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MOLECULAR GENETIC MONITORING OF BACTERIAL COMMUNITIES IN MANZALA LAKE, EGYPT, BASED ON 16S RRNA GENE ANALYSIS

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ABSTRACT

A first molecular genetic study on the diversity of bacterial communities at Manzala Lake, Egypt, was determined by culture-independent 16S rRNA gene analysis. Bulk DNAs were extracted from water and sediment at two different sampling sites namely; Bashtir and Genka, in the lake. The 16S rRNA gene was positively amplified by polymerase chain reaction (PCR) from bulk DNA of each sample, cloned and sequenced. The sequence analysis of one hundred clones from each clone library obtained number of phylotypes ranged from 8 phylotypes, in Genka water, to 20 phylotypes in each of the sediment samples. The 16S rRNA gene showed diversity in sediment samples higher than in water samples. Based on Libshuff pairwise clone library comparisons, the 16S rRNA gene phylotypes from Bashtir water encompassed the phylotypes of Genka water, while the phylotypes of Genka water account only a portion of those of Bashtir water. Both water and sediment samples were dominated by phylotypes closely related to different classes of the phylum Proteobacteria. Most of the recovered water phylotypes from the two sites were localized in the branches of Cyanobacteria, Proteobacteria and Bacteroidetes. Genka water was differentiated from Bashtir by harbouring a phylotype related to the phylum Firmicutes. Several phylotypes from Bashtir and Genka sediments were affiliated to species from the phyla Proteobacteria, Chloroflexi, Bacteroidetes, and Planctomycetes. Genka sediment sample had two phylotypes affiliated to Fusobacteria, a character differentiated this sample from that of Bashtir, which characterized by a phylotype belonging to Actinobacteria. Other sediment phylotypes constituted unique phylogenetic lineages, suggesting new bacterial phyla. The obtained results clearly demonstrated that each studied site has unique bacterial phylotypes that can be used as site-characterizing biomarkers. The successes of this study were the determination of the actual composition of bacterial populations in the studied samples and the discovery of novel bacterial phylotypes, never recorded by traditional techniques.

1. INTRODUCTION

Bacteria account efficient biomarkers for many natural and anthropogenic activities in the aquatic environment. Consequently, the need to identify bacteria is a major impetus for the development of techniques.

The Gram stain, developed in 1884 by Hans Christian Gram, characterizes bacteria based on the structural characteristics of their cell walls. Culture techniques have been designed to promote the growth and identify particular bacteria, while restricting the growth of the other bacteria in the sample. Hence, microbial culture and isolation processes, often, suffer from bias in the growth of microbes and are neither quantitatively nor qualitatively represent the total microbial community in the respective environment (Wagner *et al*., 1993). In

addition, most of environmental bacteria can not be cultured in the laboratory (Suzuki *et al*., 1997).

The identification and classification of bacteria is increasing using molecular genetic methods. Bacterial identification using such DNA-based tools, such as polymerase chain reaction, is increasingly popular due to their specificity and speed, compared to culture-based methods (Louie *et al*., 2000). These molecular methods also allow the detection and identification of "viable but unculturable" bacteria that are metabolically-active but non-dividing (Oliver, 2005).

With the introduction of molecular methods based on sequencing of the small-subunit ribosomal RNA (SSU rRNA) genes, it becomes possible to make a complete analysis of microbial diversity in an environmental sample (Olsen *et al*., 1986). The retrieval of the 16S rRNA genes directly from the environment has made it possible to estimate the phylogenetic diversity of a natural bacterial community without preceding cultivation steps. In addition, the phylogenetic information obtained by applying the rRNA gene approach to a microbial community may often be helpful in the directed isolation or identification of novel microorganisms from the respective ecosystem (Olsen *et al*., 1986). Numerous studies based on this cultivation-independent methodology, have been carried out to investigate microbes as a key player in many environmental processes in the biosphere (Head *et al*., 1998; Moeseneder *et al*., 2005 and Gentile *et al*., 2006).

Based on 16S rRNA gene analysis, bacteria were classified into approximately 23 known phyla; most of them live in aquatic environment (Whitman *et al*., 1998). Also, non-estimated huge numbers of unclassified, mostly uncultivable, environmental bacteria have been detected in both freshwater and marine environments (Spring *et al*., 2000; Oliver, 2005; Gentile *et al*., 2006). Cyanobacteria, a long with the Alpha (α)-Proteobacteria, constitute the major fraction of bacteria in the studied lake surface waters (Pandey and Pandey, 2002; Eiler and Bertilsson, 2004; Mesbah *et al*., 2007). Most of lake sediments are quite heterogeneous ecosystems, which give rise to many different environmental niches even on millimeter scale. They, therefore, harbor highly complex microbial communities with regard to species composition and metabolic activity (Spring *et al*., 2000).

Egyptian lakes are important sources of water and food. Nutrient cycles in these environments are largely influenced by metabolic processes localized in the surface layers of water and sediments. Lake Manzala has an important impact on fish production due to its water content zone variability, from marine to fresh, suitable conditions for varieties of aquaculture. Unfortunately, the lake suffers from high load of anthropogenic pollutants (Badawy *et al*., 1995; Osfor *et al*., 1998; Abbassy *et al*., 2003). The flow of water into the lake comes mainly from two drains. The sewage Bahr El-Baqar drain discharges highest pollutant load at a lake site called as Bashtir. The Bahr Hados drain feeds the lake with agriculture waste waters at the area of Genka. So, Bashtir and Genka zones represent two different reservoirs of bacterial communities affected by anthropogenic pollution. In this respect, it is of great interest to compare the composition of bacterial populations in both water and sediment from the two sites, in order to reveal what sort of bacteria is indeed restricted to each habitat.

The previous studies on identification of microbial communities in Egyptian lakes focused on microscopic morphological examination and some traditional cultured methodologies of phytoplankton (El-Naggar *et al*., 1998; Abdel-Monem, 2001; Abdel-Karim *et al*., 2006; Sabae, 2006). This study used molecular tools based on the 16S rRNA gene to investigate, for the first time, the bacterial community structures in the most two heavy polluted zones, Bashtir and Genka, of Manzala Lake (Fig. 1). Molecular statistical analyses based on gene diversity and Libshuff analyses of clone libraries were

used to compare bacterial community structures between the two zones. Phylogenetic trees based on 16S rRNA gene sequences were constructed to show the phylogenetic position of each recovered bacterial phylotype.

2. MATERIALS AND METHODS

2.1. Sampling and preparation for DNA extractions

The sampling sites were Bashtir, N31° 11.938′; E32 12º.419′, and Genka, N31º 08.815′; E32º 05.429′, located at the eastern part of Manzala Lake, Egypt (Fig. 1). Surface water and bottom sediment were collected from each site. Approximately, 10 L of surface water, from each site, were filtered on cylindrical 0.2 µm filter membrane units (type Sterivex-GS, Millipore Corp., USA), 500 ml per each filter unit. Filters were washed with 10 ml sterile SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris–HCl, pH 7.6). The inlet and outlet of the filters were capped, and the filters were stored at -30ºC until processed for DNA extraction. Sediments were collected by a clean sterile Ekman grab. At each site, about 100 g were taken from the middle part of the collected sediment, put inside a clean sterile propylene tube and covered with Tris-EDTA buffer, with high concentration of EDTA (100 mM), to chelate Mg^{++} and other nuclease co-enzymes. The sediment samples were stored at -30ºC for DNA extraction.

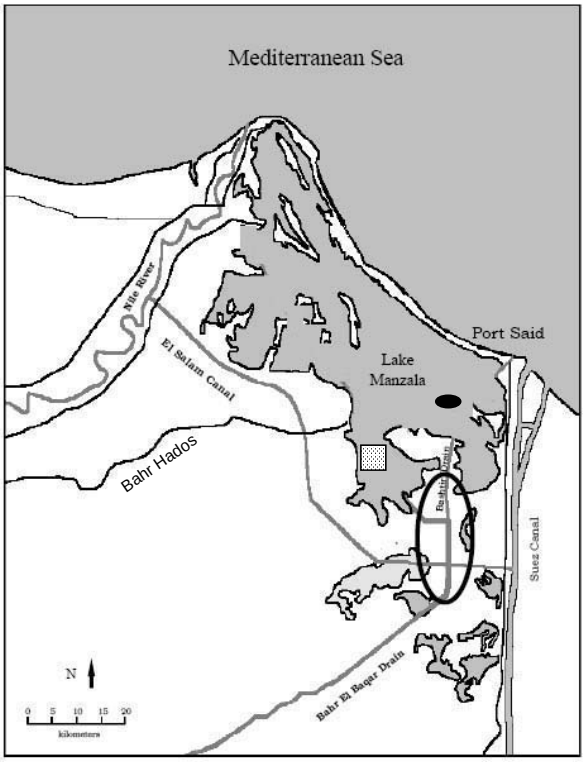
2.2. DNA extraction and PCR amplification of the 16S rRNA gene

In case of water samples, bulk microbial DNA was extracted essentially within the Sterivex-GS filters housing according to the method of Somerville *et al*. (1989) and modification of Elsaied and Naganuma (2001). For each site, the extracted DNAs from filters were combined to obtain DNA of the 10 L filtered water. DNA was extracted from 100 g of sediment, from each sampling site, using the method of Porteuous *et al.* (1994).

PCR amplifications of the 16S rRNA gene, from the purified genomic DNAs, were carried out using the primer sets 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane *et al*., 1985). PCR mixture and thermal cycler conditions were carried out according to Zwart *et al*. (1998). PCR was performed with an initial denaturation step of 3 min at 95°C. The standard reaction continued with 30 cycles of 1 min at 95°C, 40 sec. at the desirable annealing temperature and 1 min extension at 72°C. The 30 thermal cycles were followed by a final extension of 10 min at 72°C to allow 3′-A overhangs for the amplified PCR product to facilitate TA-cloning.

2.3. Construction of clone libraries and sequence analyses

The products of triplicate PCR reactions for 16S rRNA gene amplification were combined and cloned into TOP10 *Escherichia coli* using a TOPO TA-cloning kit according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). White transformed clones were grown overnight in Luria–Bertani broth prior to plasmid extraction with a Qiagen plasmid purification kit (Qiagen, USA). Inserts in the plasmids were sequenced using vector primer T7 and an ABI model 377 sequencer (Applied Biosystems, Foster City, USA). Clone sequences were analyzed by FASTA screening to determine their similarity to known sequences in the DNA database (http:// ddbj.nig.ac.jp). The recovered sequences were aligned using CLUSTAL W software (DDBJ, http://clustalw.ddbj.nig.ac.jp). The sequences that had > 97% nucleotide identity within each clone library were grouped into a phylotype (Godon *et al*., 1997).



Bashtir sampling site

Genka sampling site

Showed the old and new discharge sites of Bahr El-Baqar

Figure 1

2.4. Statistical analyses and construction of phylogenetic trees

The diversity of the resulted phylotypes was analyzed by two statistical methods. (1) Gene diversity within each clone library was estimated using the freeware program Arlequin (Schneider *et al*., 2000), where phylotype pairwise nucleotide differences were measured to extrapolate similarities or differences within the constructed clone libraries. (2) The computer program LIBSHUFF (Singleton *et al*., 2001) was used to estimate homologous and heterologous coverage of clone libraries as a function of evolutionary distance for pairwise reciprocal comparisons (library A compared with library B and vice versa). Differences in coverage were considered significant at P values of < 0.05.

The phylogenetic analyses of the resulted phylotypes and corresponded sequences from the databases were performed by applying the neighbour-joining algorithm and drawing the trees using the MEGA 3.1 software (http://www.megasoftware.net/). The branching patterns of the constructed phylogenetic trees were confirmed by reconstruction of the phylogenies using two other methods of analysis, namely; maximum-parsimony and maximum-likelihood, contained within the PHYLIP package (http://evolution.gs.washington.edu/phylip.html).

2.5. Nucleotide sequence accession numbers and nomenclature of phylotypes

The rRNA gene sequences resulting from this study were deposited in the DNA international database (http://www.ddbj.nig.ac.jp/Welcome-e.html) under the accession no. AB355027-AB355041 for Bashtir water phylotypes, AB355042-AB355049 for Genka water phylotypes, AB355050-AB355069 for Bashtir sediment phylotypes; and AB355070-AB355089 for Genka sediment phylotypes.

The direct recovery of 16S rRNA genes from environmental samples (eg. water and sediment) means that the source organism can not be identified. Consequently, we have adopted a nomenclature whereby each phylotype had the descriptor of the names of sampling site followed by sample type and the number of the recovered phylotype, for example, Bashtir.water.1, Genka.sediment.1 etc.

3. RESULTS AND DISCUSSION

3.1. PCR amplification of 16S rRNA gene revealed diversity in amplicon size in water and sediment samples.

All samples showed positive PCR amplifications of the bacterial 16S rRNA gene. The amplified products (amplicons) from each sample had sizes ranged from 1443 to 1495 bp for Bashtir water; from 1448 to 1492 bp for Genka water; from 1457 to 1515 bp for Bashtir sediment and from 1426 to 1521 for Genka sediment. These variations in amplicon size within each sample indicated the possibility of bias in PCR amplification was minimized. This is because the PCR was tested on the bulk DNA extracted from each sample by A) Increasing the annealing temperature 2°C every 10 PCR cycles to give annealing temperatures ranged from 56 to 60°C, and B) Doing PCR using number of cycles ranged from 26 to 30 cycles, and the amplicons were combined for cloning. These conditions gave the flexibility for the primers to anneal with various 16S rRNA gene templates with different GC contents (Suzuki and Giovannoni, 1996). Hence, the PCR could screen as much as possible of the actual composition of bacterial 16S rRNA gene variants in the samples, as presented in this work.

3.2. Diversity of water bacterial communities

The fixed number of one hundred clones from each clone library was screened by direct sequencing. This methodological analysis is more efficient than restriction fragment length polymorphism (RFLP) analysis due to the accuracy in the analysis of the 16S rRNA gene by showing the minimum difference, one nucleotide, in pairwise sequence comparisons. In addition, several clones belonging to the same group of RFLP may show differences when analyzed by direct sequencing (Kent *et al*., 2003). The analysis of two hundred clones, representing all water clone libraries, produced 15 and 8 phylotypes for Bashtir and Genka water, respectively (Table 1). This result was reflected on the water sample intra diversities, where the gene diversity in Bashtir water (0.206) was approximately double than that in Genka water (0.148) (Table 1).

The results from LIBSHUFF analyses confirmed the highly diverse Bashtir water bacterial communities. Paired reciprocal library comparisons showed that the Bashtir water clone library differed significantly (P= 0.002) from that of Genka water, but Genka water clone library did not differ from that of Bashtir (P = 0.898) (Table 2). One explanation for this pattern is that the diversity of Bashtir water bacterial community encompassed and described some species in the Genka water community, while Genka water community accounted for only a portion of the Bashtir water diversity.

Table (1): Gene diversities and average pairwise nucleotide position differences θ(π) between phylotypes within each clone library (intra-population diversity).

Sample	Clone library	No. phylotypes	θ(π)	Gene diversity
Water	Bashtir	15	276.15 (119.05)	0.206 (0.09)
	Genka	8	111.21 (48.14)	0.148 (0.07)
Sediment	Bashtir	20	278.55 (120.08)	0.21 (0.10)
	Genka	20	287.95 (124.13)	0.22 (0.10)

Both θ(π) and gene diversity are expressed as means (± standard deviation) for each library.

Table (2): LIBSHUFF comparison of clone libraries.

Sample	Clone library	Covhom (%)	Cov_{het} (%)	*P*
Water	Bashtir	78.0	66.0	0.002
	Genka	80.0	82.0	0.898
Sediment	Bashtir	63.9	62.4	0.970
	Genka	62.4	61.0	0.486

Homologous (Covhom) and heterologous (Covhet) coverage percentages of libraries are given. Probability values (P) for the significance of differences between homologous and heterologous coverage in reciprocal comparisons as a function of evolutionary distance are also given.

3.3. Phylogenetic distribution of the recovered water phylotypes

The phylogenetic analysis of 23 phylotypes recovered from Bashtir and Genka water samples revealed their distribution within the branches of four major bacterial phyla (Figs. 2 and 3). The phylotypes located at the phylogenetic branch of Cyanobacteria represented 41 and 38% of the total recovered phylotypes from Bashtir and Genka waters, respectively (Fig. 2). The majority of these phylotypes were closely related to the cyanobacterial species *Anabaenopsis* sp., *Synechococcus* sp. and *Prochlorales* sp. (Fig. 3). *Anabaenopsis* belongs to the heterocystous Cyanobacteria that show a remarkable degree of 16S rRNA gene sequence conservation (Giovannoni *et al*., 1988; Iteman *et al*., 2002) and this was clear in the high homology between this species and the phylotype Bashtir.water.6. (99% nucleotide identity percentage), forming a strong clad represented by high bootstrap value (Fig. 3). The phylotypes Bashtir.water.2 and 11; and Genka.water.1 had nucleotide identity percentage averages, 90%, among each others, and 80% with the closest Cyanobacterium *Prochlorales* sp., and formed a unique phylogenetic cluster, suggesting a new class of Cyanobacteria (Fig. 3). Unclassified Cyanobacterial species have been recorded in several tropical freshwater lakes and characterized specific photosynthetic communities within these lakes (Pandey and Pandey, 2002).

The major part of water phylotypes was localized at the phylogenetic branch of the phylum Proteobacteria. Most of the Bashtir Proteobacteria-like phylotypes, were belonged to the class Epsilon (ε)-Proteobacteria and showed phylogenetic affiliation with the species *Arcobacter* sp. and *Sulfurospirillum halorespirans*. Genka phylotypes were distributed in the clusters of the classes α-, Beta (β) - and Delta (δ)-Proteobacteria. Sequences affiliated to the β-Proteobacteria were recovered from freshwater lakes and could be detected as a dominant fraction of the microbial community by fluorescence in situ hybridization (FISH) (Glockner *et al*., 1999). The phylotypes Bashtir.water.9 and Genka.water.8 represented *Bacteroidetes*-like members (Fig. 3).

There were several phylotypes characterizing the sampling sites (Fig. 3). For example, the ε-Proteobacteria-like phylotypes Bashtir.water.1, 3, 8 and 10 differentiated Bashtir from Genka, which lacks these phylotypes. On the other hand, the phylotypes Genka.water. 5 and 7, which belonged to Firmicutes and δ-Proteobacteria, respectively, characterized Genka water.

3.4. Diversity of sediment phylotypes

The sequence analyses of two hundred clones, representing total sediment clone libraries, produced 20 phylotypes from each sediment sample (Table 1). Both Bashtir and Genka clone libraries showed high population coverage similarities (Table 2). This similarity coverage was clearly shown in the phylogenetic tree (Fig. 4). Most of the sediment phylotypes from the two studied sites were localized closely related to each others; some of them formed monophyletic clusters, in the phylogenetic branches of the phyla Chloroflexi, Proteobacteria, Firmicutes, Bacteroidetes, and Planctomycetes, while the percentages of appearance of these phylotypes in their corresponding phyla varied between the two sediment samples (Fig. 2). The percentage average of nucleotide identities between sediment phylotypes of the two sites within each phylogenetic cluster (Fig. 4) ranged from 85 to 92%. An important characteristic of freshwater lake sediments is that they are not spatially separated from adjacent habitats, but are part of complex ecosystems. The composition of microbial communities in freshwater sediments may therefore be

largely influenced by interactions with surrounding aquatic and terrestrial habitats (Nealson, 1997), making biogeographic similarity in community structures.

However, some phylotypes were used as sediment site biomarkers. For example, the phylotypes Bashtir.sediment.4 and Bashtir.sediment. 9, locating at clusters of Gamma (γ)-Proteobacteria and Actinobacteria, respectively, could differentiate Bashtir sediment from that of Genka. On the other hand, the phylotypes Genka.sediment.3 and 14, inserted at the cluster of Fusobacteria, differentiated Genka sediment community from that of Bashtir. Several sediment phylotypes formed unique phylogenetic lineages. These phylotypes were Genka.sediment.2, 7 and 16; and Bashtir.sediment.10. These phylotypes showed nucleotide identity percentage averages 70% between each others and 69% between them and neighbour corresponding cluster members. These 16S rRNA gene nucleotide identities represented the level for differentiation of bacteria phyla (DeLong and Pace, 2001), suggesting that these sediment phylotypes accounted new bacterial phyla. These molecular results should be supported by other studies like culture, isolation and physiological analyses of these phylotypes. However, these unique phylotypes have never been recoded in any previous studies on lake sediments and consequently may represent finger printings for Bashtir and Genka sediment bacterial flora. Generally, it has been found that the diversity of bacteria belonging to unknown phylogenetic groups, containing no or very few cultured species, is quite high in freshwater sediments (Ludwig *et al*., 1997).

3.5. Biogeographical distribution of bacterial communities in the studied sites of Manzala Lake

The presented molecular study showed that each studied site of Manzala Lake has a specific fraction of bacterial community, characterizing the sampling site. This phylogenetic bacterial distribution may correlate with the physico-chemical characteristics of each site. The distance between the two sampling sites was 12.7 Km and interrupted with zones of dense emergent vegetation, and hoshas (illegal fish enclosures), which limit water circulation and form basins with different water characteristics and consequently distinct bacterial communities, as presented in this work. In contrast to water, the bacterial phylotype similarities between the sediment samples were correlated with the topography of the sampling sites. Genka was the ancient dischargeable site of Bahr El-Baqar drain. As the sediment structure and community is more stable than water, Genka may have a sediment bacterial flora similar to that of Bashtir, the new dischargeable site of Bahr El-Baqar.

Many recovered phylotypes were related to anaerobic autotrophic and heterotrophic species. The average depth of the lake is 3-4m, facilitating the penetration of the light to the bottom and consequently the occurrence of sediment phylotypes closely related to some photosynthetic Chloroflexi species (Fig. 4). These Chloroflexi-like phylotypes were not recorded in the surface water samples due to the anoxygenic character of that species (Frigaard and Bryant, 2004). Phylotypes of δ-Proteobacteria were more frequently retrieved from anoxic lake sediments than the water column (Glockner *et al*., 1999). Occurrence of δ-Proteobacteria-like phylotypes in addition to some phylotypes related to anaerobic ε-Proteobacteria and Bacteroidetes (Luijten *et al*., 2003; Ueki *et al*., 2006) in both collected waters and sediments (Figs. 3, 4) may indicate the extreme anoxygenic character of the current studied sites. Chemical analysis of water and sediment is needed to support these molecular ecological observations.

The polluted topography of the sampling sites facilitated the occurrence of several phylotypes closely related to environmental

useful and harmful bacterial species. The water Cyanobacteria-like phylotype Bashtir.water.6 relating to *Anabaenopsis* sp., can be considered as a biomarker for sewage eutrophication (Pandey and Pandey, 2002). The samples, especially those recovered from sediments, harboured abundant phylotypes that were similar to several species used in the term of bioremediation (Figs. 3, 4) such as sulfate-reducing δ-Proteobacteria and ε-Proteobacterium *Sulfurospirillum halorespirans* (Luijten *et al*., 2003), in addition to the Bacteroidete, *Paludibacter propionicigenes*, a producer of bioactive useful compound, propionate (Ueki *et al*., 2006).

On the other hand, several phylotypes recorded in this study were affiliated to some human pathogenic bacteria such as the Firmicute species *Helcococcus kunzii* and *Catabacter hongkongensis* (Collins *et al*., 1993; Lau *et al*., 2007) and the Fusobacterium *Cetobacterium somerae* (Finegold *et al*., 2003) (Figs. 3, 4). Transfer of clinical bacterial pathogens to natural aquatic ecosystems through waste drains was recorded previously (Faruque and Nair, 2002), and gave a biological alarm for the sustainability of aquatic ecosystem. Generally, Manzala Lake is a big reservoir for bacterial communities due to its different water suppliers and heavy anthropogenic activities. However, this work is the first molecular genetic study for monitoring bacterial communities in Egyptian lakes. Future study will focus on other sites in Manzala Lake and also other lakes in order to put a full concept on the phylogenetic distribution of bacterial flora in Egyptian lakes, and their contribution in biotechnological applications.

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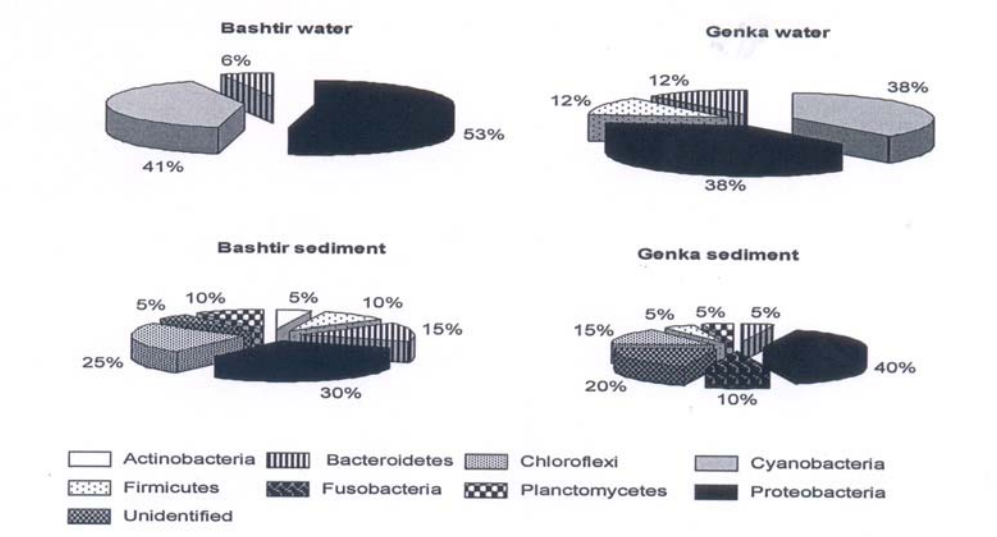

Figure 2

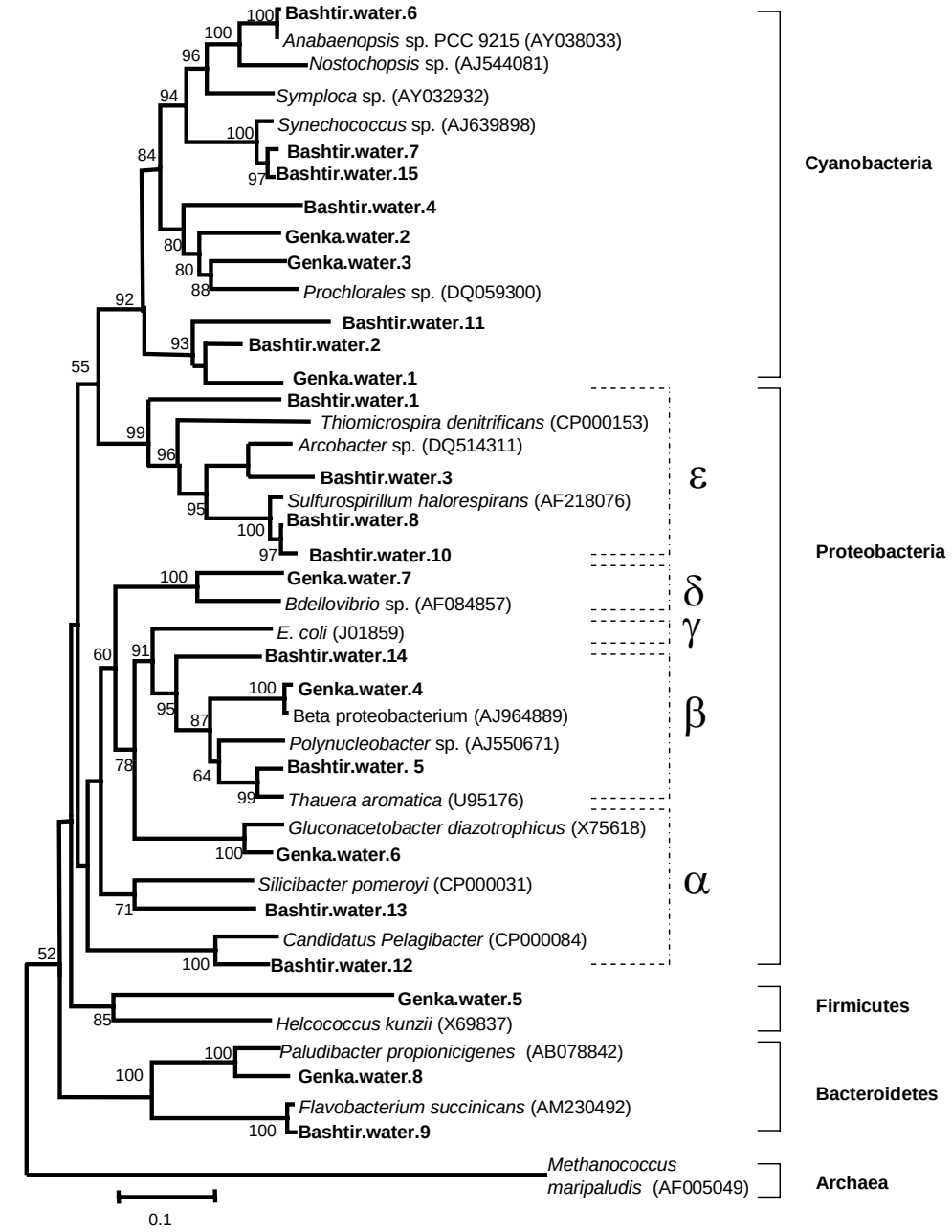
Bashtir.water.6
Anabaenopsis sp. PCC 9215 (AY038033)
Nostochopsis sp. (AJ544081)
Symploca sp. (AY032932)
Synechococcus sp. (AJ639898)
Bashtir.water.7
Bashtir.water.15
Bashtir.water.4
Genka.water.2
Genka.water.3
Prochlorales sp. (DQ059300)
Bashtir.water.11
Bashtir.water.2
Genka.water.1
Cyanobacteria
Bashtir.water.1
Thiomicrospira denitrificans (CP000153)
Arcobacter sp. (DQ514311)
Bashtir.water.3
Sulfurospirillum halorespirans (AF218076)
Bashtir.water.8
Bashtir.water.10
ε
Genka.water.7
Bdellovibrio sp. (AF084857)
δ
E. coli (J01859)
γ
Bashtir.water.14
Genka.water.4
Beta proteobacterium (AJ964889)
Polynucleobacter sp. (AJ550671)
Bashtir.water. 5
Thauera aromatica (U95176)
β
Gluconacetobacter diazotrophicus (X75618)
Genka.water.6
Silicibacter pomeroyi (CP000031)
Bashtir.water.13
Candidatus Pelagibacter (CP000084)
Bashtir.water.12
α
Proteobacteria
Genka.water.5
Helcococcus kunzii (X69837)
Firmicutes
Paludibacter propionicigenes (AB078842)
Genka.water.8
Flavobacterium succinicans (AM230492)
Bashtir.water.9
Bacteroidetes
Methanococcus maripaludis (AF005049)
Archaea
0.1

Figure 3

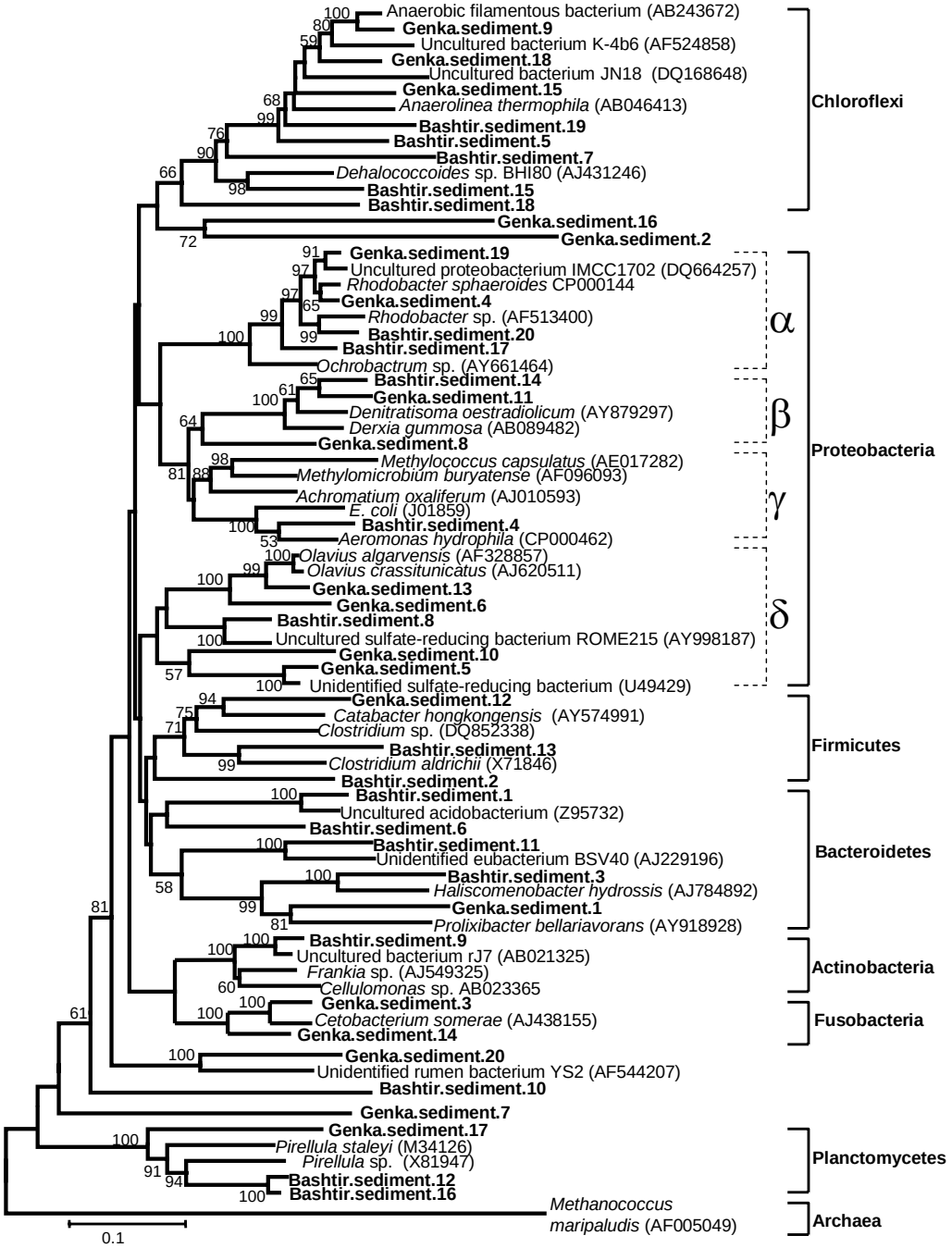
Anaerobic filamentous bacterium (AB243672)
Genka.sediment.9
Uncultured bacterium K-4b6 (AF524858)
Genka.sediment.18
Uncultured bacterium JN18 (DQ168648)
Genka.sediment.15
Anaerolinea thermophila (AB046413)
Bashtir.sediment.19
Bashtir.sediment.5
Bashtir.sediment.7
Dehalococcoides sp. BHI80 (AJ431246)
Bashtir.sediment.15
Bashtir.sediment.18
Genka.sediment.16
Genka.sediment.2
Genka.sediment.19
Uncultured proteobacterium IMCC1702 (DQ664257)
Rhodobacter sphaeroides CP000144
Genka.sediment.4
Rhodobacter sp. (AF513400)
Bashtir.sediment.20
Bashtir.sediment.17
Ochrobactrum sp. (AY661464)
Bashtir.sediment.14
Genka.sediment.11
Denitratisoma oestradiolicum (AY879297)
Derxia gummosa (AB089482)
Genka.sediment.8
Methylococcus capsulatus (AE017282)
Methylomicrobium buryatense (AF096093)
Achromatium oxaliferum (AJ010593)
E. coli (J01859)
Bashtir.sediment.4
Aeromonas hydrophila (CP000462)
Olavius algarvensis (AF328857)
Olavius crassitunicatus (AJ620511)
Genka.sediment.13
Genka.sediment.6
Bashtir.sediment.8
Uncultured sulfate-reducing bacterium ROME215 (AY998187)
Genka.sediment.10
Genka.sediment.5
Unidentified sulfate-reducing bacterium (U49429)
Genka.sediment.12
Catabacter hongkongensis (AY574991)
Clostridium sp. (DQ852338)
Bashtir.sediment.13
Clostridium aldrichii (X71846)
Bashtir.sediment.2
Bashtir.sediment.1
Uncultured acidobacterium (Z95732)
Bashtir.sediment.6
Bashtir.sediment.11
Unidentified eubacterium BSV40 (AJ229196)
Bashtir.sediment.3
Haliscomenobacter hydrossis (AJ784892)
Genka.sediment.1
Prolixibacter bellariavorans (AY918928)
Bashtir.sediment.9
Uncultured bacterium rJ7 (AB021325)
Frankia sp. (AJ549325)
Cellulomonas sp. AB023365
Genka.sediment.3
Cetobacterium somerae (AJ438155)
Genka.sediment.14
Genka.sediment.20
Unidentified rumen bacterium YS2 (AF544207)
Bashtir.sediment.10
Genka.sediment.7
Genka.sediment.17
Pirellula staleyi (M34126)
Pirellula sp. (X81947)
Bashtir.sediment.12
Bashtir.sediment.16
Methanococcus maripaludis (AF005049)
0.1
Chloroflexi
α
β
γ
δ
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
Fusobacteria
Planctomycetes
Archaea

Fig. 4

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FIGURE LEGENDS

Figure 1. A map showing the sites of sampling.

Figure 2. Identification and percentages of representative bacterial phyla detected from the 16S rRNA gene clone library of each studied sample.

Figure 3. Phylogenetic tree based on 16S rRNA gene nucleotide sequences. The tree shows the phylogenetic positions of the recovered water phylotypes, marked with bold, among bacterial species sequences, from different phyla, collected from DNA databases. An out-group sequence from the archaeon *Methanococcus maripaludis* was used to define the bacterial groups. Bootstrap values were calculated by neighbour-joining algorithm of > 50% and indicated at the roots of each cluster. α, β, γ, ε and δ are different classes of *Proteobacteria*. The bar represents 0.1 changes per nucleotide.

Figure 4. Phylogenetic tree based on 16S rRNA gene nucleotide sequences. The tree shows the phylogenetic positions of the recovered sediment phylotypes, marked with bold, among bacterial species sequences, from different phyla, collected from DNA databases. An out-group sequence from the archaeon *Methanococcus maripaludis* was used to define the bacterial groups. Bootstrap values were calculated by neighbour-joining algorithm of > 50% and indicated at the roots of each cluster. α, β, γ, and δ are different classes of *Proteobacteria*. The bar represents 0.1 changes per nucleotide.