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# Apparent richness and community composition of Bacteria and Archaea in geothermal springs

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ABSTRACT: The archaeal and bacterial 16S rRNA gene diversities of the Polihnitos (89°C), Edipsos (81.8°C), Thermopiles (38.9°C), Eleftheres (41.2°C) and Lagadas (35.2°C) geothermal springs in Greece were investigated. The abundance of prokaryotic cells varied between $0.02 \times 10^5$ and $0.92 \times 10^5$ cells $ml^{-1}$. A total of 227 archaeal and 501 bacterial clones were analysed, which were attributed to 85 and 121 operational taxonomic units (OTUs), respectively. Library clone coverage, based on Good's C estimator, was satisfactory (>75%), except for the Archaea in Thermopiles (~40%) and Eleftheres (~60%). Most of the archaeal phylotypes were related to sequences of yet-uncultivated microorganisms retrieved from terrestrial geothermal springs, deep-sea hydrothermal vents and the subsurface. A much higher number of bacterial phylotypes was related to cultivated microorganisms from similar environments. The thermophilic nature of most of the discovered phylotypes was also supported by their high G+C content, which was positively correlated with the springs' temperatures. The springs showed different diversity patterns for Bacteria and Archaea, with Bacteria having higher diversity only in Polihnitos and Lagadas springs. The Shannon diversity index *H'* showed larger variation for Archaea (0.23 to 3.44) than for Bacteria (1.22 to 3.03) and was unrelated to the prevailing temperature, pH, salinity and dissolved oxygen content. Archaeal and bacterial clone libraries respectively contained 50 to 94.1 and 68.8 to 96.2% rare phylotypes (i.e. those that appear only once or twice in the clone library), indicating the importance of rare phylotypes in shaping community diversity.

KEY WORDS: Bacteria · Archaea · 16S rRNA · Geothermal springs · Greece



## INTRODUCTION

Natural geothermal springs are widespread on the Earth's surface. Excluding deep-sea hot vents, the most accessible ones are terrestrial geothermal springs, solfataras and geothermally heated soils. Greece harbours many terrestrial and coastal sites where geothermal springs occur, with temperatures between ~30 and 90°C, due to the geology of the country. Geothermal areas in the country are related to recent volcanic activity and active tectonics. Magmatic and volcanic processes, along with the high mountain chains and active fault systems, favour the rise of deep waters which are discharged at the surface as geothermal springs. The origin of the fluid movement results from a thermal gradient closely related to volcanic activity, leading to convection, for the majority of springs within areas of Tertiary basins in northern Greece and the Aegean island arc. These springs occur mainly in the post-orogenic basins of the northern Aegean and the Aegean island arc. In western Greece, where volcanic activity does not exist, the thermal gradient is due only to depth. The prokaryotic diversity of the shallow hydrothermal vents of Milos Island on the Aegean island arc has been previously studied (Sievert et al. 1999, 2000). Geothermal spring waters in islands

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and coastal areas are typical of marine solutions and result from the mixing of deep thermal reservoir water with meteoric water. In continental areas, thermal water rising from deep reservoirs is frequently localized in aquifers of neogene sediments such as conglomerates and sandstones, or may be mixed with karstic surface water. In general, the chemistry of Greek geothermal springs is due to the interaction of groundwater with silicic rocks bearing alkaline elements and some carbonates (mostly marbles), or with shallow aquifers and seawater, e.g. Thermopiles springs (Duriez et al. 2008). Geothermometric analysis, i.e. inferring temperature ranges from the occurring minerals in spring water, suggests that the mean temperatures of hot spring waters are between 149 and 187°C. These temperatures are probably due to a normal gradient, since the depth of the post-orogenic basin of Greece is thought to be >4000 m (Lambrakis & Kallergis 2005).

Although the geochemical features of the majority of geothermal springs in Greece have been the focus of several studies (reviewed by Lambrakis & Kallergis 2005), the available information on the microbiology of such springs is remarkably scarce. Except for the Milos Island shallow vents, where diverse archaeal and bacterial communities dictated by temperature and geochemical gradients have been revealed (Sievert et al. 1999, 2000), to our knowledge, only one study that focused on the isolation of thermophilic strains that are able to degrade crude oil originating from geothermal soils, sediments and waters on the volcanic island of Santorini exists (Meintanis et al. 2006).

It is well accepted that geothermal springs have site-specific biological, physical and chemical diversities. In addition, solid relationships of the existing microorganisms with the prevailing geochemical parameters are usually difficult to unravel as the majority of the retrieved sequences are new or unknown, making understanding of the microbial ecophysiology a really challenging task. One way of overcoming this problem is to discover trends of microbial diversity in order to determine whether temperature, substrates, toxic compounds or viruses play key roles in shaping the microbial communities in geothermal springs. Patterns of bacterial diversity give us a better understanding of how bacteria are distributed spatially and temporally (van der Gast 2008), and geothermal springs are considered to be among the extreme environments of pristine quality. In geothermal springs, temporal changes are expected to be much lower than in other surface habitats (Staley & Reysenbach 2002); thus, changes in bacterial diversity most probably depict changes dictated by spatial factors, like different origin depth and/or different available electron acceptors and donors for microbial metabolism. The aims of the present study were to investigate (1) archaeal and bacterial diversities based on 16S rRNA gene phylogenetic relationships in the waters of 5 terrestrial geothermal springs from 4 different geothermal fields with temperatures ranging from ~36 to 89°C, and (2) the pattern of microbial diversity in geothermal springs with a wide range of different physical and chemical characteristics.

## MATERIALS AND METHODS

**Sampling and prokaryotic cell abundance.** Water was collected from the Polihnitos (Pol), Edipsos (Edi), Thermopiles (Thp), Eleftheres (Ele) and Lagadas (Lag) geothermal springs in Greece (Fig. 1) between January and May 2005. Water samples of 20 l were collected in pre-sterilised carboys either from 20 to 30 cm below the surface in the case of pools (Pol, Thp, Lag, Ele) or by direct filling in the case of seeps (Edi). The samples were transferred immediately (<12 h) to the laboratory and filtered upon arrival. Water was filtered under low vacuum (<150 mm Hg) through polycarbonate filters of 0.2 µm pore size and the filters were kept at –80°C until further analysis. *In situ* measurements of temperature, pH, salinity and dissolved oxygen content were conducted using probes (YSI). Bacterial counts were measured using epifluorescence microscopy as described by Turley (1993).

**PCR amplification and cloning.** DNA was extracted using a soil DNA isolation kit (UltraClean, MoBio Laboratories) according to the manufacturer's protocol after slicing the filters with a sterile scalpel. For each 16S rRNA PCR amplification, 0.5 µl of the DNA template (~90 to 160 ng $\mu l^{-1}$) was used. To decrease PCR bias related to high numbers of cycles and minimize differences in clone library representation between rare and abundant phylotypes, PCR cycle optimization was performed, i.e. each PCR was performed at the minimum number of cycles where a positive PCR signal occurred. For all samples, a mixture of archaeal and bacterial universal primers was used in order to cover possible mismatches (Table 1).

Each 50 µl PCR reaction consisted of a 9 min pre-PCR hold at 95°C, followed by *x* number of cycles as determined by cycle optimisation (Table 1) consisting of a 1 min denaturation step at 95°C, a 1 min annealing step at 50°C, a 2 min elongation step at 72°C, and a final 10 min finishing step at 72°C at the end of *x* number of cycles. All PCR ingredients were prepared with twice-autoclaved ultra pure water, using Ampli Taq Gold polymerase (Applied Biosystems). The PCR products were checked on a 1.2% agarose gel at 100 V for 35 min, and were purified using a purification kit (Montage, Millipore). The purified PCR products were cloned with a T-vector kit (pT7 Blue, Novagen). The

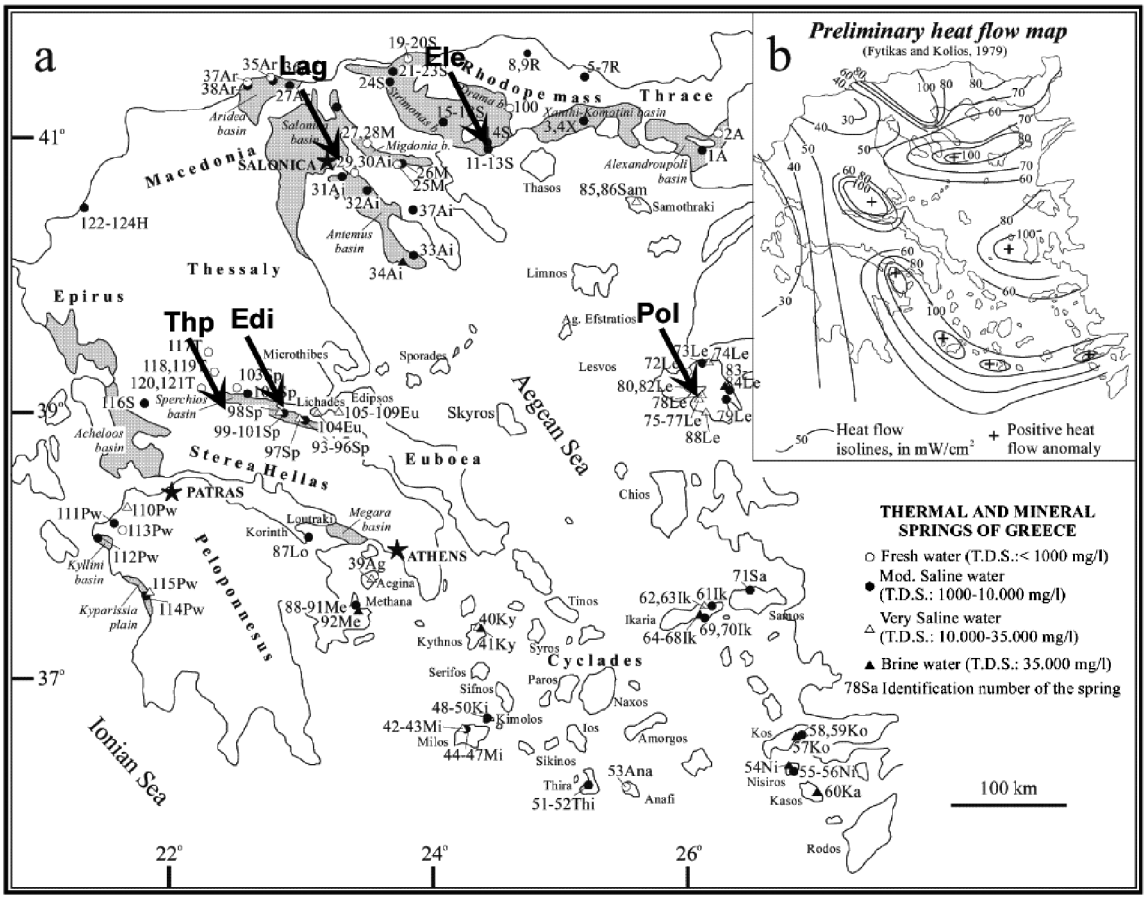


Fig. 1. Distribution map of the principal geothermal waters of Greece and location of the studied springs (arrows): Polihnitos (Pol), Edipsos (Edi), Eleftheres (Ele), Thermopiles (Thp) and Lagadas (Lag). For a full description of the map see Lambrakis & Kallergis (2005)

clonal DNAs were amplified from randomly selected recombinants by direct PCR with M13 primers (M4, 5′-GTT TTC CCA GTC ACG AC-3; RV, 5′-CAG GAA ACA GCT ATG AC-3′), purified with an HR column (MicroSpin S-400), and then used as templates for sequencing. Sequencing was performed with primers 907R (5′-CCG YCA ATT CMT TTR AGT TT-3′), a quick start kit (DTCS, Beckman), and an automated sequence analyzer (CEQ-2000, Beckman).

**Phylogenetic and diversity analysis.** Sequences of ~500 to 600 bp from the same sample were compared with those in the DDBJ/EMBL/GenBank databases using the FASTA search programs (www.ddbj.nig.ac.jp/search/). Sequences with ≥98% similarity were grouped as identical operational taxonomic units (OTU). For unique OTUs, additional sequencing was performed with M4 and RV primers, and after contig construction of the whole amplified region, detection of chimeric DNAs using the CHECK-CHIMERA program (Maidak et al. 2001) of the Ribosomal Database Project was performed and all chimeric sequences were discarded. Sequences from the present study were assigned GenBank accession numbers EF444594 to EF444797.

The sequences were automatically aligned with their closest relatives data using the Clustal X program (Jeanmougin et al. 1998) and revised by manual removal of ambiguously aligned regions. Phylogenetic trees were constructed using the neighbor-joining method (Saitou & Nei 1987) with the Clustal X program. Bootstrap analyses for 1000 replicates were performed to assign confidence levels to the tree topology by using PAUP* (phylogenetic analysis using parsimony) version 4.08b (Swofford 2000). Clone coverage was calculated using the equation $C = [1 \times (n_1/N)] \times 100$, where $n_1$ is the number of single-occurrence OTUs and $N$ is the number of 16S rRNA sequences examined (Good 1953, Kemp & Aller 2004). The Shannon-Wiener index $H'$ was used as the diversity index and was calculated as follows: $H' = \Sigma(p_i)\ (\log p_i)$, where the summation is over all OTUs, and $p_i$ is the proportion of OTU $i$ relative to the sum of all OTUs. The Shannon evenness index $E$ was calculated as $E = H'/\ln S$, where S is the number of OTUs (Shannon & Weaver 1949, Pielou 1969).

Table 1. Sequences of oligonucleotide primers used for PCR amplifications. Pol: Polihnitos, Edi: Edipsos, Thp: Thermopiles, Ele: Eleftheres, Lag: Lagadas geothermal springs. $T_m$: annealing temperature

| | Primer sequence (5′ – 3′) | Optimum number of cycles, $T_m$ = 50°C |
|---|---|---|
| **Archaea** | | |
| Forward: Arc109f-mix[a] | ACGGCTCAGTAACACGT | Pol: 30 |
| | ACTGCTCAGTAACACGT | Edi: 38 |
| | AAGGCTCAGTAACACGT | Thp: 32 |
| Reverse: Univ1492r[b] | TACGGTTACCTTGTTACGACTT | Ele: 38 |
| | TACGGCTACCTTGTTACGACTT | Lag: 35 |
| **Bacteria** | | |
| Forward: EUB338mix[c] | ACTCCTACGGGAGGCAGC | Pol: 32 |
| | ACTCCTACGGGAGGCTGC | Edi: 28 |
| | ACACCTACGGGTGGCTGC | Thp: 22 |
| | ACACCTACGGGTGGCAGC | Ele: 25 |
| Reverse: Univ1492r[b] | TACGGTTACCTTGTTACGACTT | Lag: 26 |
| | TACGGCTACCTTGTTACGACTT | |

[a]Grosskopf et al. (1998), Kamagata et al. (unpubl.)
[b]Lane (1991)
[c]Amann et al. (1990), Daims et al. (1999)

## RESULTS

### Abiotic parameters and prokaryotic cell abundance

The highest temperature (89°C) was noted in Pol while the lowest was in Lag (35.2°C) (Table 2). The most acidic spring was Thp (pH 5.14) while the rest of the springs were slightly acidic to neutral (pH 6.30 to 6.98). Edi had the highest salinity (33 PSU) with the rest of the springs being either brackish (Thp 12.6, Pol 5.1 PSU) or fresh water (Ele, Lag). Lag was the only spring with oxygen saturated waters, followed by Thp with 37% and the rest of the springs with ≤12%. Abundance of prokaryotic cells varied between $0.02 \times 10^5$ (Edi) to $0.92 \times 10^5$ cells $ml^{-1}$ (Thp) (Table 2).

Table 2. Abiotic parameters and prokaryotic cell abundance in Greek geothemal spring waters. PSU: practical salinity units

| Spring | Temp. (°C) | pH | Salinity (PSU) | Dissolved oxygen (mg $l^{-1}$) [$O_2$ saturation, %] | Prokaryotic cell abundance (cells $ml^{-1}$ ± SD) |
|---|---|---|---|---|---|
| Polihnitos | 89.0 | 6.79 | 5.1 | 2.96 [2.1] | 15,593 ± 777 |
| Edipsos | 81.8 | 6.30 | 33.0 | 3.30 [6.3] | 2,189 ± 633 |
| Thermopiles | 38.9 | 5.14 | 12.6 | 1.35 [37.0] | 91,733 ± 1,199 |
| Eleftheres | 41.2 | 6.80 | 0.0 | 0.60 [12] | 90,591 ± 1,292 |
| Lagadas | 35.2 | 6.98 | 0.0 | 5.50 [105.0] | 24,696 ± 893 |

### Clone libraries analysis

A total of 227 archaeal and 501 bacterial clones were analysed from the 5 geothermal springs, which belonged to 85 and 121 OTUs, respectively (Table S1, available as Supplementary material at www.int-res.com/suppl/a057p113_app.pdf). Clone library coverage based on Good (1953) was at least ~75%, except for the archaeal clone libraries for Thp and Ele (~40 and 60% respectively, Fig. 2), indicating that at least the most prevalent archaeal and bacterial groups in each clone library were identified.

### Phylogenetic and diversity analyses

All results are shown in Table S1 and Figs. S1a–c, available as Supplementary material at www.int-res.com/suppl/a057p113_app.pdf. In Pol, the archaeal community consisted of only 2 phylotypes. Thus, the low archaeal diversity was attributed to one Crenarchaeota phylotype (Pol-A-2, 93.9% dominance) related to tropical estuarine sediments, while the same spring showed the highest bacterial diversity of all the springs studied. The other phylotype (Pol-A-1) was closely related to *Archaeoglobus fulgidus*. The most abundant (17.1%) bacterial phylotype (Pol-B-97) was related to environmental sequences derived from Taiwan hot springs and *Hydrogenophilus thermoluteolus*. Other phylotypes were related to known thermophilic or uncultivated representatives of the phyla Chloroflexi, Deinococcus-Thermus, Bacteroidetes, Thermotogae, Aquificae, Planctomycetes and candidate division OP11.

In Edi, the dominant (43.2%) archaeal phylotype (Edi-A-1) was the same as the dominant one in Pol. The rest of the archaeal phylotypes belonged to other Crenarchaeota (total 16.0%) and Euryarchaeota (41.0%) from similar systems or the deep subsurface. The dominant (61.9%) bacterial phylotype (Edi-B-3) was related to *Persephonella hydrogeniphila*, while the rest of the phylotypes belonged to several phyla such as Nitrospirae, Proteobacteria (classes γ and δ), Cyanobacteria, Deferribacteres, Firmicutes, 'Termite group' and OP11, with representatives mostly from the deep subsurface and hot springs.

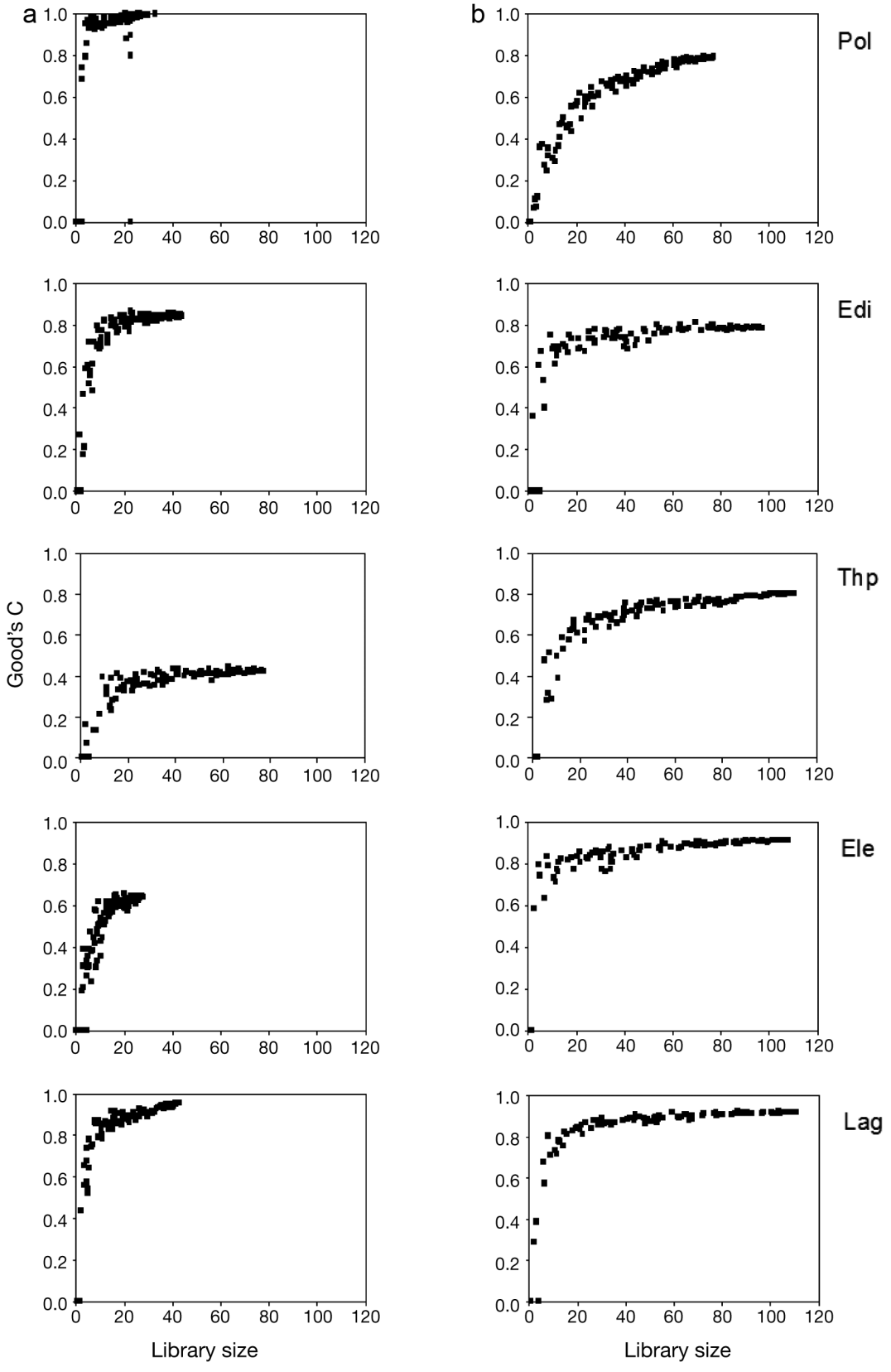


Fig. 2. Clone library coverage of (a) archaeal and (b) bacterial 16S rRNA genes (based on Good's C estimator) from the geothermal spring waters of Polihnitos (Pol), Edipsos (Edi), Thermopiles (Thp), Eleftheres (Ele) and Lagadas (Lag), Greece

In Thp, the high archaeal diversity was attributed to the high number (47 or 61.1% of all phylotypes) of singleton phylotypes. The most dominant phylotype, Thp-A-14 (20.3%), belonged to the Crenarchaeota and was related to a phylotype from a South African gold mine. Of all the archaeal phylotypes, only 6.5% belonged to the Euryarchaeota. The bacterial community in Thp was dominated by the γ-Proteobacteria (>57.9%), with phylotype Thp-B-2 being the dominant one (35.8%), which was related to a rhizosphere biofilm. The rest of the phylotypes belonged to the *gamma*, β-proteobacteria, OP11, Bacteroidetes, Thermotogae, Chlorobi, WS6, and Firmicutes, with their closest relatives originating from hot springs, anaerobic systems, hydrothermal vents and the subsurface.

In Ele, all archaeal phylotypes belonged to the Crenarchaeota. The 2 most abundant (25 and 17.9%) phylotypes (Ele-A-22 and Ele-A-6, respectively), as well as the rest of the phylotypes, were related to phylotypes from deep subsurface environments. The low diversity of the Ele bacterial community was due to the high dominance (72.2%) of phylotype Ele-B-1, which was related to a rhizosphere biofilm and belonged to the γ-Proteobacteria. This subphylum was also dominant in the rest of the phylotypes, followed by OP11 and Chloroflexi.

In Lag, the 2 dominant (46.5 and 34.9%) phylotypes (Lag-A-2 and Lag-A-9, respectively) belonged to the Crenarchaeota and were related to soil phylotypes. All but one of the rest of the phylotypes also belonged to the Crenarchaeota and were related to phylotypes from subsurface anaerobic environments. The only Euryarchaeota phylotype (Lag-A-82) was related to deep-sea sediments.

The Shannon diversity index $H'$ (Fig. 3) showed higher variation for Archaea (between 0.23 in Pol and 3.44 in Thp) than for Bacteria (between 1.22 in Ele and 3.03 in Pol). In Pol and Lag, archaeal was lower than bacterial diversity, with the former being higher in the rest of the springs. The Shannon evenness index $E$ varied between 0.33 and 0.88 for Archaea and 0.44 and 0.89 for Bacteria, and was higher for Bacteria only in Pol (Fig. 3).

All clone libraries contained rare phylotypes (sensu Aller & Kemp 2008, Fig. 4). Archaeal and bacterial libraries respectively contained 50 to 94.1 and 68.8 to 96.2% rare phylotypes. A positive relationship was observed between the number of rare and observed phylotypes, for both archaeal ($p < 0.01$) and bacterial ($p < 0.02$) clone libraries. There was a significant relationship between library size and the number of rare phylotypes (positive linear regression slope = 0.87, $r^2$ = 82.5%, $p < 0.01$) but only for the archaeal clone libraries.

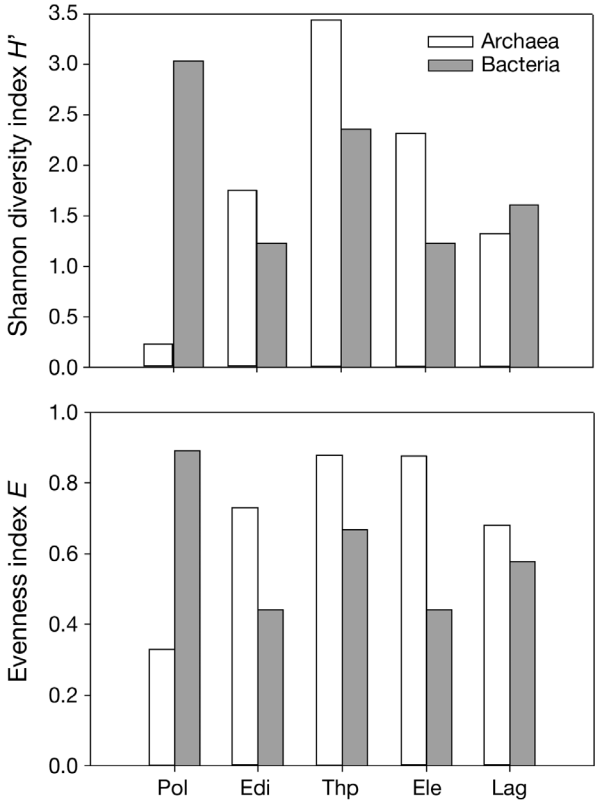


Fig. 3. Shannon diversity ($H'$) and evenness ($E$) indices for the bacterial and archaeal 16S rRNA gene clone libraries, from the geothermal spring waters of Polihnitos (Pol), Edipsos (Edi), Thermopiles (Thp), Eleftheres (Ele) and Lagadas (Lag), Greece

## DISCUSSION

Our experimental approach targeted on revealing the largest extent of the prokaryotic 16S rRNA genetic diversity in 5 geothermal springs in Greece, with temperatures ranging from 36 to 89°C. This was achieved by (1) using primer mixtures at low annealing temperature (50°C) to avoid any mismatches that could exclude some microorganisms (Sipos et al. 2007), (2) PCR cycle optimization in order to eliminate innate PCR artifacts (v. Wintzingerode et al. 1997) and reduce relative differences in abundance between the more abundant and rarer OTUs, and (3) examining the clone library coverage of each library before closing the library, without the limitation of a pre-determined minimum clone number to be analysed. Kemp & Aller (2004) have already stressed the scarcity of high clone coverage studies and the importance of knowing the full extent of diversity in environmental clone libraries for a better understanding of the role of diversity in the functioning of the ecosystem. The Shannon diversity index $H'$ causes overestimation in samples with low

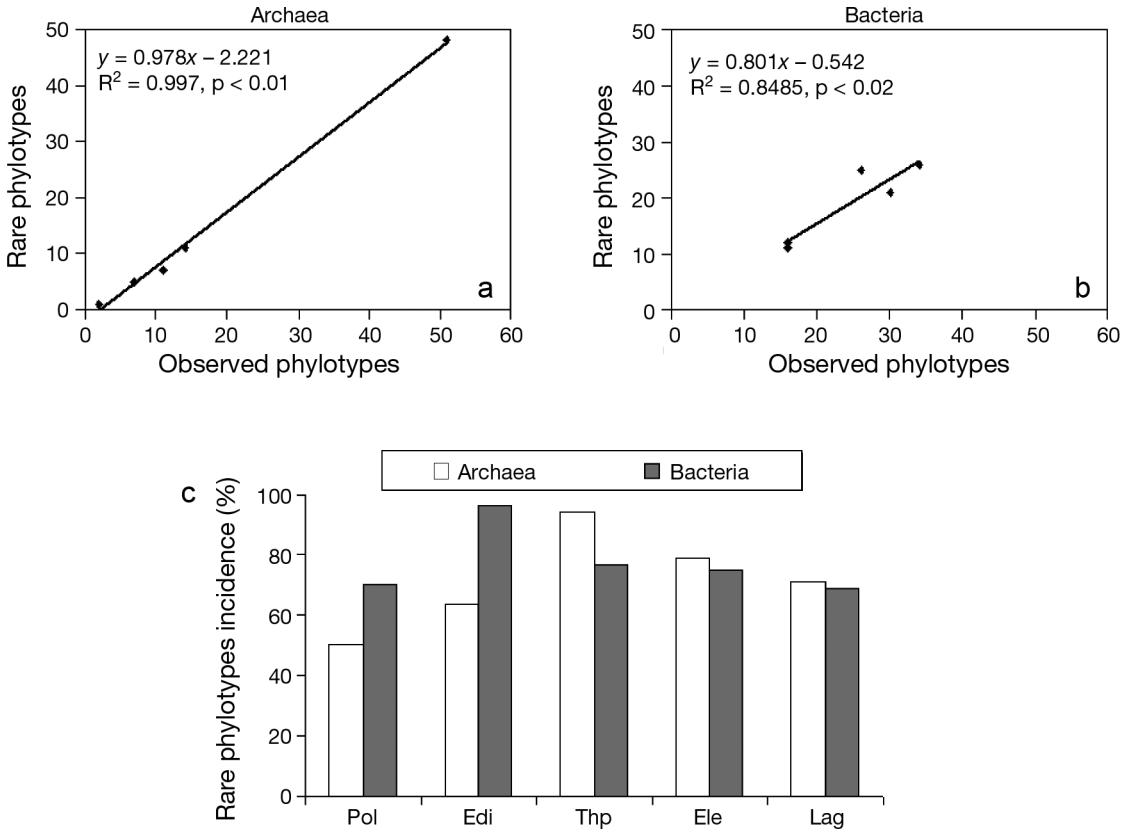


Fig. 4. Comparison of the number of rare phylotypes (i.e. those which occur only once or twice in a clone library) to the total number observed in all (a) archaeal and (b) bacterial libraries. Libraries which fall below the 1:1 line tend to have uniformly abundant phylotypes, while libraries above the line have a skewed distribution with many rare phylotypes. (c) % Contribution of rare phylotypes in each of the clone libraries from the geothermal spring waters of Polihnitos (Pol), Edipsos (Edi), Thermopiles (Thp), Eleftheres (Ele) and Lagadas (Lag), Greece

coverage (Hill et al. 2003). Our satisfactory clone library coverage allowed us to reveal the 'vast' majority of the existing prokaryotic diversity in most of the studied springs, albeit to a lesser degree for the Archaea in Thp and Ele, making the Shannon diversity index *H'* applicable and realistically informative.

The prokaryotic cell abundance was low compared with other geothermal springs (Kimura et al. 2005, Belkova et al. 2007, Hetzer et al. 2007, Mathur et al. 2007) but comparable with values from geothermal subsurface environments (Schulze-Makuch & Kennedy 2000, Kieft et al. 2005). The lack of correlation of cell abundance with temperature, pH, salinity and dissolved oxygen content (data not shown), suggests that the observed low cell numbers are controlled by other factors, such as the availability of electron acceptors and/or viral infection (Lee et al. 2007).

It is known that Crenarchaeota dominate in geothermal springs (Barns et al. 1994, Huang et al. 2007). In the present study, only 10 of the 85 archaeal OTUs belonged to the Euryarchaeota, while one spring (Ele) had no euryarchaeotal phylotypes. In addition, most of the discovered Euryarchaeota phylotypes were not abundant in the clone libraries where they came from, except for Edi-A-46 which represented 18.2% of all existing archaeal phylotypes in this specific library. The dominance of the Crenarchaeota is not so surprising since, at least in terms of cultured representatives, practically all members of this group are thermophiles; in contrast, Euryarchaeota are more diverse both in metabolism and habitats, and include mesophilic, thermophilic, and hyperthermophilic organisms as well as extreme halophiles in terms of temperature tolerance. The possibility that Crenarchaeota and Euryarchaeota possess different ecological niches (i.e. spatial or temporal dominance of the former or latter) in the same ecosystems has also been suggested for planktonic communities in Antarctic regions (Massana et al. 1988, Murray et al. 1998), in the deep Atlantic Ocean (Herndl et al. 2005), the North Sea (Herfort et al. 2007) and deep hypersaline anoxic basins (van der Wielen et al. 2005). In Yellowstone geothermal springs (Barns et al. 1994, 1996) and in the hot, reducing, and iron and zinc sulfide-rich interior regions of a deep-sea

hydrothermal vent black smoker chimney, the Crenarchaeota dominated the whole prokaryotic community (Takai et al. 2001, Schrenk et al. 2003); however, the Euryarchaeota dominated in a white smoker spire in the East Pacific Rise (9° N, Kormas et al. 2006) and in a geothermal aquifer (Kimura et al. 2005). Nevertheless, the actual relative abundance of Crenarchaeota and Euryarchaeota in Greek geothermal springs remains to be confirmed, preferably using fluorescence *in situ* hybridization (FISH).

In the present study, the physiological features of the discovered Crenarchaeota OTUs cannot be inferred with great confidence since most of them were related to phylotypes of yet-uncultivated species. In addition, physiologic traits are not necessarily always coherent within phylogenetic groups. However, these environmental sequences originated from hydrothermal, geothermal, or anaerobic environments suggesting that the discovered phylotypes could represent indigenous thermophilic organisms of the geothermal springs. Such uncultivated thermophilic Crenarchaeota are abundant in geothermal areas which are rich in hydrogen, Fe(II) and sulfur at many oxidation states, and may also be chemolithotrophic. Overall, the majority of the Archaea we found are possible members of an indigenous hot subsurface community except for those in Lag, Lag being a shallow spring with upcoming water that is mixed with meteoric water (Traganso et al. 1995). This spring is more susceptible to 'contamination' from soil microorganisms, which are similar to its several soil-related Archaea phylotypes, while its low temperature may inhibit or eliminate thermophilic microorganisms coming from the deep subsurface. Finally, it is possible that there is a higher variety of substrates originating from the more physically and chemically complex nature of soil in Lag.

The finding of nonthermophilic Crenarchaeota in warm and hot environments is becoming increasingly common (Jurgens et al. 1997, Kanokratana et al. 2004, Kvist et al. 2005, 2007, Huang et al. 2007). We also found several such OTUs that are especially related to tropical estuarine sediments and fresh water. Kvist et al. (2005) suggested that DNA from nonthermophilic Archaea is not amplifiable; thus, the phylotypes found in such clone libraries represent Archaea that may also tolerate or even grow at higher temperatures. Considering our stringent PCR protocols and the large enough clone libraries, we believe that the nonthermophilic OTUs we have found could also be thermophilic.

Unlike the archaeal phylotypes, several of the bacterial phylotypes were related to described bacterial species. The dominance of phylotypes related to thermophilic phylogenetic groups and/or environmental sequences retrieved from habitats with moderate to high temperatures falls from at least 68.2 and 78.5% for Pol and Edi, respectively, to 35.6, 14.7 and 1.8% for Thp, Ele and Lag, respectively. As most of the studied springs are subject to the mixing of geothermal fluids with meteoric waters at some point in their fluid ascension (Lambrakis & Kallergis 2005, Duriez et al. 2008), it is believed that microorganisms originating from cooler environments will have less chances of surviving in the waters of those springs with the highest temperatures, like Pol and Edi, unless they have protective adaptations, like spore formation (e.g. Baker et al. 2001). Geothermometer calculations (Lambrakis & Kallergis 2005) suggested that the temperature of the deep subsurface waters of the studied springs are 142–180, 109–152, 124–164, 218–241 and 131–171°C for Pol, Edi, Thp, Ele and Lag, respectively. Based on the measured surface temperatures in the present study, Pol and Edi showed the lowest difference between the temperatures of deep and surface waters (53 to 91 and 27 to 70°C, respectively). Such small differences between deep subsurface and surface waters imply either a faster flow (i.e. less chances for contamination from non-subsurface microorganisms) and/or less mixing with meteoric water. This renders the Pol and Edi springs more likely of hosting an indigenous hot subsurface community. Indeed, only in Pol and Edi are the most abundant phylotypes closely related to the thermophilic microorganisms *Hydrogenophilus thermoluteolus* and *Persephonella hydrogeniphila*, respectively; in the rest of the springs, mixtures of thermophiles and mesophiles prevail. Pol and Edi also had the highest G+C contents (60.1 and 55.7%, respectively; Fig. 5). It is known that the G+C content of 16S rRNAs is positively correlated with $T_{opt}$ for bacterial species (Galtier & Lobry 1997). Within the same springs, the archaeal 16S rRNAs also had the highest G+C contents (60.2 and 58.1%, respectively).

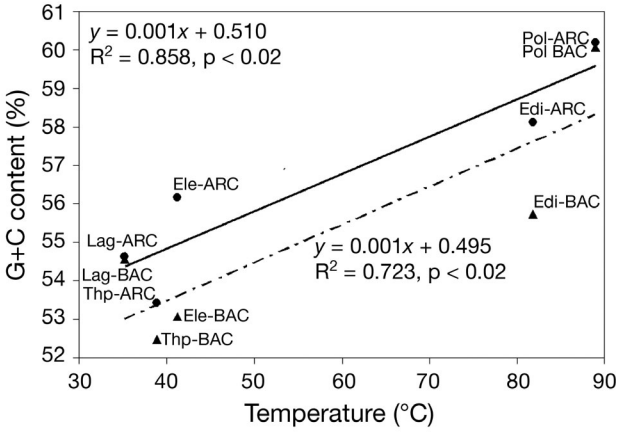


Fig. 5. Relationship of the G+C content of the retrieved bacterial (BAC) (–·–·–) and archaeal (ARC) (——) 16S rRNA gene sequences with the prevailing temperature in the geothermal spring waters of Polihnitos (Pol), Edipsos (Edi), Eleftheres (Ele), Thermopiles (Thp) and Lagadas (Lag), Greece

Members of the γ-Proteobacteria dominate in sulfidic and marine environments and are mostly involved in ecosystem function via chemolithoautotrophic metabolic pathways (Campbell et al. 2006). Recently, the first nonmarine natural system where this group dominates has been reported (Porter & Engel 2008). Here we also report their dominance in a freshwater geothermal spring (Ele) and additionally, their dominance in a brackish spring (Thp), thus supporting the cosmopolitan distribution of this group.

The importance of phylogenetically rare prokaryotic phylotypes in environmental samples has been recently highlighted (Sogin et al. 2006, Aller & Kemp 2008). The occurrence of rare phylotypes in all of our investigated archaeal and bacterial clone libraries started at 50% and reached 96.2%. Such high numbers of rare phylotypes indicate the high species richness of the systems where they are found. It has been suggested that in microbial populations, rare species can possess a survival advantage by directly competing with the dominant ones (Sogin et al. 2006). For the Archaea in the geothermal springs studied here, we suggest that rare phylotypes play a more central role in enhancing phylogenetic diversity as revealed by the linear relationship between the number of rare of phylotypes and library size.

In conclusion, the apparent richness and community composition of Bacteria and Archaea in the studied geothermal springs showed different patterns. Diversity was unrelated to the prevailing temperature, pH, salinity and dissolved oxygen concentrations. The inferred ecophysiology of at least the dominant phylotypes, coupled with geothermometer estimations, suggests that at least for the 2 hottest springs, an indigenous deep and hot subsurface community reaches the surface. In all other springs, microbial contamination due to the mixing of geothermal water with meteoric water, shapes different prokaryotic communites in terms of species richness and diversity.

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