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Polyphasic characterization of a thermo-tolerant filamentous cyanobacterium isolated from the Euganean thermal muds (Padua, Italy)

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In this paper we report a morphological, ultrastructural, biochemical and molecular (16S rRNA, 16S–23S ITS, *rbc*L and *rpo*C1 gene sequencing) survey on a very thin, non-heterocystous, filamentous cyanobacterium, isolated from mats covering several mud maturation tanks of the Euganean Thermal District, at temperatures ranging from 26 to 59°C. Denaturing gradient gel electrophoresis results, obtained using cyanobacterial primers targeting the 16S rRNA gene, confirmed that this cyanobacterium is one of the commonest taxa growing in the mud tanks. Comparison with *Geitlerinema* sp. PCC 8501 (=*Phormidium laminosum* Gomont ex Gomont strain OH-1-p Cl 1), a thin thermobiotic species isolated from hot springs of Oregon and morphologically similar to our isolate, led us to hypothesize that the Euganean and PCC 8501 strains are either very similar sister species or ecotypes of the same species in a yet to be defined clade, clearly distinct within the paraphyletic *Leptolyngbya* group.

Key words: cyanobacteria, morphology, pigment composition, thermal springs, ultrastructure, 16S rRNA, 16S–23S ITS, *rbc*L, *rpo*C1

Introduction

Thermal hot springs are unusual environments, distributed in widely separated geographical areas, characterized by different physical and chemical features of the waters that severely limit the survival of photoautotrophic organisms. In the acidic, high temperature conditions around hot springs (pH < 3) the presence of photoautotrophic organisms is restricted to the Cyanidiales, a group of asexual unicellular red algae (Ciniglia *et al.*, 2004), but in hot springs with alkaline and neutral pH, cyanobacteria are dominant. In the latter environments, cyanobacterial diversity is rather low, since most species are not able to tolerate temperatures higher than 50–60°C (Edwards *et al.*, 1997; Miller & Castenholz, 2000; Balme *et al.*, 2001), apart from some unicellular *Synechococcus* spp. that can live at 73–74°C (Castenholz, 1969; Miller & Castenholz, 2000; Steunou *et al.*, 2006). Moreover, most thermotolerant cyanobacteria from neutral and alkaline pH waters are excluded by high (>1 mg l^{-1}) soluble sulphide concentrations (Castenholz, 1976, 1977).

The specificity of these thermal environments makes the cyanobacteria living there endemic (Castenholz, 1996; Papke *et al.*, 2003; McGregor & Rasmussen, 2008). Therefore, apart from a few cosmopolitan thermophilic cyanobacterial species (e.g. *Mastigocladus laminosus* Cohn) (Castenholz, 1996; Miller *et al.*, 2007), most cyanobacteria described from this environment are new operational taxonomic units (OTUs) (Ward *et al.*, 1998; Taton *et al.*, 2006; McGregor & Rasmussen, 2008).

During recent studies to evaluate the biodiversity of the cyanobacterial mats of the Euganean Thermal Springs (Padua, Italy), an Oscillatorialean *Leptolyngbya*-like cyanobacterium was found in several tanks with mud temperatures ranging from 26 to 59°C. Denaturing gradient gel electrophoresis (DGGE) analyses, carried out on the cyanobacterial mats of different spas of the Euganean Thermal District, revealed that this is one of the commonest organisms.

To ascertain the taxonomic position of this organism, a study was undertaken to compare its morphological, ultrastructural and biochemical

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features with those of strain PCC 8501, presently listed as *Geitlerinema* sp. (= *Phormidium laminosum* Gomont ex Gomont strain OH-1-p Cl 1 = *Leptolyngbya laminosa* (Gomont) Anagnostidis et Komárek 1988), isolated from Hunter's Hot Spring, Oregon, USA. This strain was previously identified by Castenholz (1970) as the same taxon as CCMEE 5345, isolated from the Abano Terme (Padua) in 1969.

Accordingly, the genetic similarity and phylogenetic relationships of the Euganean strain (ETS-08) and the Oregon strain (PCC 8501) were investigated by sequencing the *rbc*L gene, encoding the large subunit of the ribulose 1,5-bisphosphate carboxylase-oxygenase, the *rpo*C1 gene, encoding the γ subunit of RNA polymerase, the 16S rRNA gene and the 16S–23S internal transcribed spacer (ITS) region. Based on our results, ETS-08 could either be a very similar sister species to PCC 8501 or an ecotype of the same species in a currently undefined clade within the paraphyletic *Leptolyngbya* group.

Materials and methods

Samples for isolation of ETS-08 were collected in February 2004 from microbial mats on the thermal muds of the Garden Hotel (45°19′0″N, 11°46′0″E) in Montegrotto Terme (Padua, Italy). Other samples were collected from five different spas in the same thermal area of Abano–Montegrotto between 2004 and 2007. At all sampling sites deep blue–green cyanobacterial biofilms, formed of thin filaments, and spotty brownish mats, formed by diatoms, develop under attenuated light conditions at the surface of sedimentary blue clay during the preparation of mud for therapeutic purposes. Mud is collected regularly from the bottom of the thermal lakes, Arquà Petrarca and Lispida (Padua, Italy), and transported to the health spa for processing. Thermal spring water, ranging from 30 to 50°C, pH of 6.8–7, flows continuously into the mud tanks to ensure appropriate mixing of dissolved salts with clay particles and mud maturation (Galzigna *et al.*, 1996). After 4–5 weeks, thick cyanobacterial mats are usually fully developed on the mud surface.

The predominant cations ($mg\,l^{-1}$) in the spring water flowing at 70°C at the sampling sites were the following: Ca^{2+} (310–340), Na^{+} (1000–1200), Mg^{2+} (60–63) and K^{+} (75–83). The anions ($mg\,l^{-1}$) were: Cl^{-} (1900–2100), HCO_3^{-} (170–180), SO_4^{2-} (680–780), while silica and hydrogen sulphide concentrations were around 58 and 0.09–0.2 $mg\,l^{-1}$ respectively.

Experimental growth conditions

Axenic cultures of ETS-08 and of PCC 8501, obtained from the Pasteur Culture Collection of Cyanobacteria (Paris), were cultured in BG11 medium (Rippka *et al.*, 1979) in a growth chamber at 30–35°C, with a photosynthetic photon flux density of 35 μmol photons $m^{-2}\,s^{-1}$ under a 12-h:12-h light–dark cycle. Petri dishes with 1% agar solidified BG11 medium were used to test the gliding ability under the same culture conditions. All morphological observations and biochemical analyses were carried out on cultures in exponential growth. A living culture of ETS-08 is available from the laboratory of Professor Carlo Andreoli.

Light microscopy

Observations of cultured cells were made with a DMR Leica (Sweden) microscope equipped with a digital image acquisition system. The morphological identification of the strain was based on the diacritical traits proposed by Komárek & Anagnostidis (2005).

Electron microscopy

For scanning electron microscopy (SEM) cultured cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) for 4 hours at 4°C and then treated according to Moro *et al.* (2007). For transmission electron microscopy (TEM), cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) for 4 hours at 4°C. After washing in cacodylate buffer, the specimens were post-fixed in 1% OsO_4 in the same buffer for 2 hours and dehydrated in a graded ethanol series followed by propylene oxide. The specimens were stained with uranyl acetate while undergoing dehydration in 75% ethanol and samples were embedded in Araldite resin. Ultrathin sections were cut with an Ultracut S, Reichert ultramicrotome (New York, USA), post-stained with lead citrate and examined with a HS9 Hitachi (Tokyo, Japan) transmission electron microscope operating at 75 kV.

Water-soluble pigment analyses

Phycobiliproteins (PBPs) were extracted from pellets of cultured cells ground finely in a mortar with liquid nitrogen. The extract absorbance was measured with a DU530 Beckman Coulter spectrophotometer (Fullerton, California, USA) at 562 nm (phycoerythrin), 615 nm (phycocyanin) and 652 nm (allophycocyanin), and PBP concentrations were calculated using the extinction coefficients proposed by Bennett & Bogorad (1973).

Lipid-soluble pigment analyses

The chlorophyll *a* and carotenoid analyses were carried out by reversed phase high performance liquid chromatography (HPLC) on cultured cell extracts according to Komárek *et al.* (1999). The Agilent HPLC system (Waldbronn, Germany) consisted of a Rheodyne valve (Rhonert Park), a reversed-phase column (5 μm particle size; 25 × 0.4 cm; 250/4 RP 18 Lichrocart), a binary pump and an Agilent 1100 series diode array detector that allows VIS pigment absorption spectra recording during the chromatogram run.

Genetic analyses

Genomic DNA was extracted from cell pellets, using the Genomic DNA purification kit (Fermentas©, Burlington, Ontario, Canada). The 16S rRNA gene and 16S–23S ITS region were amplified from DNA extracts by PCR, using the primers and the conditions reported in Moro *et al*. (2007). Amplification of the *rbc*L and *rpo*C1 genes was carried out with the primers reported in Tomitani *et al*. (2006) and Seo & Yokota (2003), respectively. The PCR products were visualized with ethidium bromide staining after electrophoresis in a 1% agarose gel, purified with the ExoSAP-IT^{TM} kit (Amersham Biosciences, Piscataway, New York, USA) and directly sequenced. Sequencing was performed at the BMR-Genomics Sequencing Service (Padua University) on both strands to ensure accuracy of the results. The final consensus sequences were assembled using the SeqMan II program from the Lasergene software package (DNAStar©, Madison, WI) and analysed by similarity search using the BLAST program (Altschul *et al*., 1990), available at the NCBI web server (www.ncbi.nlm.nih.gov/blast) and the ARB database (Ludwig *et al*., 2004). The sequences were aligned for phylogenetic analyses using the ClustalW computer program (Thompson *et al*., 1994) and nucleotide positions containing gaps or missing data were deleted. The alignments are available upon request from Professor Carlo Andreoli.

To infer phylogenetic relationships among the taxa, different analytical methods were employed. Maximum likelihood (ML) with the PHYML 2.4.4 program (Guindon & Gascuel, 2003) was used for each gene. Non-parametric bootstrap re-sampling (Felsenstein, 2003) was performed to test the robustness of the tree topology (1000 replicates). In addition, ML, neighbor-joining (NJ) and maximum parsimony (MP) trees were also generated using the PAUP*4.0b10 software package (Swofford, 2001) to confirm the 16S rRNA results. The program Modeltest version 3.7 (Posada & Crandall, 1998) was used to determine parameters for ML analyses.

A Bayesian analysis was performed using MrBayes version 3.0 (Ronquist & Huelsenbeck, 2003). The substitution model was the GTR + I + G. Bayesian analysis was performed using four search chains for 1 000 000 generations, sampling trees every 100 generations. The first 1000 trees were discarded as burn-in. Parameter stability was estimated by plotting log-likelihood values against generation time, and a consensus tree with posterior probabilities was then generated.

After similarity searches, the most similar sequences were selected. A data set of 52 sequences was created including, besides strain ETS-08, other isolates identified and described as members of the family Pseudanabaenaceae. *Gloebacter violaceus* PCC 7421 (GenBank accession no. AF132790) was used as the outgroup. The comparison was limited to sequences longer than 1200 bp available in GenBank. After eliminating gaps or sites whose nucleotides had not been determined, the final matrix included 1089 nucleotides and 257 parsimony-informative sites (23.6%). A threshold of 97.5% identity among sequences was used to delimit species (Stackebrandt & Goebel, 1994).

The 16S rRNA, ITS region, *rbc*L and *rpo*C1 gene sequences of strain ETS-08 were deposited in DDBJ/GenBank^{TM}/EBI Data Bank with the accession numbers, FM210757 (16S rRNA + ITS region), FM955229 and FM955230, respectively. The 16S rRNA, ITS region and *rpo*C1 gene sequences of PCC 8501 were deposited with the access codes, FM210758 (16S rRNA + ITS region) and FM955231, respectively. The *rbc*L sequence of PCC 8501 obtained in this work was identical to that already available in GenBank (accession no. EU119380).

Denaturing gradient gel electrophoresis

DNA from four cyanobacterial cultures and six mat samples from different spas was extracted by Wizard Magnetic DNA Purification System For Food kit (Promega, cat. #FF3752) and amplified by PCR using the primers and the conditions reported in Nübel *et al*. (1997). DGGE was performed as described by Nübel *et al*. (1997). Briefly, 1.5 mm-thick 8% polyacrylamide gels with a denaturing urea-formamide gradient from 20 to 60% were used for 16S rRNA gene fragments. Gels were run for 5 h at 220 V and a constant temperature of 60°C. Each gel, stained with ethidium bromide, was visualized on a UV transilluminator, and individual bands were excised from the gel. Eluted DNA was reamplified using the same primers and purified PCR products were sequenced. A mixture of PCR products derived from ETS-08 and three other cyanobacterial strains [*Spirulina* sp. ETS-09, *Cyanobacterium aponinum* ETS-03 (Moro *et al*., 2007), *Phormidium* sp. ETS-05 (Ceschi-Berrini *et al*., 2004)] isolated from the Euganean Thermal Springs and cultured in our laboratory was applied on each gel as a standard to allow gel-to-gel comparison.

Results

Cytomorphological characters of field and cultured strains

Field samples were mainly formed of densely packed Oscillatorialean cyanobacteria with inter-twined filaments and some diatom cells. Filaments of the strain ETS-08 were variously curved, flexuous, densely packed and entangled, frequently spirally coiled, forming expanded blue-green mats on the substratum. In older liquid cultures, the mats formed large membranaceous aggregates floating at the air-liquid interface. The individual isopolar filaments were surrounded by a thin colourless sheath (Figs 1–3). Trichomes were formed of cylindrical cells, longer than wide (1.2–3 μm long, 0.8–1 μm wide), with distinct constrictions at the cross walls (Fig. 2). The apical cells were rounded. Cell division took place perpendicular to the longitudinal axis of the trichome. The daughter cells divided and grew to the size of

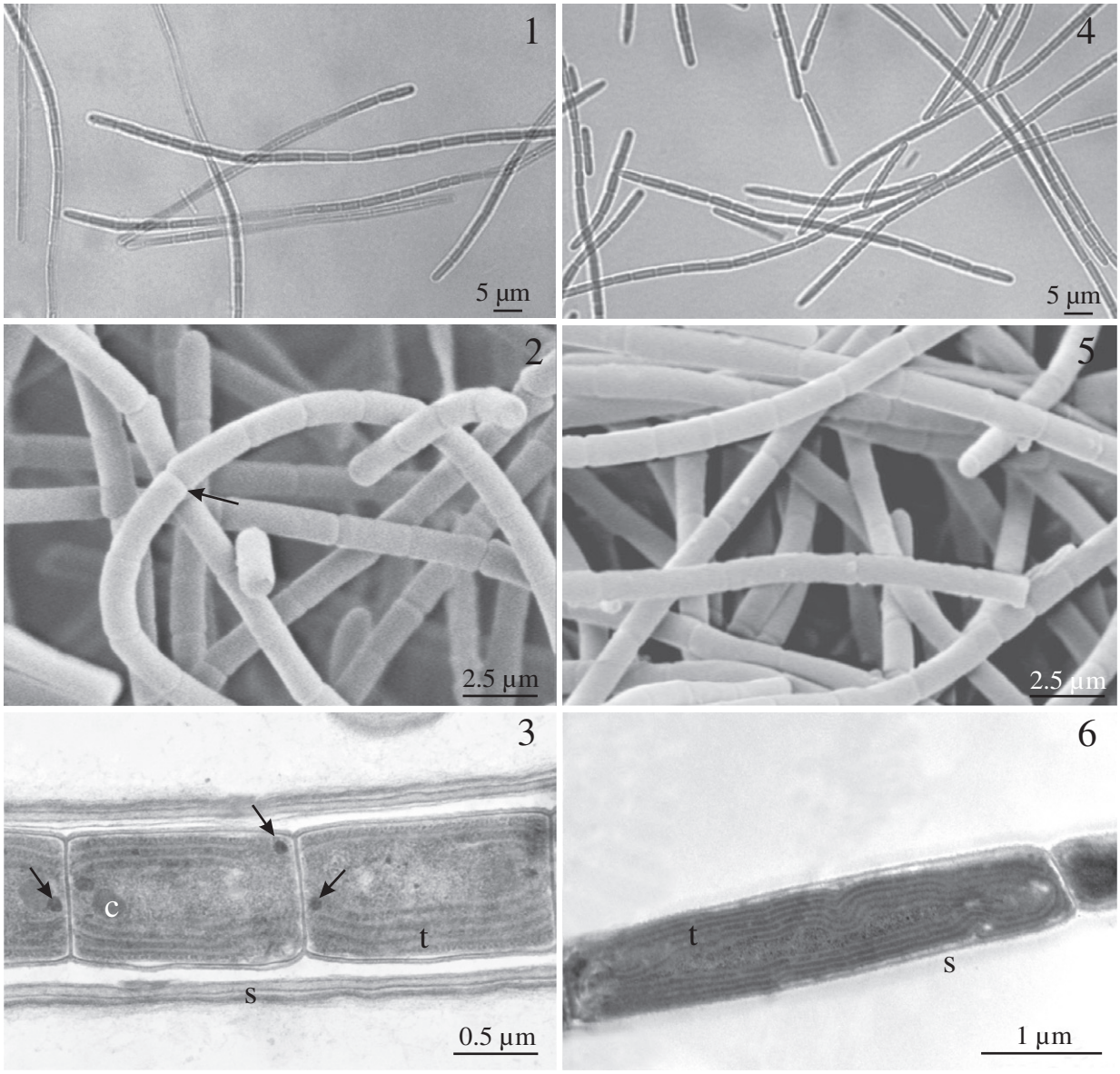
the mother cell before the next division. Reproduction was by trichome fragmentation into short hormogonia (Fig. 2) without necridia.

Transmission electron microscopical observations (Fig. 3) revealed the multilayered structure of the sheath (about 0.15 µm thick) and the presence of 3–4 thylakoids arranged in parallel at the periphery of the cells. Large carboxysomes, cyanophycin granules and a few small polyphosphate bodies were present in the cytoplasm of most cells. It was sometimes possible to see granules at the cross walls. No gas vesicles were observed.

Most of these features were also observed in PCC 8501 (Figs 4–6) although this isolate sometimes had longer cells (2–6.5 µm long, 0.8–1.8 µm wide) and no multilayered sheath (Fig. 6). Like ETS-08, PCC 8501 showed slow gliding motility on solid medium but appeared non-motile in liquid culture.

Biochemical characters of cultured strains

Spectrophotometric measurements of buffered saline PBP extracts of ETS-08 revealed the presence of allophycocyanin (36%), phycocyanin (52%) and phycoerythrin (12%). The HPLC analyses produced peaks corresponding to two different myxoxanthophylls (*sensu* Karsten &



Figs 1–6. Figs 1–3. Strain ETS-08. Fig. 1. Light micrograph showing flexuous, densely packed trichomes. Fig. 2. Scanning electron micrograph of trichomes formed of cylindrical cells and characterized by distinct constrictions at the cross walls (arrow). Fig. 3. Transmission electron micrograph of cells showing parallel thylakoids (t) arranged at the periphery of the cells, carboxysomes (c) and cyanophycin granules (arrows). Note the multilayered structure of the sheath (s). Figs 4–6. Strain PCC 8501. Fig. 4. Light micrograph of variously curved trichomes. Fig. 5. SEM micrograph showing the cyanobacterial filaments. A rounded apical cell can be seen. Fig. 6. Transmission electron micrograph of a cell with peripheral parallel thylakoids (t) and an unlayered sheath (s).

Garcia-Pichel, 1996), isozeaxanthin, zeaxanthin, chlorophyll *a*, echinenone and *β*-carotene (Fig. 7*a*). On the other hand, PCC 8501 showed slightly different PBP content (allophycocyanin 42%, phycocyanin 44%, phycoerythrin 14%) and lacked one myxoxanthophyll (Fig. 7*b*).

Genetic analyses of strains

Almost the full length of the 16S rRNA gene was sequenced for ETS-08 and PCC 8501 (1437 and 1443 nucleotides, respectively). These sequences showed a 99.2% pair-wise identity (10 substitutions and 2 insertions/deletions). The NJ, MP (not shown) and ML (Fig. 8) phylogenetic trees had the same topology and showed that ETS-08 and PCC 8501 fell in clade A, with five filamentous, thermophilic strains (Ob05, OS Type I, tBTRCCn 408, tBTRCCn 302, and tBTRCCn 102), supported by a bootstrap value of 98%.

The type species of *Leptolyngbya*, *L. boryana,* PCC 73110 (89.4% pair-wise identity to ETS-08 and 89.3% to PCC 8501), and *L. boryana* (ex *L. foveolarum*) Komárek, 1964/112 (90.8% pair-wise identity to ETS-08 and 90.7% to PCC 8501), fell in clade B, which formed a paraphyletic group with clade A (Fig. 8). This underlines the polyphyletic nature of *Leptolyngbya*. Other members of the family Pseudanabaenaceae, such as the halophilic strains of *Geitlerinema* (clade C) and the two *Halomicronema* (clade D), formed distinct groups. The Bayesian analysis produced a consensus topology that was highly congruent with those obtained by ML and MP analyses.

Three more data sets were created to infer the phylogenetic relationships amongst the strains using the 16S–23S ITS region, *rbc*L and *rpo*C1 sequences (Figs 9–11). Unfortunately, very few sequences of these genes are available for cyanobacteria. Thus, the data sets included 16, 10 and 15 sequences for the ITS rRNA region, *rbc*L and *rpo*C1 genes, respectively. The ML trees showed close relationships between ETS-08 and PCC 8501, supported by very high bootstrap values (100% for 16S–23S ITS, 100% for *rbc*L and *rpo*C1 genes) and 70.9% sequence identity for ITS, 86.6% for *rbc*L and 90.1% for *rpo*C1. In the ITS tree ETS-08 and PCC 8501 clustered together with Antarctic *Leptolyngbya*, while *Geitlerinema* and other *Leptolyngbya* strains formed a sister group (Fig. 9).

In the *rbc*L tree (Fig. 10), ETS-08 and PCC 8501 again clustered together, while the closest related strain, *L. boryana* PCC 73110, showed 85.0% pair-wise identity with ETS-08 and 80.9% with PCC 8501. *Phormidium persicinum* (CCMP638)

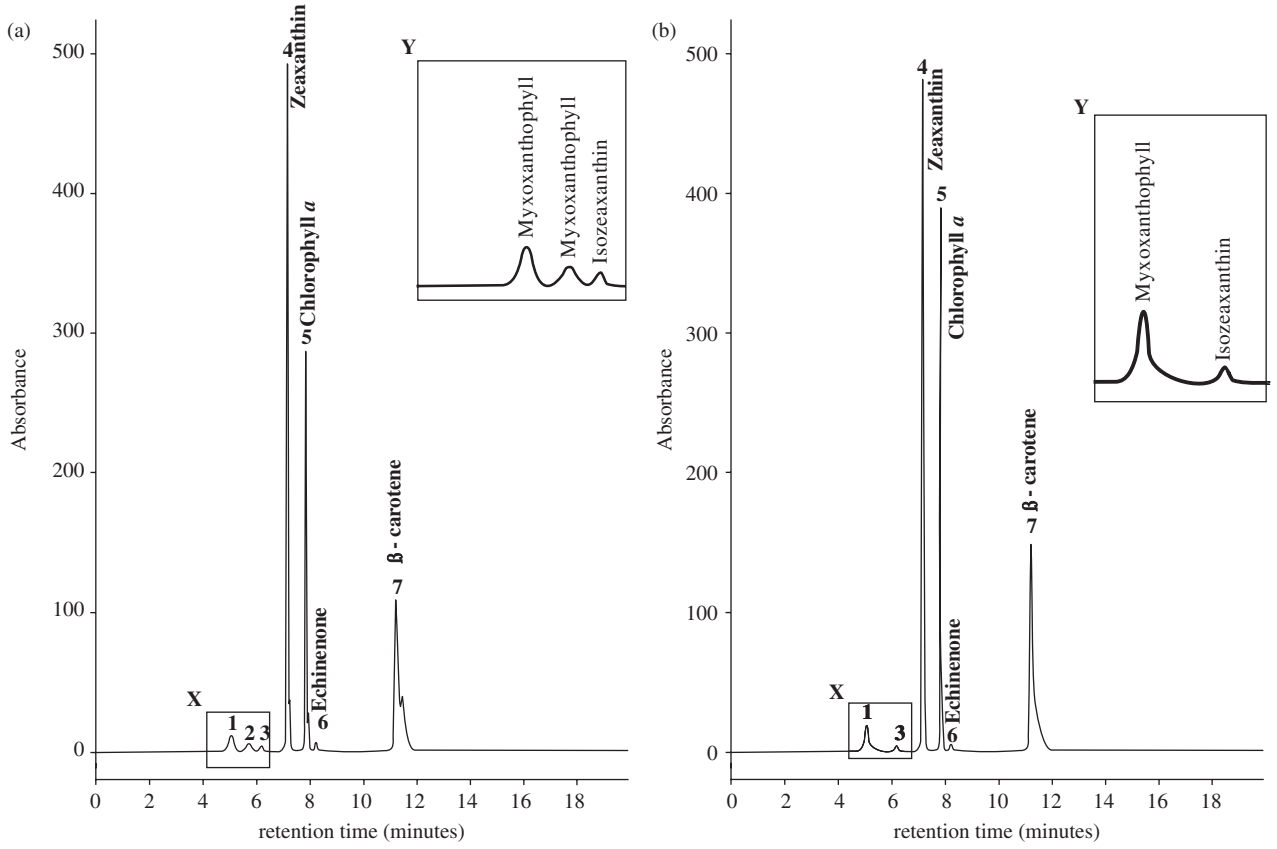


Fig. 7. HPLC chromatograms (450 nm) of pigments from ETS-08 (a) and PCC 8501 (b). In both chromatograms sections inserted in box X are amplified in box Y.

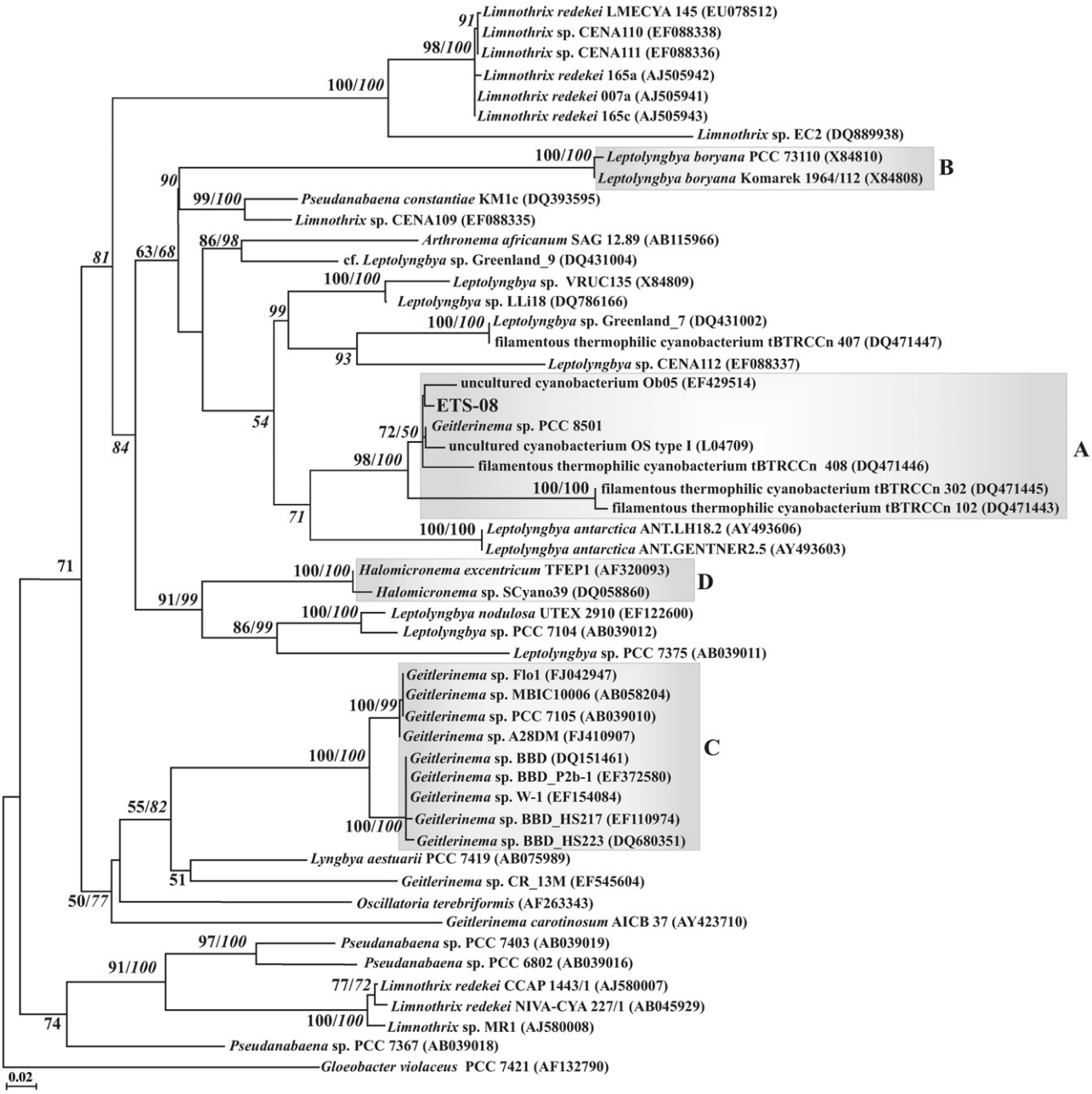


Fig. 8. Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using maximum-likelihood (ML) analysis. Numbers above branches indicate, in order, ML bootstrap value and Bayesian posterior probability (BI), in italics. When ML values are ≤50% only the BI number is indicated. Sequences determined in this work are indicated in bold and larger font. GenBank accession numbers are indicated in brackets. Bar represents 0.02 nucleotide substitutions per site.

was sister taxon (95% bootstrap) to the above-mentioned clade. However, another strain belonging to the Pseudanabaenaceae (*Pseudoanabaena* sp., PCC 7403) grouped in a different cluster together with other Oscillatoriales. Comparison of the *rbc*L sequence of PCC 8501 obtained in our study with that previously reported in GenBank (EU119380) showed that the strain has not been contaminated.

The *rpo*C1 tree (Fig. 11) showed *Phormidium* IAM M-99 and *Geitlerinema* sp. Flo 1 as the closest related strains to ETS-08 and PCC 8501, with pair-wise identities of about 78–80%.

Genetic analysis of cyanobacterial mats

The DGGE profile of the four cyanobacterial strains from the Euganean mats, ETS-08, *Phormidium* sp. (ETS-05), *Cyanobacterium aponinum* (ETS-03) and *Spirulina* sp. (ETS-09), and the six field samples from different Abano and Montegrotto Terme thermal spas, produced bands that migrated to identical positions (Fig. 9). Excision and sequencing of the bands obtained from field samples gave sequences identical to that of ETS-08, showing that this cyanobacterium was one of the commonest organisms in mud maturation tanks of the Euganean Thermal spas.

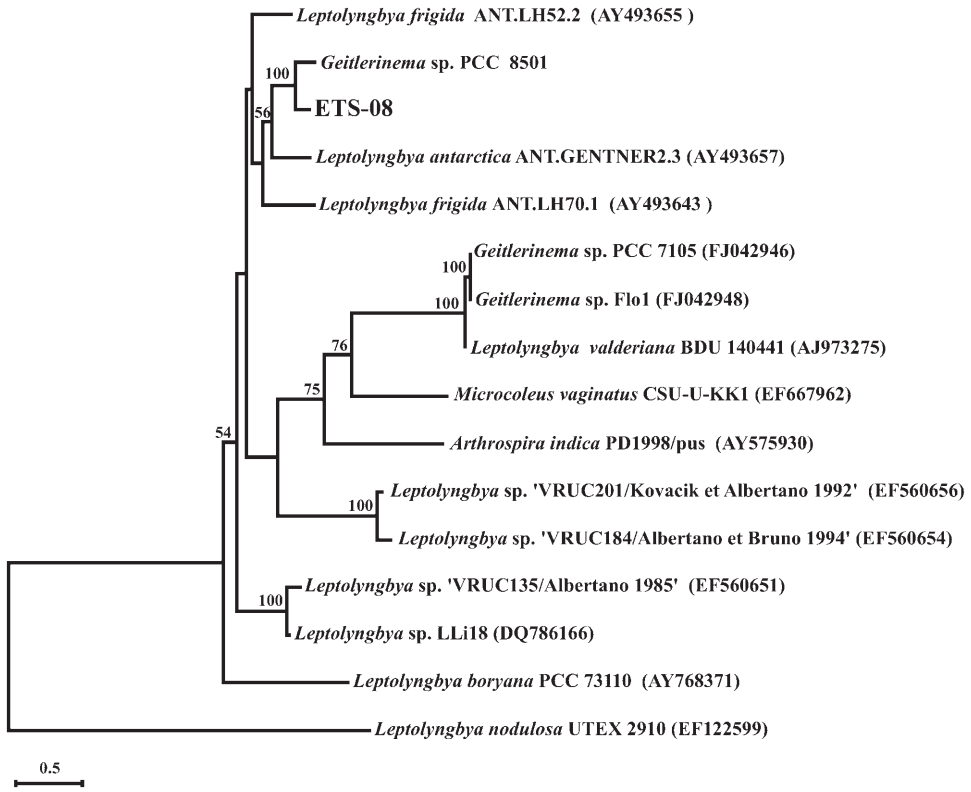


Fig. 9. Phylogenetic tree based on 16S–23S ITS region sequences, reconstructed using the maximum-likelihood analysis of evolutionary distances determined by the GTR + I + G model. Numbers above branches indicate bootstrap values greater than 50%. Sequences determined in this work are indicated in bold and larger font. GenBank accession numbers are indicated in brackets. Bar represents 0.5 nucleotide substitutions per site.

Discussion

The morphological and ultrastructural features observed in the Euganean strain, ETS-08, and the Oregon strain, PCC 8501, would place them in the family Pseudanabaenaceae, sub-family Leptolyngbyoideae, genus *Leptolyngbya sensu* Komárek & Anagnostidis (2005). These features are long, thin, slightly constricted, cylindrical trichomes, surrounded by individual thin sheaths, phenotypic traits common to several *Leptolyngbya* species from a variety of aquatic and sub-aerial environments (Albertano & Kovacik, 1994; Bellezza *et al*., 2003; Asencio & Aboal, 2004; Casamatta *et al*., 2005; Komárek & Anagnostidis, 2005; Li & Brand, 2007). Among *Leptolyngbya* species typical of thermal waters with <1 μm wide trichomes, ETS-08 and PCC 8501 are quite similar to *L. granulifera* (Copeland) Anagnostidis and *L. laminosa* (Gomont) Anagnostidis et Komárek, but differ in having a round apical cell and lacking granules on either side of the cross walls (Komárek & Anagnostidis, 2005), and to *L. thermalis* Anagnostidis in Anagnostidis & Komárek (1988), which lacks cell constrictions. There is also low sequence identity (88.44%, 71 substitutions) with the 614 bp of the 16S rRNA gene sequence of a *L. thermalis* strain from extreme seawater environments of Mexico (GenBank accession no. AF410932) and ETS-08.

The 16S rRNA phylogenetic analysis confirms the results of the most recent phylogenetic analyses on *Leptolyngbya* (Casamatta *et al*., 2005; Yoshida *et al*., 2008; Bruno *et al*., 2009). Furthermore, most of the sequences reported in GenBank for strains identified as *Leptolyngbya* species have not been accompanied by morphological, ultrastructural, biochemical or eco-physiological data (Komárek & Anagnostidis, 2005; McGregor, 2007). In addition no sequences of *L. granulifera* are available for comparison and the partial 16S rRNA gene sequence (628 bp, GenBank accession no. EU057151) of the sole strain identified as *L. laminosa* (Sorokovikova & Belykh, pers. comm), isolated from the hot springs of Baikal Rift Zone, showed very low sequence identity (89.33%, 67 substitutions) with the corresponding sequence of ETS-08.

Thus, the inclusion of ETS-08 and PCC 8501 in *Leptolyngbya* is unlikely. Similarly the inclusion of ETS-08 in the genus *Geitlerinema*, sub-family Pseudanabaenoideae, is unconvincing given its slow motility in natural samples and on agar. This gliding behaviour differs from that described for PCC 8501 (http://www.pasteur.fr/recherche/banques/PCC/docs/pcc8501.htm). However, it should

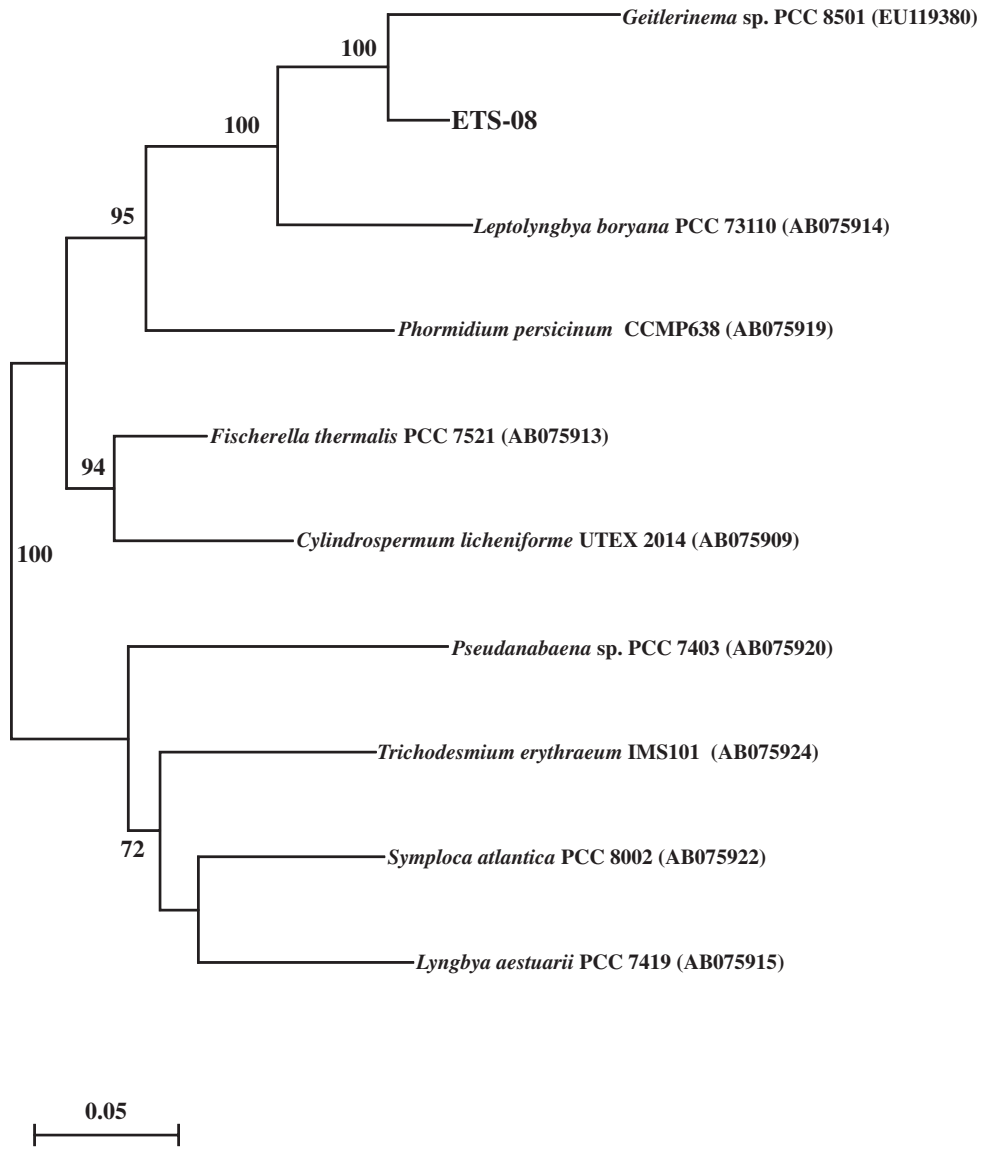


Fig. 10. Phylogenetic tree based on *rbc*L gene sequences, reconstructed using the maximum-likelihood analysis of evolutionary distances determined by the GTR + I + G model. Numbers above branches indicate bootstrap values greater than 50%. Sequence determined in this work is indicated in bold and larger font. GenBank accession numbers are indicated in brackets. Bar represents 0.05 nucleotide substitutions per site.

be noted that reduction or lack of intense motility, observed in ETS-08 and PCC 8501 (Castenholz, 1970), might be a loss occurring during growth in liquid medium as reported for *Pseudanabaena* and *Geitlerinema*, field material or freshly isolated cultures of which show active gliding (Wilmotte & Herdman, 2001; Castenholz *et al*., 2001*a*, *b*). The nine sequences available for marine *Geitlerinema* strains of clade C had showed 87.8–89.0% sequence identities and are therefore evolutionarily distant, assuming a 95% threshold for the inclusion in the same genus (Ludwig *et al*., 1998; Řeháková *et al*., 2007).

The <95% sequence similarity with representatives of *Leptolyngbya* in clade B, *Geitlerinema* in clade C, and *Halomicronema* in clade D, will allow us to consider clade A, containing ETS-08, PCC 8501, and five other unidentified thermophilic filamentous cyanobacteria, as a new OTU within the family Pseudanabaenaceae.

The six thermal strains, Ob05, from Luzon Island in the Philippines (Lacap *et al*., 2007), PCC 8501 from Hunter's Hot Spring, Oregon, USA, OS Type I, from Octopus Spring in Yellowstone National Park (Ward *et al*., 1992), tBTRCCn 408, tBTRCCn 102 and tBTRCCn 302 from Zerka Mà in Jordan (Ionescu *et al*., 2007), grouped in the same clade as ETS-08**,** suggesting that they could belong to the same genus**.**

None of the representatives of the other genera in the Pseudanabaenaceae showed >95% sequence identity with our strain. Taking into consideration that the systematics of most cyanobacteria characterized by very thin trichomes still needs a full revision (Johansen *et al*., 2008), the present work contributes to the definition of a separate OTU,

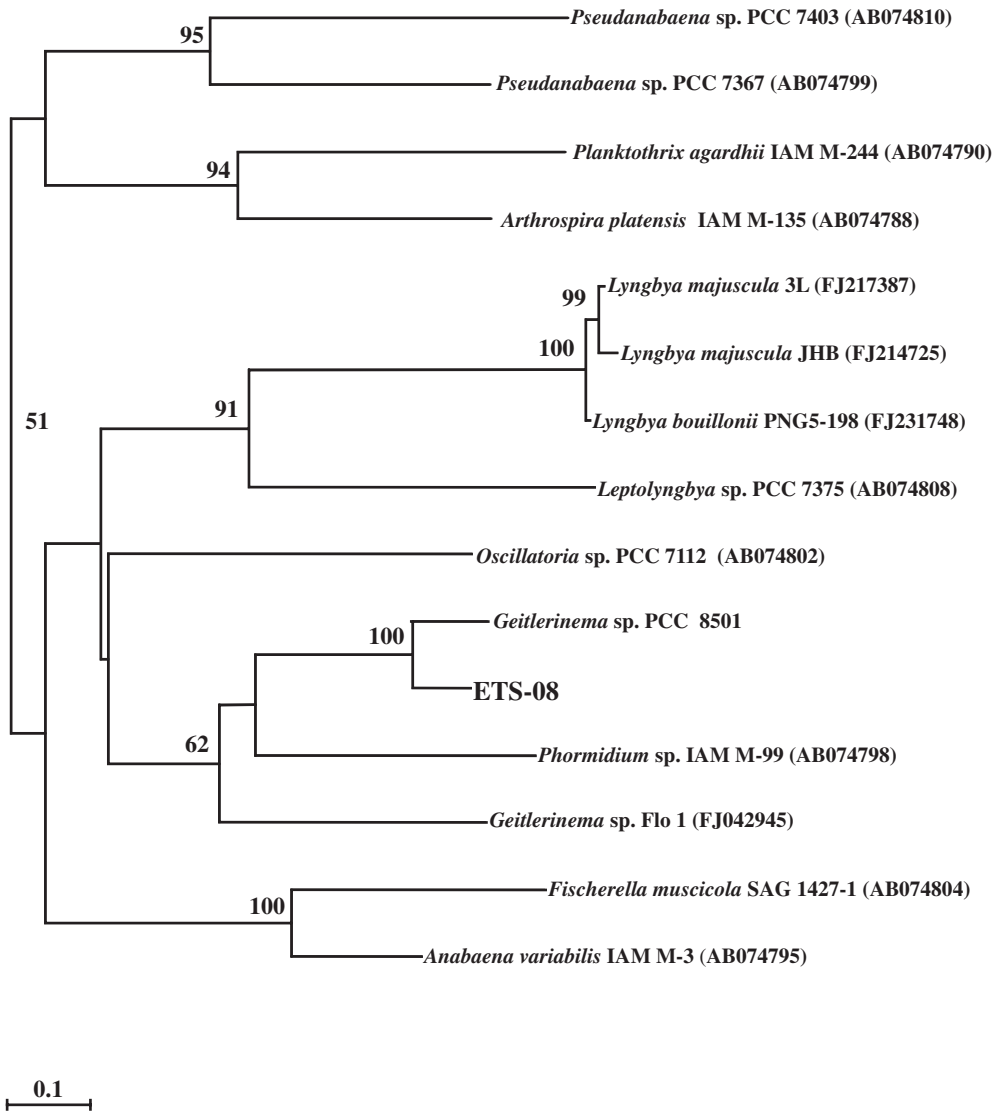


Fig. 11. Phylogenetic tree based on *rpo*C1 gene sequences, reconstructed using the maximum-likelihood analysis of evolutionary distances determined by the GTR + I + G model. Numbers above branches indicate bootstrap values greater than 50%. Sequences determined in this work are indicated in bold and larger font. GenBank accession numbers are indicated in brackets. Bar represents 0.1 nucleotide substitutions per site.

like the recent genera *Halomicronema* and *Coleofasciculus,* distinguished on the basis of 16S rRNA sequence identity of <92% and 93–94%, respectively (Abed *et al*., 2002; Siegesmund *et al*., 2008).

Taking 97.5% similarity for 16S rRNA gene sequence identity as the criterion for collapsing strains into a single species (Stackebrandt & Goebel, 1994; Casamatta *et al*., 2005; Palińska & Marquardt, 2008), the high identity (99.17%) between ETS-08 and PCC 8501 for this locus suggests that the two entities could be ecotypes, as reported for different isolates of *Mastigocladus laminosus* (Miller *et al*., 2007). That is they are 'genetically determined phenotypes of a species that are found as local variants associated with certain ecological conditions' living in geographically distant areas (Ward *et al*., 1998). In our case, however, the 70.9% sequence identity of the 16S–23S region suggests greater intraspecific variation between the strains, indicating that ITS is more useful for closely related strains of different geographic distribution (Boyer *et al*., 2001; Bruno *et al*., 2009). By combining ecological and evolutionary patterns, it was shown that 16S rRNA gene sequences are too conserved to detect all ecologically specialized populations and that ITS analysis provided evidence of localized geographical patterning, e.g. nine ITS variants within a single 16S rRNA genotype (Ward *et al*., 1998). However, because of the possible presence of multiple rRNA operons in some cyanobacteria there are potential pitfalls that should be considered when using ITS to infer phylogeny (Boyer *et al*., 2001). Nevertheless, the agarose gel-electrophoresis analyses of the amplified PCR products from the current study (both 16S rRNA and ITS) consistently produced a

Fig. 12. DGGE profile obtained from mat samples from six thermal spas (lanes 2 to 7). Lane 1, top to bottom: *Spirulina* sp., *Cyanobacterium aponinum* (ETS-03), ETS-08, *Phormidium* sp. (ETS-05). Note that ETS-08 and *Spirulina* sp. (ETS-09) are commonest in the mat samples.

single band from the two strains, indicating the presence of a single operon (data not shown).

The results of the 16S–23S ITS sequencing, showing differences between the two strains, support the recognition of different lineages within the clade. This was also found for some *Leptolyngbya* species in which ITS sequencing agreed with the 16S rRNA results and allowed discrimination at inter- and intraspecific levels. It also showed the relatedness of *Leptolyngbya* strains from hypogean environments with strains from subaerophytic and geothermal environments (Bruno *et al.*, 2009).

The *rbc*L phylogenetic analyses show that *L. boryana*, which is the type species of *Leptolyngbya*, is a sister taxon to the clade including ETS-08 and PCC 8501. However the low number of *Leptolyngbya rbc*L gene sequences in GenBank limits further interpretation of this result.

However, we have found good correlation between ITS region and *rpo*C1 phylogenetic analyses that could support the 16S rRNA results. Similar data have been obtained with *M. aeruginosa* strains by Yoshida *et al.* (2008) using a polyphasic approach, combining phenotypic and genetic characterization. This approach is effective for defining distinct lineages and discriminating the complexity of intra-specific populations. Nevertheless, 90.1% *rpo*C1 sequence identity between ETS-08 and PCC 8501 does not prove unambiguously either that they belong to the same species or that they should be separated as two species. According to Wilson *et al.* (2000), the *rpo*C1 locus was ineffective for distinguishing among several strains of *Cylindrospermopsis raciborskii* with sequence identities from 99 to 100%, while Toledo & Palenik (1997) recognized different strains of *Synechococcus* sp. based on 83% *rpo*C1 sequence identity.

ETS-08 might be one of the 80 cyanobacteria reported from the Euganean thermal environment by Trevisan (1870). Unfortunately, it has not been possible to find dry specimens or isolates for comparison and it is also difficult to establish the synonymy of several Oscillatorialean taxa reported in Komárek & Anagnostidis (2005).

Interestingly, ETS-08 differs from two other filamentous cyanobacteria (ItalyCy04 and ItalyCy05) recently found in the Euganean Thermal District and identified by partial 16S rRNA gene sequences (AF505886 and AF505887) (Papke *et al.*, 2003). There is about 94.3% sequence identity between them and our strain. This suggests that the cyanobacterial composition of phototrophic mats in the rather unusual environment of the Euganean Thermal District is variable, depending on the physico-chemical features of the different spa waters. In fact, recent surveys carried out on ninety spas suggest that the cyanobacterial diversity might be related to the thermal mud processing, which is carried out in different maturation tanks using thermal waters at various temperatures. As expected, while cyanobacterial diversity can range from 2 to 12 taxa between 26 and 40°C, only *Leptolyngbya* (ETS-08) and *Spirulina* (ETS-02) are able to thrive above 50–55°C (unpublished data).

Based on a polyphasic approach combining morphological, ultrastructural and genetic tools, we have characterized and established the taxonomic position of the Euganean strain, ETS-08, which, together with six other isolates, represents a new OTU in the family Pseudanabaenaceae. In order to erect a new systematic group to which these strains belong, further studies involving comparison of phenotypic features as well as sequences from a wider number of strains from the Leptolyngbyoideae and Pseudeanabaenoideae will be necessary. It will also be necessary to determine what is the representative generitype for any new genus.

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