination with AluI provided the best results and could be used as a starting point for future investigation dealing with the patterns and drivers of Archaeal diversity in deep-sea sediments. A throughout comparison with clone library and/or with pyrosequencing will allow to fully assess the reliability of the T-RFLP approach.

Acknowledgements

This work has been carried out within the frame of the HERMES and HERMIONE research projects, both financially supported by EU.

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Fig. 1. Multi Dimensional Scaling (MDS) ordination of Archaeal community composition obtained using A109/915 (a) and A21/958 (b) primer sets in combination with AluI. On MDS plots, samples sharing the highest similarity in Archaea community composition are grouped together, following results of the Cluster Analysis test.

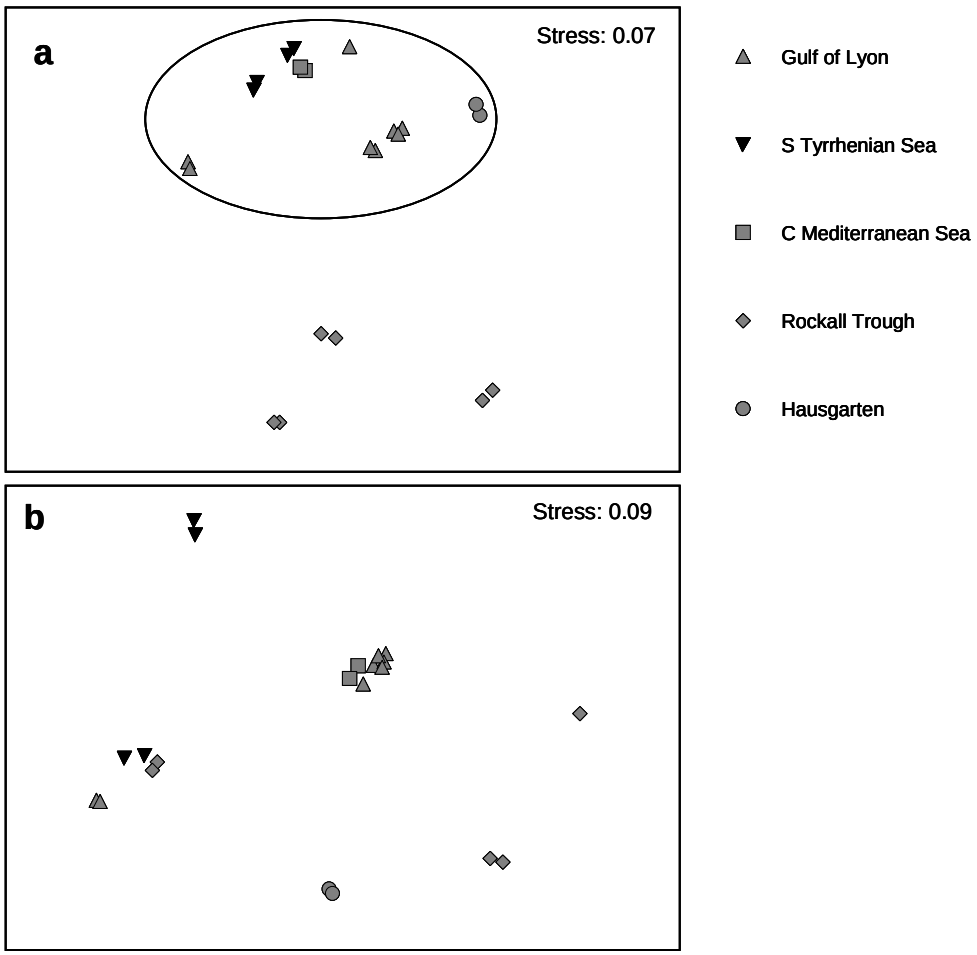

Table 1. Name, geographic area, position (as latitude and longitude), depth, temperature and biopolymeric carbon concentrations (+ standard deviation of three replicate analyses) at all investigated benthic sites.

Station	Sampling Area	Latitude (N)	Longitude (E)	Depth (m)	Temperature (°C)	Biopolymeric carbon (mgC g^{-1})
SV 242	Hausgarten (Nordic Margin)	79° 08' 06''	2° 50' 32''	5571	0.1	5.94 + 0.83
Rock 2	Rockall Trough (Atlantic Ocean)	55° 39' 00''	15° 56' 00'' (W)	469	10.0	0.59 + 0.08
Rock 3	''	55° 22' 24''	15° 39' 11'' (W)	1488	6.0	0.32 + 0.02
Rock 10	''	55° 04' 55''	15° 46' 20'' (W)	2459	2.0	2.35 + 0.29
M500T	Gulf of Lyon (Med. Sea)	42° 22' 24''	3° 21' 45''	498	12.9	2.96 + 0.25
TM 750	''	42° 20' 41''	3° 27' 52''	736	12.8	0.25 + 0.03
CC1835	''	42° 10' 8''	4° 04' 58''	1836	12.8	1.11 + 0.31
M571	''	42° 21' 51''	4° 23' 23''	506	12.9	1.32 + 0.09
CIESM 7	South Tyrrhenian Sea (Med. Sea)	39° 52' 52''	12° 46' 07''	3587	12.8	0.41 + 0.15
CIESM 4	''	39° 32' 52''	13° 22' 14''	3507	12.9	1.92 + 0.24
Malta 26	Central Mediterranean Sea	36° 49' 06''	15° 21' 75''	2325	13.0	2.77 + 0.43

Table 2. The number of Archaeal ribotypes in the investigated samples obtained using the different combinations of primers sets and restriction enzymes. The number of total OTUs (Operational Taxonomic Units) refers to the number of different OTUs observed from all investigated stations.

	Archaeal Richness					
	AluI		***RsaI***		***HaeIII***	
Station	**A21/958**	**A109/915**	**A21/958**	**A109/915**	**A21/958**	**A109/915**
SV 242	2	8	1	2	1	1
Rock 2	2	11	5	11	2	10
Rock 3	10	43	6	27	1	19
Rock 10	13	29	4	8	0	13
M500T	3	18	2	5	0	3
TM 750	12	28	10	7	1	6
CC1835	11	25	6	3	1	1
M571	6	21	3	7	0	6
CIESM 7	7	18	4	10	1	11
CIESM 4	9	35	4	15	1	17
Malta 26	11	22	13	4	6	2
Total OTUs	21	81	12	41	8	32
Ratio A109/915 vs. A21/958	3.9		3.4		4.0	

Table 3. The results of the two-way analysis of variance testing differences in Archaeal OTUs richness using the different combinations of primer sets and restriction enzymes. Reported are also the results of the SNK test indicating the combination of primer set and restriction enzyme resulting in the highest OTUs richness. df = degree of freedom, MS = mean square; *** = P<0.001, ns = not significant.

	OTUs richness				
Source	**Df**	**MS**	**F**	**P**	**SNK test**
Primer (PR)	1	85,01	117,23	***	**AluI**: A109/915 > A21/958 **Rsa**: A109/915 > A21/958 **HaeIII**: A109/915 > A21/958
Restriction Enzyme (RE)	2	50,85	70,13	***	**A109/915**: AluI > Rsa > HaeIII **A21/958**: AluI > [Rsa, HaeIII]
PR x RE	2	7,80	10,76	***	
Residuals	192	0,73			
Total	197				

Table 4. Effects of environmental variables on Archaeal OTUs richness. Reported are the results of the forward selection procedure with the conditional tests, (i.e. fitting each variable one at a time, conditional on the variables that are already included in the model). % Var is the percentage of variance explained by each variable. The Cumulative percentage of variance is also reported.

		OTU's richness			
Primer Set	**Variable**	**F**	**P**	**% Var**	**Cumulative**
A21/958	**Latitude**	11.40	0.002	0.27	0.27
	Bottom temperature	19.13	0.001	0.28	0.55
	Biopolymeric C	1.40	0.250	0.02	0.57
	Water depth	1.99	0.137	0.03	0.60
	Longitude	2.37	0.096	0.03	0.63
A109/915	**Latitude**	14.50	0.001	0.32	0.32
	Bottom temperature	17.91	0.001	0.25	0.57
	Biopolymeric C	5.68	0.234	0.07	0.64
	Water depth	0.43	0.543	0.01	0.65
	Longitude	1.83	0.182	0.02	0.67