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Diversity of freshwater *Thioploca* species and their specific association with filamentous bacteria of the phylum *Chloroflexi*

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Abstract

Phylogenetic diversity among filamentous sulfur-oxidizing bacteria of the genus *Thioploca* inhabiting freshwater/brackish environments was analyzed in detail. The 16S rRNA gene sequence of *Thioploca* found in a freshwater lake in Japan, Lake Okotanpe, was identical to that of *Thioploca* from Lake Ogawara, a brackish lake. The samples of two lakes could be differentiated by the sequences of their 23S rRNA genes and 16S-23S rRNA internal transcribed spacer (ITS) regions. The 23S rRNA-based phylogenetic relationships between *Thioploca* samples from four lakes (Lake Okotanpe, Lake Ogawara, Lake Biwa, and Lake Constance) were similar to those based on the 16S rRNA gene sequences. In addition, multiple types of the ITS sequences were obtained from *Thioploca* inhabiting Lake Okotanpe and Lake Constance. Variations within respective *Thioploca* populations were also observed in the analysis of the *soxB* gene, involved in sulfur oxidation. As major members of the sheath-associated microbial community, bacteria of the phylum *Chloroflexi* were

consistently detected in the samples from different lakes. Fluorescence *in situ* hybridization (FISH) revealed that they were filamentous and abundantly distributed within the sheaths of *Thioploca*.

Introduction

Members of the genus *Thioploca* are filamentous sulfur-oxidizing bacteria that have a common sheath surrounding a bundle of trichomes [19]. Populations of *Thioploca* species have been found in aquatic sediments from marine, brackish, and freshwater environments. They obtain energy from a combination of nitrate and/or oxygen in overlaying water and sulfide available in deeper sediment [16, 33]. They glide vertically within their sheaths to take up electron donors and acceptors, which are spatially separated [19, 21].

The genus *Thioploca* belongs to a monophyletic group consisting of nitrate-storing sulfur oxidizers [38] that may play important roles in nitrogen and sulfur cycles in their habitats [8]. They can accumulate large amounts of nitrate and elemental sulfur, and use them for respiration. In addition to the elements mentioned above, nitrate-storing sulfur oxidizers may influence the phosphorus cycle, as it has been shown that some of them accumulate phosphorus as polyphosphate in their cells [4, 39]. The intracellular

accumulation of polyphosphate to overcome physical and chemical stress is widespread among microorganisms [5, 12, 23, 28], although absence of polyphosphate granules in cells of *Thioploca* has been repeatedly reported [14, 15, 16, 22].

Since lakes, in contrast to marine environments, are closed water bodies, the influence of *Thioploca* on the local biogeochemical processes may be more significant there. However, the knowledge about *Thioploca* species living in freshwater/brackish lakes is still limited compared to that regarding marine species. *Thioploca* species in nonmarine habitats are apparently different from their marine counterparts, both morphologically and phylogenetically [15, 21, 22, 45]. In fact, a new independent candidate genus, *"Candidatus Marithioploca"* was recently proposed to encompass marine species [38]. This proposal for reclassification was based mainly on analysis of the 16S rRNA gene sequence of nitrate-storing sulfur oxidizers, which ultimately emphasized distinctive features of freshwater *Thioploca*. In comparison to their marine relatives, genetic diversity of the reported freshwater/brackish *Thioploca* is quite low, and very similar 16S rRNA gene sequences have been obtained from samples of

geographically separated sites [15, 21, 22].

The sheaths of *Thioploca* species act as habitats for other bacteria [20, 44]. It is thought that marine *Thioploca* cells are supplied with sulfide from sheath-associated sulfate-reducing bacteria of the genus *Desulfonema* [9, 44]. Metabolic interactions between *Thioploca* and other attaching bacteria, involving exchange of compounds containing alternative elements such as nitrogen and carbon, are also possible. To reveal the conclusive function of the communities consisting of *Thioploca* and sheath-associated bacteria, identifying major members comprising such communities is essential.

In the present study, phylogenetic relationships among members of the genus *Thioploca* inhabiting freshwater/brackish environments, including one representing a newly found population were analyzed in detail. In addition, the bacterial community associated with *Thioploca* sheaths was analyzed, focusing on the specific association of filamentous bacteria belonging to the phylum *Chloroflexi*.

Methods

Characterization of *Thioploca* and its habitat in Lake Okotanpe

Lake Okotanpe is a freshwater lake on Hokkaido Island, in northern Japan [6]. Its surface area is 0.41 km^2, and its maximum depth is 21 m. Samples were collected at a site 2.4 m deep near the eastern lakeshore. There were no inflow rivers near the site. Samples of *Thioploca* filaments (sheaths with trichomes) were collected by sieving sediment. Immediately after collection with an Ekman–Birge grab sampler, sediment was sieved with a 0.25 mm mesh in lake water on site. Materials retained on the mesh were transferred to a plastic tray, and *Thioploca* bundles were picked up with forceps and transferred to the lake water. Sediment samples for the quantitative analysis were collected using a core sampler (Type MT-2; Rigosha, Tokyo, Japan) in October 2010. Three large cores (inside diameter, 110 mm) were collected. From each large core, three sub-cores were sampled immediately. Three sub-cores obtained from the same large core were used for three different measurements, i.e., ignition loss, pore-water

analysis, and quantification of *Thioploca* biomass. Thus, three values were obtained from the three large cores for each measurement. The *in situ* pH and dissolved oxygen concentration in overlying water were measured using pH and DO meters (ProODO; YSI, OH, USA). All samples obtained were maintained on ice in the dark for a few hours until they were transferred to the laboratory.

Upon returning to the laboratory, sub-core samples were sectioned into five layers: 0–2 cm, 2–5 cm, 5–8 cm, 8–11 cm, and 11–15 cm. A portion of sediment was dried at 110°C for 24 h and then baked at 550°C for 2 h. Ignition loss was calculated from the change in sediment weight during this procedure. Samples of interstitial water were extracted by centrifuging sediment samples at 2°C. Immediately after collection, the dissolved ferrous iron concentration was determined using the 1,10-phenanthroline method as described previously [43]. The remaining portion of interstitial water and the samples of lake water were filtered through a 0.2 μm filter and kept at -20°C until chemical analysis. Ammonium concentration was determined using the indophenol method [36]. Concentrations of chloride, nitrate, and sulfate were measured using an

ion chromatograph (DX-120; Dionex, Sunnyvale, CA, U.S.A.) equipped with a column for anion analyses (IonPac AS4ASC; Dionex).

To measure the abundance of *Thioploca* from Lake Okotanpe in its habitat, the length of *Thioploca* trichomes was estimated as described previously [31]. Three sediment sub-cores (inside diameter, 46 mm) retrieved from three different cores were sectioned into five layers in the same manner as that for sediment characterization. *Thioploca* filaments in each section were collected by sieving with a 0.149 mm pore size mesh and fixed with 2% formaldehyde solution. The trichomes packed in the sheaths were released by breaking the sheaths with a blender (Physcotron; Nichion-Irika-Kikai Co. Ltd., Chiba, Japan). The lengths of released trichomes were measured under an optic microscope using the method outlined by Olson [32]. *Thioploca* biomass was calculated from the measured trichome length, the average trichome diameter, and a estimated cell density of 1 $g \cdot cm^{-3}$ [15, 18].

Before each physiological experiment, sample viability was ascertained by observing gliding mortality under an optical microscope. For intracellular

polyphosphate staining, intact *Thioploca* filaments were immersed in toluidine blue solution as described previously [39]. To detect glycogen and other polyglucose storage molecules, Lugol staining was also carried out [39]. These tests were performed on samples obtained in October 2009.

To analyze salinity tolerance, *Thioploca* filaments obtained in June 2010 were incubated in lake water amended with NaCl. Twenty *Thioploca* filaments were put in bottles containing 50 ml of filter-sterilized lake water supplemented with 0%, 0.2%, 0.5%, 1.0%, 2.0% or 3.5% NaCl (w/v). The bottles were incubated at 4°C in the dark, and the filaments were observed with an optical microscope after 24, 48, 72, and 96 h. Survival was examined by observing of the gliding motility of several tens of trichomes.

Phylogenetic analysis based on the 16S and 23S rRNA genes and the 16S–23S rRNA ITS region

Genomic DNA was extracted from washed *Thioploca* filaments from Lake

Okotanpe sample obtained in September 2006. The clumps of *Thioploca* filaments were rinsed with filter-sterilized lake water, and the residual contaminating materials were removed with forceps. DNA extraction was performed as described previously [22].

The 16S rRNA gene sequence of *Thioploca* from Lake Okotanpe was determined using specific primers as described previously [22]. For further detailed phylogenetic analysis, DNA fragments covering the region that included a part of the 16S rRNA gene, the internal transcribed spacer (ITS), and a part of the 23S rRNA gene were amplified by PCR. In addition to the sample from Lake Okotanpe, *Thioploca* samples obtained from Lake Biwa, Lake Constance, and Lake Ogawara were also analyzed. The sample of Lake Biwa for this analysis was collected from station C (coordinates: 35°23.40N, 136°7.70E; water depth, 50 m) in May 2000. The samples of *Thioploca* from Lake Ogawara and Lake Constance were identical to those analyzed in a previous study [20]. For *Thioploca*-specific PCR amplification, the primer pair 829F/GAM42a was used. The 829F primer is the forward version of Biwa829,

designed as a specific primer for the 16S rRNA gene of *Thioploca* from Lake Biwa [22]. The other primer, GAM42a (*E. coli* 23S rRNA position 1027 to 1043), was originally designed as a probe targeting the 23S rRNA of gammaproteobacteria for fluorescence *in situ* hybridization [26]. The PCR conditions were as follows: initial denaturation for 2 min at 94°C; 27 cycles of denaturing for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 150 s at 72°C; and final extension for 10 min at 72°C. The regions of the genes for 16S and 23S rRNA were directly sequenced (without cloning) from the resultant PCR products. To determine the ITS region sequences that include genetic heterogeneity, a cloning analysis was performed using the same PCR products. The purified amplicons were cloned into pCR2.1-TOPO plasmid vectors using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After white/blue selection, the inserts were directly amplified from cells of randomly selected colonies with the vector primers M13F and M13R. By analyzing the resultant PCR products, 19 ITS sequences were obtained from each of the samples of the four lakes. ITS sequences with nucleotide sequence

similarities of >98.8% were grouped into the same operational taxonomic unit (OTU). The sequences were aligned with reference sequences from the public database using the ClustalX program [46]. A phylogenetic tree was constructed by the neighbor-joining method using the MEGA4 software [42]. Bootstrap analysis was performed for 1000 replicates.

Analysis of *SoxB* gene

From the same genomic DNA samples used for the analysis of rRNA genes, fragments of the *soxB* gene, encoding a sulfate thioesterase/sulfate thiohydrolase, were amplified with the primer pair soxB432F/soxB1446B and the PCR conditions previously described [34]. Subsequent cloning was carried out as described above, and 7 clones were sequenced from the samples of each of the four lakes. On the basis of peptide sequences deduced from the nucleotide sequences obtained, clones sharing more than 336 amino acids in 342 sites were grouped into the same OTU.

PCR-based analyses of the bacterial community of the *Thioploca* sheaths

To analyze the entire bacterial community attached to the sheaths of *Thioploca* from Lake Okotanpe, PCR-denaturing gradient gel electrophoresis (DGGE) was performed as described previously [20]. In brief, fragments of the bacterial 16S rRNA gene were amplified with the 341F-GC/907R primer set [29] from the genomic DNA used for the phylogenetic analysis. For PCR amplification, the annealing temperature was set at 45°C.

In addition to the analysis using the universal primer pair described above, an analysis targeting members of the phylum *Chloroflexi* was also performed on samples from Lake Okotanpe (obtained in June 2009), Lake Biwa (obtained in December 2009 from station A [20]), and Lake Constance (sample described above). The GNSB941 probe, originally designed as a FISH probe for the phylum *Chloroflexi* [10], was used as a reverse primer in combination with the 518F-GC [29]. The PCR program for amplification with this primer pair was as follows: initial denaturation at 95°C for 5

min; followed by 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min; and final elongation at 72°C for 5 min. The resultant PCR products were subjected to DGGE as described above. To obtain longer sequences of the predominant *Chloroflexi* bacteria, fragments of the 16S rRNA gene were also amplified with another primer pair. The CFX1223 probe, also designed as a *Chloroflexi*-targeted FISH probe [3], was used as a primer in combination with the 27F [24]. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 2 min; and final elongation at 72°C for 5 min. The resultant PCR products were sequenced directly, without cloning.

From the *Thioploca* samples of Lake Okotanpe and Lake Biwa used for the *Chloroflexi*-targeted analysis, fragments of *aprA* gene, encoding an adenosine-5'-phosphosulfate reductase, were amplified using the primer pair AprA-1-FW/AprA-5-RV [27]. The PCR amplification was performed with the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1.5 min, and a 10 min final extension step at 72°C.

The cloning analysis was carried out as described above for the *soxB* gene, but the OTUs were defined in such a way that the clones of the same OTU shared more than 115 amino acids in 121 sites.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was mainly performed on the sample obtained from Lake Biwa in October 2010. The samples for FISH were individually picked up with a pair of tweezers from the sediment of the site of previous studies (station A [20, 21]), and fixed with 4% paraformaldehyde in PBS. Oligonucleotide probes used in this study are given in Table 1. The probes were labeled with fluorescein isothiocyanate (FITC) or cyanine (Cy3). The probes CoSRB385, alf19a, CFB319a, and ARC915 were selected as they were previously used in the analysis of sheath-associated bacteria of marine *Thioploca* [44]. All procedures were performed as described previously [1], and the specific conditions for hybridization and washing were determined based on the descriptions in the references

given in Table 1. The hybridized samples were mounted on slide glasses with the Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4', 6-diamidino-2-phenylindole (DAPI). Fluorescence images were acquired with a confocal laser scanning microscope (ECLIPSE 80i; Nikon, Tokyo, Japan).

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were assigned the DDBJ/EMBL/GenBank accession numbers **********_********** (accession numbers will be provided at a later stage).

Results

***Thioploca* sp. and its habitat in Lake Okotanpe**

The trichomes of *Thioploca* from Lake Okotanpe had a mean diameter of 3.9

μm (SD = 0.51 μm, range = 2.9–5.7 μm, n = 288). Numerous globules of elemental sulfur but no vacuoles were observed in the trichomes. The tip of each trichome was tapered, and independent gliding motility of each trichome was observed. The sheath diameters ranged from 13.7–80.6 μm, with a mean of 41.6 μm (SD = 15.8 μm; n = 38). Unlike *Thioploca* from Lake Biwa and Lake Constance [22], the sheaths had no constricted zones.

No intracellular polyphosphate granules were observed with toluidine blue staining. Lugol staining was negative, indicating the absence of glycogen and other polyglucose granules. In the salinity tolerance test, *Thioploca* from Lake Okotanpe was alive in 0.5% NaCl after 96 h. It survived 72 h of incubation in 1.0% NaCl; however, no trichome motility was observed after 96 h incubation.

The properties of overlaying water of the habitat of *Thioploca* were measured in October 2010. The temperature was 13.9°C, pH was 6.82, and the dissolved oxygen concentration was 325.6 μM. The concentrations of ammonium (15.0 μM), nitrate (13.0 μM) and sulfate (488.9 μM) were almost the same as those observed in the

surface water, reflecting the shallow water depth of the sampling site (2.4 m). The vertical profiles of the sediment characteristics are shown in Fig. 1. The nitrate concentration was below the detection limit (0.8 μM). The ammonium concentration increased with depth. In the layers deeper than 5–8 cm, the ferrous ion concentration was lower than that in the upper layers. The sulfate concentration was greatest in the top layer of sediment and gradually decreased with depth. Ignition loss values ranged from 14.3–23.1%.

Thioploca biomass was greatest in the top layer of sediment, but it varied greatly among the cores (Fig. 1); the values ranged from 0.18–11.10 $\mu g \cdot cm^{-3}$, corresponding to $1.5 \times 10^4 - 9.3 \times 10^5$ $\mu m \cdot cm^{-3}$. The magnitude of the biomass was similar to that at stations 9, 11, and 12 in Lake Biwa (the sites with relatively high biomass in that lake) [31] but was markedly lower than that in Hjarbæk fjord (approximately 500 $\mu g \cdot cm^{-3}$) [15]. No populations were observed in layers deeper than 8–11 cm.

Phylogeny of *Thioploca* sp. from Lake Okotanpe and its closest relatives

The 16S rRNA gene sequence of *Thioploca* from Lake Okotanpe was successfully obtained using the specific PCR primers. The obtained sequence (1378 bp) was identical to that of *Thioploca* from Lake Ogawara, a brackish lake in northern Japan [20].

In the direct sequencing of PCR products of the DNA region spanning from the 16S rRNA gene to the 23S rRNA gene, the regions of genes coding rRNA were successfully sequenced without ambiguity for all samples from four lakes. The sequences of the 16S rRNA gene region perfectly matched those of the known sequences of *Thioploca* from each lake, indicating that the PCR products were specifically amplified from genomic DNA of *Thioploca* species. In the analysis based on the partial 23S rRNA gene sequence, *Thioploca* from Lake Okotanpe could be differentiated from that from Lake Ogawara (4 mismatches in 1023 nucleotide positions). The 23S rRNA-based phylogenetic relationships of *Thioploca* species were similar to those based on the 16S rRNA gene (Fig. 2A).

In contrast to the 23S rRNA gene, the ITS region of the sample from Lake Okotanpe could not be sequenced directly. Therefore, cloning analysis was carried out. The results of the cloning analysis are shown in Fig. 2B. As expected, sequences of the ITS region from Lake Okotanpe sample included variations and could be divided into two distinct types with different numbers of nucleotides (459 and 480). The diversity of the ITS sequence was also observed in clones originating from the sample from Lake Constance. The ITS sequences of the 19 clones from this lake had various lengths ranging from 444 to 461, and they were grouped into 5 OTUs (Fig 2B). No OTU was shared across two or more lakes.

In the analysis of the *soxB* gene, all 28 clones obtained from the four lakes were very closely related to each other (Fig. 3). Their closest relative in the public database was uncultivated marine *Beggiatoa* sp. [30]; they were also closely related to *Beggiatoa alba*. All 7 clones from Lake Biwa had almost identical sequences, and two or three phylotypes were recognized in other lakes. The clones from Lake Constance and Lake Ogawara formed monophyletic clusters, but one of the clones obtained from

Lake Okotanpe was distinct from other clones from that lake (Fig. 3).

Chloroflexi associated with *Thioploca* sheaths

In the PCR-DGGE analysis, the band originating from *Thioploca* was observed as a major band; four other bands were also sequenced (Fig. 4A). Bands OK1 and OK2, belonging to the phylum *Chloroflexi*, were closely related to the sequences detected as *Thioploca*-associated bacteria from freshwater/brackish lakes [20]. The sequence of band OK3 was grouped with cyanobacteria, and band OK4 was closely related to clones from biomat in the sediment of a cenote, a phreatic limestone sinkhole, located at low latitude [37].

As the *Chloroflexi* lineage has been consistently detected in *Thioploca* samples of various lakes, *Chloroflexi*-targeted analysis was performed. In the PCR-DGGE analysis using the specific primer, one each predominant band was observed in the samples of the three freshwater lakes (Fig. 4B). The sequence of the dominant band from Lake Okotanpe sample (O-ch1) was identical to that of OK2. The sequences of

the most intense bands from Lake Biwa (B-ch1) and Lake Constance (C-ch1) were identical to those previously detected from in same lakes with different primers (HYs-2 and BO9, respectively) [20]. The other sequenced bands labeled in Fig. 4B all belonged to the subphylum I in the phylum *Chloroflexi*.

By direct sequencing of the PCR products obtained with the primer pair 27F/CFX1223, longer sequences (1160 bp) of predominant *Chloroflexi* bacteria were successfully obtained (only one nucleotide position of Lake Biwa and two positions of Lake Okotanpe samples were ambiguous) from the samples of the three lakes. The sequences obtained were identical to those of the major DGGE bands of respective lakes. Based on the longer sequences of 16S rRNA gene, phylogenetic positions of the dominant sheath-associated bacteria were inferred. As shown in Fig. 5, bacteria of the three lakes were very closely related to each other and grouped with the family *Anaerolineaceae*. It was also confirmed that these sequences perfectly match to the probes GNSB941 and EUB-III.

Under a fluorescent microscope, numerous DAPI-stained filaments were observed

within the sheaths of *Thioploca* (Fig. 6) Since *Thioploca* trichomes were strongly autofluorescent [22], observation of the thin filaments was easier in regions where the trichomes were absent from the sheaths. These thin filaments were generally oriented parallel to the trichomes. The filaments hybridized positively with the probe EUB338-III (Fig. 6) but not with EUB338-I, EUB338-II or ARC915. They were also stained with the probe GNSB941, not with the probes CoSRB, alf19b, or CFB319a.

Cloning analysis of *aprA* gene

The results of *aprA* gene analysis are summarized in Fig. 7. A total of 100 clones from the libraries of the two lakes were grouped into 26 OTUs. The clone library of Lake Okotanpe was dominated by a specific OTU, apr15, which was also detected in the sample of Lake Biwa. The clones of this OTU were closely related to vacuolated *Beggiatoa* sp. [30], suggesting that they originated from *Thioploca*. The library of Lake Biwa was dominated by another OTU, apr8. This OTU was distinct from apr15 but also affiliated with a cluster comprising sulfur-oxidizing bacteria.

Including these two predominant ones, 5 OTUs appeared in both libraries, but none of them were closely related to the cultivated sulfate reducers (Fig. 7).

Discussion

In Lake Okotanpe, the biomass of *Thioploca* markedly differed between sampled cores (Fig. 1), indicating a highly heterogeneous distribution of *Thioploca* in the study site. The core samples used for the quantification were obtained within a small area (approximately 10 m^2), and the investigated sediment characteristics, except for the ferrous ion concentration, were not significantly different among them in the surface layers (Fig. 1). The ferrous ion concentration in the 0–2 cm layer largely varied among the cores (CV = 84.0), but there was no correlation between the ferrous ion and *Thioploca* abundance. These results suggest the presence of unidentified prominent factors controlling the abundance of *Thioploca* other than the measured parameters. The heterogeneous distribution highlights the difficulty in estimating the total biomass

of *Thioploca* in this lake and indicates that it is problematic to evaluate the contribution of the *Thioploca* population to local material cycles, even if physiological properties are fully elucidated.

The 16S rRNA gene analysis revealed that *Thioploca* from Lake Okotanpe and Lake Ogawara had identical sequences. In addition to the trichome diameter, the sheath shape without constrictions was also shared by *Thioploca* of the two lakes. In the salinity tolerance test, *Thioploca* from Lake Okotanpe exhibited tolerance to a NaCl concentration corresponding to that observed in the brackish lake water of Lake Ogawara. Thus, differences between *Thioploca* of the two lakes were revealed only by the sequencing of the 23S rRNA gene and the ITS region. Very high sequence similarities of rRNA gene among *Thioploca* of distant freshwater/brackish habitats were reported in previous studies [15, 20, 22, 38]. Even though such high similarities between the gene sequences from geographically separated sites were also reported in other bacteria [13, 50], the case of freshwater *Thioploca* seems notable, considering

their extraordinarily large size in comparison to general bacteria. High dispersal rates linked to small size are often assumed in discussions on biogeography of bacteria, but it is debatable whether this assumption is fully applicable to the long and thick filaments of *Thioploca*. Recent studies of nitrate-storing sulfur oxidizers revealed that their diversity was greater than previously thought [11, 38], but *Thioploca* still remains unique because it can thrive in freshwater environments, whereas all other members of the group are marine bacteria. For nitrate-storing sulfur oxidizers, it might be difficult to adapt to freshwater environment, and unspecified selective pressure may restrict their diversity in such habitats.

The analysis of the ITS region suggested that multiple phylotypes of *Thioploca* coexisted in Lake Okotanpe, as well as Lake Constance. Until now, the diversity within a single freshwater *Thioploca* population was hardly discussed. The physiological properties can differ among bacteria that have the same 16S rRNA gene sequences and different ITS sequences [17, 49]. Possible niche separation or habitat segregation among different types of *Thioploca* living in freshwater habitats may be

subjects of future investigations.

Although there is no solid evidence, the results of our phylogenetic analysis suggested that the obtained sequences of the *soxB* gene have originated from *Thioploca*. From the sample of Lake Biwa, only one type of *soxB* gene sequence was obtained, whereas some variations were observed in the samples from other lakes (Fig. 3). The differences in diversity among the four lakes were also observed in the analysis of the ITS, lowest in Lake Biwa and highest in Lake Constance. This pattern seems consistent with the results of the *soxB* gene analysis.

In a previous study, bacteria belonging to the phylum *Chloroflexi* were detected in samples of *Thioploca* filaments from three lakes [20]. In the present study, DGGE bands closely related to these bacteria were also detected from *Thioploca* in Lake Okotanpe (Fig. 4A). Except for the sample of Lake Biwa, these bacteria were detected as the most intense DGGE bands second to *Thioploca* itself [20]. As revealed by direct sequencing of the longer PCR products, there was one mismatch between the

gene sequences of these bacteria and the forward primer 341F, used for the DGGE analysis. Considering that negative PCR bias might have been imposed on the DGGE results, dominance of these bacteria in sheath-associated bacterial communities is strongly suggested. Accordingly, numerous filaments embedded in the sheaths were specifically stained with the probes perfectly matched to these bacteria, GNSB941 and EUB-III (Fig. 6). The results of the FISH were consistent with their phylogenetic positions within the lineage of filamentous bacteria.

The specific association observed suggests the presence of some interplay between *Chloroflexi* and *Thioploca*, but it still remains unclear how they interact. The cultivated relatives of the sheath-associated bacteria, belonging to the family *Anaerolineaceae*, are strict anaerobes growing fermentatively on sugar [47, 48]. As the closest partially characterized relative, a filamentous bacterium was reported as a symbiont of *Balneomonas flocculans* [41]. As some members of this family degrade polysaccharide [48], the sheath-associated bacteria might utilize the sheath material as growth substrate.

As an interaction between marine *Thioploca* and bacteria inhabiting their sheaths, sulfide supply from sulfate reducers of the genus *Desulfonema* has been suggested [9, 44]. In the previous study on the sheath-associated bacteria of freshwater *Thioploca*, clone libraries of dissimilatory sulfite reductase gene were analyzed to identify primal sulfide supplier inhabiting the sheaths [20]. In that analysis, diverse sulfate reducers were detected but no specific lineage was found to be dominant. These results agree well with the *aprA* gene-based analysis performed in this study (Fig. 7). In the FISH with the probe CoSRB385 targeting sulfate reducers of *Desulfobacteraceae*, only very sparse unicellular bacteria were observed (data not shown). All these findings consistently suggest absence of dominating sulfate reducer on sheaths, in contrast to the case of marine counterparts. In the bacterial communities associated with *Thioploca*, sulfate reducers might be less dominant in freshwater lakes because of reduced sulfate supply. At present, it is still unclear how *Thioploca* manage with the poor sulfide supply from low sulfate reduction rate in freshwater habitats. There might be unknown symbiotic sulfate reducers which are difficult to detect by

conventional methods, or mechanism completely different from the case of marine counterparts might be involved.

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Table 1. Oligonucleotide probes used in this study.

Probe	Target	Probe sequence (5'-3')	FA (%)[a]	Temp. (°C)[b]	Reference
CoSRB385	*Desulfobacteraceae*	CGGCGTTGCTGCGTCAGG	30	37	[35, 44]
alf19b	*Alphaproteobacteria*	CGTTCGYTCTGAGCCAG	30	37	[26, 44]
CFB319a	*Cytophaga-Flavobacter-Bacteroides*	TGGTCCGTGTCTCAGTAC	30	37	[25, 44]
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	30	37	[40, 44]
EUB338-I	Most *Bacteria*	GCTGCCTCCCGTAGGAGT	35	46	[2, 26]
EUB338-II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	35	46	[7, 26]
EUB338-III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	35	46	[7, 26]
GNSB941	*Chloroflexi*	AAACCACACGCTCCGCT	35	37	[3, 10]

[a] Formamide concentration in the hybridization buffer.
[b] Hybridization temperature.

Figure lagend

Figure 1 Vertical profiles of *Thioploca* biomass and sediment characteristics determined for three sediment cores, A, B, and C.

Figure 2 Phylogenetic relationships of *Thioploca* from four lakes and their closest relatives based on the sequences of 23S rRNA gene (A), and 16S-23S rRNA gene ITS region (B). The OTUs of ITS are named after lake where they were detected, Lake Okotanpe (OK), Lake Ogawara (OG), Lake Constance (CN), and Lake Biwa (BW), respectively. In each parenthesis, name of representative clone used for phylogenetic analysis and the number of clone belonging to the OTU is given. Boot strap values above 50% are shown.

Figure 3 Phylogenetic relationships of the *soxB* OTUs obtained in this study. Boot strap values above 50% (1000 replicates) are shown. The name of representative clone of each OTU and the number of clone within the OTU are given in parentheses. Name of clone prefixed “sOko”, “sOga”, “sCon”, and “sBiwa” indicate the clones from Lake Okotanpe, Lake Ogawara, Lake Constance, and Lake Biwa, respectively.

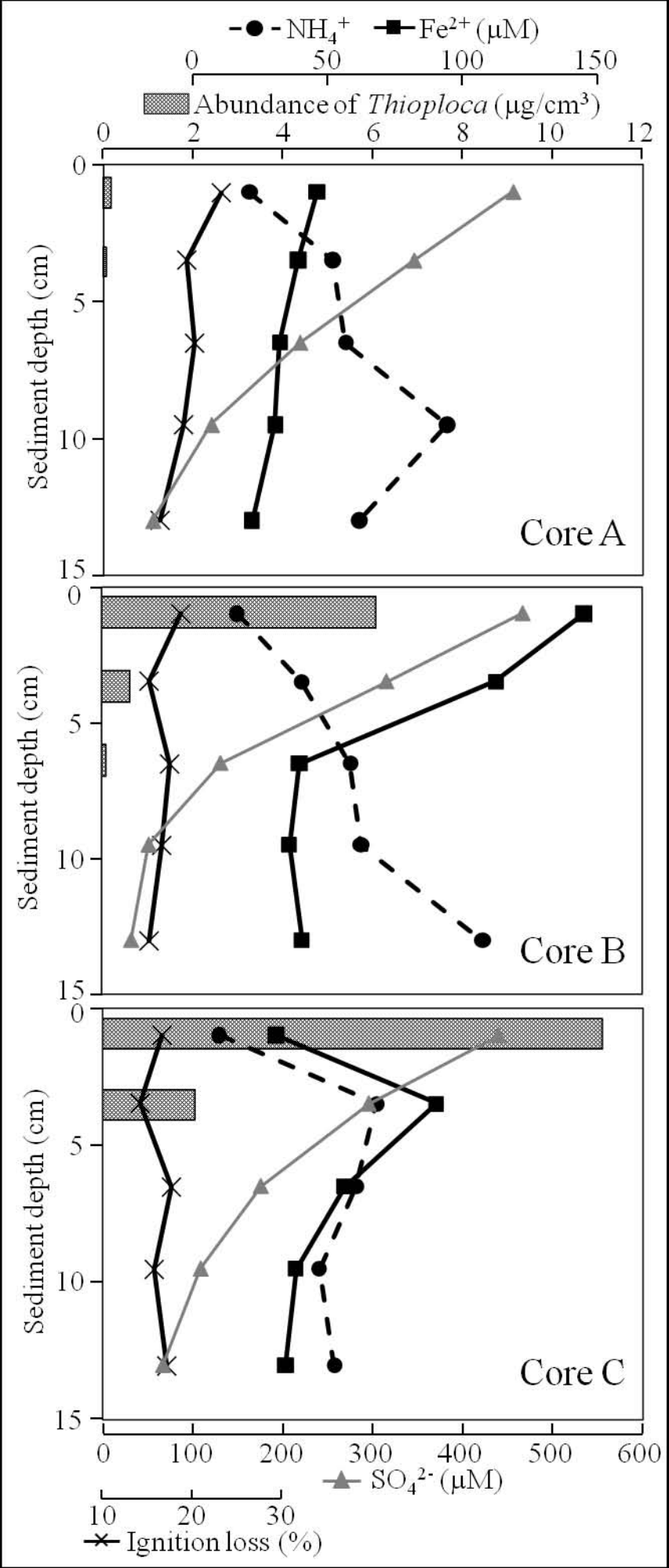
Figure 4 DGGE band profiles of 16S rRNA gene fragments, obtained from Lake Okotanpe with the universal primer pair (A), and with the *Chloroflexi*-specific primer pair (B), from Lake Okotanpe (lane 1), Lake Biwa (lane 2) and Lake Constance (lane3).

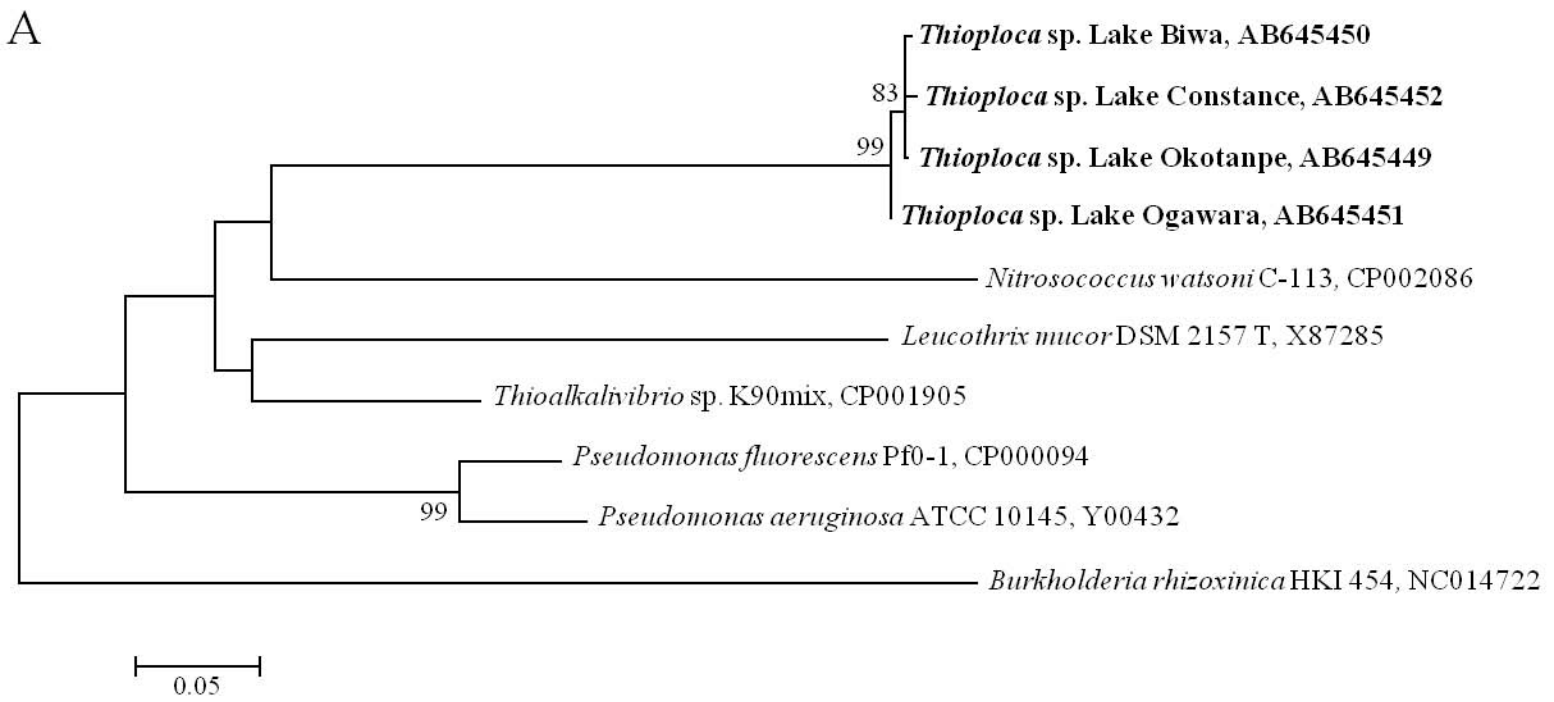
Figure 5 Phylogenetic affiliation of the predominant *Chloroflexi* on the sheaths of

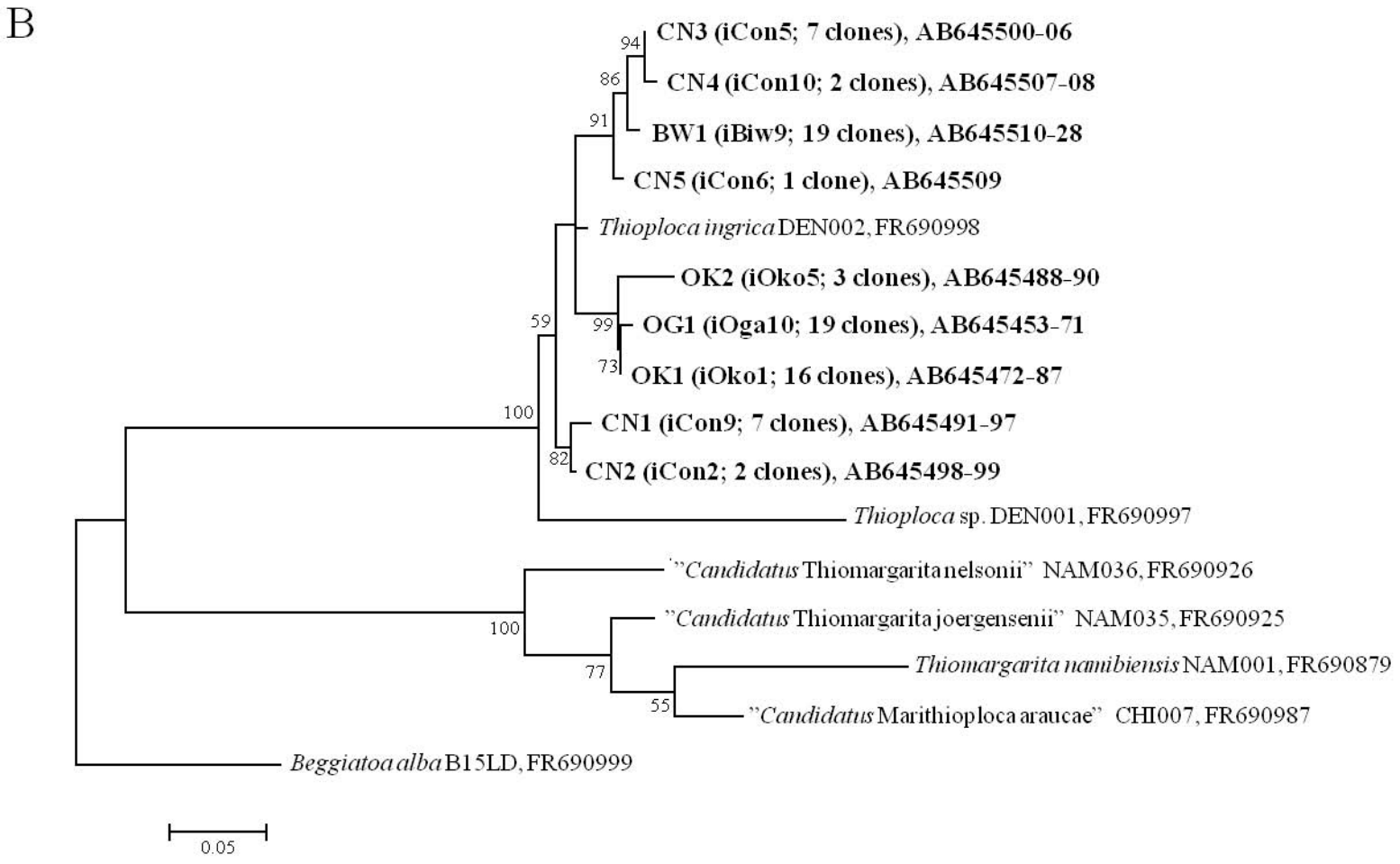
Thioploca. Boot strap values above 50% are shown.

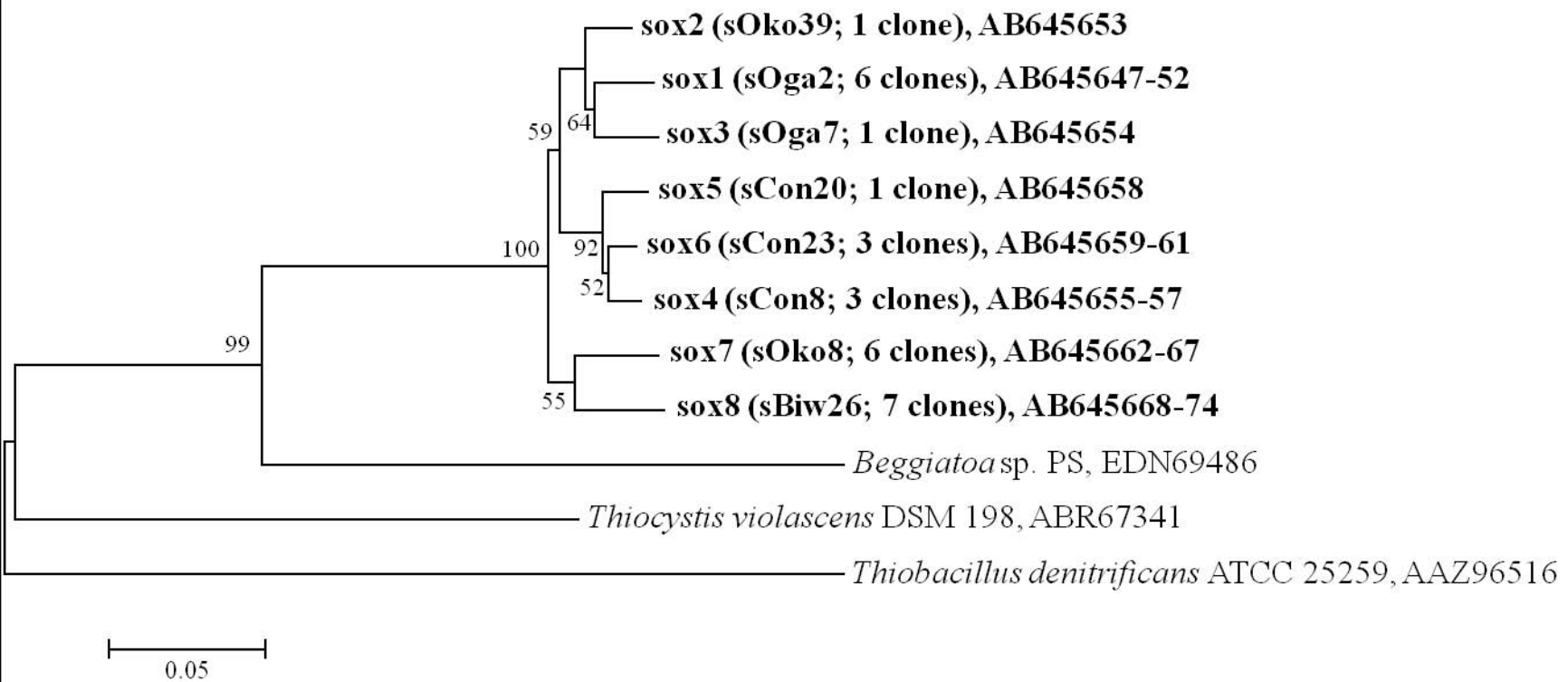
Figure 6 Microscopic image showing the presence of numerous thin filamentous bacterium (indicated by arrow) within the sheath of *Thioploca* from Lake Biwa using phase-contrast microscope (A and E) and confocal laser scanning microscope (B-D and F-H). (B and F) DAPI staining. (C) FISH using Cy3-labeled EUB338-III. (D) Combined image with the images of DAPI staining and FISH using EUB338-III. (G) FISH using Cy3-labeled GNSB941. (H) Combined image with the images of DAPI staining and FISH using GNSB941. T, trichomes of *Thioploca*. Scale bar, 10 μm.

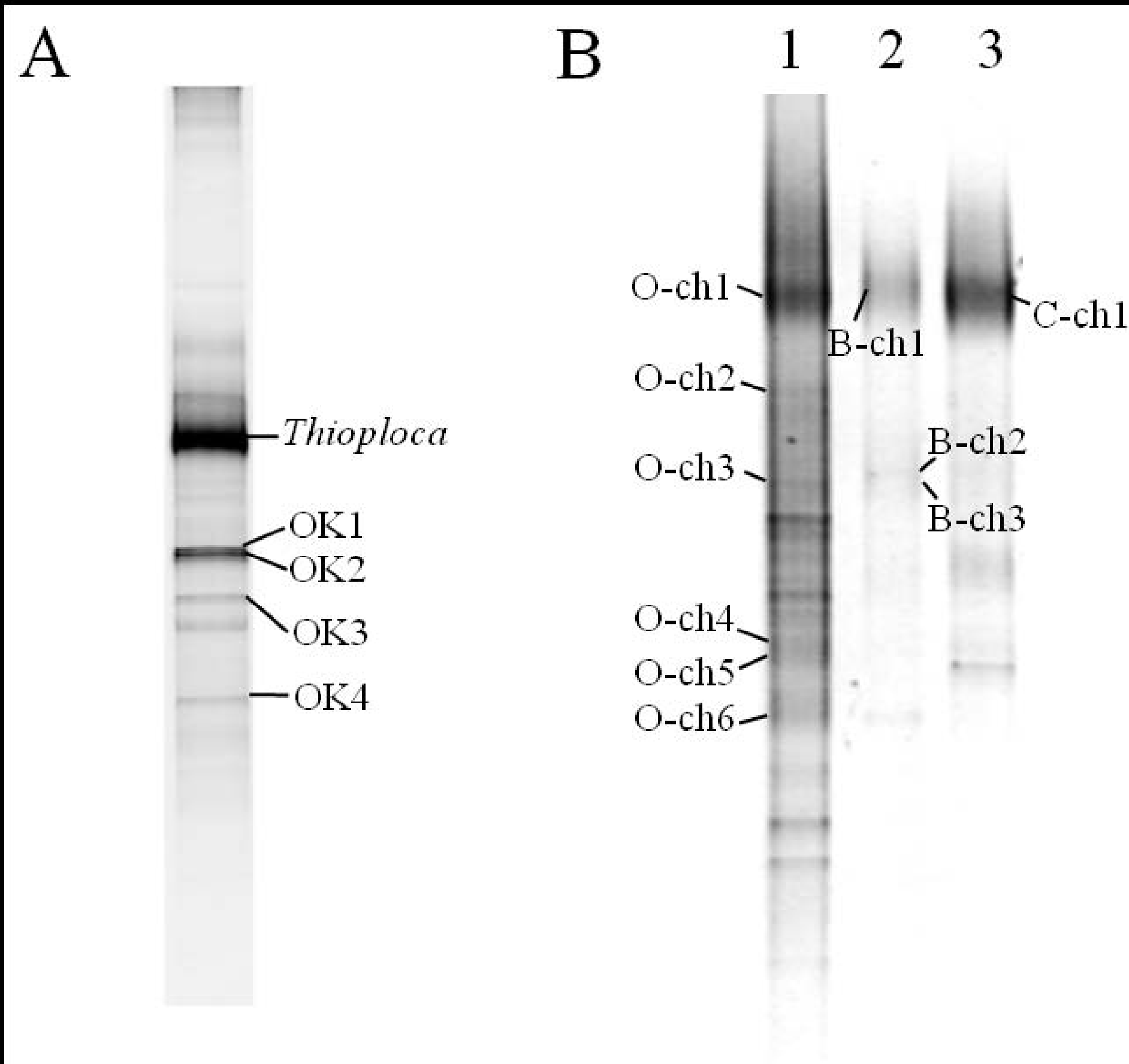
Figure 7 Phylogenetic tree of OTUs of *aprA* gene sequences obtained in this study. Boot strap values above 50% are shown. The numbers in parentheses are the number of clones belonging to each OTU in the libraries of Lake Biwa (left) and Lake Okotanpe (right), respectively.

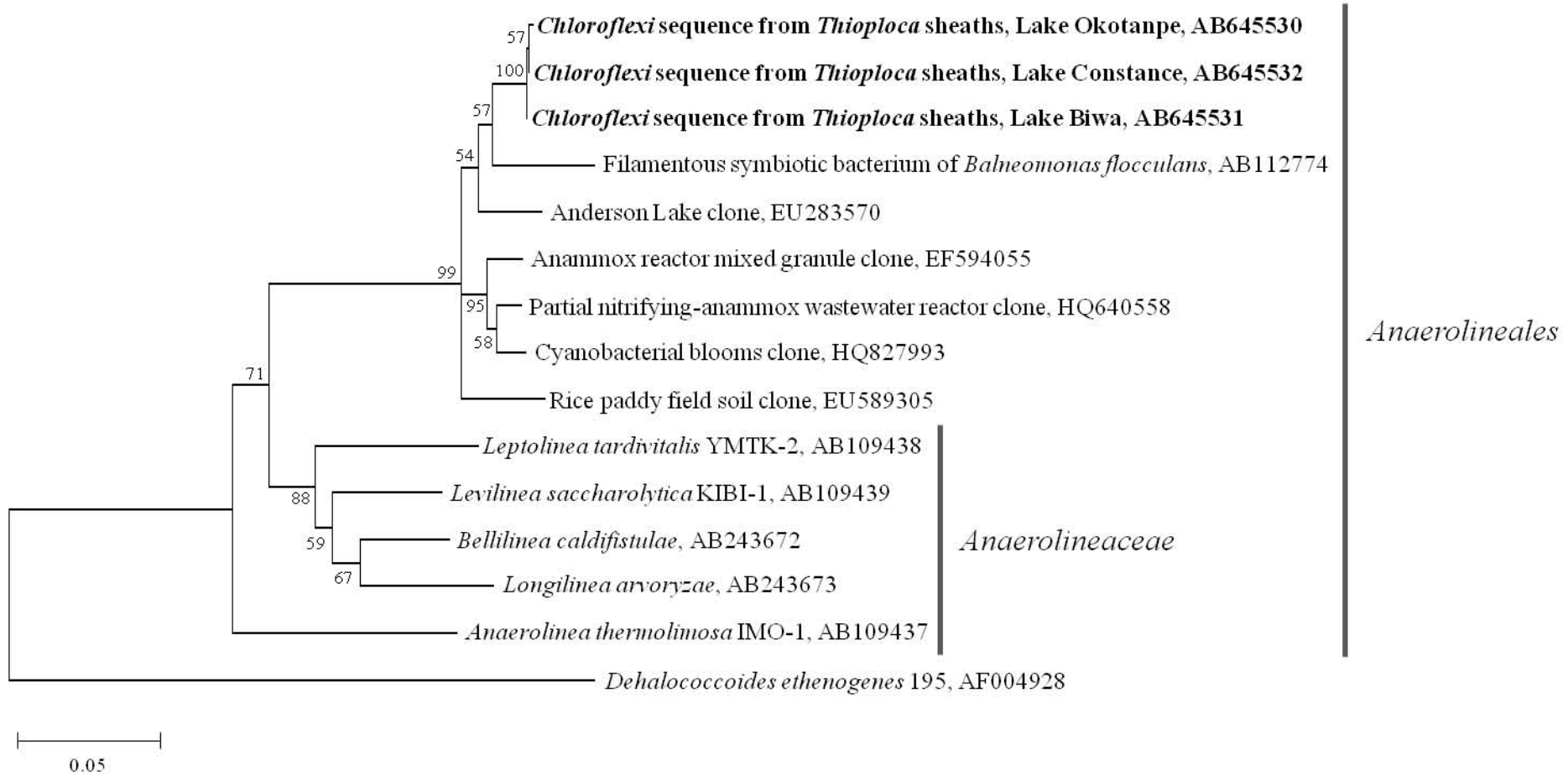

NH_4^+
Fe^{2+} (μM)
0
50
100
150
Abundance of *Thioploca* (μg/cm³)
0
2
4
6
8
10
12
Sediment depth (cm)
0
5
10
15
Core A
Core B
Core C
0
100
200
300
400
500
600
SO_4^{2-} (μM)
10
20
30
Ignition loss (%)

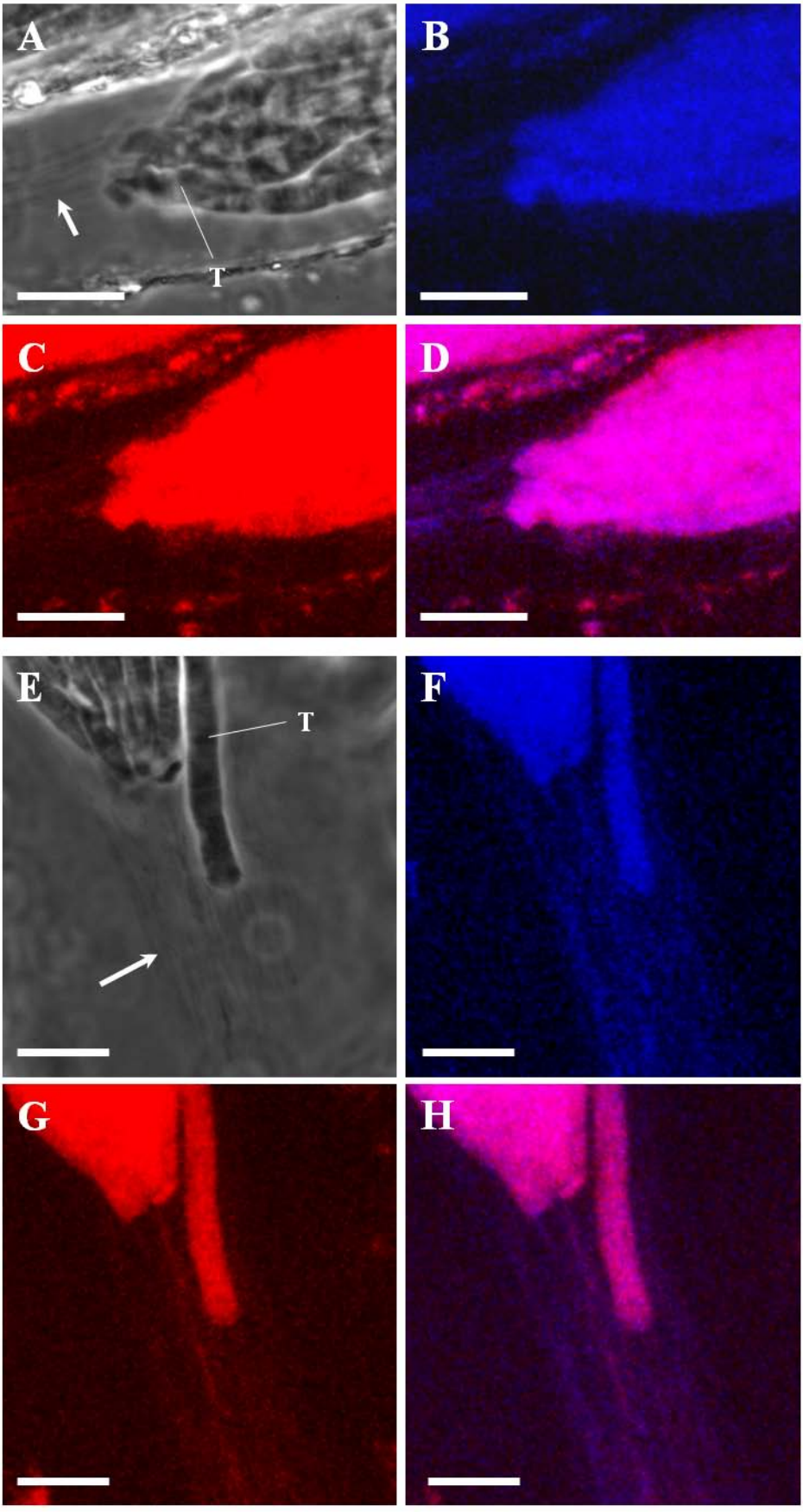

A
Thioploca sp. Lake Biwa, AB645450
83
Thioploca sp. Lake Constance, AB645452
99
Thioploca sp. Lake Okotanpe, AB645449
Thioploca sp. Lake Ogawara, AB645451
Nitrosococcus watsoni C-113, CP002086
Leucothrix mucor DSM 2157 T, X87285
Thioalkalivibrio sp. K90mix, CP001905
Pseudomonas fluorescens Pf0-1, CP000094
99
Pseudomonas aeruginosa ATCC 10145, Y00432
Burkholderia rhizoxinica HKI 454, NC014722
0.05

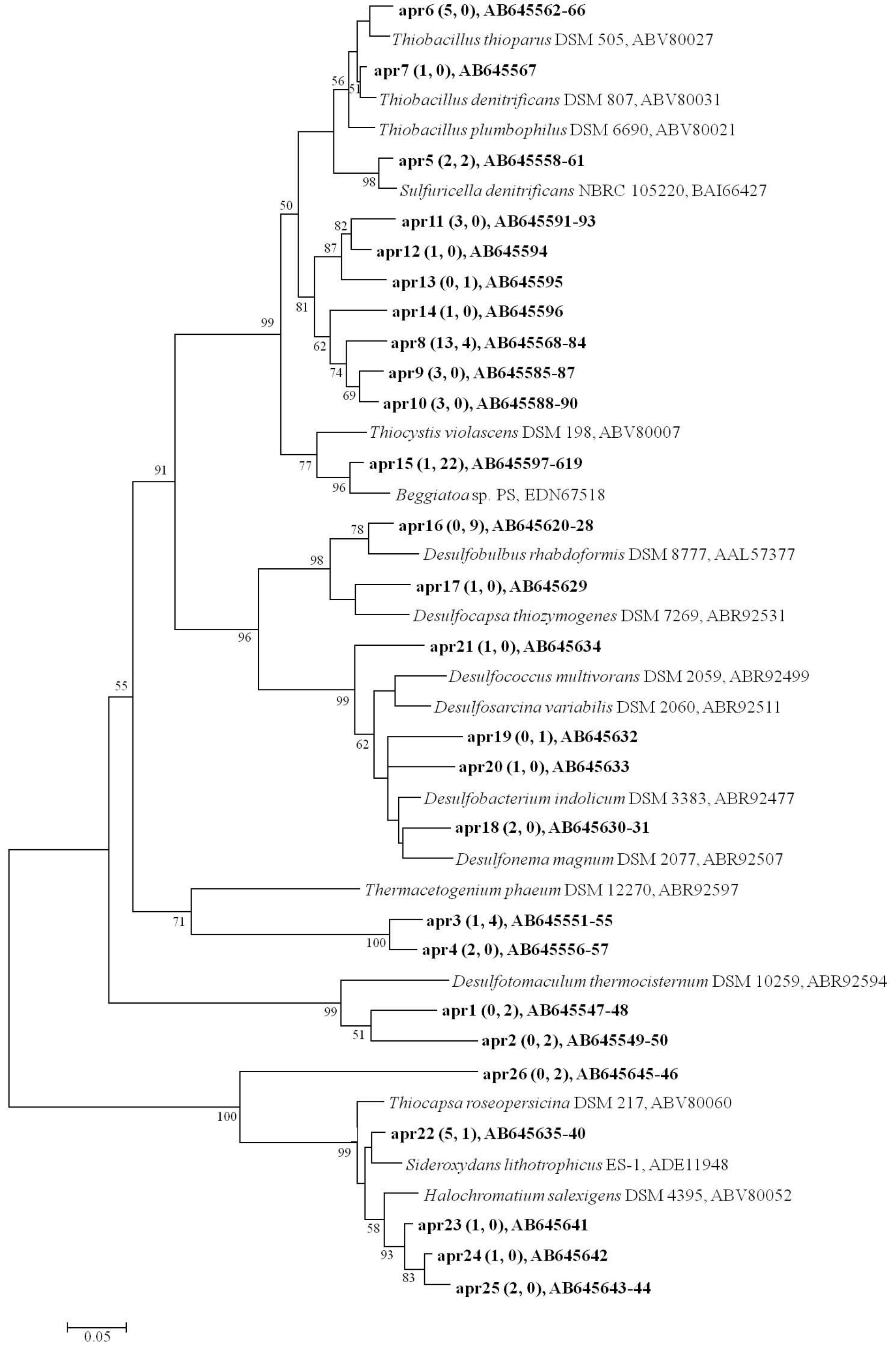

B
94
CN3 (iCon5; 7 clones), AB645500-06
86
CN4 (iCon10; 2 clones), AB645507-08
91
BW1 (iBiw9; 19 clones), AB645510-28
CN5 (iCon6; 1 clone), AB645509
Thioploca ingrica DEN002, FR690998
OK2 (iOko5; 3 clones), AB645488-90
59
99
OG1 (iOga10; 19 clones), AB645453-71
73
OK1 (iOko1; 16 clones), AB645472-87
100
CN1 (iCon9; 7 clones), AB645491-97
82
CN2 (iCon2; 2 clones), AB645498-99
Thioploca sp. DEN001, FR690997
"Candidatus Thiomargarita nelsonii" NAM036, FR690926
100
"Candidatus Thiomargarita joergensenii" NAM035, FR690925
77
Thiomargarita namibiensis NAM001, FR690879
55
"Candidatus Marithioploca araucae" CHI007, FR690987
Beggiatoa alba B15LD, FR690999
0.05


sox2 (sOko39; 1 clone), AB645653
sox1 (sOga2; 6 clones), AB645647-52
sox3 (sOga7; 1 clone), AB645654
sox5 (sCon20; 1 clone), AB645658
sox6 (sCon23; 3 clones), AB645659-61
sox4 (sCon8; 3 clones), AB645655-57
sox7 (sOko8; 6 clones), AB645662-67
sox8 (sBiw26; 7 clones), AB645668-74
Beggiatoa sp. PS, EDN69486
Thiocystis violascens DSM 198, ABR67341
Thiobacillus denitrificans ATCC 25259, AAZ96516
59
64
100
92
52
99
55
0.05


A
Thioploca
OK1
OK2
OK3
OK4
B
1
2
3
O-ch1
O-ch2
O-ch3
O-ch4
O-ch5
O-ch6
B-ch1
B-ch2
B-ch3
C-ch1


57
100
57
54
99
95
58
71
88
59
67
Chloroflexi sequence from Thioploca sheaths, Lake Okotanpe, AB645530
Chloroflexi sequence from Thioploca sheaths, Lake Constance, AB645532
Chloroflexi sequence from Thioploca sheaths, Lake Biwa, AB645531
Filamentous symbiotic bacterium of Balneomonas flocculans, AB112774
Anderson Lake clone, EU283570
Anammox reactor mixed granule clone, EF594055
Partial nitrifying-anammox wastewater reactor clone, HQ640558
Cyanobacterial blooms clone, HQ827993
Rice paddy field soil clone, EU589305
Leptolinea tardivitalis YMTK-2, AB109438
Levilinea saccharolytica KIBI-1, AB109439
Bellilinea caldifistulae, AB243672
Longilinea arvoryzae, AB243673
Anaerolinea thermolimosa IMO-1, AB109437
Dehalococcoides ethenogenes 195, AF004928
Anaerolineales
Anaerolineaceae
0.05


A
T
B
C
D
E
T
F
G
H


apr6 (5, 0), AB645562-66
Thiobacillus thioparus DSM 505, ABV80027
apr7 (1, 0), AB645567
Thiobacillus denitrificans DSM 807, ABV80031
Thiobacillus plumbophilus DSM 6690, ABV80021
apr5 (2, 2), AB645558-61
Sulfuricella denitrificans NBRC 105220, BAI66427
apr11 (3, 0), AB645591-93
apr12 (1, 0), AB645594
apr13 (0, 1), AB645595
apr14 (1, 0), AB645596
apr8 (13, 4), AB645568-84
apr9 (3, 0), AB645585-87
apr10 (3, 0), AB645588-90
Thiocystis violascens DSM 198, ABV80007
apr15 (1, 22), AB645597-619
Beggiatoa sp. PS, EDN67518
apr16 (0, 9), AB645620-28
Desulfobulbus rhabdoformis DSM 8777, AAL57377
apr17 (1, 0), AB645629
Desulfocapsa thiozymogenes DSM 7269, ABR92531
apr21 (1, 0), AB645634
Desulfococcus multivorans DSM 2059, ABR92499
Desulfosarcina variabilis DSM 2060, ABR92511
apr19 (0, 1), AB645632
apr20 (1, 0), AB645633
Desulfobacterium indolicum DSM 3383, ABR92477
apr18 (2, 0), AB645630-31
Desulfonema magnum DSM 2077, ABR92507
Thermacetogenium phaeum DSM 12270, ABR92597
apr3 (1, 4), AB645551-55
apr4 (2, 0), AB645556-57
Desulfotomaculum thermocisternum DSM 10259, ABR92594
apr1 (0, 2), AB645547-48
apr2 (0, 2), AB645549-50
apr26 (0, 2), AB645645-46
Thiocapsa roseopersicina DSM 217, ABV80060
apr22 (5, 1), AB645635-40
Sideroxydans lithotrophicus ES-1, ADE11948
Halochromatium salexigens DSM 4395, ABV80052
apr23 (1, 0), AB645641
apr24 (1, 0), AB645642
apr25 (2, 0), AB645643-44
56
51
98
50
82
87
81
99
62
74
69
91
77
96
78
98
96
55
99
62
71
100
99
51
100
99
58
93
83
0.05