Universidade de Aveiro Departamento de Biologia
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Estudo do Efeito "rizosfera" nas Comunidades de *Archaea* em Plantas de Mangal

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Mangrove Rhizosphere Effect on Sediment Archaeal Communities

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Newton Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro e co-orientação do Doutor Daniel Cleary, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

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o júri

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palavras-chave *Archaea*, comunidades de *Archaea*, genes 16S rRNA, mangal, *Nanoarchaeum*

resumo

Os mangais são económica e biologicamente importantes. Sendo, contudo, cada vez mais ameaçados. Com o intuito de recuperar estes ecossistemas, têm sido desenvolvidos programas de reabilitação. Todavia, geralmente, estes programas não consideram a importância e os possíveis efeitos das interacções entre microrganismos e plantas no ecossistema dos mangais, devido ao número limitado de estudos em ecologia microbiana neste ecossistema. Sabe-se que as raízes de espécies de plantas terrestres influenciam a composição das comunidades bacterianas do solo. Por sua vez, os microrganismos podem contribuir no crescimento e saúde das plantas. Este estudo teve como objectivos desenvolver um sistema primers para reacção em cadeia da polimerase -electroforese em gel com gradiente desnaturante (PCR-DGGE) para o domínio *Archaea* e o género *Nanoarchaeum* e determinar se as raízes de plantas de mangal (*Rhizophora mangle* e *Laguncularia racemosa*) afectam a composição das comunidades de *Archaea* e *Nanoarchaeum* que habitam o solo que está sob a influência das raízes de mangal (efeito "rizosfera").

As comunidades de *Archaea* e *Nanoarchaeum* foram analisadas por métodos moleculares, como a PCR e o DGGE. Foi desenvolvido um sistema de primers para PCR-DGGE adequado para o domínio *Archaea* e para o género *Nanoarchaeum* com base em novas sequências do gene 16S rRNA recentemente publicadas. Os perfis de DGGE foram analisados com a análise de similaridades (ANOSIM), o método de escalonamento multidimensional não paramétrico (MDS) e o índice de Shannon-Wiener. Os resultados de MDS e ANOSIM sugerem que existem diferenças significativas entre as amostras de sedimento e as amostras de rizosfera de *R. mangle* e *L. racemosa*. Por sua vez, a análise de MDS sugere que as raízes de *L. racemosa* afectam mais a composição da comunidade de *Archaea* do sedimento do que as raízes de *R. mangle*. Pelo contrário, os resultados de MDS e ANOSIM sugerem que as plantas de *L. racemosa* e *R. mangle* não exercem qualquer efeito na composição de *Nanoarchaeum* e que não existem diferenças entre as amostras de sedimento e as de rizosfera. A diversidade das populações de *Archaea* e *Nanoarchaeum* foi estimada pelo índice de Shannon-Wiener; e mostrou que a diversidade de *Archaea* era mais elevada do que anteriormente descrito em sedimentos marinhos.

Com o propósito de completar este estudo algumas bandas dominantes das amostras de rizosfera vão ser clonados e sequenciados, vão ser criadas bibliotecas de clones para *Nanoarchaeum* e serão efectuadas análises de pirosequenciação às comunidades de *Archaea*.

keywords *Archaea*, archaeal communities, 16S rRNA genes, mangrove, *Nanoarchaeum*

abstract

Mangrove forests are economically and biologically important; however, they are also increasingly threatened. In order to recuperate these ecosystems, rehabilitation programs have been developed. However, in general these programs have no knowledge about the importance of plant-microbe interactions in mangrove ecosystem. This happens also because the limited numbers of studies on microbial ecology in this ecosystem. Therefore, they do not consider the possible effects of plant-microbe interactions in mangrove reforestation approaches. It is known that roots of terrestrial plant species influence the composition of soil bacterial communities. In turn, microorganisms can contribute to plant growth and health. In this study we aimed to develop a polymerase chain reaction -denaturing gradient gel electrophoresis (PCR-DGGE) primer system suitable for *Archaea* domain and *Nanoarchaeum* genus and to determine if roots of mangrove plants (*Rhizophora mangle* and *Laguncularia racemosa*) affect the composition of *Archaea* and *Nanoarchaeum* communities inhabiting the sediment under influence of mangrove roots (rhizosphere effect).

Archaea and *Nanoarchaeum* communities were analyzed using molecular methods, such as PCR and DGGE. A PCR-DGGE primer system suitable for *Archaea* domain and *Nanoarchaeum* genus was developed based on new 16S rRNA gene sequences recently published. DGGE profiles were analyzed with analysis of similarities (ANOSIM), non-metric multidimensional scaling (MDS) and Shannon-Wiener index. Both MDS and ANOSIM results suggest that there are significant differences between bulk and rhizosphere samples of *R. mangle* and *L. racemosa*. In turn, MDS analyses suggest that roots of *L. racemosa* affect more the composition of *Archaea* community from bulk sediment than roots of *R. mangle*. On the opposite, ANOSIM statistics and MDS analyses suggest that *L. racemosa* or *R. mangle* plants do not influence the nanoarchaeal composition and that there are no differences between bulk and rhizosphere samples. Diversity of *Archaea* and *Nanoarchaeum* populations was estimated by using the Shannon-Wiener index; and showed that diversity of *Archaea* was higher than previously reported in marine sediments.

With the purpose to complete this study some dominant bands of rhizosphere samples will be cloned and sequenced, clone libraries for *Nanoarchaeum* will be generated and pyrosequencing analysis of archaeal communities will be performed.

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1. INTRODUCTION

1.1. MANGROVE FORESTS

Mangroves are coastal forests with a tropical and subtropical distribution (Fig. 1). These forests represent an important ecotone between terrestrial and marine environments. Mangroves are inundated on daily with seawater and can also receive important freshwater inputs, sediments, and nutrients from rivers (Lacerda 2002; Lewis 2004; FAO 2007).

As of 2007, 124 countries have been identified with one or more mangroves species, mainly growing in soft sediments (FAO 2007). According to Lacerda (2002 and references therein), the largest contiguous mangrove system in the world covers the coast of the northern Brazilian states of Pará and Maranhão and covers approx. 700000 ha.

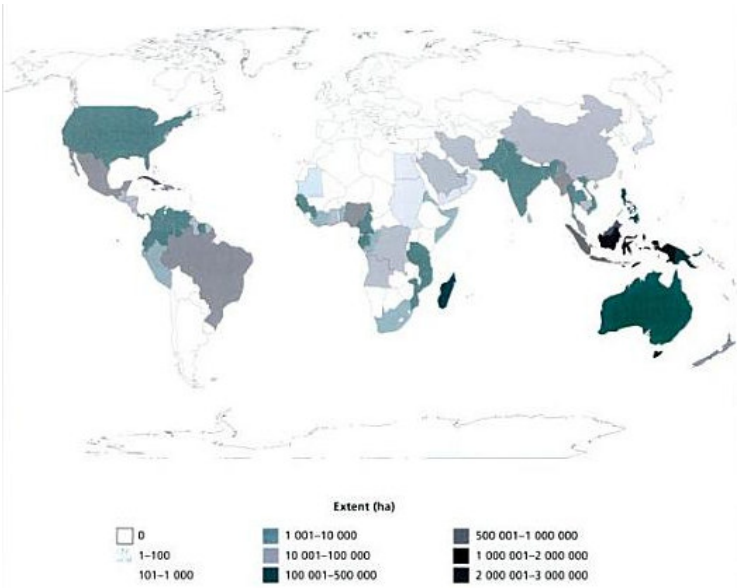

Figure 1 – Mangrove area worldwide, in 2005. Extracted from FAO (2007).

Mangroves are economically and biologically important, since they provide protection against erosion of coastal areas by stabilizing sediments, decreasing the impact of waves and tsunamis, provide nursing grounds and habitat for fish and shellfish, and a niche for several commercially-important aquatic animals during some part of their life cycle, including birds (Valiela et al. 2001; Lacerda 2002; Lewis 2004; Kathiresan and Rajendran

2005; FAO 2007; Aburto-Oropeza et al. 2008). Mangrove forests are also used in the production of chemicals, medical and wood products (Lewis 2004).

However, mangroves are increasingly being threatened and as a result of global changes are quickly disappearing (see Table 1) (Valiela et al. 2001; Duke et al 2007).

Table 1 – Rates of mangrove forests losses worldwide. Adapted from Valiela et al. (2001).

Region	Present mangrove area (km^2)	Area of mangroves for countries with available multiyear data		Percentage of total present mangrove area represented mangrove in loss estimates	Percentage loss of mangrove forest area	Annual rate of loss (km^2 y^{-1})	Percentage of original area lost per year
		Present area (km^2)	Original area (km^2)				
Asia	77.169	26.193	41.208	34	36	628	1.52
Africa	36.259	14.903	21.847	41	32	274	1.25
Australia	10.287	10.000	11.617	97	14	231	1.99
Americas	43.161	38.472	62.242	89	38	2251	3.62
World total	166.876	89.568	136.914	54	35	2834	2.07

1.2. MANGROVE MANAGEMENT AND REHABILITATION

Despite the use of mangroves as a source of wood, the main causes of habitat loss are pollution and competition for land. Mangroves have been lost due to hydrological modifications including the construction of roads or dams, creation of aquaculture ponds, dredging or even for urban development, tourism or agriculture (Lewis 2004; FAO 2007). Thus, directly and indirectly, human activity represents a major threat to mangroves.

According to Field (1998) and Lewis (2004) the best way to avoid, or at least partially mitigate, problems like these is by creating protected areas in undisturbed sites, like National parks. It has, however, not always has been possible to create these parks before prior to mangrove destruction. To supplement protective measures, rehabilitation programs have been established (Toledo et al. 2001; Lacerda 2002; Hogarth 2007; Kirui et al. 2008).

Before considering a rehabilitation program, it is important to understand the reasons that natural mangrove regeneration was hampered; under normal conditions mangroves have a self-renewal period of 15-30 years when the normal hydrology has not been disturbed and the availability of seeds is not limited or blocked (Lewis 2004). A successful case of natural regeneration, and unfortunately an exception, is the case of *Avicennia* patches, in New Zealand and Australia, that have regrown spontaneously (Hogarth 2007).

However, ecologists and ecological engineers often fail to understand mangrove hydrology and initiate rehabilitation programs without first determining why natural recovery has been hampered (Lewis 2004). Once it has been established that natural regeneration will not be successful, it is necessary to institute programs of restoration (Toledo et al. 2001; Hogarth 2007; Kirui et al.2008).

To establish a successful rehabilitation program Lewis and Marshall (1997) suggested the following steps:

1 Understand the autoecology of each mangrove species at the site;
2 Understand the normal hydrology patterns;
3 Evaluate the alterations of the initial mangrove that inhibited renewal;
4 Create the rehabilitation program to restore the correct hydrological conditions;
5 Utilize planting after determining through the previous steps that natural renewal is not possible.

Most rehabilitation programs often fail because they are only based on the step 5 without considering the steps 1-4 (Lewis 2004).

Despite efforts to set up successful mangrove rehabilitation programs there is a lack of knowledge about the importance of plant microbe interactions in mangrove ecosystem. It is well known that roots of terrestrial plant species influence the composition of soil bacterial communities (Neumann and Römheld 2001; Smalla et al. 2001). In turn, microorganisms can contribute to plant growth and health. Previous studies have already investigated the ecological role and importance of microbial communities inhabiting bulk sediments. However, it is apparent that the study of plant microbe interaction in this ecosystem is still in its infancy.

1.3. MANGROVE MICROBIAL COMMUNITIES

The ecological role and importance of microbial communities inhabiting bulk sediments of mangroves is well known, however, the impact of mangrove plants on microorganisms and vice versa is still poorly understood (Elster 2000; Höflich et al. 1994).

Several studies have shown that many terrestrial plant species are able to influence the microorganisms inhabiting their roots and the rhizosphere (soil portion under plant

influence) also known as the “rhizosphere effect” (Höflich et al. 1994; Grayston et al. 1998; Gomes et al. 2001; Neumann and Römheld 2001; Smalla et al. 2001). In turn, microorganisms contribute to nutrient cycling, soil structure generation and decomposition (Höflich et al. 1994; Holguin et al. 2001). However, due to the frequency and duration of mangrove forests flooding it is hard to understand if mangrove roots are able exert such pressure on microbial communities as it happens with terrestrial plants.

Ananda and Sridhar (2002) have demonstrated that the mangrove environment combines terrestrial, marine and freshwater microorganisms. Several studies have shown that *Archaea* are more diverse and inhabit more habitats than previously assumed. Unlike bacteria and fungi, however, little is known about *Archaea* in mangrove habitats (Robertson et al. 2005; Chaban et al. 2006; Yan et al. 2006).

Archaea are responsible for several steps in nitrogen cycle; removing nitrogen from ecosystems by denitrification and nitrate-based respiration, and introducing it by nitrate assimilation and fixation (Cabello et al. 2004; Mehta and Baross 2006). Francis et al. (2007) and Coolen et al. (2007) discovered that *Archaea* are also involved in ammonia oxidation reactions. These reactions are important in oceans and in terrestrial environments since plants and other organisms consume nitrate, a product of nitrite oxidation (Leininger 2006; Coolen et al. 2007; Francis et al. 2007).

According to Baker and Banfield (2003) *Archaea* oxidize sulfur compounds from rocks, thereby enabling its availability to other organisms. However, sulfuric acid is also produced, contributing to acid mine drainage (abandoned mines) and environment damage due to the increasing of metals and sulfur.

Methanogenic *Archaea* removes hydrogen and decrease the amount of organic matter in anaerobic ecosystems, such as sediments, marshes and sewage treatment plants. Methanogenic *Archaea* are anaerobic and are the source of circa 70% of global methane produced (Schimel 2004).

In 2002, a new archaeal phylum was discovered and identified as Nanoarchaeota. It is known from the isolation of single chemolithotrophic *Crenarchaeote Ignicoccus* cells (Huber et al. 2002), two environmental DNA samples from Yellowstone and Kamchatka (Hohn et al. 2002) and 19 DNA sequences (McCliment et al. 2006). Waters et al. (2003) and Jahn et al. (2008) suggest that nanoarchaeal microorganisms have a parasitic lifestyle associated to *Ignicoccus* cells, since they do not have several core metabolic pathways. At the molecular level, a first study carried out by Huber et al. (2002) demonstrated that *Nanoarchaeum* genome has only 500 kilobases (kb).

Any functions of *Nanoarchaeum* are still unknown. However, unlike its *Ignicoccus* host, *Nanoarchaeum equitans* has no genes to support chemolithoautotrophic physiology inhibiting it to gain energy through hydrogen to reduce elemental sulfur (Waters et al. 2003). Since nanoarchaeal microorganisms only have been identified from hyperthermophilic environments and are typically found associated with the *Crenarchaeote Ignicoccus hospitalis*, that lives in temperature range of 70-98 ℃ (optimum around 90 ℃) (Huber et al. 2002), they are considered as hyperthermophilic microorganism; suggesting that *Nanoarchaeum* just can be identified in this temperature range. In order to improve the knowledge of *Nanoarchaeota* much more studies have to be done; allowing the knowledge of the different environments they inhabit or their functions. Nevertheless, the discovery of the *Nanoarchaeota* shows that the microbial diversity may be greater than expected (Stetter et al. 2005).

1.4. OBJECTIVES

The objectives of this study were to develop a polymerase chain reaction -denaturing gradient gel electrophoresis (PCR-DGGE) primer system suitable for *Archaea* domain and *Nanoarchaeum* genus based on new sequences recently submitted to public data banks and to assess *Archaea* and *Nanoarchaeum* community composition in the bulk sediment and rhizosphere samples in order to ascertain if the roots of mangrove plants affect sediment archaeal composition (rhizosphere effect). In addition we aimed to evaluate the rhizosphere effect on archaeal community by two different mangrove species - *Rhizophora mangle* and *Laguncularia racemosa*.

2. MATERIALS AND METHODS

2.1. SAMPLING AND TOTAL COMMUNITY DNA EXTRACTION

Sample processing and total community DNA extraction was performed as described by Gomes et al. (in preparation). Briefly, four composite replicates of bulk sediment (~20 cm of top sediment with 4 cm diameter) samples and roots of individual mangrove plants (4 replicates) – species *R. mangle* and *L. racemosa* – were sampled. Each rhizosphere sample consisted of the total root system with tightly adhering sediment of four individual plants, which were cut and thoroughly mixed. To detach bacterial cells from the bulk and rhizosphere sediments, the samples were treated as previously described in Gomes et al. (2007) for sediment samples.

Total community DNA extraction was performed from microbial cell pellets retrieved from sediment and rhizosphere samples as previously described in Gomes et al. 2007.

2.2. PRIMER DESIGN

The newly developed PCR-DGGE primer systems were designed by using the PROBE DESIGN and MATCH PROBE subroutines in the ARB software (Ludwig et al. 2004). The Probe Match function of the Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu/) was used for *in silico* analysis of the primer specificity based on the last ten 3'-end nucleotides (primer region with the greatest template discrimination). The searches were done with sequence data in the specified 16S rRNA gene of *Escherichia coli* region. Therefore 16S rRNA gene sequences available in the RDP database were searched for perfect match against the forward and reverse primers targeting *Archaea* and *Nanoarchaeum*. The primers were optimized using the program Oligo 4.0 (National Biosciences Inc.) and empirically tested against environmental samples.

2.3. PCR-AMPLIFICATION OF 16S RRNA GENE FRAGMENTS

Archaea

In this study a nested approach suitable for DGGE fingerprint analyses of *Archaea* community was developed, and performed using Bio-Rad My Cycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Initially, the primers ARC344f (Bano et al. 2004) and Arch958R (DeLong 1992) used in the first PCR were modified to cover most of

the sequences recently deposited in the GenBank database (Table 2) (fragment size with approximately 624bp).

Aliquots from each replicate were diluted 1:10 and used as a template for the first PCR. For this PCR, a reaction mixture for 25 µl was prepared containing 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates (dNTP's), 2.75 mM MgCl2, 2.5 µg BSA, 4% (v/v) formamide, 0.2 µM primers 334f and Arch958R mod and 0.5 U Dream Taq Polymerase (Fermentas) and template DNA (c. 10 ng). After 5 min denaturation at 94°C, 30 thermal cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C were carried out. A final extension step at 72°C for 7 min was performed to finish the reaction.

One µl of the first PCR was used as template for a second PCR using the mini primer 524F-10 (Isenbarger et al. 2008) and Arch958R mod with a GC clamp attached to the 5' end (Table 2). These primers target shorter regions within 16S rRNA gene sequences amplified in the first PCR round. The aim of this approach was to enhance the number of environmental archaeal 16S rRNA gene amplicons and simultaneously attach a GC-clamp to the amplified sequences to prevent complete melting of double-strand DNA during DGGE analyses (fragment size with approximately 425bp). The GC clamp sequence was published elsewhere (Heuer et al. 1997). Reaction mixtures (25 µl) 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM dNTP's, 2.5 mM MgCl2, 4% (v/v) acetamide, 0.2 µM primers 524F-10 and Arch958R mod-GC and 0.5 U Dream Taq polymerase (Fermentas). Denaturation for 5 min at 94°C was carried out, after which 36 thermal cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C were performed. A final extension step of 7 min at 72°C finished the reaction. Taq polymerase, buffer and dNTP's were acquired from MBI Fermentas (Vilnius, Lithuania).

Nanoarchaeum

Here a PCR-DGGE fingerprint system using primer sets targeting 16S rRNA gene fragments of *Nanoarchaeum* was developed. Similarly to what was described for *Archaea* fingerprint, the *Nanoarchaeum* -DGGE system is also a nested based approach. The first primer pair nano1-358F and nano1-920R (Table 3) amplify 16S rRNA gene fragments with approximately 562bp. A PCR reaction mixture for 25 µl was prepared containing 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM dNTP's, 2.5 mM MgCl2, 2% (v/v) dimethylsulfoxide (DMSO), 0.2 µM primers nano1-358F and nano1-920R and 0.5 U Dream Taq polymerase (Fermentas) and template DNA (c. 10 ng). After 5 min denaturation at 94°C, 35 thermal cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C

were carried out. A final extension step at 72ºC for 7 min was performed to finish the reaction.

One µl of the first PCR was used as template for a second PCR using the primer pair nano2-364F and nano2-778R with a GC clamp attached to the 5' end (Table 3). These primers target shorter regions within 16S rRNA gene sequences amplified in the first PCR round. This nested approach was applied to enhance the number of environmental *Nanoarchaeum* 16S rRNA gene amplicons and simultaneously attach a GC-clamp as explained above (fragment size with approximately 414bp). The GC clamp sequence was published elsewhere (Heuer et al. 1997). Reaction mixtures (25 µl) 1x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM dNTP's, 3.75 mM MgCl2, 4% (v/v) acetamide, 0.2 µM primers nano2-364F and nano2-778R and 0.5 U Dream Taq polymerase (Fermentas). Denaturation for 5 min at 94ºC was carried out, after which 35 thermal cycles of 1 min at 95ºC, 1 min at 50ºC and 1 min at 72ºC were performed. A final extension step of 10 min at 72ºC finished the reaction.

2.4. DENATURING GRADIENT GEL ELECTROPHORESIS

DGGE gels of the amplified 16S rRNA gene sequences were performed using the CSB System (CBS Scientific Company, Del Mar, CA, USA). The run was performed in 1 x Tris-acetate–EDTA buffer with a denaturant gradient 22-57%, at 60 ºC and a constant voltage of 220 V for 8 h; 8µl of each PCR product were loaded with 5µl of loading buffer for DGGE.

The DGGE gels were silver-stained according to Heuer et al. (1997) with slight differences. The solutions used were 10% (vol/vol) ethanol plus 0.5% acetic acid for fixation, 0.1% (wt/vol) silver nitrate for staining, freshly prepared developing solution containing 0.15% formaldehyde, 1.5% (wt/vol) NaOH and, finally, 0.75% (wt/vol) sodium carbonate solution to stop the development. Gels were scanned using a Molecular FX apparatus (Molecular Image FX apparatus, Bio-Rad Hercules, CA).

2.5. STATISTICAL ANALYSIS

DGGE gels were analyzed with the software package Gelcompar 4.0 program (Applied Maths) as described by Smalla et al. (2001).

Briefly, both band position and intensity were processed in Excel (Microsoft); and the band intensity was converted to relative intensity by dividing its intensity by the sum of all

band intensities in a lane (sample). This treatment was done with a tolerance and optimization of 5pts (0.5%).

A Bray-Curtis similarity index was calculated based on relative intensity of each band. Analysis of similarities (ANOSIM), non-metric multidimensional scaling (MDS) and Shannon-Wiener index were used to analyze DGGE profiles with PRIMER 5 (Primer-E Ltd, Plymouth UK). The ANOSIM was used to test if there is complete (R=1) or no (R=0) separation between archaeal communities from different samples (Clarke 1993). In ANOSIM, R varies between 0 and 1, where higher values correspond to more differentiation; the null hypothesis is that there are no differences among different groups of samples. Significance was tested with a permutation test using 999 permutations (Clarke and Gorley 2001). Differences in archaeal community structure of sediments and rhizosphere (*R. mangle* and *L. racemosa*) samples were assessed graphically using MDS (Yannarell et al. 2005). Shannon-Wiener index (H') was used to estimate the diversity of archaeal and nanoarchaeal communities in each sample (Zar 1984; Krebs 1999).

3. RESULTS

3.1. SPECIFIC PRIMERS FOR *ARCHAEA*

Table 2 shows *Archaea*-specific 16S rRNA gene sequence/PCR primers; and shows that primers 524F-10, ARCH958R, 334f and ARCH958R mod-GC, used in this work, are the best when compared with other primers with approximately the same target position. Table 2 also shows that the modification made to primer ARCH958R enhanced its specificity by the alteration of the percentage of matches from 61% to 87%.

Table 2 – *Archaea*-specific 16S rRNA gene sequence/PCR primers. M-Matches, PM-Possible Matches, %M-Matches%; B – Bacteria, A – Archaea, N – Nanoarchaeota.

Primer	Sequence 5'-3'	Specificity									Reference
		B			A			N			
		PM	M	%M	PM	M	%M	PM	M	%M	
524F-10	GCCGCGGTAA	1016347	**952221**	**93,7**	50192	**48707**	**97**	6	0	0	Isenbarger et al. 2008
334f[a]	ACGGGGYGCASSAGKCGVGA	1063884	**44570**	**4,2**	44624	**41079**	**92**	22	0	0	This study
ARCH958R mod[b]	YCCGGCGTTGAVTCCAATT	802105	**363**	**0,1**	19512	17020	**87**	16	2	13	This study
Arch958R mod-GC	CCGGCGTTGAVTCCAATT	802105	363	0,1	19512	17020	87	16	2	13	This study
A934R	GTGCTCCCCCGCCAATTCCT	811114	**5696**	**0,7**	19299	18943	98	15	0	0	DasSarma and Fleischmann, 1995
533f	GTGCCAGC(AC)GCCGCGGTAA	1013672	**949790**	**93,7**	50140	**48665**	**97**	6	0	0	Lane DJ 1991
UA1406R	ACGGGCGGTGWGTRCAA	303241	296467	97,8	7475	7253	97	3	3	100	Baker et al. 2003
1513uR	ACGGHTACCTTGTTACGACTT	10864	10326	95,1	1205	1144	95	1	0	0	Eder et al. 1999
A357f	CCCTACGGGGCGCAGCAG	1064138	307896	28,9	44776	42026	94	22	0	0	Yu et al. 2008
A1040F	GAGAGGWGGTGCATGGCC	794190	915	0,1	19120	17833	93	16	12	75	Reysenbach and Pace, 1995
Arch915R	GTGCTCCCCCGCCAATTCCT	815403	**5739**	**0,7**	26291	24495	93	28	0	0	Stahl and Amann 1991
Arch1381R	GCGGTGTGTGCAAGGRGCAGGG	309369	278	0,01	7658	7123	93	3	3	100	Kublanov et al. 2009
A340F	CCCTACGGGGYGCASCAG	1064029	**296466**	**27,9**	44684	**41286**	**92**	22	18	82	Vetriani et al., 1999
Ab127R	CCACGTGTTACTSAGC	990039	5942	0,6	31395	28921	92	23	18	78	DasSarma and Fleischmann, 1995
Ab927R	CCCGCCAATTCCTTTAAGTTTC	812299	**2907**	**0,4**	19760	17929	91	28	24	86	Jurgens et al., 2000
Arch338F	GGCCCTAYGGGGYGCASCAGGC	1064274	**176**	**0,02**	44427	**38199**	**86**	22	0	0	Kublanov et al. 2009

A24f	TCYGKTTGATCCYGSCRGA	26751	16	0,1	2953	2498	85	2	0	0	Baker et al. 2003
A751F	CCGACGGTGAGRGRYGAA	983439	52936	5,4	50986	40689	80	33	0	0	Baker et al. 2003
A348R	CCCCGTAGGGCCYGG	1064045	82	0,01	44726	34956	78	22	6	27	Barns et al., 1994
A109F	ACKGCTCAGTAACACGT	879881	327721	37,3	29529	22563	76	22	19	86	Whitehead and Cotta, 1999
1114aR	GGGTCTCGCTCGTTRCC	786701	13028	1,7	17518	12652	72	16	0	0	Huber et al., 2002
1119aR	GGYRSGGGTCTCGCTCGTT	786471	4330	0,6	17494	12061	69	16	0	0	Hohn et al. 2002
ARC344f	ACGGGGCGCAGCAGGCGCGA	1064056	**167**	**0,02**	44701	**29947**	**67**	22	0	0	Bano et al. 2004
A3Fa	TCCGGTTGATCCYGCCGG	26751	18	0,07	2953	1906	65	2	0	0	McInnery et al., 1995
Arch21F	TTCCGGTTGATCCYGCCGGA	26751	2	0,01	2953	1875	63	2	0	0	DeLong 1992
A2Fa	TTCCGGTTGATCCYGCCGGA	26751	2	0,01	2953	1875	63	2	0	0	Reysenbach and Pace, 1995; Martinez-Murcia et al., 1995;
8aF	TCYGGTTGATCCTGCC	26751	19	0,07	2953	1837	62	2	0	0	Eder et al. 1999
A1F	ATTCCGGTTGATCCTGC	26751	60	0,22	2953	1826	62	2	1	50	Tajima et al., 2001
ARCH958R	YCCGGCGTTGAMTCCAATT	802105	330	0,04	19512	11810	61	16	2	13	DeLong 1992
A333F	TCCAGGCCCTACGGG	1063879	35191	3,3	44621	25417	57	22	19	86	Reysenbach and Pace, 1995
A1098F	GGCAACGAGCGMGACCC	787263	1161	0,2	17549	9229	53	16	15	94	Reysenbach and Pace, 1995
A3Fb	TCYGKTTGATCCYGSCRGAG	26751	32	0,1	2953	1521	52	2	0	0	Lopez-Garcia et al., 2001
Ar3F	TTCCGGTTGATCCTGCCGGA	26751	1	0,00	2953	1515	51	2	0	0	Jurgens et al. 1997
A1115R	GGGTCTCGCTCGTTG	786651	417	0,1	17509	7857	45	16	0	0	Reysenbach and Pace, 1995
A329r	TGTCTCAGGTTCCATCTCCG	1063786	2357	0,22	44584	17526	39	22	0	0	Yu e tal. 2008
A693r	GGATTACARGATTTC	1028307	1249	0,12	51772	14686	28	33	2	6	Yu et al. 2008
Ar4f	TCYGGTTGATTCTGCCRG	26751	2	0,01	2953	52	2	2	0	0	Hershberger et al. 1996
- GC clamp	CGCCCGGGGCGCGCCCCGGGC GGGGCGGGGGGCACGGGGGG										Heuer et al. 1997

Primers were submitted to the 'check probe' facility of the Ribosomal Database Project (http://www.rdp.cme.msu.edu/) to check for archaeal specificity. All forward primers were submitted as 'target sequence' and reverse primers were submitted as probes.
[a]Modified from ARC344f Bano et al. 2004; [b]Modified from ARCH958R DeLong 1992

In turn, table 3 shows *Nanoarchaeum*-specific 16S rRNA gene sequence/PCR primers; and shows that primers nano1-358F, nano1-920R, nano2-364F and nano2-778R-GC, used in this work, are the best considering the percentage of matches with the existent 16S rRNA gene sequence/PCR primers.

Table 3 – *Nanoarchaeum*-specific 16S rRNA gene sequence/PCR primers. M-Matches, PM-Possible Matches, %M-Matches%; B – Bacteria, A – Archaea, N – Nanoarchaeota.

Primer	Sequence 5'-3'	Specificity									Reference
		B			A			N			
		PM	M	%M	PM	M	%M	PM	M	%M	
nano1-358F	ACCAGGGGCGAAACCTC	1064138	48	0,00	44776	11446	25,6	**21**	**21**	100	This study
nano2-364F	GGCGAAACCTCCGCAATG	1064138	318	0,03	44776	6500	14,5	**22**	**22**	100	This study
nano1-920R	DTCCMATTAAACCGCRCAC	873614	358	0,04	41960	63	0,2	**33**	**31**	94	This study
nano2-778R-GC	CCCGCAGCGTTGACAGC	952483	119	0,01	49480	42	0,1	**33**	**32**	97	This study
1044aF	GAGAGGWGGTGCATGGCCG	794023	17190	2,16	19107	17609	92,1	16	12	75	Stetter e tal. 2005
9bF	CCCGTTGATCCTGCGGGAG	26751	96	0,36	2953	8	0,3	2	1	50	Eder et al. 1999
N3F	TCCCGTTGATCCTGCG	26751	7	0,03	2953	14	0,5	2	1	50	Huber et al., 2002
N1510R	ACGGCTACCTTGTGTCGACTT	25874	0	0,00	1206	1	0,1	1	1	100	Huber et al., 2002
N1406R	ACGGGCGGTGAGTGCAA	303240	400	0,13	7475	16	0,2	3	3	100	Huber et al., 2002
N961R	CMATTAAACCGCRCACCC	802105	920	0,11	19512	20	0,1	16	15	94	Casanueva et al. 2008
934mcR	GTGCTCCCCCGCCTATTCCT	826520	797	0,10	29945	91	0,3	**32**	**30**	94	Huber et al., 2002
515mcR	CCCCTCTTGCCCACCGCT	1019155	159	0,02	50259	25	0,1	6	4	67	Huber et al., 2002
511mcR	CTTGCCCACCGCTT	1019155	1608	0,16	50259	51	0,1	6	4	67	Huber et al. 2002
N989R	GGTTTCCGGTGTCAGTTC	795534	1567	0,20	20035	8	0,04	16	7	44	Casanueva et al. 2008
1114mcR	GGGTCTCGCCTGTTTCC	785977	653	0,08	17487	1	0,01	16	1	6	Huber et al., 2002
- GC clamp	CGCCCGGGGCGCGCCCCGGGC GGGGCGGGGGCACGGGGGG										Heuer et al. 1997

Primers were submitted to the 'check probe' facility of the Ribosomal Database Project (http://www.rdp.cme.msu.edu/) to check for nanoarchaeal specificity. All forward primers were submitted as 'target sequence' and reverse primers were submitted as probes.

The most *Nanoarchaeum*-specific primers in Table 3 are primers nano1-358F, nano2-364F, nano1-920R, nano2-778R-GC and 934mcR, because they cover 21-33 "possible matches" with nanoarchaeal sequences. However, comparing primers nano1-920R and 934mcR, the last is less specific since it covers about two times more Bacteria and Archaea sequences than the first primer.

3.2. STRUCTURAL DIVERSITY OF ARCHAEAL COMMUNITIES

The influence of mangrove roots of *R. mangle* and *L. racemosa* in archaeal and nanoarchaeal communities inhabiting intertidal sediments is evaluated by the number and position of bands in the different DGGE profiles.

The DGGE gel of *Archaea* 16S rDNA PCR products shows the differences between archaeal communities from bulk and rhizosphere sediment (Figure 2). Some ribotypes are present dominantly in both *R. mangle* and *L. Racemosa* samples (indicated with an arrow in Figure 2) and others are present in bulk sediment samples and in *R. mangle* or *L. Racemosa* samples (an example is marked in red, Figure 2).

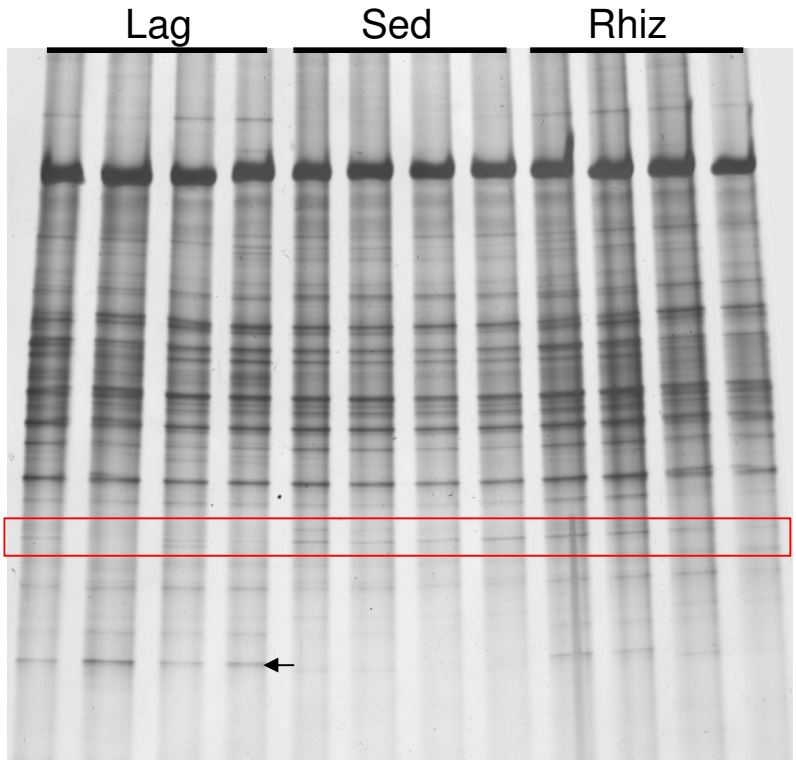

Figure 2 – Comparison of DGGE patterns of 16S ribosomal RNA gene fragments of *Archaea* amplified from bulk (Sed) and rhizosphere sediment of two mangrove species (Lag – *L. racemosa* and Rhiz – *R. mangle*). The arrow indicates a DGGE ribotypes present dominantly in mangrove roots but not in bulk sediment.

The fact of bulk sediment samples and *R. mangle* or *L. Racemosa* samples have groups of ribotypes not common to all the groups of samples, indicate the difference among groups of samples. Table 4 shows that samples from bulk and rhizosphere sediment of *R. mangle* or *L. Racemosa* have significant differences (global R=0.512); and that there are significant differences among different groups of samples.

Table 4 – ANOSIM statistics analysis of Bray-Curtis similarity measures (R) of bulk (Sed) and rhizosphere sediment of mangroves *L. racemosa* (Lag) and *R. mangle* (Rhiz) samples of A*rchaea*.

	Global R	Significance level %
	0.512	0.1
Group of samples	R Statistic	Significance level %
Lag, Sed	0.719	2.9
Lag, Rhiz	0.479	2.9
Sed, Rhiz	0.417	2.9

Non-metric multidimensional scaling (MDS) analyses corroborate ANOSIM results, indicating that samples from bulk and rhizosphere sediments are clearly separated (Figure 3). It is also visible that bulk sediment samples form a cluster; are more separated from samples of *L. racemosa* than from samples of *R. mangle*; and even are more similar to samples Rhiz C, Rhiz D and Lag B, confirming the ANOSIM results that bulk and rhizosphere sediment of *R. mangle* sample are moderately separated.

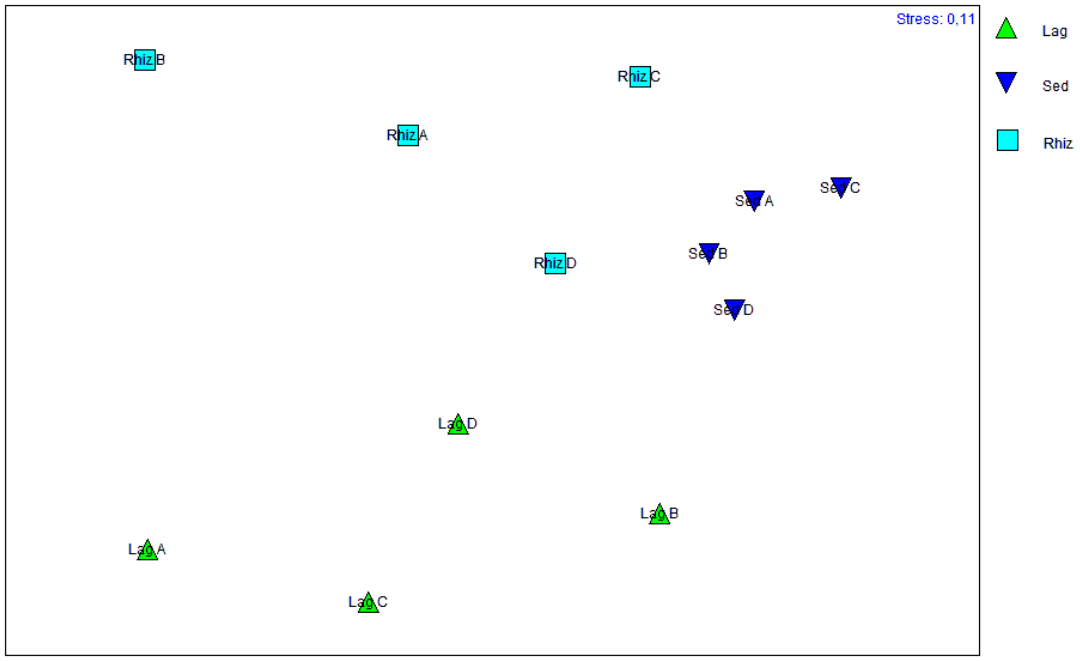

Figure 3 – MDS diagram of *Archaea* DGGE profiles.

Since one of the objectives of this study is to determinate if the roots of mangrove plants affect bulk sediment archaeal composition (rhizosphere effect). Through ANOSIM statistics and MDS analyses, it is clear that roots of *L. racemosa* influence sediment archaeal composition (R=0.719). On the other hand, roots of *R. mangle* do not have the same significant influence in sediment archaeal composition (R=0.417).

3.3. STRUCTURAL DIVERSITY OF NANOARCHAEAL COMMUNITIES

The DGGE gel of Nanorchaeum 16S rDNA PCR products shows that nanoarchaeal communities from bulk and rhizosphere sediment samples are similarly distributed, with slight differences (Figure 4).

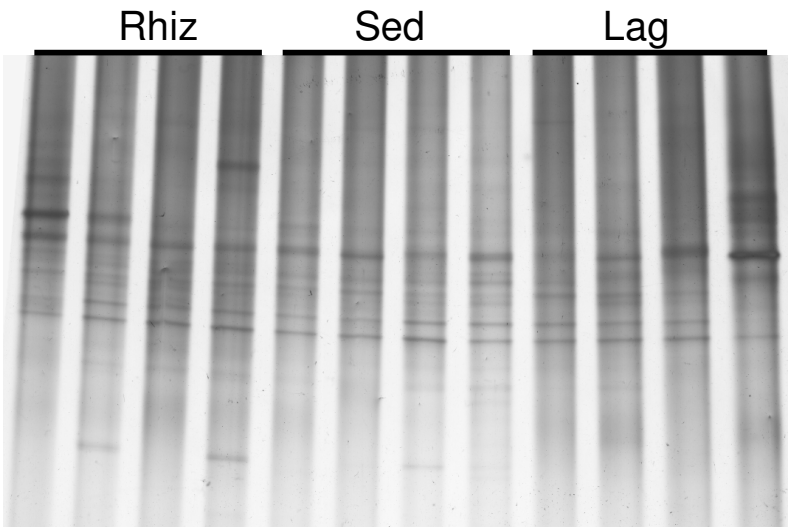

Figure 4 – Comparison of DGGE patterns of 16S ribosomal RNA gene fragments of *Nanoarchaeum* amplified from bulk (Sed) and rhizosphere sediment of two mangrove species (Lag – *L. racemosa* and Rhiz – *R. mangle*).

This first evaluation is corroborated by the ANOSIM statistics (Table 5) and MDS analyses (Figure 5). Table 4 shows that no significant difference (global R=0.069) was found between samples from bulk and rizhospere sediment of *R. mangle* or *L. racemosa* and among different groups of samples

Table 5 – ANOSIM statistics analysis of Bray-Curtis similarity measures (R) of bulk (Sed) and rhizosphere sediment of mangroves *L. racemosa* (Lag) and *R. mangle* (Rhiz) samples of *Nanoarchaeum*.

	Global R	Significance level %
	0.069	27.8
Group of samples	R Statistic	Significance level %
Rhiz, Sed	0.094	22.9
Rhiz, Lag	0.094	34.3
Sed, Lag	0.125	25.7

MDS analyses indicate that samples from bulk and rhizosphere sediments are barely separated (Figure 5) and just one of the samples of *L. racemosa* is separated from the rest.

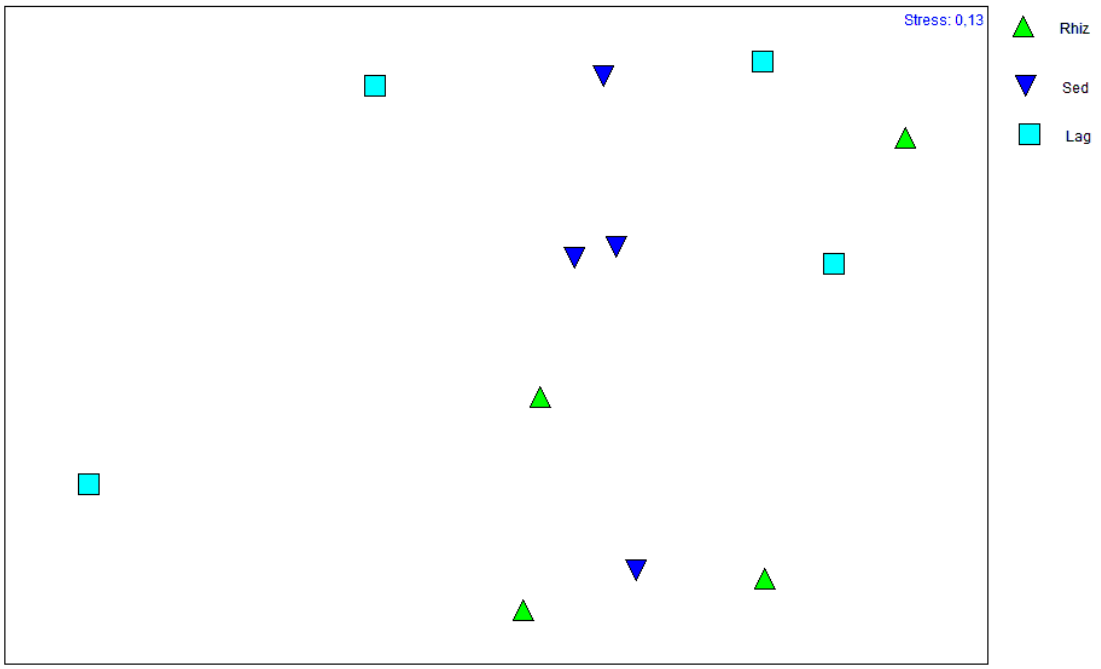

Figure 5 – MDS diagram of *Nanoarchaeum* DGGE profiles.

Concerning to the determination of the possible effect of mangrove plants in nanoarchaeal composition, through ANOSIM statistics and MDS analyses it is obvious that neither roots of *L. racemosa* nor *R. mangle* influence sediment nanoarchaeal composition.

Shannon-Wiener index (H') was used to estimate the diversity of *Archaea* and *Nanoarchaeum* populations in each sample. Table 6 shows that the diversity of *Archaea* and *Nanoarchaeum* is high.

Table 6 – Shannon-Wiener index (H') of *Archaea* and *Nanoarchaeum* in each sample.

Sample	Diversity (H') *Archaea*	Diversity (H') *Nanoarchaeum*
Lag A	3,144	1,881
Lag B	3,056	1,968
Lag C	3,15	2,248
Lag D	3,197	1,84
Sed A	3,103	2,147
Sed B	3,079	2,311
Sed C	2,985	2,142
Sed D	2,998	2,238
Rhiz A	3,283	2,274
Rhiz B	3,113	2,348
Rhiz C	3,146	2,472
Rhiz D	3,092	2,234

4. DISCUSSION

According to Amann et al. 1995, the analysis of 16S rRNA genes, by the use of PCR, has provided better estimation of bacterial diversity. Despite the high availability of PCR primers for 16S rRNA genes amplification (Lane 1991), they have been constructed based on incomplete database of 16S rRNA sequences and have not been tested systematically (Marchesi et al. 1998). Thus, is very important to adapt existent primers or to design new ones based on the most recent sequences to enhance the efficiency of PCR amplification. In turn, Aurelius et al. (1991) defend that both specificity and sensitivety of PCR are increased by the nested primer. This specificity is guaranteed by the most specific primers, i.e. the primers that exclude the maximun of non target and amplify the maximum of target sequences. Besides the increase of specificity and sensitivety, the main advantage of the nested PCR is the decrease of nonspecific amplification compared to traditional PCR (Albert and Fenyo 1990). Here, the most specific primers used on nested PCR for *Archaea* are both ARCH958R mod and ARCH958R mod-CG, that cover 0.1% of non target sequences and 87% of target sequences.

In the first PCR primer 524F-10 was used combined with ARCH958R mod. Primer 524F-10 sequence matches with the last 10 nucleotides of primer 533f; suggesting that, acording to the “possible matches” on archaeal sequences, primer 533f is more specific than 524F-10. However, when the all sequence of primer 533f is submitted to the ‘check probe’ facility of the RDP, its specificity for *Archaea* greatly decreases from 97% to less than 1% (0.002%). Compared to primer ARCH958R, primers A934R, Arch915R and Ab927R cover between 8 and 16 times the amount of *Bacteria* sequences, confirming that they are less specific for *Archaea*. In turn, the second PCR was carried out with primers 334f and ARCH958R mod-GC. Thus, primer 334f is best than primers 340F, Arch338F and ARC334f because, comparing with primer 334f:

- primer 340F covers more than six times *Bacteria* sequences;
- primers Arch338F and ARC334f cover less *Archaea* sequences.

Nakatsu et al. (2000) study represents one of many studies that also was carried out by the use of a nested approach to analyze soil community. In this study, the pairs of primers PRA46F/PREA1100R and PARCH340F/PARCH519R were used in first and second PCR amplification, respectively. The analyses of DGGE profiles of the 16S rDNA PCR-DGGE products from *Archaea* revealed that they were distinct but had less then ten

populations in any of the examined soils. Thus, comparing these results with ours (Figure 2), we can say that our primers, ARC344f/Arch958R and 524F-10/Arch958R mod-GC, are more specific for *Archaea*.

On the other hand, most *Archaea*-specific primer sequences are not specific for the *Nanoarchaeum* (Baker and Cowan 2004; Hohn et al. 2002; McCliment et al. 2006); as is shown in Table 2, 22 of 36 *Archaea*-specific primers do not show any match with nanoarchaeal sequences. Thus, there was a great need to develop *Nanoarchaeum*-specific primers to cover more nanoarchaeal sequences deposited in the GenBank database. And, when compared to already published *Nanoarchaeum*-specific primers, the primers we developed are more specific for *Nanoarchaeum*, since they cover between 94-100% of the "possible matches" of nanoarchaeal 16S rRNA sequences.

In this study, the statistical analysis and the analysis of DGGE profiles of archaeal community were done to evaluate if mangrove species *L. racemosa* (Lag) and *R. mangle* influence or not sediment archaeal composition. The analysis of similarities (ANOSIM) of DGGE profiles, based on Bray-Curtis measure of similarity, was used to assess differences between different groups of samples, i.e. bulk and rhizosphere sediment of mangrove plants of *R. mangle* and *L. racemosa*. In the ANOSIM R value varies between 0 and 1, where higher values correspond to higher differences (Clarke 1993); $R \leq 0.25$ – no significant differences, $0.25 < R < 0.50$ – low significance and $0.50 < R \leq 1.00$ – high significance (Clarke and Gorley 2001).

The results obtained reveal that *Archaea* community fingerprints from bulk and rhizosphere sediment of *L. racemosa* samples have significant differences ($R=0.719$). On the other hand, *Archaea* communities from bulk and rhizosphere sediment of *R. mangle* samples have lower significant differences ($R=0.417$). These results suggest that the roots of *L. racemosa* exert a greater influence on the composition of archaeal communities inhabiting bulk sediment than roots of *R. mangle*. Moreover, these results also show that roots of mangrove plants may also influence the composition of archaeal communities from bulk sediment, as happens with terrestrial plants – such as maize, strawberry, oilseed rape or potato – whose roots influence the composition of microbial community inhabiting the bulk sediment (Gomes et al. 2001; Smalla et al. 2001). This is the first time that is shown that an archaeal population is enhanced by roots of mangrove plants. More information about this population can improve of our knowledge of the importance of these organisms in the system plant microbe interactions in mangrove ecosystems.

On the opposite of what occurs with archaeal communities, the nanoarchaeal communities from bulk and rhizosphere samples are barely separated (global R=0.069). Since the samples are greatly similar to each other, the groups of samples cannot be separated significantly, as is shown.

Giving the importance of archaean microorganisms in biogeochemical cycles, such as carbon, nitrogen and sulphur cycles, it is important to extent the knowledge of the diverse environmental impacts on archaeal communities. It is also important to better know the ecology, habitats and functions of nanoarchaean microorganisms, since they are considered as being hyperthermophilic and our results demonstrate their detection in an environment with temperatures not extreme. This findings support the idea that microbial diversity and the range of environments inhabits by nanoarchaeal communities, is higher than ever estimated (Huber et al. 2992; Stetter et al. 2005).

Furthermore, the newly developed PCR-DGGE system for archaeal communities unravel higher diversity of DGGE band types than previous molecular fingerprints used for sediment marine archaeal (Wang et al. 2009; Harrison et al. 2009). The diversity of *Archaea* and *Nanoarchaeum* populations in each sample was estimated using the Shannon-Wiener index (H'). Previous studies estimated H'=1.5 (Wang et al. 2009) and H'= 2.427 (Harrison et al. 2009) as the highest values of diversity of *Archaea* in marine sediments. Here, we show that diversity of *Archaea* is higher than reported previously (Table 6). On the other hand, there are no similar studies concerning *Nanoarchaeum*. So, it is surprisingly to achieve high values of diversity of *Nanoarchaeum* compared to reported values of *Archaea* in marine sediments (Wang et al. 2009; Harrison et al. 2009).

The technique PCR-DGGE has been used frequently in the evaluation of microbial community. However, the choice of primers set can be considered a critical step; as is shown in tables 2 and 3, even of two primers just differ in one base they can increase or decrease considerably their efficiency. A great advantage of PCR-DDGE is the possibility to combine it with other analyses, such as excision of bands followed by its re-amplification, cloning and sequencing (van Elsas et al. 2006). In order to complete this study and confirm the specificity of the PCR-DGGE systems developed in this study we aim to clone and sequence the band indicated in Figure 2, generate clone libraries for *Nanoarchaeum* and perform pyrosequencing analysis of archaeal communities. The pyrosequencing will be done with the same PCR system developed here for *Archaea* community. The second round PCR primers will be adapted for pyrosequencing analysis using the pyrosequencing platform 454 Life Siences (Roche Diagnostics).

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