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NOTE

Filamentous sulfur-oxidizing bacteria of the genus *Thioploca* from Lake Tonle Sap in Cambodia

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ABSTRACT: Members of the genus *Thioploca* are uncultured filamentous sulfur-oxidizing bacteria that live in freshwater/brackish sediments and have the ability to store nitrate in high concentrations in their cells. Their close relatives that inhabit marine sediments, such as *Thiomargarita* and '*Candidatus* Marithioploca', are thought to greatly influence cycles of sulfur, nitrogen, and phosphorus. To date, the genus *Thioploca* has been reported only from temperate and subarctic areas. Our demonstration of *Thioploca* in Lake Tonle Sap, Cambodia, is the first report of this genus in a tropical lake. The filaments obtained from Lake Tonle Sap were morphologically similar to those of other lakes. Phylogenetic analysis based on genes for 16S rRNA, 23S rRNA, and the 16S-23S rRNA internal transcribed spacer (ITS) region revealed that 3 distinct lineages coexist in this lake. These results indicate that the geographical distribution and phylogenetic diversity of the genus *Thioploca* is greater than previously thought.

KEY WORDS: Sulfur-oxidizing bacteria · Freshwater lake · Sediment · Phylogeny

INTRODUCTION

Members of the genus *Thioploca* are uncultured sulfur-oxidizing bacteria that live in freshwater and brackish water sediments. They are visible to the naked eye as white filaments because of abundant elemental sulfur granules in their cells and their conspicuous morphology. Several multicellular filaments (trichomes) form bundles surrounded by a common sheath, and each trichome can glide independently within the common sheath. Early descriptions of *Thioploca* used the diameter of the trichome as a characteristic to discriminate species (Lauterborn 1907, Wislouch 1912, Maier 1984); most of the recent studies were performed on samples with trichome diameters of ~4 µm (Zemskaya et al. 2001, Kojima et al. 2003, 2006, Høgslund et al. 2010, Nemoto et al. 2011). Until recently, this genus also included marine species with much thicker trichomes, but a new independent candidate genus, '*Candidatus* Marithioploca', has now been proposed to encompass these species (Salman et al. 2011).

Thioploca spp. have the ability to accumulate nitrate at high concentrations within their large cells; nitrate concentrations in the cells have been reported to be 2 or 3 orders of magnitude higher than ambient concentrations (Zemskaya et al. 2001, Kojima et al. 2007, Høgslund et al. 2010). This feature is more pronounced in closely related marine species in the genera *Thiomargarita* and '*Candidatus* Marithioploca', among others, some of which are thought to greatly influence cycles of sulfur, nitrogen, and phosphorus in marine environments (Otte et al. 1999, Sayama 2001, Schulz & Schulz 2005).

The first description of *Thioploca* species was of a population found in Lake Constance in Europe

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(Lauterborn 1907). Subsequently, populations were reported from Lake Erie and Lake Ontario in North America (Maier & Murray 1965, Dermott & Legner 2002), Lake Baikal in Siberia (Zemskaya et al. 2001), and Lake Biwa, Lake Ogawara, and Lake Okotanpe in Japan (Nishino et al. 1998, Kojima et al. 2006, Nemoto et al. 2011). These lakes are located in areas of mid-range to high latitudes in the northern hemisphere. The 16S rRNA gene sequences of *Thioploca* in these lakes are very closely related, even though they are separated geographically.

In the present study, we report the occurrence of the genus *Thioploca* in Lake Tonle Sap (Cambodia), which is located in a tropical monsoon climatic zone, and show results of phylogenetic analyses.

MATERIALS AND METHODS

Samples were obtained in May 2005, from the south basin of Lake Tonle Sap. This lake is characterized by a dramatic seasonal change of water level, and the samples were obtained in the season of shallow water depth. The sampling site, Site S3 (12° 33' 57'' N, 104° 22' 10'' E; 0.4 m deep), corresponds to the site of a previous study (Ohtaka et al. 2010). At the sampling time, the pH and specific conductivity of surface water were 8.0 and 155 µS cm^{-1} respectively (Ohtaka et al. 2010). The lake water was characterized by very low transparency (0.02 m). The sediment samples, taken using an Ekman-Birge grab sampler, were passed through a mesh with a pore size of 0.25 mm. The retained filamentous materials were immediately fixed in 100% ethanol.

Before microscopic observation, the filamentous samples were soaked in distilled water for 30 min to replace the ethanol with water. The shapes of the filaments were observed, and the diameters of 200 trichomes were measured under an optical microscope (Axioplan 2, Zeiss).

Thioploca filaments (sheaths with trichomes) were carefully sorted from residual contaminating materials using tweezers and then rinsed with sterile water. DNA extraction was performed as described previously (Kojima et al. 2003). For *Thioploca*-specific polymerase chain reaction (PCR) amplification of the DNA region spanning from the 16S rRNA gene to the 23S rRNA gene, the primer FWTpl131F (*Escherichia coli* 16S rRNA positions 131–148; Salman et al. 2011) was used in combination with the probe GAM42a for *Gammaproteobacteria* (*E. coli* 23S rRNA positions 1027–1043; Manz et al. 1992) as a reverse primer. The following PCR conditions were used: 94°C for 5 min; 32 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 3.5 min; and 68°C for 7 min. The PCR product was cloned using the TOPO TA cloning kit (Invitrogen). The regions including the cloned inserts were directly amplified from the cells by PCR, using the vector-specific primer pair M13F/M13R. Nucleotide sequences obtained from 12 clones were aligned with reference sequences from the public database using the ClustalX program (Thompson et al. 1997). Independent phylogenetic analyses were performed for each of the 3 gene regions (16S rRNA, 23S rRNA, and 16S-23S rRNA ITS) because the availability of reference sequences varied greatly between them. Neighbor-joining trees were constructed using MEGA4 software (Tamura et al. 2007). Bootstrap analysis was performed for 1000 replicates.

The nucleotide sequences obtained were submitted to GenBank under the accession numbers AB699673 to AB699684.

RESULTS AND DISCUSSION

The filaments analyzed in the present study were not abundant in the sediment of Lake Tonle Sap, and their occurrence was noticed only after sieving. Microscopic observation revealed the presence of trichomes with tapered ends, covered with a common sheath (Fig. 1). The trichome diameters ranged from 2.6 to 5.8 µm, with a mean of 3.9 µm (Fig. 2). These morphological characteristics are similar to those of *Thioploca* species of other lakes. The sheaths had no constricted zone, which was observed in samples collected from Lake Biwa and Lake Constance (Kojima et al. 2003).

All of the 16S rRNA gene sequences obtained were related to published *Thioploca* sequences (Fig. 3).

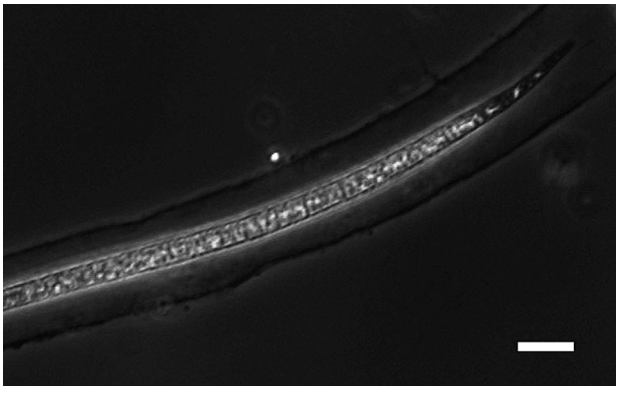


Fig. 1. Microphotograph of the tip of a trichome within a sheath. The tapered ends are characteristic for the genera *Thioploca* and *Candidatus* Marithioploca. Scale bar = 10 µm

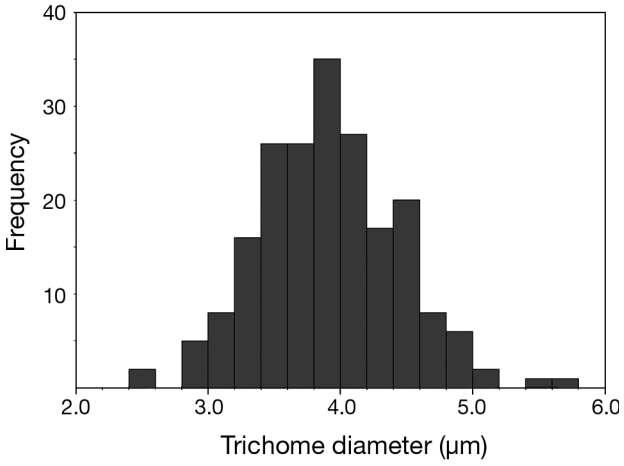
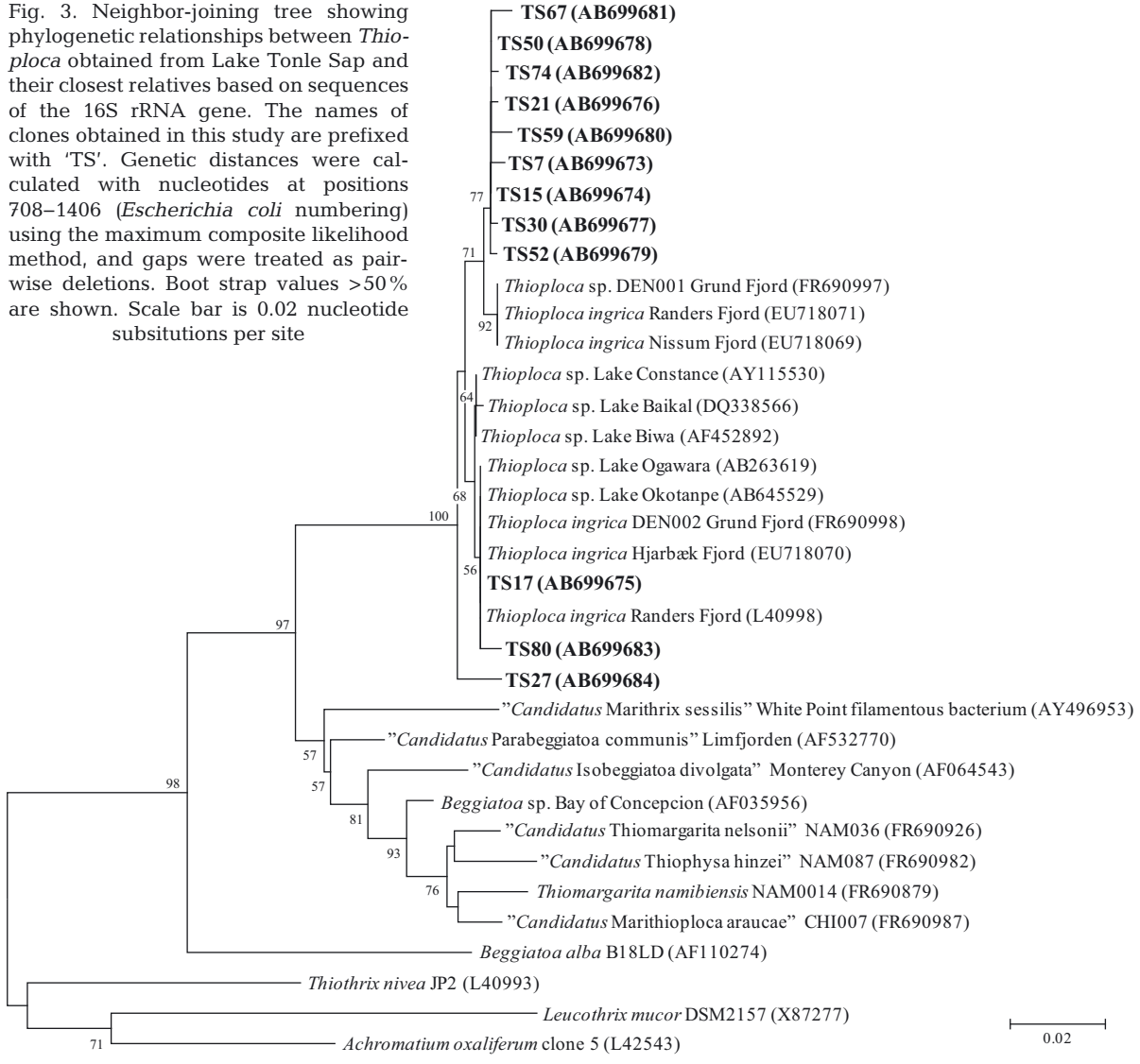


Fig. 2. Distribution of *Thioploca* trichome diameters (n = 200)

Sequences of 9 clones were most closely related to a sequence from Grund Fjord, Denmark, and formed a distinctive cluster. Within this cluster of 9 clones, the 16S rRNA gene sequence identities were >99%. The sequence identities between these 9 clones and the other clones clustered with sequences from limnetic sediments, TS17 and TS80, were ~98%. The other clone, TS27, represented another independent lineage. The lowest identity, 97.4%, was observed between TS27 and TS59. The results from the analysis of the ITS region were consistent with those of the 16S rRNA gene (Fig. 4), and similar results were also obtained in the analysis of the 23S rRNA gene (Fig. 5).

Based on morphological and phylogenetic characterizations, the filamentous organisms from Lake Tonle Sap were members of the genus *Thioploca*. According

Fig. 3. Neighbor-joining tree showing phylogenetic relationships between *Thioploca* obtained from Lake Tonle Sap and their closest relatives based on sequences of the 16S rRNA gene. The names of clones obtained in this study are prefixed with 'TS'. Genetic distances were calculated with nucleotides at positions 708–1406 (*Escherichia coli* numbering) using the maximum composite likelihood method, and gaps were treated as pairwise deletions. Boot strap values >50% are shown. Scale bar is 0.02 nucleotide subsitutions per site



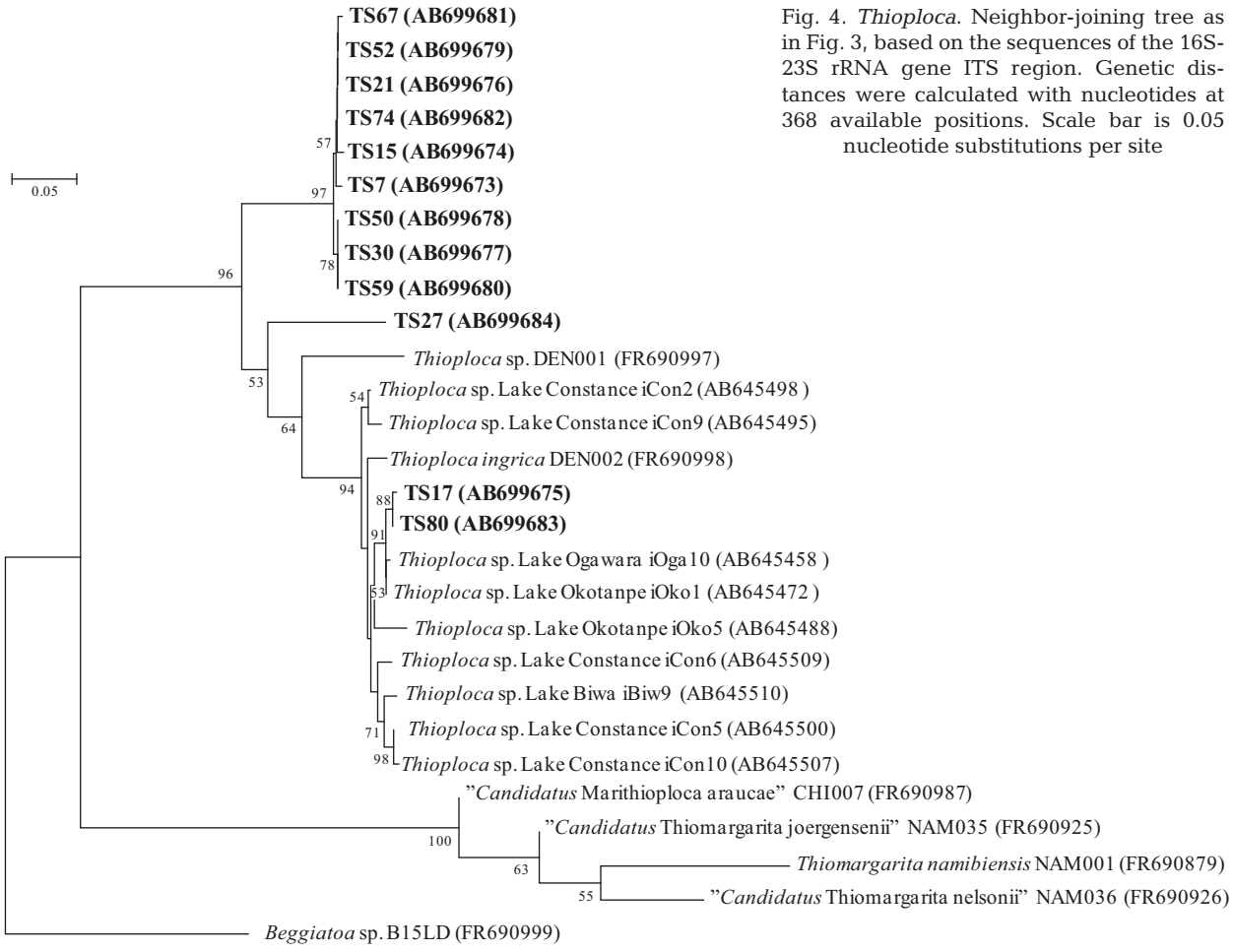


Fig. 4. *Thioploca*. Neighbor-joining tree as in Fig. 3, based on the sequences of the 16S-23S rRNA gene ITS region. Genetic distances were calculated with nucleotides at 368 available positions. Scale bar is 0.05 nucleotide substitutions per site

to the classification recently proposed (Salman et al. 2011), the present study is the first report of the genus *Thioploca* from a tropical area. To date, members of the genus *Thioploca* have been reported only from temperate and subarctic areas (Lauterborn 1907, Maier & Murray 1965, Zemskaya et al. 2001, Dermott & Legner 2002, Kojima et al. 2003, 2006, Høgslund et al. 2010, Nemoto et al. 2011). Among these lakes and fjords, Lake Biwa is located at the lowest latitude. In this lake, the 16S rRNA gene sequence of *Thioploca* was obtained from a sample taken from a profundal site, with temperatures ranging between 7 and 8°C throughout the year (Nishino et al. 1998, Kojima et al. 2003). In Lake Biwa, *Thioploca* was also detected in a site where sediment temperatures reached 30°C in summer but decreased to 7°C in winter. In contrast, the bottom water temperature of the sampling site in Lake Tonle Sap was 28.1°C in December 2004 and 30.9°C in May 2005 (Ohtaka et al. 2010).

The phylogenetic diversity within *Thioploca* of Lake Tonle Sap was consistently shown in the analyses of 16S rRNA, 23S rRNA, and the ITS region. In other habitats, only a single 16S rRNA gene sequence of *Thioploca* has been reported from each lake (Kojima et al. 2003, 2006, Zemskaya et al. 2009, Nemoto et al. 2011), although the coexistence of different sequences was reported in a brackish fjord (Høgslund et al. 2010, Salman et al. 2011). In the present study, DNA was extracted from a clump of tangled filaments obtained by sieving bulk sediment. Therefore, there is no way to relate each sequence to morphology or localization in the sediment. Although further investigation is needed to evaluate the ecological significance of the coexistence observed, some sequences from Lake Tonle Sap were very closely related to those from other lakes and fjords, while others were distinct from those sequences. The former finding suggests that this lineage can sustain populations under a wide range of environmental conditions, whereas the latter result may indicate the presence of novel types of *Thioploca* specifically adapted to tropical lakes.

A previous study reported specific primers for the 16S rRNA gene of *Thioploca* (Kojima et al. 2003), but

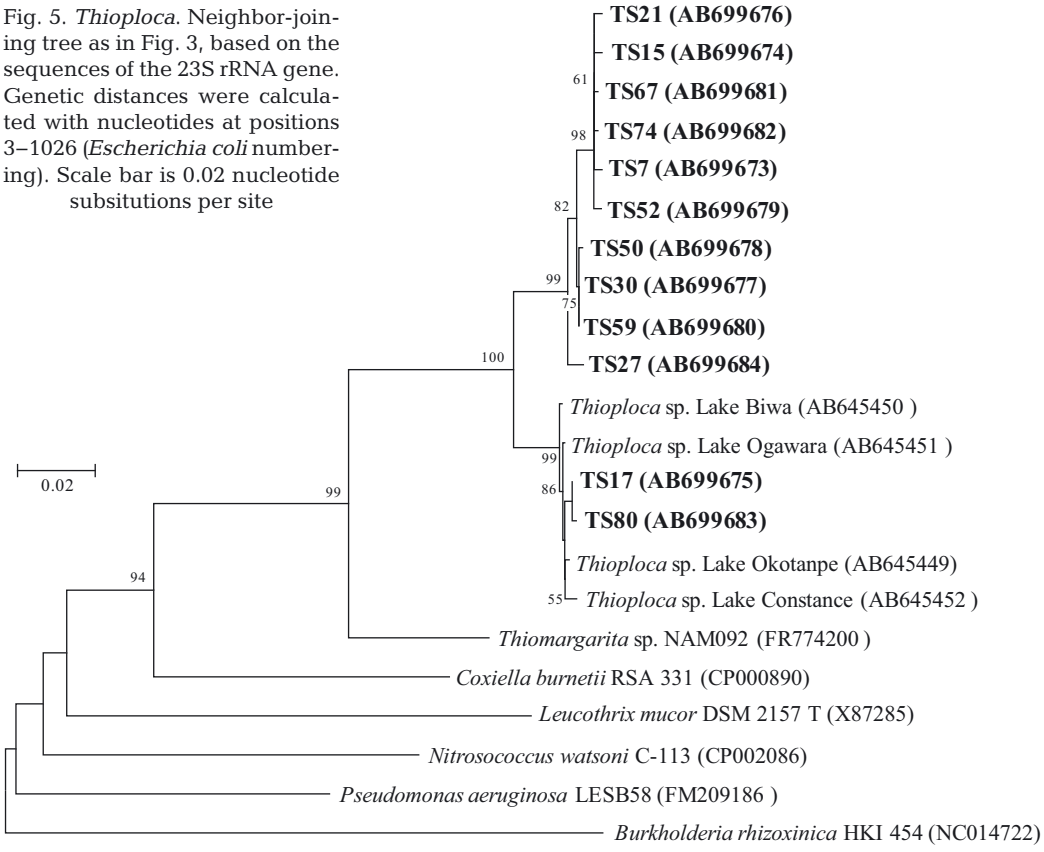


Fig. 5. *Thioploca*. Neighbor-joining tree as in Fig. 3, based on the sequences of the 23S rRNA gene. Genetic distances were calculated with nucleotides at positions 3–1026 (*Escherichia coli* numbering). Scale bar is 0.02 nucleotide subsitutions per site

there are some mismatches between these primers and sequences of *Thioploca* from Lake Tonle Sap. The primer used in the present study, FWTpl131F, may be more suitable for detection of diverse *Thioploca*, although its specificity is lower than those of the above-mentioned primers.

The present study shows that the geographical distribution and phylogenetic diversity of the genus *Thioploca* are greater than previously known. Further detailed analysis of the phylogenetic relationships and ecological characteristics of this genus in different habitats will provide more insights into *Thioploca* biogeography and the ecological interactions among coexisting strains.

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