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Original Article

The influence of primer choice on archaeal phylogenetic analyses based on 16S rRNA gene PCR

A influência da escolha de iniciadores na filogenia de *Archaea* em ensaios de PCR para o gene rRNA 16S

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Abstract

Polymerase chain reaction (PCR) assays targeting 16S rRNA genes followed by DNA sequencing are still important tools to characterize microbial communities present in environmental samples. However, despite the crescent number of deposited archaeal DNA sequences in databases, until now we do not have a clear picture of the effectiveness and specificity of the universal primers widely used to describe archaeal communities from different natural habitats. Therefore, in this study, we compared the phylogenetic profile obtained when Cerrado lake sediment DNA samples were submitted to 16S rDNA PCR employing three *Archaea*-specific primer sets commonly used. Our findings reveal that specificity of primers differed depending on the source of the analyzed DNA. Furthermore, archaeal communities revealed by each primer pair varied greatly, indicating that 16S rRNA gene primer choice affects the community profile obtained, with differences in both taxon detection and operational taxonomic unit (OTU) estimates.

Keywords: *Archaea*, PCR, universal primers, environmental microbiology.

Resumo

A amplificação de genes que codificam o rRNA 16S por reação em cadeia da polimerase (PCR) e o seu subsequente sequenciamento consistem em uma ferramenta importante na caracterização de comunidades microbianas presentes em amostras ambientais. No entanto, apesar do crescente número de sequências de DNA de *Archaea* depositadas em bancos de dados, a especificidade e efetividade dos iniciadores de PCR descritos como universais e amplamente utilizados na descrição desse grupo ainda não está clara. Neste estudo foram comparados os perfis filogenéticos de comunidades de arqueias obtidos a partir amostras de DNA de sedimentos lacustres do Cerrado submetidas a ensaios de PCR empregando três pares de iniciadores específicos para Archaea, comumente utilizados neste tipo de estudo. Nossos resultados indicam que as comunidades de arqueias detectadas com cada par de iniciadores apresentaram grande variação filogenética, sugerindo que a escolha de iniciadores dirigidos ao gene de rRNA 16S tem efeito significativo no perfil da comunidade descrita, com diferenças tanto em relação aos táxons detectados, como nas estimativas de unidades taxonômicas operacionais (OTU).

Palavras-chave: *Archaea*, PCR, iniciadores universais, microbiologia ambiental.

1. Introduction

It has been extensively documented that the vast majority of existing microbial diversity consists of yet uncultured organisms (Rappé and Giovannoni, 2003; Lloyd et al., 2018). To access this diversity, molecular biology techniques have become a valuable asset to explore microbial communities in environmental samples. Several methods have been employed throughout the years, such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), clone libraries of polymerase chain reaction (PCR) amplicons, quantitative PCR and, more recently, metagenome assembly using next generation sequencing (Streit and Schmitz, 2004; Garrido-Cardenas and Manzano-Agugliaro, 2017). As a result, our knowledge on the diversity and metabolic potential of yet uncultured groups has greatly improved in the last years, with the description of several novel taxa and the proposal of entire phyla with no cultured representatives, greatly affecting both bacterial and archaeal phylogeny (Hug et al., 2016).

Among the methods most frequently employed in environmental studies, PCR assays targeting conserved genes have played pivotal roles both in pioneer and

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recent studies (Muyzer et al., 1993; Bahram et al., 2018). Indeed, reports using both clone libraries and next generation sequencing of PCR amplicons are still frequently published (Tupinambá et al., 2016; Antranikian et al., 2017; Wu et al., 2017; Belmok et al., 2019). However, it is widely acknowledged that this approach is not devoid of potential biases, with steps such as DNA extraction, inhibition of, or unspecific DNA amplification, generation of PCR artefacts and differential amplification all playing a crucial role in result analyses (Delmont et al., 2013).

It has been demonstrated that the primers used in 16S rRNA gene PCR assays greatly affect the microbial taxa detected in environmental samples (Frank et al., 2008; Hong et al., 2009) and factors such as the DNA flanking the template region may also result in preferential amplification of some sequences (Santos et al., 2019). Indeed, the effectiveness of PCR targeting bacterial 16S rRNA genes to evaluate microbial diversity is subject of a plethora of factors (Acinas et al., 2005; Engelbrektson et al., 2010). Furthermore, despite the great advances in DNA sequencing techniques, biases on 16S rRNA gene-based studies is a topic that still requires attention (Klindworth et al., 2013; Kennedy et al., 2014; Brooks et al., 2015).

Primers described as universal to prokaryotic 16S rRNA gene have been demonstrated to be not as effective for the *Archaea* domain (Kolganova et al., 2002; Baker et al., 2003), reinforcing the utmost importance of adequate archaeal-specific primer design (Baker et al., 2003; Gantner et al., 2011). Since most studies comparing prokaryotic 16S rDNA-based methods were performed with *Bacteria*, little is known about the biases associated with the *Archaea* domain. Therefore, in the present study we compared the phylogenetic profile obtained when a Cerrado lake sediment DNA sample was submitted to 16S rDNA PCR employing widely used *Archaea*-specific primers.

2. Material and Methods

2.1. DNA Samples

Lake sediment samples were collected in native Cerrado (a Brazilian savannah-like biome) and used in this study. Sediment samples were obtained from a lake known as "Lagoa Rio Preto Alto" (LRPA) in the "Sempre Vivas" National Park, located in the state of Minas Gerais, Brazil, on May 2010 (FAP-DF project 2009/00086-7). Sampling was performed using 10 cm diameter PVC tubes, by introducing the tube up to 5 cm into sediments 1 m below the lake`s water level.

2.2. DNA extraction, PCR conditions and 16S rRNA genes libraries construction

DNA was extracted from 0.5 g of each sample with PowerSoil DNA Isolation Kit (MO Bio Laboratories Inc.) according to the manufacturer's instructions. PCR assays were conducted for each sample with three different primer combinations: 21f/958r (5´TTCCGGTTGATCCYGCCGGA-3´/ 5´YCCGGCGTTGAMTCCAATT3´) (DeLong, 1992), 109f/915r (5´ACKGCTCAGTAACACGT-3´/ 5´GTGCTCCCCCGCCAATTCCT-3´) (Großkopf et al., 1998) and 340f/1000r (5′CCCTAYGGGGYGCASCAG3′/ 5′GGCCATGCACYWCYTCTC-3′) (Gantner et al., 2011), all proposed as specific and universal for the 16S rRNA gene of *Archaea*. These primer sets amplify overlapping regions of the 16S rRNA gene. All PCR assays were performed in 30 µL or 50 µL reaction mixtures, containing 1 to 100 ng of template DNA, 1X reaction buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 400 ng/µL bovine serum albumin (BSA), 0.5 µM of each primer, 200 µM dNTPs (Invitrogen), 1.5 U *Taq* DNA Polymerase (Invitrogen). The following PCR conditions were used for primers 21f-958r and 109f-915r: initial denaturation at 94°C for 1 min, followed by 30 cycles consisting of denaturation (94°C for 1 min), annealing (1 min) and extension (72°C for 1 min and 30 s) and a final extension step at 72°C for 5 min. Annealing temperatures were 55 and 57°C for primer pairs 21f-958r and 109f-915r, respectively. PCR conditions used for amplification of LRPA with primers 340f-1000r were the same described by Gantner et al. (2011). All PCR experiments were performed in a MJ PTC-100 (Peltier Thermal Cycles) thermocycler.

Amplified DNA was visualized on 1% agarose gels electrophoresis stained with ethidium bromide (0.5 µg/mL). Amplicons were purified using GeneJET PCR Purification kit (Thermo Scientific), cloned into pGEM-T Easy® (Promega) vector, according to manufacturer's instructions, and transformed into *Escherichia coli* DH5α competent cells by heat shock treatment. Plasmidial DNA of the recombinant clones was extracted by phenol-chloroform-isoamyl alcohol at 25:24:1 (vol/vol/vol) and sequenced by Sanger method at Macrogen Inc. (Korea).

2.3. DNA Sequences analyses and primers coverage in silico analysis

The quality of the DNA sequences obtained was checked with Phred algorithm (http://asparagin.cenargen.embrapa.br/phph/) and only those with quality superior to 20 in more than 400 nucleotides were considered for further analyses. Chimeric sequences were identified by UCHIME 2, through the NCBI platform. Taxonomic classification of the sequences was performed with the latest releases of Greengenes (13_8) (DeSantis et al., 2006) and SILVA (v132) (Quast et al., 2013) taxonomical databases, both using Mothur v.1.24.1 (Schloss et al., 2009), and with the Ribosomal Data Project (RDP) tool on https://rdp.cme.msu.edu/classifier/classifier.jsp. Only identity thresholds of 90% or higher were considered.

Multiple alignments of 16S rRNA gene sequences amplified with different primers sets were performed separately with Clustal X v. 2.1 (Larkin et al., 2007) and gap columns generated were manually filtered. Mothur was used to calculate richness and diversity indexes, coverage estimations, as well as unique and shared OTUs at 97% identity for the construction of Venn diagrams. Shared and exclusive OTUs were estimated based on analysis of overlapping region of the sequences amplified by the different primers.

In silico evaluation of the primer pairs was performed using the online tool TestPrime1.0 (Klindworth et al., 2013) and the non-redundant SILVA database (SSU r138) (Quast et al., 2013). Aiming a more realistic simulation of

PCR behavior, one mismatch per primer at all locations except at the five bases of the 3´end was allowed (Klindworth et al., 2013).

2.4. Nucleotide sequence accession numbers

The nucleotide sequences from this study were deposited in the GenBank database under accession numbers MK527511-MK527839.

3. Results and Discussion

Three different primer pairs described as universal and specific for archaeal 16S rRNA genes were used to amplify a sediment environmental DNA sample (LRPA). Clone libraries of 16S rRNA genes amplified with each of the three selected primer combinations were obtained for the lake sediment DNA sample, totalizing three libraries (LRPA 21f-958r, LRPA 109f-915r and LRPA 340f-1000r). Despite the similar number of sequenced clones in each library, the number of sequences that were classified as *Archaea* after quality and chimera analyses was highly different among primer pairs (Table 1).

All 16S rDNA sequences detected with primers 21f-958r were classified as *Archaea* (Table 1), suggesting a high specificity of this primer combination for archaeal DNA sequences present in our sample. In contrast, primers 109f-915r were less specific, and amplified two bacterial sequences. Although these primers, originally designed to describe methanogens (Großkopf et al., 1998), have been extensively used to describe archaeal communities (Nishizawa et al., 2008; Jeyanathan et al., 2011; Carnevali et al., 2018), previous results of our group revealed that they are effective in the amplification of bacterial DNA sequences, especially when these organisms are present in higher abundance in environmental samples (data not shown).

Considering the current archaeal phylogeny and taxonomy proposed in the literature, classifications obtained with SILVA seemed more adequate, especially at the lower taxonomic levels, as it considers nomenclatures currently employed for many uncultivated groups (*e.g.* I.1c, *Bathyarchaeia*, *Woesearchaeia*), while other databases still present outdated nomenclatures or are not able to classify sequences affiliated to recently proposed taxa. Therefore, classifications obtained with SILVA were selected for further comparisons of the 16S rDNA libraries obtained with the different primer pairs.

As shown in Figure 1, the archaeal communities revealed by each primer pair varied greatly. While sequences amplified with primers 109f-915r and 340f-1000r resulted in similar phyla profiles, with most sequences affiliated to *Euryarchaeota*, amplicons obtained with primers 21f-958r were mainly associated to *Thaumarchaeota* and *Bathyarchaeota* (Figure 1A). In addition, *Woesearchaeota* could only be detected by 109f-915r and 340f-1000r. At lower taxonomic ranks, differences among the three primer pairs were also observed. With the exception of a few unclassified sequences amplified by 340f-1000r, all *Euryarchaeota* sequences were affiliated to methanogenic orders. While only *Methanocellales* and *Methanosarcinales* could be detected by primers 21f-958r, three additional methanogenic groups were amplified by 109f-915r and 340f-1000r – *Methanobacteriales*, *Methanomicrobiales* and *Methanomassillicoccales* – in different proportions (Figure 1B). Regarding *Thaumarchaeota*, 16S rDNA sequences from the yet uncultured subgroup I.1c were only revealed by primers 21f-958r and 109f-915r, while sequences belonging to I.1a (*Nitrosotaleales*) and I.1b (*Nitrososphaerales*) were amplified by all primers (Figure 1B).

The archaeal community depicted by primers 109f-915r and 340f-1000r was more comprehensive, revealing a high diversity of archaeal groups usually associated with hypoxic eutrophic freshwater sediments, similar to the one analyzed in the present study (*e.g.* methanogens) (Castelle et al., 2015; Laskar et al., 2018; Zhou et al., 2018). Interestingly, the archaeal diversity established by primers 21f-958r, however, was mainly associated to the TACK superphylum, with many thaumarchaeal sequences, a group not usually reported in lake sediment surveys (Fan and Xing, 2016; Hu et al., 2015). It is worth pointing out that this primer pair also yielded a high number of *Bathyarchaeota* sequences, a group that has been increasingly demonstrated as fundamental to the ecological dynamics in anoxic environments (Zhou et al., 2018). Thus, while generating fewer amplicons from methanogenic 16S rDNA when compared to the other two pairs, this primer pair provided important insights into this phylum`s diversity.

Despite this fact, such low methanogenic 16S rRNA gene detection in freshwater lake sediments is unusual. One of the main features of these environments is the high abundance of organic matter, especially in the

Table 1. Number of sequenced clones, quality, and classification at domain level of sequences amplified from a lake sediment (LRPA) DNA sample with three different primer pairs.

Sample/Primers	Nº of sequenced clones	High Quality sequences (PHRED>31)	Chimeras	Non-specific sequences[a]	*Bacteria*[b]	*Archaea*[b]	Percentage of sequences classified as *Archaea*[c]
LRPA 21f-958r	100	84	8	0	0	76	100.00%
LRPA 109f-915r	100	81	14	2	2	63	94.02%
LRPA 340f-1000r	183	113	1	1	0	111	99.10%

[a] Sequences not identified as 16S rDNA (no significant BLAST hits). [b] 16S rRNA gene sequences classified with 100% identity in Greengenes, RDP and SILVA databases. [c] Percentage of high quality, non-chimeric sequences classified as *Archaea.*

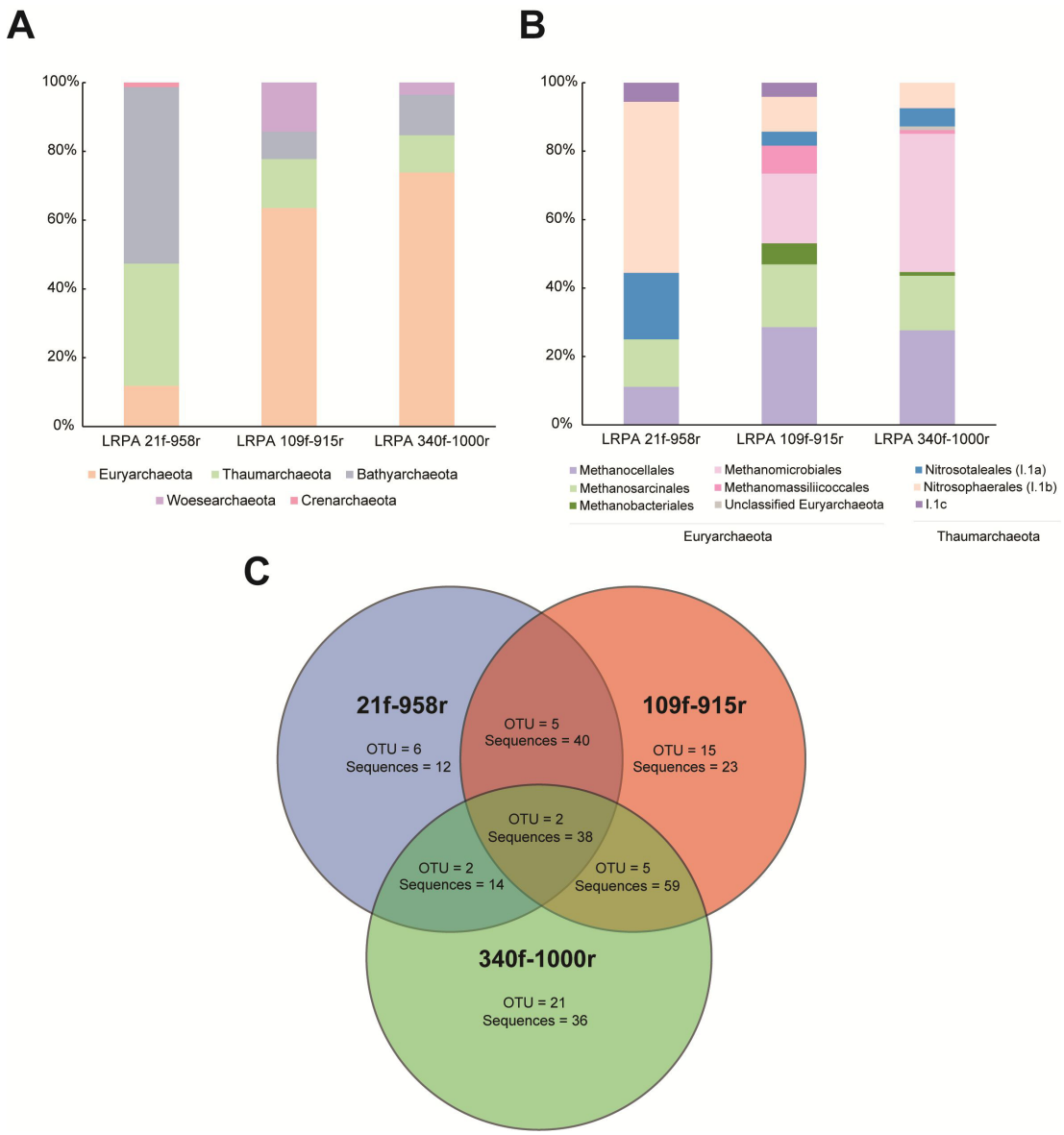

Figure 1. Classification with SILVA database of archaeal 16S rRNA sequences amplified from a lake sediment (LRPA) DNA with three different primers sets at phylum level (A) and order level (B). In (C), Venn diagram showing shared and unique OTUs, with 97% sequence similarity, among sequences amplified from LRPA sample with the three different primers sets.

upper layers (Thomaz et al., 2001; Esteves, 2011). Thus, the decomposition of organic matter plays an important ecological role, where complex molecules are degraded, leading to H_2, CO_2 and CH_4 formation through microbial activity (Sansone and Martens, 1982). In this context, methanogens are key players in this process, considering that methanogenesis serves as the final step in the anaerobic food chain while also maintaining thermodynamically favorable conditions for fermentative and acetogenic processes (Ferry, 2011). For this reason, methanogens are commonly detected in freshwater lake sediments (Zhu et al., 2012; Rodrigues et al., 2014). Thus, primers 21f-958r are possibly not the most adequate when describing these archaeal communities.

These results are reinforced when compared to *in silico* assays employing SILVA's TestPrime tool (Table 2) (Klindworth et al., 2013). While it is important to highlight that differences between *in vitro* and *in silico* assays are a common occurrence, this analysis indicates that the pair 21f-958r has the lowest taxonomic coverage, being restricted to mainly halophiles and TACK superphylum related groups (*e.g. Bathyarchaeia, Nitrososphaeria, Thermoprotei*). As previously mentioned, this primer pair yielded a notoriously higher relative abundance of TACK superphylum sequences when compared to methanogens, a result that greatly differs from what would be expected from an ecological standpoint as well as the results obtained with the 109f-915r and 340f-1000r pairs. On the other

hand, *in silico* assays indicated that the pair 340f-1000r has the highest taxonomic coverage of the three, with widespread detection across all recognized archaeal taxa by SILVA. Primer pair 109f-915r yielded similar results, though its overall taxonomic coverage was not as high as 340f-1000r. It is also worth pointing out that, as previously mentioned, this primer pair resulted in a few bacterial sequences, something that was not detected on *in silico* analysis (Table 2). Thus, considering the taxonomic coverage estimations employing the TestPrime tool as well as the results obtained in our *in vitro* assays, primer pair 340f-1000r is likely best suited to these environments.

OTU based analyses at 97% sequence similarity revealed great differences when the results obtained in LRPA with the three sets of primers are compared. A low number of shared OTUs among all three libraries and a high number of exclusive OTUs retrieved by each primer pair were observed (Figure 1C). Furthermore, α-diversity analyses showed small variations in the Shannon diversity index when each primer pair was used (Table 3), which could

Table 2. Taxonomic coverage of *in silico* assays of each primer pair used in this study according to the TestPrime tool (SILVA). Taxa covered by each primer pair are shown in bold.

21f-958r	109f-915r	340f-1000r
Archaea (5.0%)	**Archaea (55.1%)**	**Archaea (82.6%)**
Aenigmarchaeota	**Aenigmarchaeota** (13.6%)	**Aenigmarchaeota** (37.8%)
Altiarchaeota	**Altiarchaeota (64.7%)**	**Altiarchaeota** (36.0%)
Asgardarchaeota	**Asgardarchaeota** (5.1%)	**Asgardarchaeota** (6.0%)
Crenarchaeota (0.1%)	**Crenarchaeota** (63.8%)	**Crenarchaeota** (85.5%)
Bathyarchaeia (0.1%)	**Bathyarchaeia** (69.0%)	**Bathyarchaeia** (84.8%)
Methanomethylicia	**Methanomethylicia** (15.2%)	**Methanomethylicia** (100.0%)
Nitrososphaeria (0.2%)	**Nitrososphaeria** (55.5%)	**Nitrososphaeria** (87.2%)
Thermoprotei (0.3%)	**Thermoprotei** (77.3%)	**Thermoprotei** (80.7%)
Euryarchaeota	**Euryarchaeota** (80.1%)	**Euryarchaeota** (86.4%)
Methanobacteria	**Methanobacteria (80.9%)**	**Methanobacteria (87.0%)**
Methanococci	**Methanococci (88.0%)**	**Methanococci** (94.4%)
Methanopyri	**Methanopyri** (100.0%)	**Methanopyri** (100.0%)
Thermococci	**Thermococci** (77.0%)	**Thermococci** (81.5%)
Hadarchaeota	**Hadarchaeota** (2.7%)	**Hadarchaeota** (85.8%)
Halobacterota (16.2%)	**Halobacterota (44.0%)**	**Halobacterota (89.2%)**
Hydrothermarchaeota	**Hydrothermarchaeota** (86.7%)	**Hydrothermarchaeota** (96.8%)
Iainarchaeota	**Iainarchaeota** (59.4%)	**Iainarchaeota** (46.7%)
Korarchaeota	Korarchaeota	**Korarchaeota** (1.8%)
Micrarchaeota	**Micrarchaeota** (33.3%)	**Micrarchaeota** (55.3%)
Nanoarchaeota	**Nanoarchaeota** (45.1%)	**Nanoarchaeota** (61.4%)
Nanohaloarchaeota	Nanohaloarchaeota	**Nanohaloarchaeota** (84.8%)
Thermoplasmatota	**Thermoplasmatota** (67.0%)	**Thermoplasmatota** (89.4%)
Uncultured	**Uncultured (14.3%)**	**Uncultured (80.0%)**
Bacteria	Bacteria	Bacteria
Eukaryota	Eukaryota	Eukaryota
Unclassified	Unclassified	Unclassified

Table 3. α-Diversity analysis (97% sequence similarity) of archaeal 16S rRNA gene sequences amplified from a lake sediment (LRPA) sample with three different primer pairs. Archaeal sequences were normalized for the smallest library size (n = 63).

Primers	Observed OTUs	Shannon	Coverage
21f-958r	27	2.900	77.77%
109f-915r	29	3.141	80.95%
340f-1000r	21	2.522	82.54%

lead to different interpretations of a given environment's diversity. It is worth pointing out that the libraries obtained with all three primer pairs had a coverage of around 80% with the number of sequences analyzed. These results indicate that archaeal 16S rRNA gene primer choice greatly affects the community profile obtained, with differences in both taxon detection and OTU estimates.

4. Conclusions

Altogether, our results highlight the importance of primer choice when describing archaeal communities in PCR based environmental studies. Domain-specific primer design is a well-recognized challenge, and the commonly used primers analyzed in this study may yield different outlooks on archaeal diversity and phylogeny in a variety of habitats. This aspect should be considered during both experimental design and data analyses. Depending on the community composition and archaeal abundance in a given sample, entire groups could possibly be overlooked and OTU estimations at different taxonomic levels may vary. Therefore, as more 16S rRNA gene sequences are deposited and novel groups are discovered, more comprehensive domain specific primers may be designed for archaea, reducing preferential amplification issues. Thus, while PCR based environmental studies have inherent biases, future studies will surely improve archaeal detection when employing this method.

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