Identification and characterization of a new *Halomicronema* species (Cyanobacteria) isolated from the Mediterranean marine sponge *Petrosia ficiformis* (Porifera)

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Abstract: A filamentous cyanobacterium (strain ITAC101) isolated from a Mediterranean sponge (*Petrosia ficiformis*) was characterized by a combined phenotypic and genetic approach. Morphological and ultrastructural observations were performed along with growth measurements and pigment characterization. The molecular phylogenetic analyses were based on the sequencing of the 16S rRNA gene. In culture conditions, strain ITAC101 is moderately halophilic and grew in the range 0.3–7.6% (w/v) salinity with the optimum at 3.6%. Cell dimensions, thylakoid arrangement and pigment composition of this cyanobacterium fit the *Halomicronem*a genus description, and phylogenetic analyses evidenced 99.9% similarity with another strain endolithic in tropical corals. The new *Halomicronem*a *metazoicum* species was established including the two cyanobacteria associated to marine animals.

Key words: cyanobacteria, Leptolyngbya, marine sponge, Mediterranean Sea, polyphasic approach,16S rRNA

Introduction

The association between cyanobacteria and sponges is a well known and largely studied phenomenon and has been confirmed in at least 26 *Demospongiae* and 17 *Calcarea* families (Carpenter 2002; Diaz et al. 2007; Zhu et al. 2008). This mutually beneficial association is thought to be one of the oldest microbe–metazoan interactions, and is speculated to have dominated over hard corals during periods of the Paleozoic and Mesozoic (Taylor et al. 2007). Indeed, in a number of marine sponges, single–celled and filamentous cyanobacteria having both extra–cellular and intra–cellular localization (Rützler 1990; Wilkinson 1992) are frequently found. In this association, the involved cyanobacterial species mainly belong to the genera *Synechocystis* (Larkum et al. 1988), *Aphanocapsa* (Feldmann 1933), *Oscillatoria* (Wilkinson 1992; Thacker & Starnes 2003), *Anabaena* (Larkum 1999), *Cyanobacterium* (Webb & Maas 2002) and *Synechococcus* (Hentschel et al. 2002). Other cyanobacteria associated to sponges include *Prochlorococcus* (Steindler et al. 2005) and two, so far unnamed, symbionts of *Mycale hentscheli* and *Cymbastela marshae* (Hentschel et al. 2006).

Actually, the knowledge of the diversity and the ecological role of cyanobacteria living in association with sponges is in its infancy, and molecular and biochemical techniques are greatly expanding the information on their taxonomy and phylogeny, particularly in case of culturable microorganisms (Usher et al. 2004). At present, the relevance of culturing cyanobacteria derives from their ability to produce bioactive natural compounds for biotechnological application (Moore et al. 1988; Sivonen & Börner 2008; Gerçe et al. 2009). Moreover, the chemical

structures of over 13,000 novel compounds from marine organisms have been already determined and their potential use in pharmaceutics widely considered (FENICAL 1997).

Recently, eight cyanobacterial strains were isolated from the marine sponge *Petrosia ficiformis*, and among them, the strain ITAC101, firstly assigned to the genus *Leptolyngbya.* This species resulted particularly interesting because showed haemolytic activity and influenced brine shrimp vitality and sea urchin development (PAGLIARA & CAROPPO 2010, 2011). These preliminary findings encouraged further investigations to characterize and identify the strain.

The taxonomic classification of non–heterocystous, thin filamentous cyanobacteria having trichomes <3 μm is controversial. These cyanobacteria have been previously assigned to the genus *Phormidium* (GEITLER 1932) according to the botanical code, and to the *Lyngbya/Plectonema/Phormidium*–group B (RIPPKA et al. 1979) according to the bacteriological system. Lately, they have been included in the genus *Leptolyngbya* by ANAGNOSTIDIS & KOMÁREK (1988) and accordingly described in the BERGEY'S manual of systematic bacteriology (CASTENHOLZ & WATERBURY 1989). Indeed, the combined use of molecular and traditional techniques (WILMOTTE 1994; GARCIA–PICHEL et al. 1998; NÜBEL et al. 2000) subsequently revealed that cyanobacteria with *Phormidium*–like morphologies do not form a monophyletic group (GIOVANNONI et al. 1988).
Among the *Phormidium*–like cyanobacteria, a new genus of non–heterocystous, thin filamentous species has been recently described as *Halomicronema excentricum* by ABED and co–authors (2002a). This cyanobacterium has been firstly isolated from benthic microbial mats in a hypersaline pond of the Solar Lake (Sinai, Egypt), and also found in Camargue (France) (FOURÇANS et al. 2004), in a heavily polluted site of a coastal stream in Palestine (ABED et al. 2002b) and in the Arabian Gulf (ABED et al. 2006). Probably, the filamentous cyanobacterium inhabiting the skeleton of *Goniastrea aspera*, a massive reef–building coral, also belongs to *Halomicronema* genus (YAMAZAKI et al. 2006; 2008a).

The aim of this paper was the characterization and the proper identification by a polyphasic approach of the strain ITAC101 isolated from the sponge *Petrosia ficiformi*s. By combining morphological, ultrastructural, physiological, biochemical and molecular studies, the strain was analyzed with light and transmission electron microscopy, its growth measured at varying salinity, pigment content characterized and all these phenotypic traits compared to the results obtained by molecular taxonomy based on 16S rRNA gene sequence analysis (KOMÁREK 2005).

Materials and methods

Strain origin and growth conditions

Specimens of *Petrosia ficiformis* were collected from the Gulf of Taranto (Northern Ionian Sea, Mediterranean Sea), at the Porto Cesareo station (E 17° 55' 50'', N 40° 10' 40''), using SCUBA at depths between 0.5 and 10m, in October 2008. Sea temperature, pH and salinity were simultaneously measured by an Idronaut Ocean Seven 501 multiprobe. During the study period water temperature ranged from 19.70 to 21.80 °C, salinity from 3.84 to 3.87% and pH from 8.05 to 8.10.

The sponges were transferred to the laboratory, rinsed with filtered sea water and cut into small pieces. Each piece was pressed through a nylon mesh (100 μm pore size) to obtain a suspension of single cells. This suspension was centrifuged, then filtrated by using a 100 μm net and inoculated both on solid and liquid MN medium enriched with B_{12} vitamin (5 $\mu g.l^{-1}$) (RIPPKA et al. 1979). Solid media were prepared by adding 1% (w/v) of agar to liquid MN medium. Cycloheximide (100 $mg.l^{-1}$) was added to all cultures to prevent the growth of eukaryotic contaminants. The cultures were incubated at 26.0 ± 1.0 °C under white fluorescent light at a photosynthetic photon flux density of 20 $\mu mol.photon.m^{-2}.s^{-1}$ (KANA & GLIBERT 1987) and a light/dark cycle of 12:12 hours. Monospecific cultures with extremely thin filaments were isolated and further purified by successive transfers. Axenic culture was obtained after repeated self–isolation on agar (CASTENHOLZ 1988). To assess the purity of the strain, the samples were stained with 4'–6–diamidino–2–phenylindole dihydrochloride (DAPI, Sigma) and observed under an epifluorescence microscope.

The axenic strain ITAC101 is presently deposited and maintained at the Institute TAranto Cnr – ITAC culture collection.

Growth measurements

Cultures were grown in 250 ml Erlenmeyer flasks, containing 100 ml of MN medium at 0, 1.8, 3.6, 4.4, 7.2, 9.0 and 10.8% salinity under the same temperature and light conditions reported above. The different salinity concentrations, from hyposaline to hypersaline, were obtained by adding appropriate amounts of distilled water or NaCl to the natural seawater (3.8%).

Growth was followed in triplicate cultures during 10 days and measured by monitoring chlorophyll–*a* (Chl–*a*) concentration as biomass indicator. At the end of the experiments, all the cultures were kept for

additional 2 months in the same conditions in order to check slow growth rates and long–term ability to survive under nutrient starvation.

To evaluate Chl–*a* content triplicate samples (1 ml of cell suspension) from each salinity condition were collected at the indicated times, washed twice with ultrapure sterile water and harvested at 12,000 × *g* for 10 min. Extraction was performed in 1 ml of 90% (v/v) methanol at 25 °C for 15 min in the dark, followed by centrifugation at 12,000 × *g* for 10 min. Absorbance of the supernatant, was determined at 663 nm using a spectrophotometer (Beckman Coulter DU 800 UV/Vis, Spectrometer) (Fiore et al. 2000). Comparison of biomass reached at the stationary phase was done by measuring the absorbance of Chl–*a* for each salinity condition. The Chl–*a* content was estimated using the equation: C ($\mu g.ml^{-1}$) × OD_{663} × 12.7 (Meeks & Castenholz 1971).

Confocal laser scanning microscopy (CLSM)

For confocal analysis the samples were processed alive. The cells were directly placed on a microscope slide, sealed with cover slip and observed using a 488 nm Argon laser line of a C1 Nikon confocal laser scanning unit coupled to a Nikon TE300 microscope with a 100×/1.30 oil objective (Nikon, Japan). For pigment fluorescence (autofluorescence) an excitation filter at 568 nm (emission at >590 nm) was used.

Transmission electron microscopy

Samples of growing cultures were prepared for electron microscopy. Specimens were fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1 M, pH 7.2, and post–fixed with 1% osmium tetroxide in the same buffer. Then samples were dehydrated in a graded alcohol series, embedded in Spurr's resin, and sectioned at 90 nm with an ultracut (Leica Microsystem, Germany). The sections were stained with 2% uranyl acetate and lead citrate and examined in a Zeiss EM 910 electron microscope operating at 60 kV.

Pigment analysis

Pigment analysis involved chromatographic and spectrophotometric characterization and quantification of chlorophylline pigments, carotenoids and phycobiliproteins of the strain ITAC101. Samples of about 50 mg dry weight were thoroughly washed with distilled water to remove medium and then suspended in either 3 ml of absolute acetone for the extraction of chlorophylline pigments and carotenoids or in 3 mL of 0.01 M phosphate buffer, pH 7.0, plus 0.15 M NaCl for the extraction of phycobiliproteins. Samples were sonicated for 4 min in ice–bath, centrifuged for 20 min at 15,000 *g* and filtered through 0.45 µm cellulose filters. Profile of the chlorophylline pigments and carotenoids in the crude extract was obtained by HPLC–DAD on a Agilent 1100 binary system with a Luna C18(2) 250 x 4.6 mm 5 µm analytical column (Phenomenex, St. Torrents, USA), following the protocol of Airs et al. (2001). Peaks were identified on the basis of the retention time and absorbance spectra obtained using a Beckman Coulter DU 800 UV/Vis spectrophotometer. Quantification of Chl–*a* and some major carotenoids was carried out by Gauss Peak spectra analysis (Küpper et al. 2007) on the absorbance spectrum in the range 350–700 nm. After the recording of absorbance spectra of crude phycobiliproteins extracts, quantification of phycoerythrin, phycocyanin and allophycocyanin was obtained using the equations of Bennet & Bogorad (1972).

DNA extraction

DNA extraction was performed by a modified method previously described by Lotti et al. (1996). Briefly, fresh cells were harvested during exponential phase by centrifugation (11,000 × g for 20 min at 15 °C) and washed with TEN–25 buffer (25 mM Tris/HCl, 25 mM EDTA, 100 mM NaCl, pH 8). The pellets were kept frozen at –20 °C until use. To obtain spheroplasts, the cells were suspended in TEN buffer (10 mM Tris/HCl, 1 mM EDTA, 100 mM NaCl, pH 8), and incubated with 100 $\mu g.ml^{-1}$ proteinase K and 100 $\mu g.ml^{-1}$ lysozyme. The suspension was incubated for 60 min at 4 °C under slow shaking. Lyses of spheroplasts was achieved by adding 2% SDS (w/v) and incubating for 90 to 120 min at 4 °C under gentle shaking. The subsequent extraction and purification was performed by phenol–chloroform and isoamylic alcohol (25:24:1) according to standard procedures (Sambrook & Russel 2001). 40 $\mu g.ml^{-1}$ of ribonuclease A were used to remove RNA. The DNA was then precipitated with ethanol, collected with a glass rod and re–suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8). The quality and quantity of DNA was analyzed by electrophoresis on 0.8% agarose gel and spectrophotometric analysis (Maniatis et al. 1982).

16S rRNA Gene Sequence Analysis

The gene coding for 16S rRNA subunit was subjected to polymerase chain reaction (PCR) amplification using the cyanobacteria–specific universal primers CIANO1–F (5'–CTTAACACATGCAAGTCGAAC–3') and CIANO4–R (5'–CTCCTCCACAAGGGTTGGA GTAAC–3'), complementary to *Synechococcus* sp. PCC7001 16S rRNA (GenBank accession no. AB015058) sequence position 21–42 and 1356–1379 respectively. Primers were constructed by CLUSTAL W program from European Bioinformatics Institute (http://www.ebi.ac.uk/), for multiple sequence alignments of cyanobacterial sequences, and by Probe Match tool of the Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/), for specificity evaluation. PCR, reactions were performed in a 25 µl reaction mixture containing approximately 100 ηg of genomic DNA and 0.5 U of *Taq* polymerase (Amersham, Pharmacia),

according to the manufacturer's instructions, in a Perkin–Elmer GeneAmp PCR System 9600 (Perkin Elmer, USA) as follows: 5 min initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.45 min. The final extension was set at 72 °C for 7 min. PCR products were separated by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris–acetate,1 mM EDTA, pH 8.0) and recovered by using the Qiaex II DNA purification kit (QIAGEN). PCR products were subjected to DNA sequencing of both strands as a service by MWG Biotech Custom Sequencing Service (Germany) utilizing primer Ciano1–F/Ciano4–R and Com1–F (5'–CAGCAGCCGCGGTAATAC–3') / Com2–R (5'–CCGTCAATTCCTTTGAGTTT–3'), annealing position 519–536 and 927–908, respectively, of *E. coli* 16S rRNA gene (LANE et al. 1985). Processing of the DNA sequences was performed with the software GeneJockey Sequence Processor (published and distributed by Biosoft).

Phylogenetic analysis

The 16S rRNA gene sequence of the isolated strain (1358 nucleotides) was first analyzed by a similarity search using the BLAST function of GenBank at the National Center NCBI electronic site and the Seq Match tool of the Ribosomal Database Project II (http://www.ncbi.nlm.nih.gov/ and http://rdp.cme.msu.edu/, respectively). For phylogenetic analysis were considered 16S rRNA gene sequences of cyanobacteria longer than 1000 nucleotide deposited in GenBank and RDP databases and multiple sequence alignments were conducted using the CLUSTAL W program from European Bioinformatics Institute (http://www.ebi.ac.uk/). To infer phylogenetic relationships among the taxa, different analytical methods were employed. Maximum–parsimony and distance trees were generated using a heuristic search constrained using the PAUP* 4.0 b10 software package (SWOFFORD 2001). A maximum–likelihood tree was constructed using the PAUP* software with the computation of likelihood scores obtained with PHYML by JMODELTEST 0.1.1 (POSADA 2008) with the estimation of the GTR+I+G model. The robustness of the inferred phylogenies was determined by bootstrap analysis based on 1000 resamplings of data. The Bayesian inference (BI) was performed using MRBAYES 215 version 3.0 (RONQUIST & HUELSENBECK 2003). The substitution model was the GTR+I+G. Bayesian analysis was performed using four search chains for 1000000 generations, sampling trees every 100 generation. The first 1000 trees were discarded as burn–in. Parameter 220 stability was estimated by plotting log–likelihood values against generation time, and a consensus tree with posterior probabilities (PP) was then generated.

The 16S rRNA gene sequence was deposited in the Gen Bank database under accession number GU220365.

Results

Morphology

The axenic culture of the filamentous cyanobacterium ITAC101 was obtained after isolation from the sponge *P. ficiformis*. In liquid culture, filaments appeared as deep–green macroscopic aggregates covering the walls of the culture vessel. On solid media, colonies did not show gliding motility or phototaxis towards a light source, also after a long term exposition.

The filament sheath was colourless, very thin, firm and distinct (Fig. 1a). A calyptra was evident at the end of most filaments (Fig. 1b). Filaments were about 1 μm wide with slight constrictions at the cross–wall, better visible in CLSM (Fig. 1c). Cells 0.8–1.0 μm in diameter were always longer than wide and their length usually ranged from 3 to 6 μm. Seldom, shorter (around 2 μm) and longer (up to 8 μm) cells have been observed in the apical and sub–apical zone of the trichomes. Reproduction occurred by fragmentation in two–three celled hormogonia.

TEM analysis (Fig. 2) evidenced the fibrillar and compact structure of the sheath and the presence of cell wall constrictions (Figs 2a, d). Pores were present on cell walls and septa. Three peripheral thylakoids run in the peripheral cytoplasm, concentrically arranged around the long axis of the trichome (Fig. 2b). Phycobilisomes were not observed on the thylakoidal membranes. Polyhedral bodies (carboxysomes), polyphosphate and cyanophycin granules, and lipid inclusions (Figs 2b, c, d) were present in the nucleoplasm along with DNA fibrils and polyribosomes while gas vesicles were never observed.

Growth rates at varying salinity

In laboratory, significant growth was observed up to 4.4% salinity, with the optimal condition around 3.6% (Fig. 3). Growth rates at 0.3%, 1.8%, 3.6% and 4.4% were respectively 0.21, 0.26, 0.39 and 0.19 generations per day. At 7.2% NaCl growth decreased to 0.16 after 10 days and the culture remained viable for the next two months. After four days at 9.0% and 10.8% NaCl cultures died.

Pigment analysis

Strain ITAC101 has deep–green trichomes due to a complex profile of lipophilic pigments, as evidenced by the HPLC chromatogram comprising up to 43 detectable peaks (Fig. 4). The majority of the HPLC peaks were due to the homologues of few

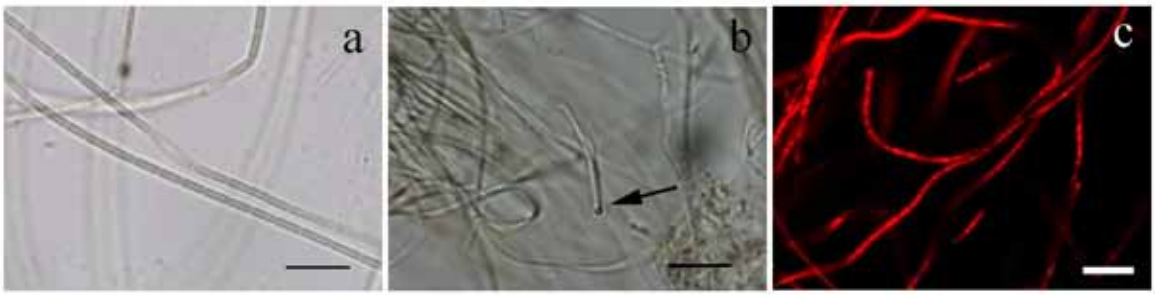

Fig. 1. Light micrographs (a, b scale bar 5 μm) and CLSM image (c, scale bar 10 μm) of *Halomicronema* ITAC101.

basic pigments: chlorophyllide–*a*, chlorophyll–*a*, pheophytin–*a*, zeaxanthin, β–cryptoxanthin and canthaxanthin. Chlorophyll–*a* was largely the major photosynthetic pigment, representing the 53.2% of the total lipophylic pigment content. Zeaxanthin and β–cryptoxanthin accounted for the 49.4% and 36.4% respectively of the total carotenoids, the rest being represented by canthaxanthin (14.3%). Some cyanobacterial biomarkers, like echinenone and myxoxanthophyll, were totally absent and other common carotenoids, such as β–carotene and neoxanthin were found only in trace.

The absorption spectra of phycobiliprotein crude extracts showed major peaks in the visible range at 560 and 615, and shoulder at 650 and 680 nm (Fig. 5). Phycoerythrin accounted for the 60% of total phycobiliproteins, followed by phycocyanin (29.0%) and allophycocyanin (11.0%).

Molecular characterization

The strain ITAC101 was characterized by 16S rRNA gene analysis and its phylogenetic relationships with other 56 strains of cyanobacteria, available in database, represented by a maximum–likelyhood tree (Fig. 6). Strain ITAC101 showed 99.9% pairwise sequence identity with *Halomicronema* sp., strain Goniastrea–1 (GenBank accession number AB257773) isolated from the skeleton of the reef–building coral *Goniastrea aspera* (Yamazaki et al. 2008a). The two strains clustered together in a different clade of that formed by the type species *H. excentricum* (GenBank accession number AF320093), isolated from a man–made hypersaline pond (Abed et al. 2002a), and other *Halomicronema* strains isolated from benthic microbial mats in the Shark Bay, SCyano39 and SCyano40 (Allen et al., unpublished). The clades were resolved with high PP (1.00) in BI and high bootstrap values 100% in ML analyses. The pairwise sequence identities shown by ITAC101 and the other *Halomicronema* strains considered in this study ranged from 94.6 to 94.8%, while with genera of thin filamentous cyanobacteria such as *Leptolyngbya*, *Limnothrix* and *Geitlerinema* were lower than 92%.

Discussion

This is the first report of a *Halomicronema* species living in association with a marine sponge and the first record of this genus for the Mediterranean Sea. A complex microbial community was recently described in association to *Petrosia ficiformis*, in which cyanobacteria are represented by coccoid and filamentous species (Pagliara & Caroppo 2011). Based on morphology, some of the latter species have been previously ascribed to the genus *Leptolyngbya* because of their very thin filaments (Pagliara & Caroppo 2010). However, the strain ITAC101 was for the first time characterized in axenic culture after isolation from the sponge *Petrosia ficiformis*, and this allowed its proper characterization and identification. Up to date, only heterotrophic bacterial cultures have been obtained from sponges (Hentschel et al. 2001; Lafi et al. 2005). The culturing of microorganisms isolated from sponges is a necessary step not only for the assessment of the physiological and biochemical features, but also to analyze the ability to produce secondary metabolites. A collection of microbial isolates makes the screening for bio–pharmaceuticals more feasible on a large scale and possible the scale–up of an *in vitro* production with no need to culture the metabolite sponge tissue itself (Haygood et al. 2000).

Among cyanobacterial symbionts of the Mediterranean sponge *Petrosia ficiformis, Aphanocapsa feldmannii* (Feldmann 1933) has been the best described (Usher 2008). This

coccoid species is responsible for the red colour of the host living in dim light environments. Previous phylogenetic analyses clarified the identity of this cyanobacterium and demonstrated that it belongs to the *Synechococcus* genus (USHER et al. 2004). This supported the need of using microscopy in combination with molecular studies since, the description of microbial species without genetic support is criticised by numerous authors. Accordingly, molecular techniques involving DNA sequencing (CASAMATTA et al. 2005; GKELIS et al. 2005; COMTE et al. 2007; BRUNO et al. 2009) are presently used to investigate evolutionary relationships within cyanobacteria. Therefore, the idea that polyphasic approach is indispensable to identify cyanobacterial strains at genus as well as at species level (e.g. MARQUARDT & PALINSKA 2007) has been supported also by our analyses.

The combination of morphological, physiological, biochemical and molecular data obtained for the strain ITAC101 allows its inclusion in the *Halomicronema* genus*,* family *Pseudanabaenaceae.*

The immobility of the benthic marine, non–heterocystous, very thin filaments, the cylindrical trichomes <1 μm wide, and the peripheral arrangement of thylakoids parallel to the plasma membrane are in agreement with the diagnostic features of the genus *Halomicronema* ABED, GARCIA–PICHEL et HERNANDEZ–MARINÉ. The presence of a colourless, firm though thin, fibrillar sheath and cell wall constrictions, the length to width ratio higher than 3–2:1, the symmetrical *versus* asymmetrical arrangement of thylakoids reported for the type species *H. excentricum,* and the absence of gas vacuoles are morphological features distinctive of the marine animal associated ITAC101 strain.

The pigment analysis of the strain ITA101 in culture revealed a profile typical of several cyanobacterial species in which chlorophyll–*a* and phycobiliproteins (phycoerythrin, phycocyanin and allophycocyanin) contribute to the absorption of almost the whole spectrum of visible light (EVANGELISTA et al. 2006). Contrary to most of the cyanobacteria, in which β–carotene is invariably present and frequently as major carotenoid (HERTZBERG et al. 1971), zeaxanthin was the most abundant followed by β–cryptoxanthin, another xanthophyll frequent in Oscillatorialean cyanobacteria (AAKERMANN et al. 1992). The carotenoid composition of ITAC 101 is rather peculiar also considering that neoxanthin detected in this strain has been detected mostly in green algae (TAIKAICHI & MIRAURO 1998). By dissipating surplus energy, zeaxanthin acts as an essential protective part of the photosynthetic apparatus, though depending on light intensity, varying amounts of zeaxanthin are to be expected (WILHELM et al. 1995).

Although the strain ITAC101 in nature is deep green in color, phycoerythrin was the most abundant pigment in culture. The same phycoerythrin has been possibly purified from the *Halomicronema* sp. A32DM isolated from rocky shores, that showed absorbance peaks at 562 nm and fluorescence emission at 580 nm (PARMAR et

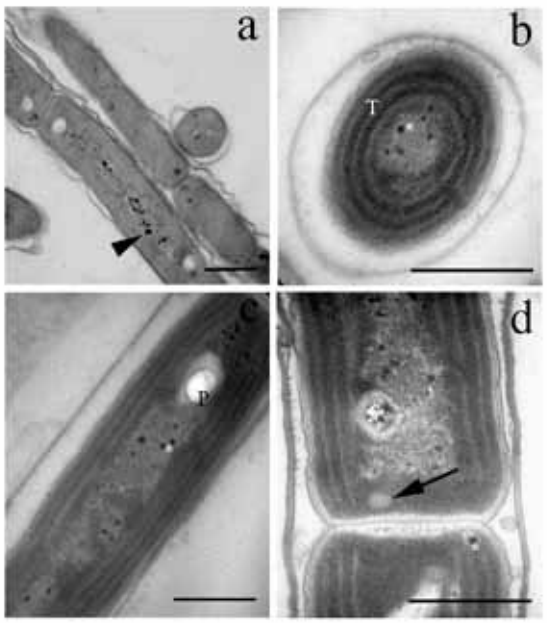

Fig. 2. TEM micrographs of *Halomicronema* ITAC101. Note the peripheral (b) and parallel (c) disposition of thylakoids; lipid droplet (asterisk), cyanophycin granules, and carboxysomes (arrows) were also observed in the cytoplasm. Scale bar 1 μm (a) and 0.5 μm (b, c; d)

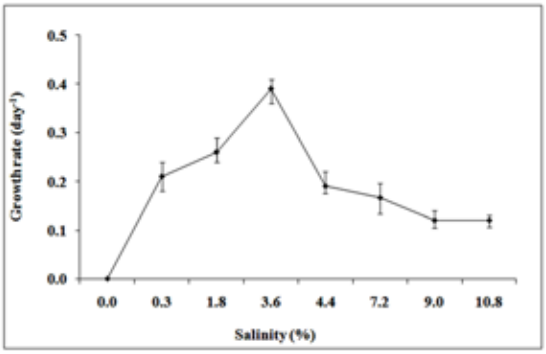

Fig. 3. Growth rates of *Halomicronema* ITAC101 at different salinity conditions. Symbols are the average of triplicate determination.

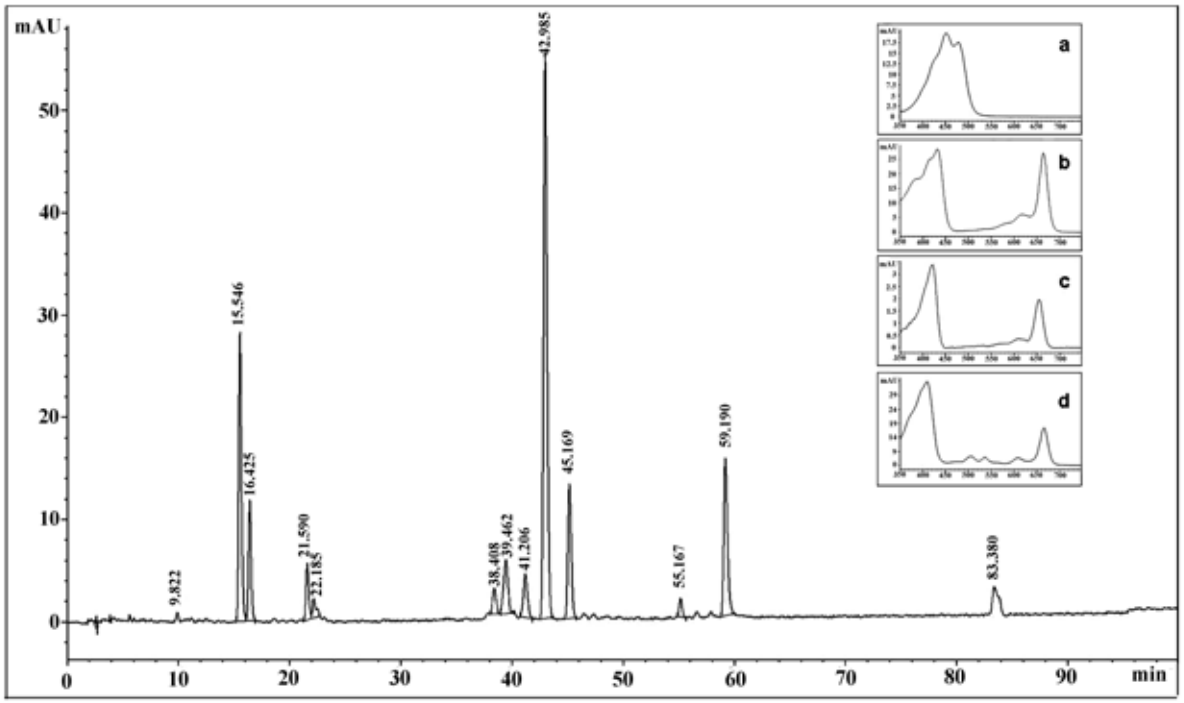

Fig. 4. HPLC chromatogramme of lipophilic pigments of *Halomicronema* ITAC101. Numbers on the peaks represent, respectively: 1=Zeaxanthin$_{(1)}$; 2=Zeaxanthin$_{(2)}$; 3=Zeaxanthin$_{(3)}$; 4=β–chryptoxanthin$_{(1)}$; 5 = β–chryptoxanthin$_{(2)}$; 6=Zn–chlorophyll–$a_{(1)}$; 7=Mg–chlorophyll–$a_{(1)}$; 8=Zn–chlorophyll–$a_{(2)}$; 9=Mg–chlorophyll–$a_{(2)}$; 10=Mg–chlorophyll–$a_{(3)}$; 11=Pheophytin–*a*; 12=β–carotene$_{(1)}$; 13=β–carotene$_{(2)}$. The inserts show the absorbance spectra of eluted zeaxanthin (a), Mg–chlorophyll–*a* (b), Zn–chlorophyll–*a* (c) and pheophytin–*a* (d).

al. 2011). In scleractinian corals, deeper bands are formed by cyanobacteria containing Chl–*a* and phycobiliproteins (Raplh et al. 2007). A pale green layer within the skeleton of *Goniastrea australensis* was dominated by an unidentified endolithic filamentous cyanobacterium extremely shade–adapted and able to efficiently use light to thrive under the optically dense coral tissue that exhibits a strong light attenuation and spectral filtering (Ralph et al. 2007).

Molecular analysis evidenced that the strain *Halomicronema* ITAC101 possesses a high (>99%) sequence identity with *Halomicronema* sp. *Goniastrea*–1 at 16S rRNA gene level, whereas only 94.8 % similarity with *H. excentricum.* The 99.9% sequence identity of ITAC101 strain 16S rRNA gene with that of *Halomicronema* sp. Goniastrea–1 is well above the molecular limits for species definition indicated by Stackebrandt & Ebers (2006). On the other hand, match analysis of other *Halomicronema* 16S rRNA gene sequences of significant length (>1000 pb) at GenBank indicated that these strains generally showed identity <95%, close to the limit for genus delimitation (Stackebrandt & Ebers 2006). Indeed, identity values between 16S rRNA of ITAC101 strain and those of other thin filamentous cyanobacteria were always below this limit, except 96% similarity with the strain *Leptolyngbya* CR_L27 (GenBank accession number EF545640) as indicated by bootstrap values and blast analysis (not shown) but unfortunately no morphological and ecological information is available to support a correct taxonomic identification of this strain.

Therefore, according to the ecological, morphological and genetic differences we concluded that strain ITAC101 and strain *Goniastrea*–1 can be considered as a new phylotype within the genus *Halomicronema*. This strain resulted well separated phylogenetically, from *H. excentricum* forming a distinct sister clade highly supported in bootstrap and Bayesian analyses, and hence it is described in this work as a new species.

Here we propose the species name *Halomicronema metazoicum* because of the association with widespread species of marine sponges and corals.

Further studies will be necessary to clarify the degree of association to the sponge and how the same species can live as endolith inside the coral skeleton. One possible explanation might be found in the biological activity exerted by *Halomicronema* ITAC101 against bacterial

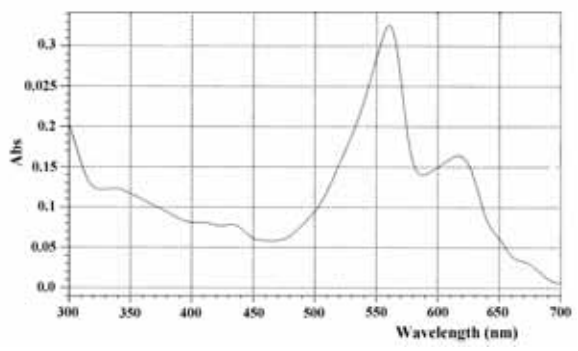

Fig. 5. The UV–visible absorption spectra of crude phycobiliprotein extracts of *Halomicronema* ITAC101. Major peaks are due the maximum absorption of phycoerythrin at 560 nm and phycocyanin at 615 nm. Allophycocyanin is visible as the shoulder at 650 nm.

pathogens. Results from studies on corals also suggested that colonization of endolithic algae within the coral skeleton provides beneficial effects on coral photosynthesis in terms of high–light tolerance (YAMAZAKI et al. 2008b).

The literature results also indicate that thin filamentous cyanobacteria of the *Halomicronema* and *Phormidium* genera contain a group of opportunistic microorganisms that tend to prevail after perturbations in ecosystems, including oil–tolerant cyanobacteria that dominate the mats immediately after oil spill events (ABED & GOLUBIC 2009). *H. metazoicum* might be one of these organisms able to acclimate to the disturbed conditions of the Gulf of Taranto and possibly the Bisezaki waters in Japan. Further studies may be aimed to investigate the role of this new species similarly to *H. excentricum* in the degradation of oil compounds (ABED & KÖSTER 2005).

Halomicronema metazoicum CAROPPO, PAGLIARA et ALBERTANO sp. nov.

Diagnosis: tenues filamenta solitaria metazoans adiuncti. Filamenta penitus viridia. Lapsus motility absit et non phototactic filamentis. Vaginae sine colore, firmae, tenues, distinctae. Trichomata leviter constricta melius visibilis ad electrum microscopium. Cellulae apicales sine calyptra. Cellulae 0.8–1.0 µm latae et 3–6 longae. Thylakoidae sunt disposita periphericorum concentricis circum axem longa trichoma. Parce halotolerant et halophilic cyanobacterium.

Habitus. Spongia et corallia.

Collectio conservata deposita in: IAMC–CNR (ITAC101) depositus (coll. Carmela Caroppo, 23.09.1999);

Type species: Halomicronema metazoicum species nova

Description of *Halomicronema metazoicum* sp. nov.

Thin filamentous marine cyanobacterium associated to metazoans. Filaments and cultures are deep–green in colour. Gliding motility is absent and filaments are not phototactic. Sheath is colourless, firm, distinct and very thin. Trichomes with slight constriction at the cross–walls, better visible in confocal and electron microscopy. The calyptra is present at the top of the apical cell. Cells are 0.8–1.0 µm in diameter and 3–6 µm long. Thylakoids are peripheral and concentrically arranged around the long axis of the trichome. Moderately halophilic and halotolerant cyanobacterium. Found as phycobiont of marine sponges in the Mediterranean Sea and scleractinian corals in Japan.

Type strain: ITAC101, isolated from the sponge *Petrosia ficiformis* sampled in the Northern Ionian Sea (Mediterranean Sea). Deposited and maintained at the Culture Collection of the Coastal Marine Environment, National Research Council, Taranto. GenBank accession number: GU220365.

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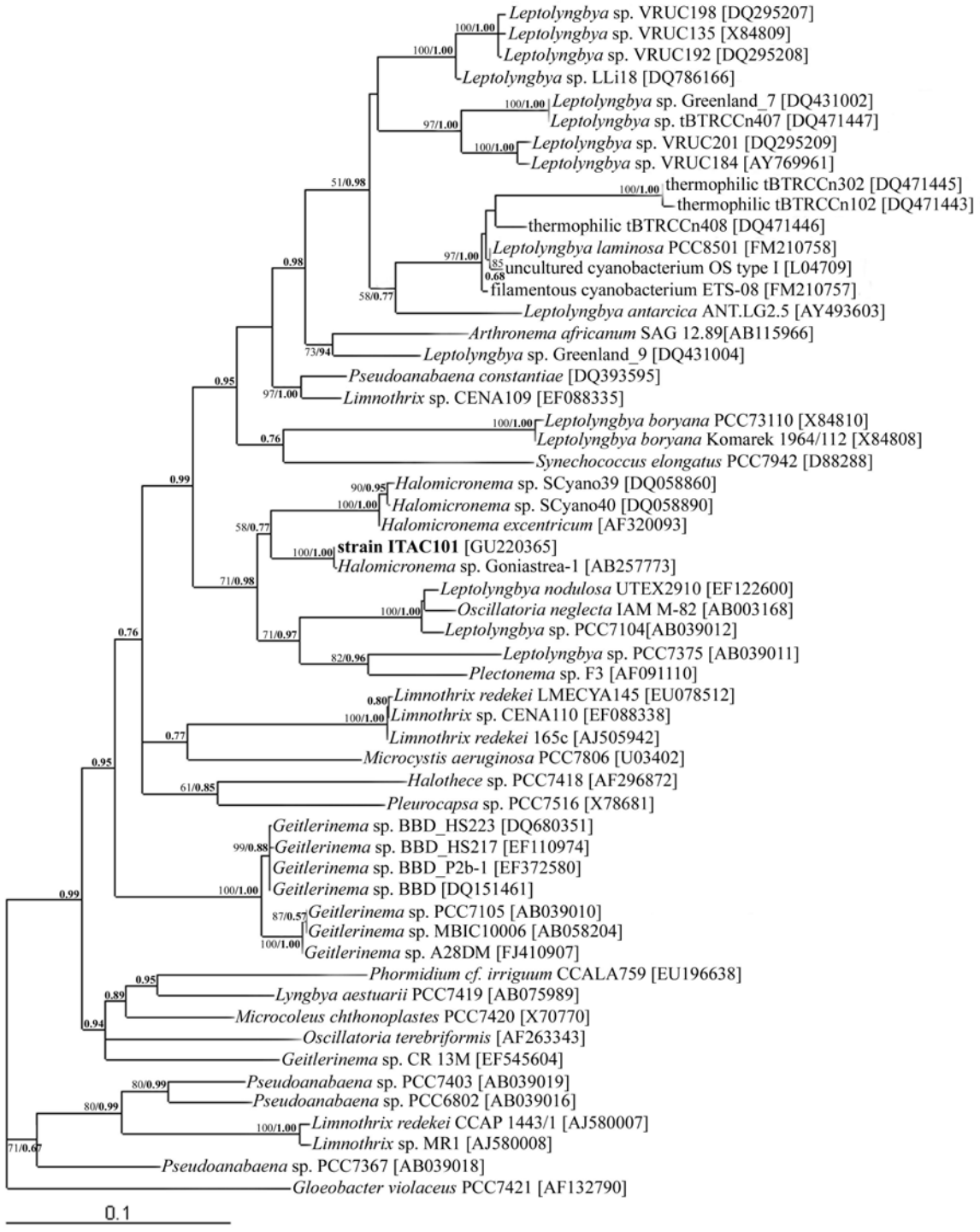

Fig. 6. Phylogenetic tree based on 16S rRNA gene sequences and reconstructed using the maximum–likelihood (ML) analysis. Numbers above branches indicate, in order, the bootstrap value of the ML (as percentages of 1,000 replications, greater than 50%) and Bayesian posterior probability (in bold). Strain ITAC101 is indicated in bold, GenBank accession numbers are indicated in brackets. Bar represents 0.1 nucleotide substitutions per site.

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In memory of Patrizia Beatrice Albertano (Sept 1952-Mar 2012), the field of phycology has lost a great scholar and the people who knew and worked with her have lost a great mentor and friend. She left us so much to keep in mind and heart.