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Archaeal and bacterial diversity in two hot springs from geothermal regions in Bulgaria as demostrated by 16S rRNA and GH-57 genes

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Summary. Archaeal and bacterial diversity in two Bulgarian hot springs, geographically separated with different tectonic origin and different temperature of water was investigated exploring two genes, 16S rRNA and GH-57. Archaeal diversity was significantly higher in the hotter spring Levunovo (LV) (82°C); on the contrary, bacterial diversity was higher in the spring Vetren Dol (VD) (68°C). The analyzed clones from LV library were referred to twenty eight different sequence types belonging to five archaeal groups from Crenarchaeota and Euryarchaeota. A domination of two groups was observed, *Candidate* Thaumarchaeota and Methanosarcinales. The majority of the clones from VD were referred to HWCG (Hot Water Crenarchaeotic Group). The formation of a group of thermophiles in the order Methanosarcinales was suggested. Phylogenetic analysis revealed high numbers of novel sequences, more than one third of archaeal and half of the bacterial phylotypes displayed similarity lower than 97% with known ones. The retrieved GH-57 gene sequences showed a complex phylogenic distribution. The main part of the retrieved homologous GH-57 sequences affiliated with bacterial phyla Bacteroidetes, Deltaproteobacteria, *Candidate* Saccharibacteria and affiliation of almost half of the analyzed sequences is not fully resolved. GH-57 gene analysis allows an increased resolution of the biodiversity assessment and in depth analysis of specific taxonomic groups. [**Int Microbiol** 18(4):217-223 (2015)]

Keywords: Archaea · hot spring · phylogenetic analysis · 16S rRNA gene · GH-57 gene

Introduction

It is commonly accepted that microorganisms isolated by conventional approaches are less than 1% of microorganisms in any given environment [9] and this value is even lower in extreme niches. The application of molecular-based methods is the only way for revealing the amazing microbial diversity in environmental niches. Terrestrial hot springs and oceanic hydrothermal vents are unique places to study microbial diversity under the pressure of one or more extreme factors. Many of the inhabitants identified in such niches refer to still unknown phylogenetic units and even groups, especially among archaea representatives. Most of the so far reported molecular analyses of microbial biodiversity are based on 16S rRNA gene. However, the expanding of analyses including additional metabolic genes have become increasingly popular. According to Xu [26], the characterization of

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biodiversity through more than one marker gene provides additional advantages including: (i) draws more convincing conclusions based on the extended information; (ii) the results are more representative for the whole genome as more genes are included; (iii) horizontal gene transfer could be ignored by analysis of more genes. Such studies usually involve simultaneous comparative analysis of 16S rRNA and enzyme genes including: *amoA* (ammonia monooxygenase gene) for characterization of ammonia-oxidizing bacteria and archaea [4]; *mcrA* (methyl coenzyme-M reductase) for methanogen diversity [11]; chitinase and glycoside hydrolase family 4 genes for heterotrophic microorganisms [2]. Accelerated genome sequencing over the last two decades has made it possible the comparative analysis of larger sets of homologous genes from wide range of cultured bacteria and archaea and metagenome sequencing assemblies [17], thus facilitating the application of the gene-specific characterization of microbial biodiversity and enhancing the efficiency of isolation of novel genes from uncultivated microorganisms growing at specific environmental conditions.

Glycoside hydrolase-57 (GH-57) family of proteins has been relatively recent described, and the number of its members has grown and now includes more than 1100 bacterial and archaeal proteins [http://www.cazyorg/Glycoside-Hydrolases.html]. Generally, five enzyme activities have been experimentally associated to the GH-57 proteins. All of them are of industrial interest, especially α-amylase, amylopullulanase, branching enzymes, 4-α-glucanotransferase and α-galactosidase. The members of the GH-57 family have been subject of several extensive bioinformatics studies pointing out the main conservative regions and sub-family clustering [5,8]. However, apart from the great interest in identification of novel enzymes with amylolytic activities and characterization and utilization of GH-57 members among them, only a very small part (less than 2%) of this protein family has been biochemically characterized.

Here we report the characterization of microbial biodiversity of two hot springs based on 16S rDNA and GH-57 sequences. The results are analyzed comparatively trying to elucidate the impact of each gene for revealing the real diversity in studied hot springs.

Materials and methods

Description of the study sites. Bulgarian hot springs investigated in this study are located in geographically different areas. The spring Levunovo (LV) is located in South-West Bulgaria (N 41°28′59.988″, E 23°18′0″), in Struma fault zone formed in Precambrian age. The spring Vetren Dol (VD) is located in Maritsa fault zone (N 42°08′60.0″, E 24°08′60.0″) along the northern margin of the Rhodope Mountains formed in the Paleozoic and Cretaceous-Paleogene. The mineral composition of water in LV spring was determined as containing (mg/l): Na^+ (89), K^+ (23.5), Ca^{2+} (10), Mg^{2+} (0.9), HCO_3^- (66), F^- (3.6), Cl^- (1.0), SO_4^{2-} (33), SiO_3^{2-} (1.6), pH around neutral (7.6); water for VD contained higher ion concentrations: Na^+ (97.8), K^+ (45.6), Ca^{2+} (0), Mg^{2+} (1.08), F^- (4.2), Cl^- (20.59), SO_4^{2-} (259), HCO_3^- (179.96), SiO_3^{2-} (18.74), pH around neutral (6.8).

16S rDNA and GH57 clone libraries. Effluent water samples were collected aseptically from the spring outlet on the ground surface, transported to the lab in thermostat bags and used for isolation of a total DNA. Five-liter water samples were concentrated by cross-flow filtration through sterile hollow fiber cartridges (1.2 μm pore-size glass fiber prefilter and 0.2 μm membrane filter; Millipore). Environmental DNA was isolated from environmental samples according to a previously described procedure [7]. Bacterial 16S rDNA were amplified by two universal primers: 8F (EUB008) and 1492R (EUB1492). Archaeal 16S rDNA were amplified using two universal archaeal primers, 21F and 958R. The 16S rDNA fragments were PCR amplified using DreamTaq PCR master mix (Thermo Scientific) and PCR conditions: denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and final extension at 72°C for 5 min. The degenerated primers pairs used for PCR amplification of fragment of GH-57 genes were: AMBF (5′- TTYGAASTNCAYCARCC) × AMBR (5′- TGYTCNCCRAANGTYTCRTARTC) and AMBF × ABDRI3 (5′- CACATATAATARAAATGRTC). The 16S rDNA fragments were PCR amplified using Phusion High-Fidelity DNA Polymerase master mix (Thermo Scientific) and PCR conditions: 30 s denaturation at 98°C, followed by 35 cycles at 98°C for 10 s, 46°C for 30 s and 72°C for 45 s and final extension at 72°C for 7 min. The obtained PCR products were subject of agarose gel electrophoresis and purification using NucleoSpin Extract II Kit (Macherey-Nagel). The purified 16S rDNA and GH-57 fragments were cloned into a pJET 1.2 vector of a CloneJet PCR Cloning kit (Thermo Scientific). The DNA fragments cloned into the pJET 1.2 vector were PCR amplified using vector primers and digested separately by the *Msp I* and *Hae III* endonucleases. The products from each digestion were separated following electrophoresis in 2% agarose gel. The analyzed clones were grouped according to their restriction profiles. Representative clones of each group were sequenced at both ends using the pJET forward and reverse primers by the Macrogen Sequencing Service.

Sequence analysis. The obtained sequences were assembled and manually edited using Vector NTI v. 10 software package (Life Technologies). The obtained 16S rDNA sequences were first checked for chimera sequences by 'DECIPHER Find Chimeras' web tool [25] and those detected ones were not used for further study. The clones containing correct inserts were compared to the known sequences by using BLAST search [1] and Ribosomal Database Project resources [12] to determine their close relatives and approximate phylogenetic affiliations. The putative amino acid sequence for each GH-57 clone was determine based on the GH-57 nucleotide sequences using AMBF primer sequence as starting point of the reading frame. The generated GH-57 sequences were used to search sequences in GenBank database using blastp, DELTA-BLAST and Tblastn search tools. The 16S rDNA and GH-57 sequences together with selected pools of retrieved sequences were subject of phylogenetic analyses using MEGA software [20]. The sequences obtained in this study were deposited in the GenBank database under accession numbers KJ465919 to KJ465965 (bacterial 16S rDNA clones), HF922629 to HF922668 (archaeal 16S rDNA clones), and KJ465966 to KJ465987 (GH-57 clones).

Results and Discussion

Archaeal diversity based on 16S rRNA gene. 16S rRNA gene clone libraries were successfully constructed using environmental DNA from two Bulgarian hot springs and almost the same number of clones was analyzed (86 for LV and 84 for VD,Fig. 1A). The comparison of biodiversity in the two springs, LV (temperature of water 82°C) and VD (temperature of water 64°C), confirmed the observation of increasing archaeal diversity in thermal environments with temperature rising [16].

On the basis of the gene analysis, significantly higher archaeal diversity was observed in LV hot spring and high abundance of phylotypes was established. The analyzed clones were realated to twenty eight different sequence types belonging to five archaeal groups from Crenarchaeota and Euryarchaeota. A strong domination of two groups was observed—the group I.1b (40.6% of the clones, 11 sequence types) and Methanosarcinales (35.4% of the clones, 9 sequence types). Less frequent were the groups MCG (8.3%, 3 sequence types), Methanobacteriales (3.1%, 2 sequence types) and I.3b (2.1%, 2 sequence types). Most of the clones (81) from VD were related to HWCG (96.4%, 12 sequence types). Similarly to other investigations of the archaeal diversity, the retrieved clones grouped together with environmental uncultured clones confirmed the common acceptation that archaea are much more versatile than it is represented by validly recognized and *Candidate* types. Even in both recognized phyla Crenarchaeota and Euryarchaeota, most archaeal groups are still unculturable; they are formed on the basis of environmental clones and comprised putative taxons [34].

Most of the sequences from LV libraries are affiliated with soil group I.1b, resigned to *Candidate* Thaumarchaeota [19]. Clustering of the sequences from thermal environment with sequences from nonthermophilic I.1b have been reported by several authors and widening of the group I.1b to comprise thermophilic members has been suggested [13]. Domination of the sequences related to the moderate thermophiles from the genus *Nitrososphaera* in archaeal communities has been observed by other authors [4,7].

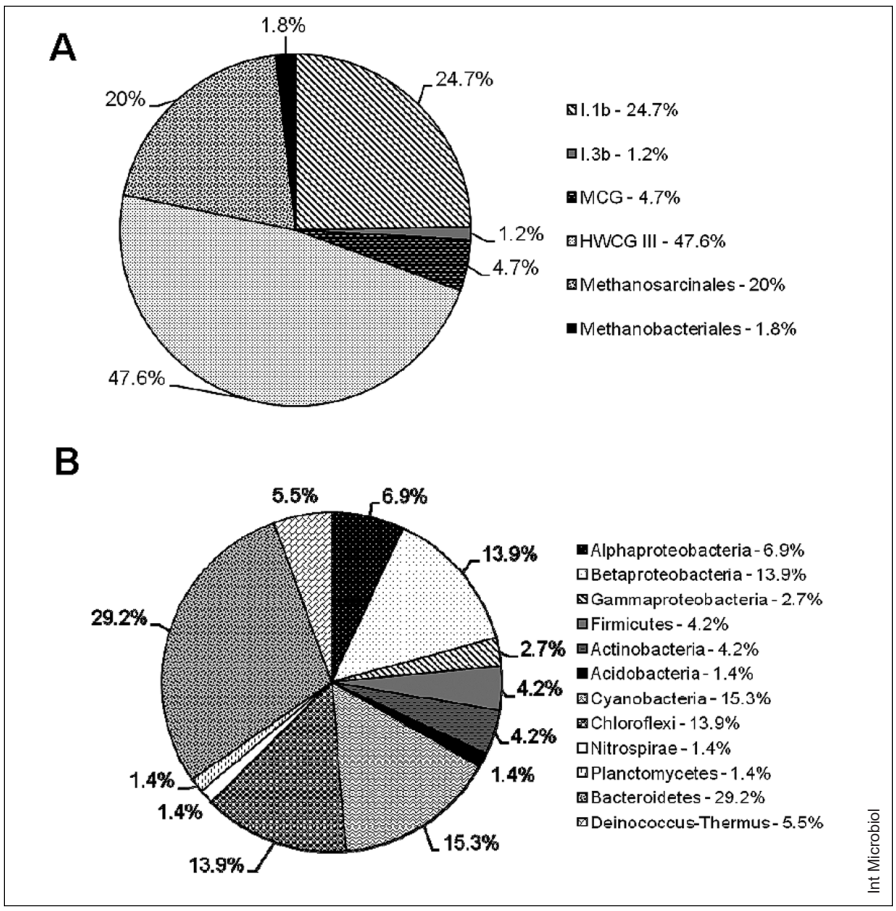

Fig. 1. Relative abundance of 16S rDNA archaeal and bacterial clone groups retrieved from Levunovo and Vetren Dol hot springs. (**A**) Archaea; (**B**) Bacteria.

Relatively small numbers of the sequences retrieved from LV were affiliated with the Miscellaneous Crenarchaeotal Group (MCG). Although MCG has only been defined by 16S rRNA sequences, it appears widespread and characterized by a large MCG intragroup diversity with 16S rRNA gene similarity values as low as 76% [45]. The diversity within most of our subgroups was in the proposed cutoff of 87.7% minimum level for family boundaries [46]. The sequences affiliated with the order *Methanosarcinales* represented the second dominant group in the archaeal community from LV. Although LV-108 grouped close to an uncultured clone related to the genus *Methanomethylovorans,* 16S rDNA sequence similarity for both clones was lower than 89%. The deep branching of the sequences retrieved from the investigated hot spring with a temperature of water higher than 80°C in the group of moderate thermophiles suggests the possibility for the formation of a group of obligate and/or extreme thermophiles in the order Methanosarcinales as it has been suggested for the order Methanobacteriales [24]. This idea is supported by the low similarity of the retrieved sequences with the closest neighbors. Out of 10 novel sequences (less than 97% similarity), six grouped in the clade of Methanosarcinales and, for 3 of them, the phylogenetic distance was more than 10%. One of the clones, LV-128, showed 84% similarity with the phylogenetic neighbor, a value lower than the proposed cutoff of 85% minimum level for phylum boundary [6]. Representatives of the order Methanobacteriales are commonly accepted as mesophiles and thermophilic strains from the genus *Methanobacterium* are included in the genus *Methanothermobacter* [24]. The LV-39 and LV-75 sequences, identified from LV sample, were close to the 16S rRNA gene of *Methanothermobacter defuvii*.

Most of the sequence types from VD were grouped around the 16S rRNA gene of *Candidatus* Nitrosocaldus yellowstonii, suggesting an active participation of archaea in ammonia-oxidation in this spring. Despite the fact that they represented 12 different sequence types, most of them (11) showed phylogenetic distances lower than 3% with the phylogenetic neighbors.

Bacterial diversity based on 16S rRNA gene. The characterization of the bacterial 16S rDNA libraries derived from the two studied hot springs also revealed a high abundance of phylotypes, totally 73 groups with different restriction profiles. The sequencing and chimera analysis of represented rDNA clones from each phylotype identified 26 chimeric sequences, which have been omitted from the subsequent microbial diversity analysis. The remaining 47 rDNA sequences branched among 10 bacterial phyla demonstrating unusual high bacterial diversity in the studied springs (Fig. 1B). Four of those sequences involved rDNAs common in both springs. Correspondingly, the number of the rDNA clones affiliated to these four phyla dominated in the studied rDNA pools of the springs, namely Bacteroidetes 29.2% (25% for LV and 32.5% for VD), Proteobacteria 23.5% (39.2% for LV and 13.8% for VD), Cyanobacteria 15.3% (7.1% for LV and 20.9% for VD), and Chloroflexi 13.9% (7.1% for LV and 18.6% for VD). The phylogeny for the dominant phyla revealed considerable diversity among phylotypes. Additionally, sequences of Firmicutes and Actinobacteria representatives were retrieved in LV, and Deinococcus–Thermus, Acidobacteria, Nitrospirae and Planctomycetes in VD. Proteobacteria, Bacteroidetes, Cyanobacteria, Chloroflexi, and Firmicutes have been found to be widely distributed in both terrrestial and aquatic environments [14,15,21]. *Meiothermus* is established to dominate among the sequences affiliated to the phylum Deinococcus–Thermus. The genera *Thermus* and *Meiothermus* are commonly present in global hot springs; however, they are not dominant in terrestrial hot springs, except for a few springs in Iceland [22] and Bulgaria [23]. Planctomycetes, Acidobacteria and Nitrospirae account for only about 1% each of phylotype abundance. The sequences phylogenetically affiliated with as-yet-uncultured Planctomycetes lineages are identified. The retrieved Acidobacteria sequence (KJ465963) shows less than 90% similarity to its phylogenetic neighbors. Acidobacteria is a vast group of organisms virtually unknown prior to rDNA sequence-based surveys and now its diversity and wide spreading are commonly accepted [3]. Actinobacteria (4.2%) are often considered as soil-borne bacteria and although various studies have focused on microbial ecology of this phylum data for the presence of its representatives in hot waters are scant. Its unexpected capability of adapting to hot spring environments was reported for the first time by Song et al. [18].

Closest phylogenetic affiliations found with a number of physiological groups suggested possible mode of metabolism or thermophily of inhabitants of LV and VD, although conclusions based on environmental sequences are only hypothetical. The presence of the autotrophic Cyanobacteria and *Chloroflexus* suggested the probable importance of primary production in providing nutrients to other taxa via photoexcretion. Chemolithoautotrophic nitrite-oxidizing bacteria affiliated with the genus *Nitrospira* are widespread in environments with elevated temperatures [10]. Organotrophic organisms probably were common in the investigated springs, supported by the primary productivity of the hydrogen metabolizers.

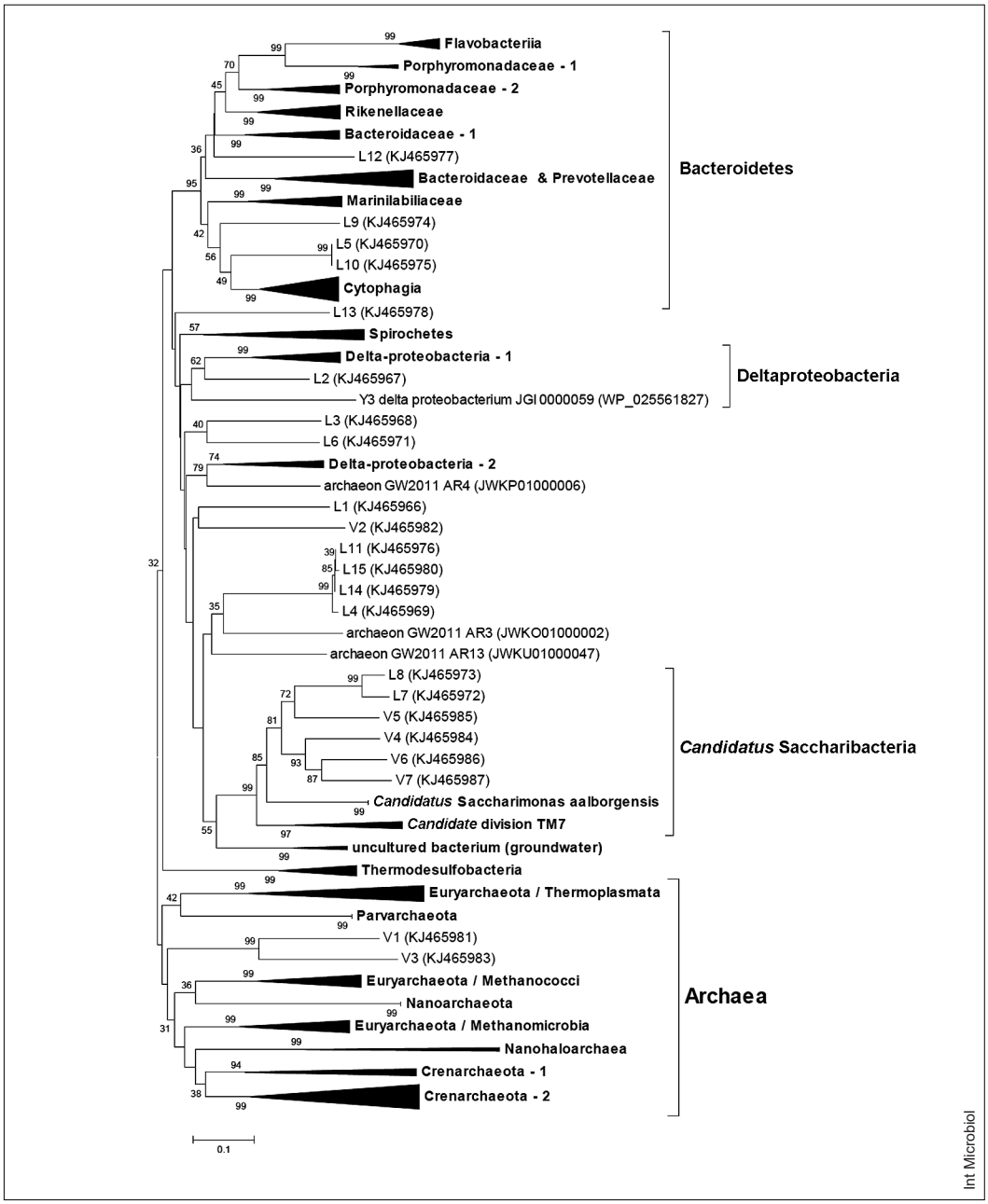

Fig. 2. Neighbor joining phylogenetic tree of GH-57 amino acid sequences constructed from GH-57 clone sequences from Levunovo and Vetren Dol hot springs and their closest homologous sequences from the archaea and bacteria taxonomic groups retrieved after BLAST search. Bootstrap values greater than 30% confidences are shown at branching points (percentage of 1000 resamplings). Sequences from the Levonovo and Vetren Dol hot springs are designated with the abbreviation 'L' and 'V'. All phylogroups without studied hot spring phylotypes are presented as subtree triangles.

About one third of the phylotypes (10) showed high to very high database matches (≥97% identity) and clustered well with their closest relatives. Half of the phylotypes (14) displayed very low identity scores (≤90%), and their respective identification remain only putative. These results suggests a presence of unknown taxa in the investigated springs.

Microbial diversity based on GH-57 gene. Among the combinations of the tested degenerated primers only two primer pairs produced abundant single band PCR products at expected sizes of approximately 700 bp for AMBF × AMBR pair and 1050 bp for AMBF × ABDRI3 pair. The AMBF forward primer matched the FExHQP sequence

corresponding to the 'conserved sequence region 1' (CSR-1) of GH-57 family [11,16]. Accordingly, the reverse primer AMBR matched DYETFGE sequence of the CSR-4 and ABDRI3 primer matched DHFYYM sequence of the CSR-5. The initial sequence characterization of the GH-57 clone libraries shows that all sequences of the shorter GH-57 clones derived from the PCR amplification with AMBF × AMBR primer pair overlaped with part of the longer GH-57 clones from AMBF × ABDRI3 primer pair and the last was used further in the study. The characterization of the entire AMBF × ABDRI3 clone libraries from the two hot springs showed higher diversity in hotter spring, totally 22 phylotypes, 15 from LV and 7 from VD. The analysis of the nucleotide sequences of representatives of each phylotype demonstrated that 20 clones contained the primer sequences at both ends and 2 clones were truncated and contained AMBF, but not the ABDRI3 primer sequence. The nucleotide sequences were further used to generate putative GH-57 amino acid sequences using the AMBF primer sequence as a starting point. The alignment of the obtained GH-57 amino acid sequences showed they shared from 43% to 98% sequence identity and 65% to 100% similarity. The BLAST search retrieved totally 448 bacterial and 116 archaeal sequences showing above 50% of amino acid sequence similarity at full length (>90%) coverage of the analyzed sequence query. The retrieved sequences have been designated as members of GH-57 protein family. Most of the retrieved GH-57 sequences originated from genome sequences of cultured bacteria and archaea, as the rest came from sequence assemblies of uncultured microorganisms following next generation sequencing of metagenomic DNA or sequencing of metagenomic clones. The main part of the retrieved homologous GH-57 sequences were originated in the phylum Bacteroidetes and were spread among its three main classes. On the contrary, all homologous sequences identified in the well characterized phylum Proteobacteria (44,046 genomes) were related only to the class of Deltaproteobacteria.

A distinct result of the performed BLAST search was that homologous GH-57 sequences were abundant among the sequence assemblies from sequencing of metagenomic DNAs related to representatives from recently coined and still poorly characterized bacterial phylum *Candidatus* Saccharibacteria, as well as unclassified members of archaeal phylum Crenarchaeota and unclassified archaea. The last supports the expectations that genomes reconstruction from metagenome sequencing would increasingly serve as rich data source for complex characterization of biodiversity and phylogenetic distribution of specific metabolic gene families, employing cultivation-independent approach. The phylogenetic distribution of the retrieved GH-57 homologous sequences among the archaeal phylum was more uniform, although no homologous sequences were retrieved from some of the better genomically characterized orders of the phylum Euryarchaeota, including the orders Halobacteria, Methanobacteria and Thermococcus. The phylogenetic tree, constructed from the analyzed GH-57 sequences and homologous representatives of different phylogenetic groups, further demonstrates the complexity of the isolated GH-57 pool (Fig. 2).

Two sequences (V1, V3) are allocated among the archaeal, and three (L2, L3, L6) among the Deltaproteobacteria GH-57 sequences. The affiliation of five analyzed sequences (L1, L4, L14, L15 and V2) is not fully resolved. Together with the GH-57 sequences of two unclassified archael clones, they have been allocated among the bacterial GH-57 sequences and entered into a group of sequences originated from the recently metagenomically characterized groundwater microorganisms—unclassified archaea, uncultured bacterium and members of phylum *Candidatus* Saccharibacteria. The complexity of the isolated GH-57 clones demonstrated by the phylogenetic analysis suggested that the AMBF × ABDRI3 degenerate primer pair allowed efficient PCR amplification of GH-57 sequences, containing FExHQP and DHFYYM conserved sequence regions, from phylogenetically diverse bacteria and archaea. The comparison of the results from 16S rDNA and GH-57 analyses of the microbial diversity of the studied hot springs showed that biodiversity based on GH-57 analysis was only a fraction of the 16S rDNA biodiversity, due to the probable lack of target GH-57 sequences in the genomes of the auxotrophic members of several bacterial and archaeal phyla and lower taxonomic groups. From the other side, the lack of the 16S rDNA clones affiliated to the *Candidatus* Saccharibacteria and the identification of relatively larger set of GH-57 clones clustered with known GH-57 sequences related to this phylum demonstrated that metabolic genes analysis would allow an increased resolution of the biodiversity assessment and in-depth analysis of specific taxonomic groups.

The lower bootstrap affiliation of part of the isolated GH-57 clones to unclassified archaeal GH-57 sequences originated from metagenomic sequence assemblies suggested that despite the large and steady growing number of sequenced microbial genomes, the currently available genomic sequence data are still insufficient to resolve fully the phylogenetic distribution of the isolated GH-57 pool and to cover the range of GH-57 diversity.

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Competing interests. None declared.

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