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PHD THESIS

PHYLOGENY AND MOLECULAR BIODIVERSITY OF BIDIRECTIONAL HYDROGENASES IN CYANOBACTERIA

- summary-

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TABLE OF CONTENTS

INTRODUCTION

Cyanobacteria, represent one of the largest and most important groups of bacteria on Earth, are able to perform oxygenic photosynthesis using water as an electron donor and may be found in almost any ecological niche from fresh to salt water, terrestrial and extreme environments (Whitton & Potts, 2000).

Knowledge about this group increased with the sequencing of the first cyanobacterial genomes (in 1996 the *Synechocystis* sp. PCC6803 was first published). Since then, many cyanobacterial genomes have been sequenced reaching today a total of 93 cyanobacterial genomes (Cyanobase – 39 genomes; CyanoGEBA database – 54 genomes (Shih et al., 2012).

In cyanobacteria, as in any diazotrophic bacteria, the reduction of N_2 to NH_3 is accompanied by the formation of molecular hydrogen. The H2 produced by the nitrogenase is rapidly consumed by an uptake hydrogenase, an enzyme that has been found in almost all the N_2-fixing cyanobacteria examined so far, with one reported exception – *Synechococcus* sp. BG 043511 (Ludwig et al., 2006). Additionally, these strains may contain a bidirectional hydrogenase, an enzyme that is generally present in the non nitrogen-fixing cyanobacteria (Tamagnini et al., 2002).

Today, in the context of the depletion of the availability of fossil fuels, new, viable alternatives are being investigated to satisfy the planet's energy needs. One of these alternatives seems to be the production of hydrogen by photosynthetic microorganisms, especially cyanobacteria. In consequence, many of today's research focuses on the study of cyanobacterial hydrogenases, especially the study of the bidirectional hydrogenase.

For a better understanding, the bidirectional hydrogenases need to be extensively studied. An important role in this endeavor is represented by the investigation of their molecular biodiversity and phylogeny. At the same time, the phylogeny of this enzyme needs to be supported by a phylogenetic study of the organisms in which the bidirectional hydrogenase is present.

The aim of this PhD thesis was the investigation of the molecular biodiversity and phylogeny of the bidirectional hydrogenases in cyanobacteria from the AICB Culture

Collection from the Institute of Biological Research Cluj Napoca, compared with the phylogeny of the organisms in which these hydrogenases are present.

The originality at national and international scale emerges from multiple aspects. In the first place, this study takes advantages of the AICB collection, unique in our country due to its impressive number and biodiversity of photosynthetic microorganisms; 70 strains, isolated from different parts of Transylvania, were used in this study.

Internationally, the novelty of this thesis is emphasized by the fact that this is the first comparative study between the phylogeny of the bidirectional hydrogenases in cyanobacteria and the phylogeny of the organisms in which these hydrogenases are present. Also we suggest for the first time a possible horizontal gene transfer (HGT) in cyanobacterial bidirectional hydrogenases. Another aspect of novelty is the first morphological, ultrastructural and phylogenetic characterization of the species *Coelomoron pusillum* strain AICB 1012.

THE AIMS OF THE STUDY

- Phylogenetic analysis based on the nuclear marker ADNr 16S-ITS in order to establish phylogenetic relations between the AICB strains studied.
- Phylogenetic analysis based on the phycocyanin locus (PC-IGS), in order to confirm the phylogenetic relations between the AICB strains established based on the ADNr 16S-ITS marker.
- Identification and sequencing of the AICB strains that contain a bidirectional hydrogenase.
- Phylogenetic analysis based on the large subunit of the bidirectional hydrogenase (*hox*H) of the AICB strains, compared to the phylogeny of the organisms (16S-ITS și PC-IGS) in which these hydrogenases are present.
- The first morphological, ultrastructural and phylogenetic characterization of the species *Coelomoron pusillum* strain AICB 1012.
- The analysis of *hox* gene expression in filamentous cyanobacteria under atmospheric oxigen and in anerobiosis in the dark and light.

A. GENERAL CONSIDERATIONS

I. CYANOBACTERIAL BIODIVERSITY

At present, cyanobacteria are found in a wide range of habitats including aquatic (saltwater and freshwater), terrestrial, and extreme environments (like frigid lakes of the Antarctic or hot springs).

Molecular phylogeny of cyanobacteria based on comparative analysis of certain conserved genes

- Molecular phylogeny of cyanobacteria based on the ARNr 16S and the ITS (Internal Transcribed Spacer)

The comparative analysis of small subunit RNA plays a central role in the microbial identificaion and taxonomy even today, in the genomic era.

The use of 16S rRNA genes in the phylogenetic studies led to a revolutionary approach and finally to the re-organization of the living world in 3 domains: Archaea, Bacteria and Eukaria (Woese, 1987; Ludwig et al., 1993).

Even though the advantages of this marker are well known, with reference to their informational content and to the complexity of the 16S rRNA sequence database, it is generally accepted that this marker does not reflect in detail the evolutionary history. Additional phylogenetic markers should be taken into consideration for a better, more detailed phylogeny.

This inconvenient can be overcomed by the use of some intergenic regions of the operon which have a much higher divergence than the 16S rRNA gene, like the ITS (16S rRNA-23S rRNA Internal Transcribed Spacer).

- Molecular phylogeny of cyanobacteria based on the cpcBA gene and the IGS (Intergenic Spacer)

A fragment of the phycocyanin operon, namely IGS (Intergenic Spacer) flanked by the two bilin subunits cpcB and cpcA is used as a molecular marker in cyanobacteria.

IGS, the same as ITS is a highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level. At the same time, the cpcB and cpcA regions are very conserved in cyanobacteria and together with the IGS fragment they can offer a good phylogenetic analysis (Neilan et al., 1995).

II. BIODIVERSITY OF CYANOBACTERIAL HYDROGENASES

Hydrogenases are enzymes that catalyze the reversible oxidation of molecular hydrogen according to the reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^-$$

According to the metal composition of the active site, hydrogenases are classified into three major groups: NiFe hydrogenases, FeFe hydrogenases (Vignais et al., 2001), and FeS-free hydrogenases (Shima et al., 2004).

II.1 NiFe – hydrogenases

The most numerous and best studied class of hydrogenases are the [NiFe]-hydrogenases from the domain of Bacteria. The core enzyme consists of a αβ heterodimer with the large subunit (α-subunit) harboring the bimetallic active site and the small subunit (β-subunit) hosting the Fe-S clusters (Volbeda *et al.*, 1995; Higuchi *et al.*, 1997; Higuchi *et al.*, 1999).

II.2 Cyanobacterial hydrogenases

Cyanobacteria possess two functionally different NiFe hydrogenases, an uptake enzyme found in nitrogen fixing strains and a bidirectional enzyme that can be present in both nitrogen-fixing and non nitrogen-fixing strains.

- Cynaobacterial uptake hydrogenase

The cyanobacterial uptake hydrogenase is found exclusively in N_2-fixing strains and encoded by the *hup*SL genes, is a heterodimeric enzyme with a large subunit containing the active site (HupL) and a small subunit playing a role in electron transfer (HupS) (Tamagnini et al., 2007).

The main physiological function of the uptake hydrogenase is to reutilize and regain the H2/electrons produced by the H2 evolution through the nitrogenase. This recycling has been suggested to have at least three beneficial functions to the organism (Schutz et al., 2004):

1. it provides ATP via the oxyhydrogen reaction, minimizing the loss of energy;
2. it removes the oxygen from nitrogenase, thereby protecting it from inactivation;
3. it supplies reducing equivalents (electrons) to various cell functions.

- Cynaobacterial bidirectional hydrogenase

The soluble or loosely membrane associated cyanobacterial bidirectional hydrogenase might be present in both N2- and non-N2-fixing strains (Tamagnini et al., 2000, 2002).

The bidirectional hydrogenase is composed of five subunits (encoded by the *hox* – *hydrogen oxidation genes*), in which *Hox*EFU constitute the diaphorase part, and *Hox*YH constitute the hydrogenase part (Schmitz et al., 1995; Appel & Schulz, 1996; Sheremetieva et al., 2002; Schmitz et al., 2002).

The physiological function of the bidirectional hydrogenase in cyanobacteria is not totally clear. It has been suggested that the enzyme acts as an electron valve during photosynthesis in Synechocystis sp. PCC 6803 (Appel et al., 2000). The enzyme has also been proposed to play a role in fermentation functioning as a mediator in the release of excess reducing power under anaerobic conditions (Stal & Moezelaar, 1997; Troshina et al., 2002). Furthermore, it has been suggested previously that the bidirectional hydrogenase could be part of the respiratory complex I (Appel & Schulz, 1996; Schmitz & Bothe, 1996). Still, because the bidirectional hydrogenase is not present in some cyanobacterial strains (Tamagnini et al., 1997, 2000; Schutz et al., 2004; Ludwig et al.,

2006), it seems that in general the bidirectional hydrogenase does not play an essential role for cell survival in the strains where it is present.

II.3. Phylogeny of the cyanobacterial hydrogenases

Phylogenetic studies based on aminoacid sequences of the uptake hydrogenase have clearly shown the monophyletic nature of the cyanobacteria but the phylogenetic relations inside the group were poorly resolved (Ludwig et al., 2006). These results stress the need of further studies which will clarify the phylogenetic relations inside this group of organisms.

B. MATERIALS AND METHODS

III. STRAINS AND CULTURE MEDIA

The biologic material studied in this thesis contained 70 cyanobacterial strains, deposited in the Algal and Cyanobacterial Collection (AICB) of the Institute of Biological Research from Cluj-Napoca (tab. 1). These strains were collected from different counties of Transylvania (Cluj, Bihor, Mureş), with one exception: AICB 51 which was collected from a water basin in Egipt. All the AICB strains investigated are incubated in BG 11 or Z (the *Arthrospira* strains) liquid medium.

Table no. 1

The 70 cyanobacterial strains studied, geographical localisation and growth medium

No.	Strain code	Species	Geographical localisation	Growth medium
1	AICB 808	*Anabaena eliptica*	Suatu, Cluj	BG 11
2	AICB 93	*Anabaena oscillatorioides*	Cluj Napoca, Cluj	BG 11
3	AICB 563	*Anabaena oscillatorioides*	Valea Ierii, Cluj	BG 11
4	AICB 742	*Anabaena* sp.	Ciurila, Cluj	BG 11
5	AICB 841	*Anabaena* sp.	Apahida, Cluj	BG 11
6	AICB 707	*Anabaena* sp.	Catina, Cluj	BG 11
7	AICB 708	*Anabaena* sp.	Catina, Cluj	BG 11
8	AICB 717	*Anabaenopsis* sp.	Ciurila, Cluj	BG 11
9	AICB 740	*Anabaenopsis* sp.	Ciurila, Cluj	BG 11
10	AICB 709	*Aphanizomenon elenkinii*	Ciurila, Cluj	BG 11
11	AICB 743	*Aphanizomenon elenkinii*	Gheorgheni, Cluj	BG 11
12	AICB 716	*Calothrix* sp.	Turda, Cluj	BG 11
13	AICB 39	*Cylindrospermum alatosporum*	Geaca, Cluj	BG 11

14	AICB 820	*Fremyella diplosiphon*	UT(SF3)	BG 11
15	AICB 718	*Gloeotrichia ecinulata*	Turda, Cluj	BG 11
16	AICB 285	*Nostoc sp.*	Martineşti, Cluj	BG 11
17	AICB 420	*Nodularia harveyana*	Fânaţele Cluj, Cluj	BG 11
18	AICB 421	*Nostoc linckia*	Turda, Cluj	BG 11
19	AICB 44	*Nostoc palludosum*	Cluj Napoca, Cluj	BG 11
20	AICB 362	*Nostoc punctiforme*	Martineşti, Cluj	BG 11
21	AICB 386	*Tolypothrix tenuis*	Fânaţele Cluj, Cluj	BG 11
22	AICB 514	*Tolypothrix tenuis*	Cluj Napoca, Cluj	BG 11
23	AICB 34	*Microcystis aeruginosa*	Radvani, Bihor	BG 11
24	AICB 35	*Microcystis aeruginosa*	Catina, Cluj	BG 11
25	AICB 36	*Microcystis aeruginosa*	Geaca, Cluj	BG 11
26	AICB 618	*Microcystis aeruginosa*	Pond, Taga Mare, Cluj	BG 11
27	AICB 619	*Microcystis aeruginosa*	Cluj Napoca, Cluj	BG 11
28	AICB 620	*Microcystis aeruginosa*	Cluj Napoca, Cluj	BG 11
29	AICB 679	*Microcystis aeruginosa*	Zau de Campie, Mures	BG 11
30	AICB 680	*Microcystis aeruginosa*	Zau de Campie, Mures	BG 11
31	AICB 681	*Microcystis aeruginosa*	Fishpond, Cefa, Bihor	BG 11
32	AICB 682	*Microcystis aeruginosa*	Zau de Campie, Mures	BG 11
33	AICB 689	*Microcystis aeruginosa*	Madaras, Bihor	BG 11
34	AICB 695	*Microcystis aeruginosa*	Zau de Campie, Mures	BG 11
35	AICB 697	*Microcystis aeruginosa*	Zau de Campie, Mures	BG 11
36	AICB 702	*Microcystis aeruginosa*	Mihes, Mures	BG 11
37	AICB 747	*Microcystis* sp.	Geaca, Cluj	BG 11
38	AICB 748	*Microcystis* sp.	Lacu, Cluj	BG 11
39	AICB 822	*Microcystis* sp.	Geaca, Cluj	BG 11
40	AICB 823	*Microcystis* sp.	Geaca, Cluj	BG 11
41	AICB 826	*Microcystis* sp.	Geaca, Cluj	BG 11
42	AICB 827	*Microcystis* sp.	Scutard, Cluj	BG 11
43	AICB 832	*Microcystis* sp.	Geaca, Cluj	BG 11
44	AICB 833	*Microcystis* sp.	Geaca, Cluj	BG 11
45	AICB 51	*Synechocystis* sp.	Egipt	Z
46	AICB 61	*Gloeocapsa turgida*	Băile Felix, Bihor	BG 11
47	AICB 62	*Synechocystis minuscula*	Băile Felix, Bihor	BG 11
48	AICB 1012	*Coelomoron* sp.	Zau de Câmpie, Mureş	BG 11
49	AICB 1013	*Gloeocapsa* sp.	Tăureni, Mureş	BG 11
50	AICB 1014	*Merismopedia* sp.	Lacul Mic, Mureş	BG 11
51	AICB 1015	*Merismopedia* sp.	Lacul Mic, Mureş	BG 11

52	AICB 1016	*Synechococcus* sp.	Apahida, Cluj	Z
53	AICB 95	*Phormidium formosum*	Cefa, Bihor	BG 11
54	AICB 97	*Oscillatoria lachneri*	Cluj Napoca, Cluj	BG 11
55	AICB 254	*Oscillatoria boryana*	Dej, Cluj	BG 11
56	AICB 343	*Phormidium fragile*	Cluj Napoca, Cluj	BG 11
57	AICB 382	*Oscillatoria lemmermannii*	Turda, Cluj	BG 11
58	AICB 384	*Phormidium formosum*	Turda, Cluj	BG 11
59	AICB 404	*Phormidium formosum*	Geaca, Cluj	BG 11
60	AICB 450	*Arthrospira jennerii*	Hoteni, Maramureş	BG 11
61	AICB 545	*Oscillatoria limnetica*	Turda, Cluj	BG 11
62	AICB 597	*Phormidium bijugatum*	Cătina, Cluj	BG 11
63	AICB 605	*Arthrospira fusiformis*	Apahida, Cluj	Z
64	AICB 627	*Arthrospira fusiformis*	Apahida, Cluj	Z
65	AICB 668	*Arthrospira fusiformis*	Apahida, Cluj	Z
66	AICB 641	*Oscillatoria amphibia*	Apahida, Cluj	D/2
67	AICB 670	*Arthrospira fusiformis*	Apahida, Cluj	Z
68	AICB 683	*Oscillatoria geminata*	Miheşul de Câmpie, Mureş	BG 11
69	AICB 728	*Lyngbya* sp.	Turda, Cluj	BG 11
70	AICB 1054	*Arthrospira jennerii*	Mogoşa, Maramureş	BG 11

IV. LIGHT AND ELECTRON MICROSCOPY METHODS

As for the light microscopy, the protocol consisted in microscopic observation of each strain using an Olympus BX-41 light microscope, digital photography and making of necessary measurements for identification (cells and colonies size, etc.).

For SEM protocol, the algal samples were analyzed with an electron microscope, Jeol JSM 5510LV, using an acceleration voltage of 10 kV with a 5 size spot.

The TEM investigations permitted the obtaining of some ultrafine sections using a TEM microscope, Jeol JEM 1010.

V. MOLECULAR METHODS USED IN THE STUDY OF THE CYANOBACTERIAL STRAINS

For the phylogenetic analyses based on the ADNr 16S-ITS, PC-IGS and *hox*H sequences, the following steps were taken: obtaining the ADNr 16S-ITS, PC-IGS and *hox*H sequences presumed the genomic DNA extraction, using the commercial kits useful in obtaining a high degree of purity. Using specific primers we proceeded for the standard PCR reaction. The DNA fragments were directly sequenced or cloned in cloning vectors (pJET1.2/blunt) prior to sequencing. We used the Applied Biosystems 3130 Genetic Analizer. The DNA fragments were assembled using the *Vector NTI Advanced v 9.0* software. The sequences validation was done using blastn (BLAST-NCBI). For multiple sequence alignment, ADNr 16S-ITS, PC-IGS and *hox*H sequences were extracted from GenBank (NCBI). In this way, the *Mega 5.1* software was used.

Phenetics or distance (Minimum Evolution) and cladistic methods (Maximum Likelihood) were two distinct methods used in constructing phylogenetic trees, based on ADNr 16S-ITS, PC-IGS and *hox*H sequences.

For the *hox* gene expression analyses, total RNA was extracted using a classic protocol adapted after McGinn et al (2003). The total RNA obtained was treated with commercial DNA-se and cDNA synthesis was performed usind also a commercial kit. The qRT-PCR reaction was then performed using specific primers.

The data obtained were analyzed using the 7000 System SDS Software 1.2.3. (Applied Biosystem), Microsoft Office Excel and Origin 8.6. According to the comparative Cq method also referred to as the $2^{-\Delta\Delta CT}$ method the Cq values of the genes of interest were double normalized (Schmittgen & Livak 2008).

C. RESULTS AND DISCUSSION

VI. PHYLOGENETIC ANALYSIS OF THE AICB STRAINS BASED ON THE SELECTED GENETIC MARKERS

- Phylogenetic analysis of the AICB strains from the Order Nostocales

22 strains belonging to AICB Culture Collection from the Order Nostocales were investigated based on the sequence length and phylogeny of the ARNr 16S-ITS gene (fig.1), the phycocyanin locus (fig.2) and the gene for the bidirectional hydrogenase *hox*H (fig.3).

The trees obtained for the ARNr 16S-ITS (fig.1) and PC-IGS (fig.2) markers were very similar which supports the validity of the phylogenetic clusters proposed. Based on the ARNr 16S-ITS (fig.1) and PC-IGS (fig.2) markers, the genera *Anabaena*, *Aphanizomenon*, *Calothrix* and *Nostoc* are polyphyletic, results supported by the data from the literature. This indicates the need for a taxonomical revision of these genera considering morphological traits but also molecular, biochemical and ecological data (polyphasic approach). The genera *Nodularia* and *Cylindrospermum* seem to be homogenous groups, with a monophyletic origin, according to both the morphological and molecular data.

The bidirectional hydrogenase (*hox*H) was detected in 18 of the 22 Nostocales AICB strains. The length of the sequences varied between 904-1278 bp (tab.2).

The topology of the phylogenetic tree obtained based on the *hox*H sequences (fig.3) was very similar to that of the 16S-ITS and cpcBA-IGS trees, which shows that the phylogeny of the *hox*H gene is very similar with the phylogeny of the organisms where it is present.

The clustering of the *Anabaena oscillatorioides* AICB 563 strain with the *Nostoc* strains is very unusual especially as the clusters N1 and N2 (fig.3) are not closely related. This clustering suggests that between the two cyanobacterial genera a lateral gene transfer could have taken place.

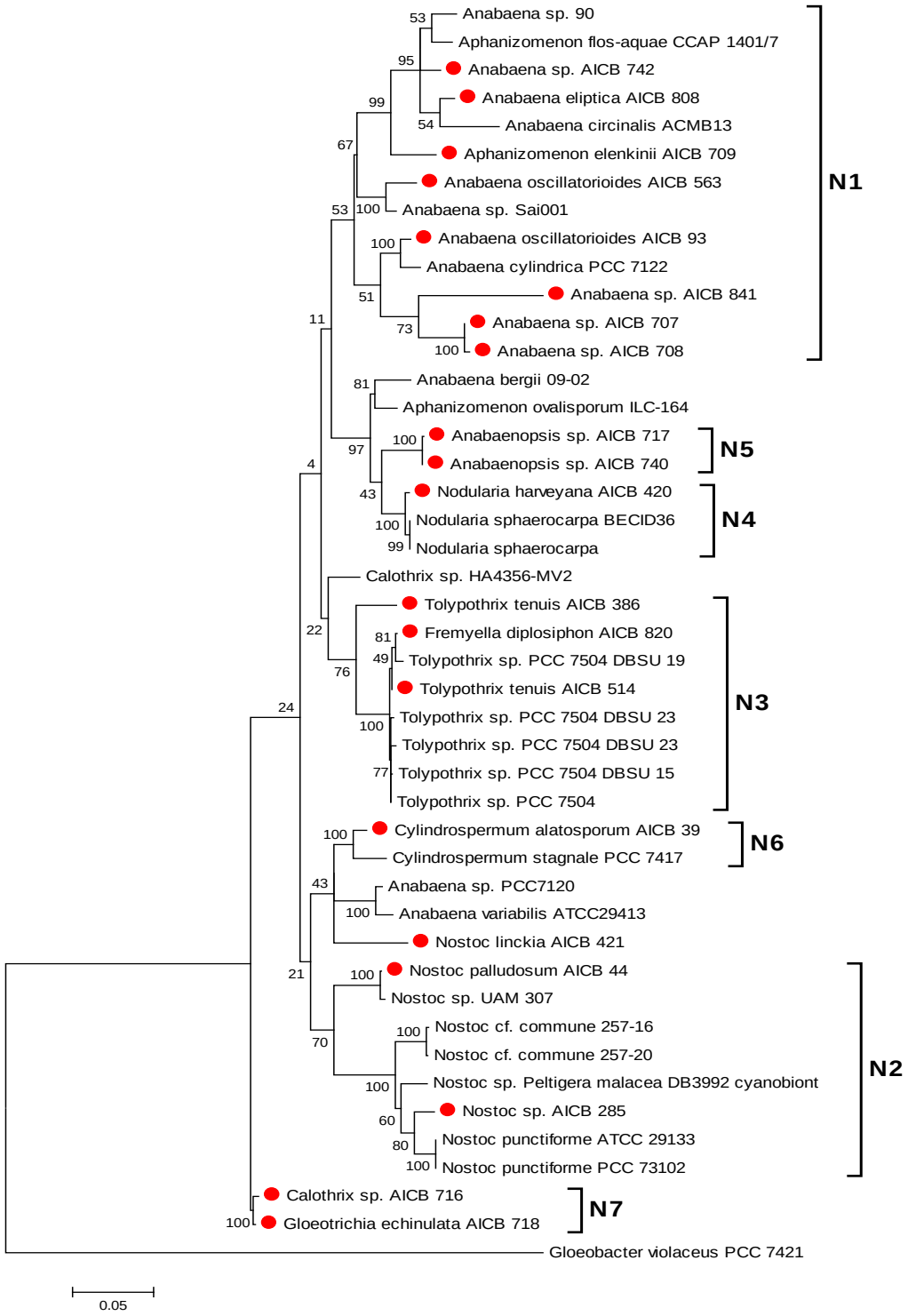

Fig. 1 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Nostocales based on 16S-ITS sequences. AICB sequences are marked with red dots. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the 16S-ITS sequence from *Gloeobacter violaceus* PCC 7421.

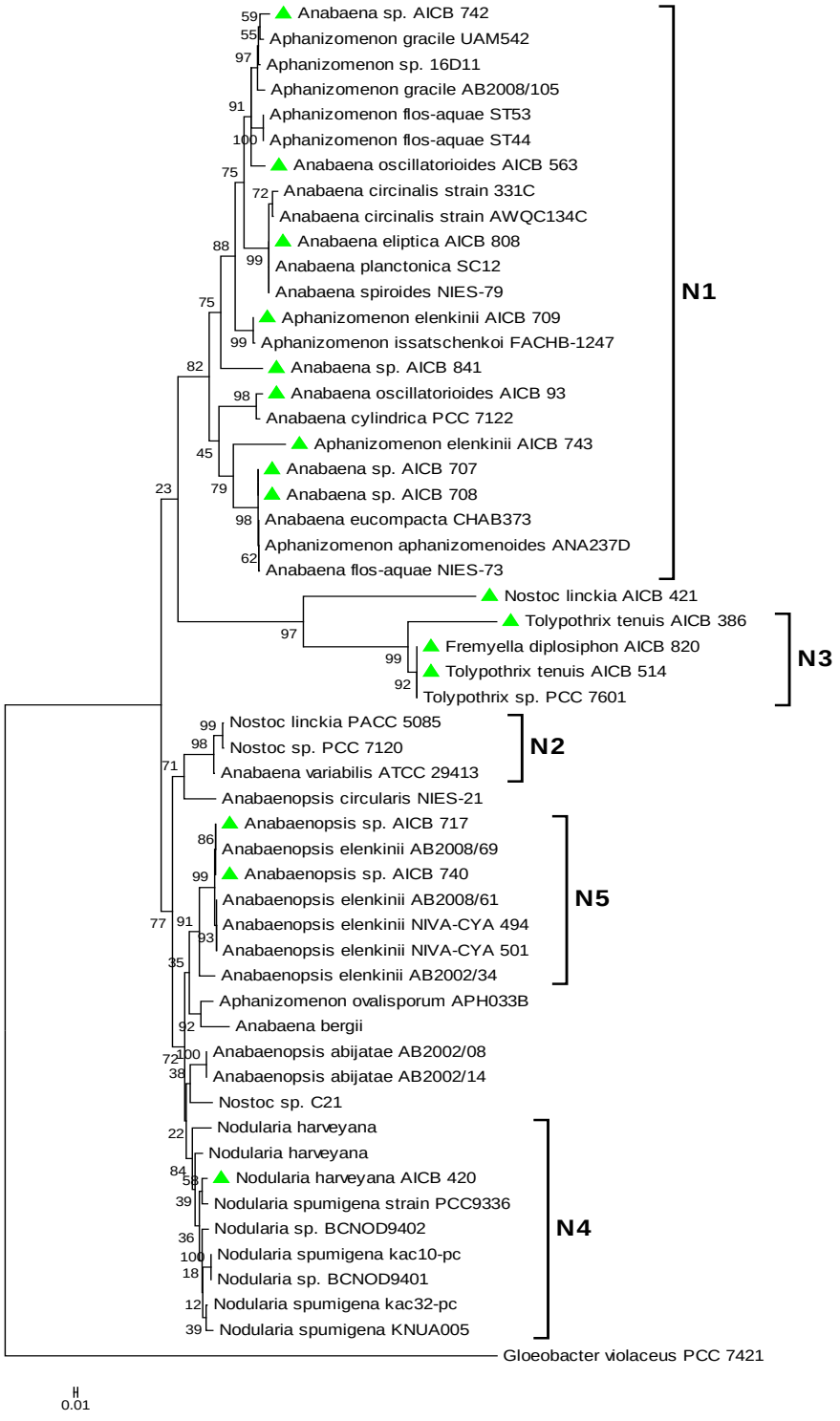

Fig. 2 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Nostocales based on PC-IGS sequences. AICB sequences are marked with red dots. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the PC-IGS sequence from *Gloeobacter violaceus* PCC 7421.

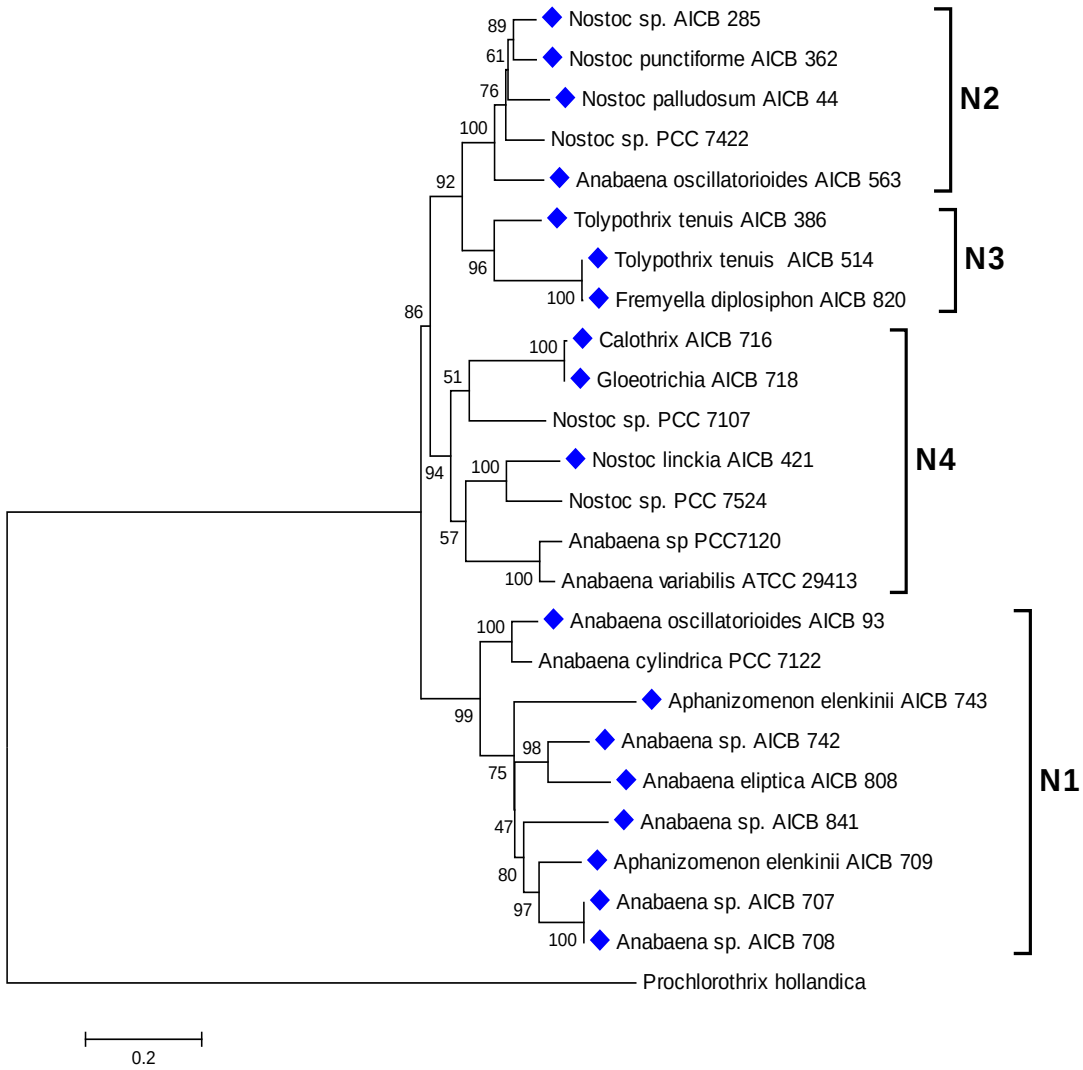

Fig. 3 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Nostocales based on the *hox*H sequences. AICB sequences are marked with blue rhombes. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the *hox*H sequence from *Prochlorothrix hollandica*.

Table no. 2

The 22 cyanobacterial strains from the Nostocales studied, the presence/absence of *hox*H and the number of *hox*H base pairs sequenced

No.	Strain	Species	Presence/absence of *hox*H	No. of base pairs
1	AICB 808	*Anabaena eliptica*	+	921
2	AICB 93	*Anabaena oscillatorioides*	+	919
3	AICB 563	*Anabaena oscillatorioides*	+	916
4	AICB 742	*Anabaena* sp.	+	904
5	AICB 841	*Anabaena* sp.	+	914

6	AICB 707	*Anabaena* sp.	+	908
7	AICB 708	*Anabaena* sp.	+	919
8	AICB 717	*Anabaenopsis* elenkinii	-	-
9	AICB 740	*Anabaenopsis* elenkinii	-	-
10	AICB 709	*Aphanizomenon elenkinii*	+	911
11	AICB 743	*Aphanizomenon elenkinii*	+	912
12	AICB 716	*Calothrix* sp.	+	1224
13	AICB 39	*Cylindrospermum alatosporum*	-	-
14	AICB 820	*Fremyella diplosiphon*	+	912
15	AICB 718	*Gloeotrichia ecinulata*	+	1223
16	AICB 285	*Nostoc sp.*	+	912
17	AICB 420	*Nodularia harveyana*	-	-
18	AICB 421	*Nostoc linckia*	+	1278
19	AICB 44	*Nostoc palludosum*	+	1226
20	AICB 362	*Nostoc punctiforme*	+	910
21	AICB 386	*Tolypothrix tenuis*	+	911
22	AICB 514	*Tolypothrix tenuis*	+	908

- Phylogenetic analysis of the AICB strains from the Order Chroococcales

30 strains belonging to AICB Culture Collection from the Order Chroococcales were investigated based on the sequence length and phylogeny of the ARNr 16S-ITS gene (fig.4), the phycocyanin locus (fig.5) and the gene for the bidirectional hydrogenase *hox*H (fig.6).

The trees obtained for the ARNr 16S-ITS (fig.4) and PC-IGS (fig.5) markers were very similar (4 and 5 clusters) which supports the validity of the phylogenetic clusters proposed. The clustering of the *Microcystis*, *Gloeocapsa* and *Merismopedia* genera indicates that they are monophyletic, their classification being supported by both morphologic and molecular data. Based on their heterogenous distribution in several clusters in the 16S-ITS (fig.4) and cpcBA-IGS (fig.5) trees, the genera *Synechocystis* and *Synechococcus* seem to be polyphyletic.

The bidirectional hydrogenase could be detected in 27 of the 30 AICB strains. The lack of amplification in the strains *Synechocystis* sp. AICB 51, *Gloeocapsa turgida* AICB 61 and *Synechocystis minuscula* AICB 62 suggest that these strains don't have a bidirectional enzyme but it is also possible that the primers used were not adequate for these strains. The length of the *hox*H sequences varied between 1125-1339 bp (tab.3).

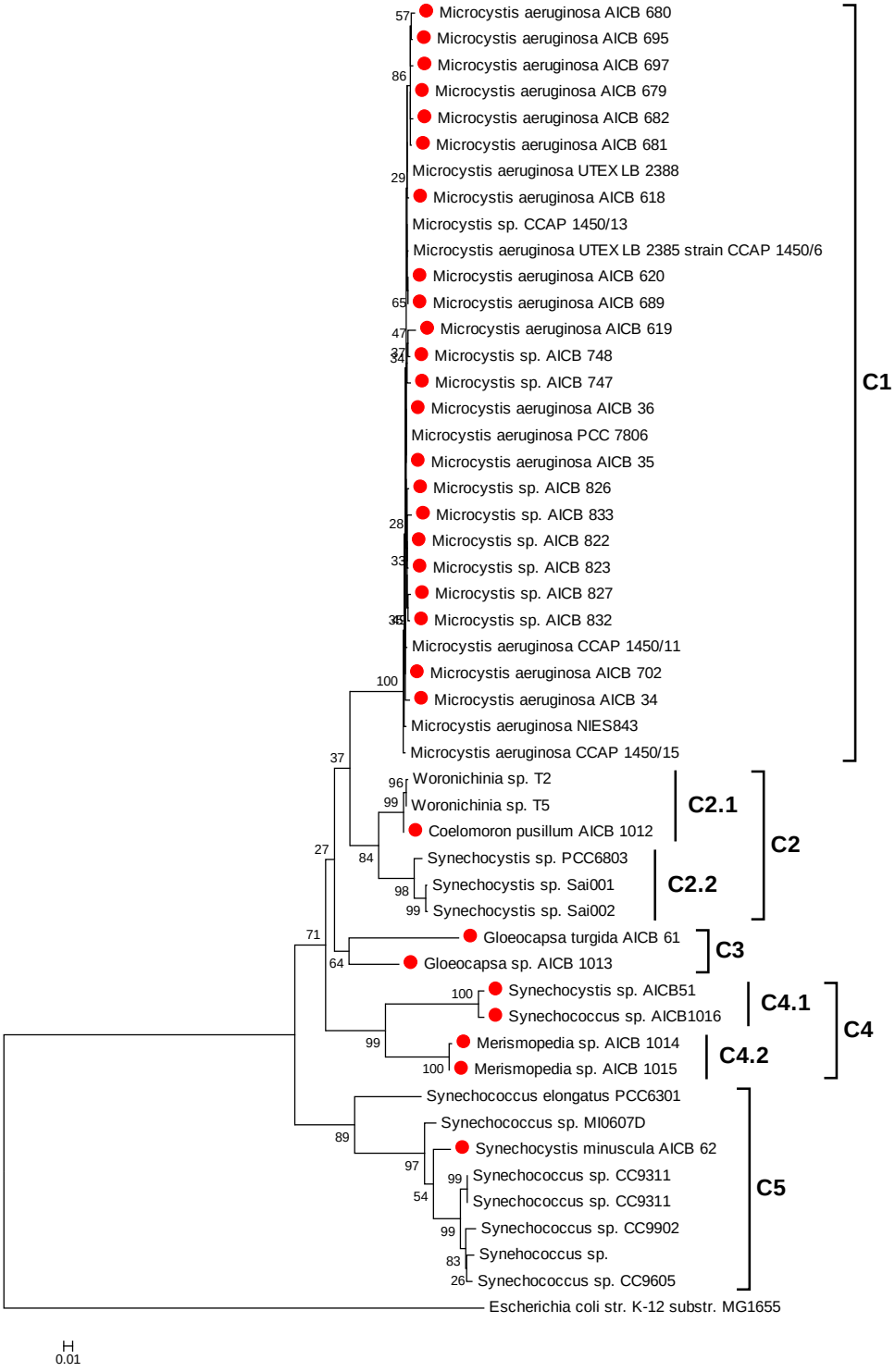

Fig.4 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Chroococcales based on 16S-ITS sequences. AICB sequences are marked with red dots. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the 16S-ITS sequence from *Escherichia coli* K-12.

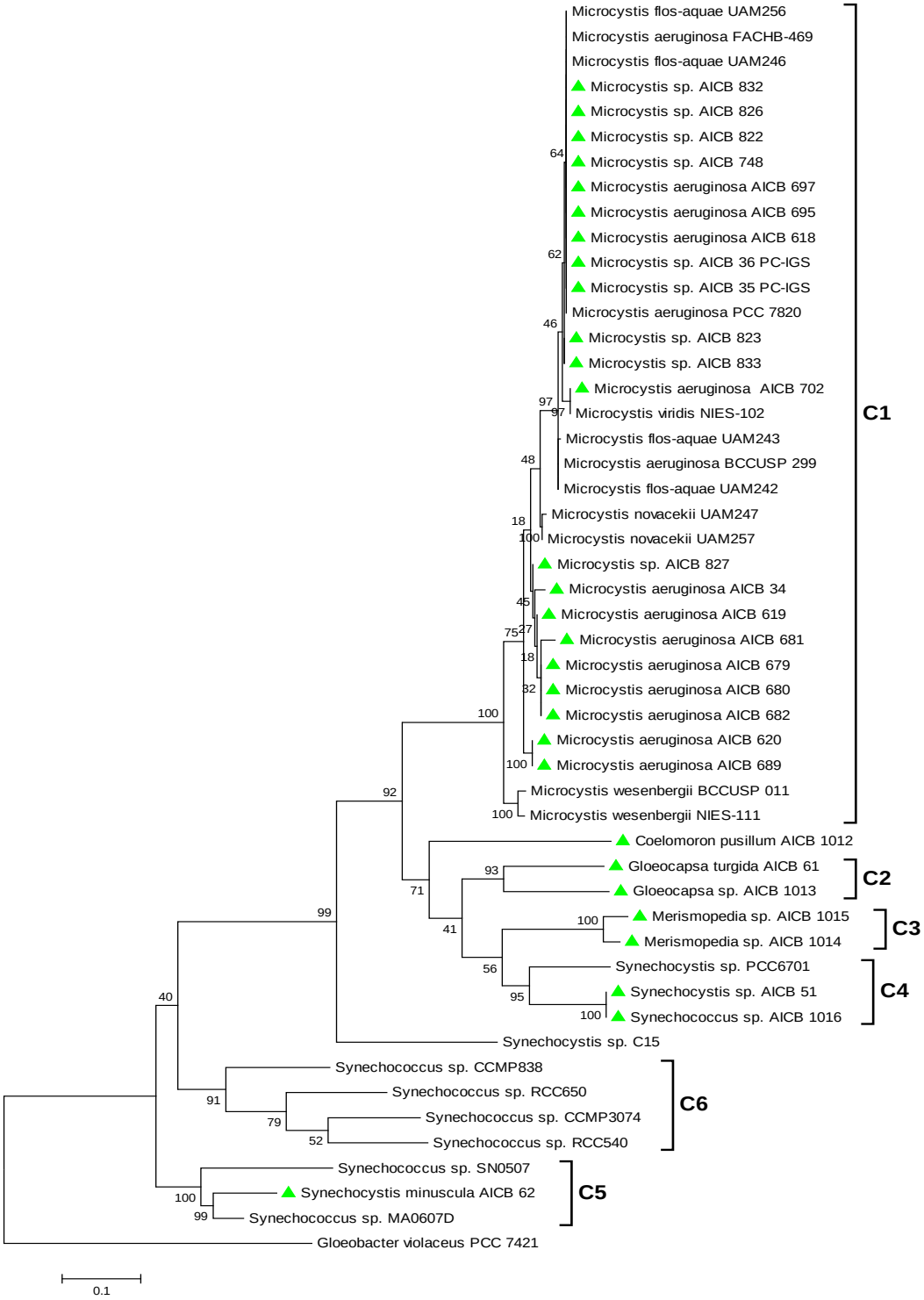

Fig. 5 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Chroococcales based on PC-IGS sequences. AICB sequences are marked with green triangles. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the PC-IGS sequence from *Gloeobacter violaceus* PCC 7421.

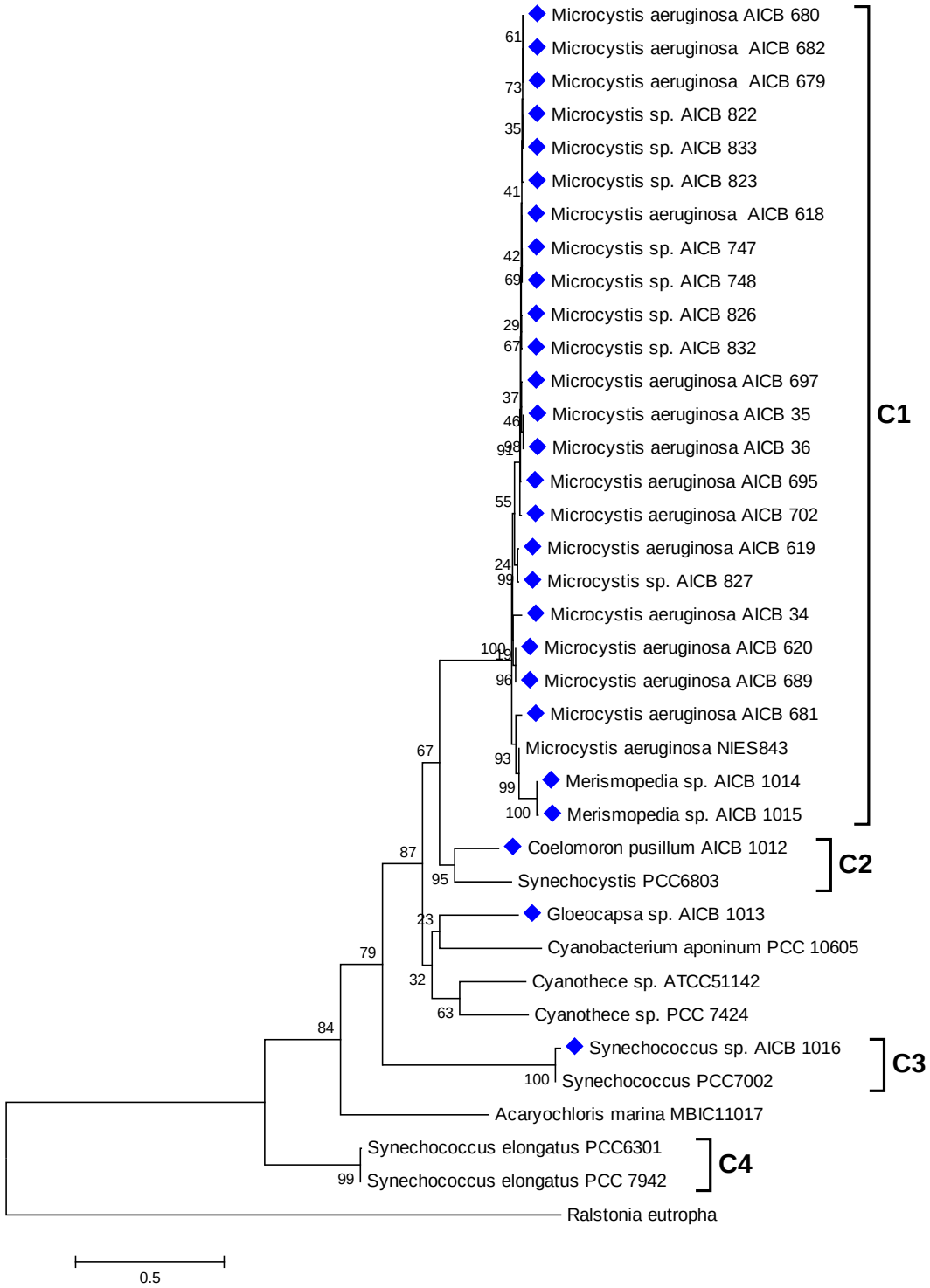

Fig. 6 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Chroococcales based on the *hox*H sequences. AICB sequences are marked with blue rhombes. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the *hox*H sequence from *Ralstonia eutropha*.

The topology of the phylogenetic tree obtained based on the *hox*H sequences (fig.6) was very similar to that of the 16S-ITS and cpcBA-IGS trees. The clustering of the *Merismopedia* strains AICB 1014 and AICB 1015 with the *Microcystis* cluster (fig.6) is probably due to a horizontal gene transfer (HGT) between the two cyanobacterial genera.

Table no. 3

The 30 cyanobacterial strains from the Chroococcales studied, the presence/absence of *hox*H and the number of *hox*H base pairs sequenced

No.	Strain	Species	Presence/absence of *hox*H	No. of base pairs
1	AICB 34	*Microcystis aeruginosa*	+	1327
2	AICB 35	*Microcystis aeruginosa*	+	1327
3	AICB 36	*Microcystis aeruginosa*	+	1339
4	AICB 618	*Microcystis aeruginosa*	+	1333
5	AICB 619	*Microcystis aeruginosa*	+	1334
6	AICB 620	*Microcystis aeruginosa*	+	1336
7	AICB 679	*Microcystis aeruginosa*	+	1336
8	AICB 680	*Microcystis aeruginosa*	+	1337
9	AICB 681	*Microcystis aeruginosa*	+	1334
10	AICB 682	*Microcystis aeruginosa*	+	1337
11	AICB 689	*Microcystis aeruginosa*	+	1336
12	AICB 695	*Microcystis aeruginosa*	+	1337
13	AICB 697	*Microcystis aeruginosa*	+	1333
14	AICB 702	*Microcystis aeruginosa*	+	1333
15	AICB 747	*Microcystis* sp.	+	1337
16	AICB 748	*Microcystis* sp.	+	1337
17	AICB 822	*Microcystis* sp.	+	1324
18	AICB 823	*Microcystis* sp.	+	1337
19	AICB 826	*Microcystis* sp.	+	1334
20	AICB 827	*Microcystis* sp.	+	1335
21	AICB 832	*Microcystis* sp.	+	1333
22	AICB 833	*Microcystis* sp.	+	1337
23	AICB 51	*Synechocystis* sp.	-	-
24	AICB 61	*Gloeocapsa turgida*	-	-
25	AICB 62	*Synechocystis minuscula*	-	-
26	AICB 1012	*Coelomoron* sp.	+	1185
27	AICB 1013	*Gloeocapsa* sp.	+	1186
28	AICB 1014	*Merismopedia* sp.	+	1164
29	AICB 1015	*Merismopedia* sp.	+	1125
30	AICB 1016	*Synechococcus* sp.	+	1237

- Phylogenetic analysis of the AICB strains from the Order Oscillatoriales

18 strains belonging to AICB Culture Collection from the Order Chroococcales (tab.4) were investigated based on the sequence length and phylogeny of the ARNr 16S-ITS gene (fig.7), the phycocyanin locus (fig.8) and the gene for the bidirectional hydrogenase *hox*H (fig.9).

The trees obtained for the ARNr 16S-ITS (fig.7) and PC-IGS (fig.8) markers were very similar with 5 clusters, which supports the validity of the phylogenetic clusters proposed. Based on their heterogenous distribution in several clusters in the 16S-ITS (fig.7) and cpcBA-IGS (fig.8) trees, the genera *Phormidium*, *Oscillatoria* şi *Lyngbya* (fig.7 and 8) seem to be polyphyletic. The genus *Arthrospira* is the only homogenous group between the AICB strains belonging to the Oscillatoriales studied. We can say that the strains belonging to this genus are valid taxonomic units (supported by morphologic and molecular data) with a monophyletic origin.

The bidirectional hydrogenase could be detected in 16 of the 18 AICB strains (tab.4) and the topology of the *hox*H tree (fig.9) was very similar to that of the 16S-ITS (fig.7) and cpcBA-IGS (fig.8) trees.

The clustering of the *Spirulina platensis* FACHB 440 with the *Oscillatoria* and *Phormidium* strains (fig.9) is probably due to a horizontal gene transfer between the two cyanobacterial genera and not an error in taxonomic classification of this strain.

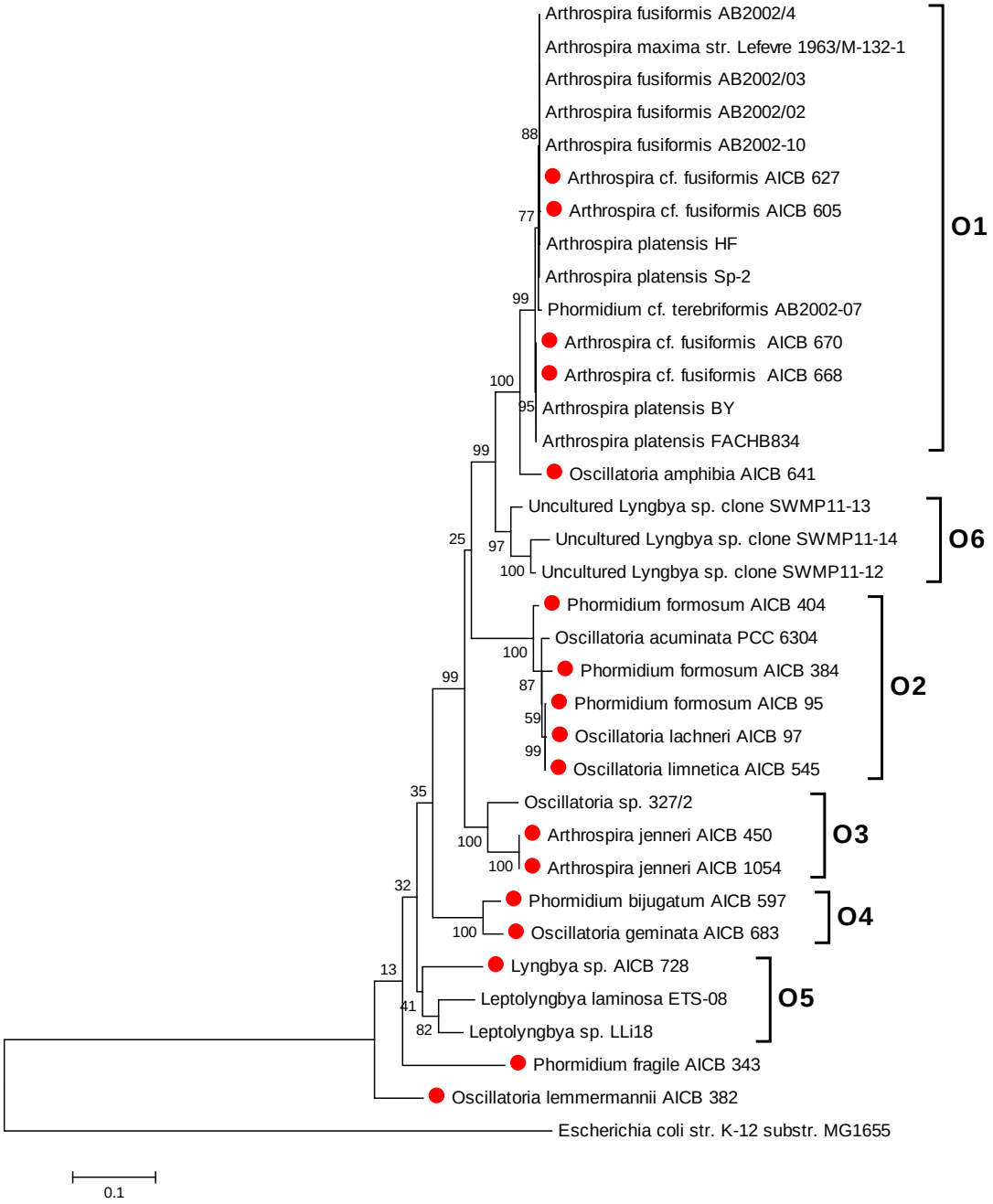

Fig. 4 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Oscillatoriales based on 16S-ITS sequences. AICB sequences are marked with red dots. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the 16S-ITS sequence from *Escherichia coli* K-12.

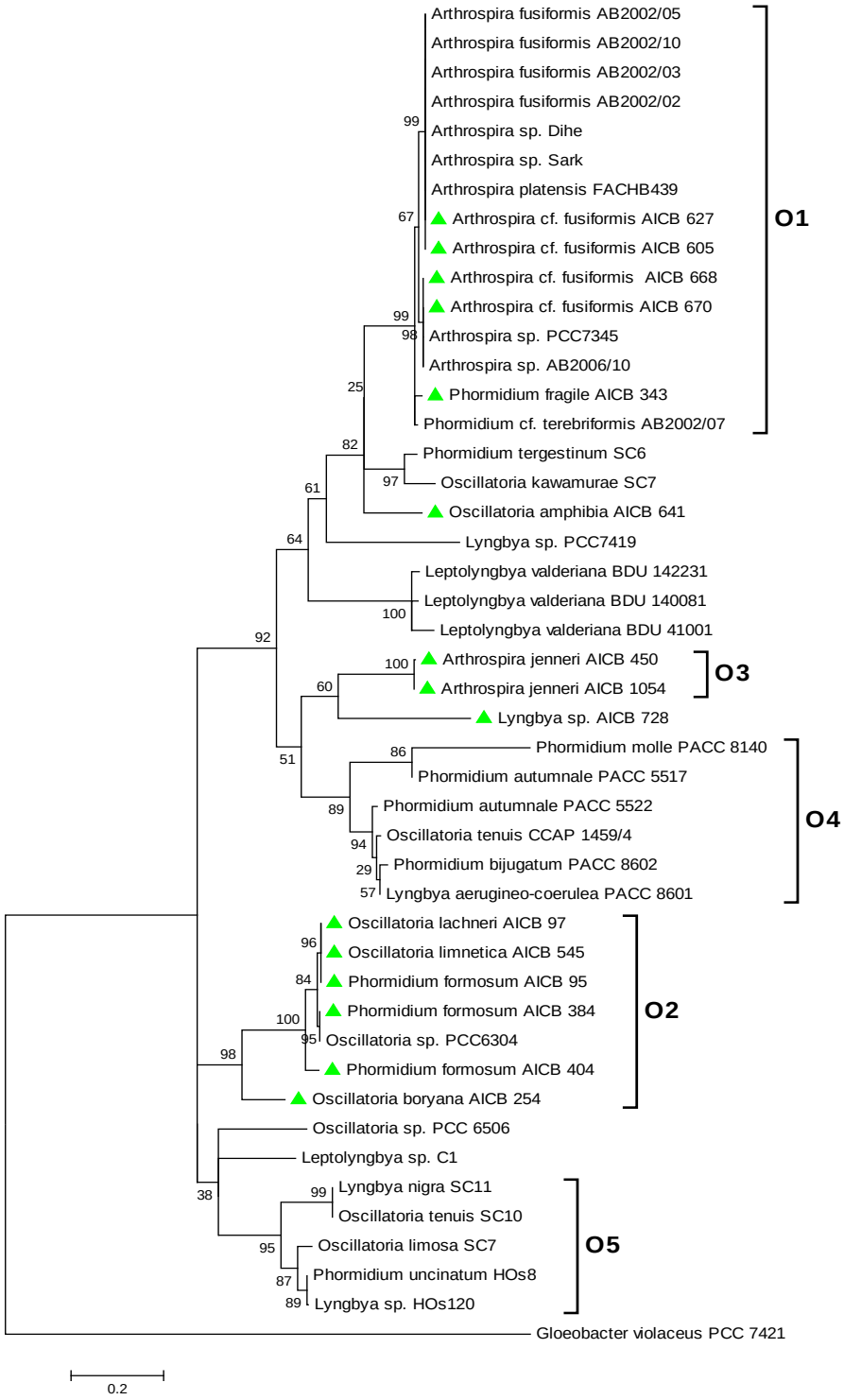

Fig. 8 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Oscillatoriales based on PC-IGS sequences. AICB sequences are marked with green triangles. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the PC-IGS sequence from *Gloeobacter violaceus* PCC 7421.

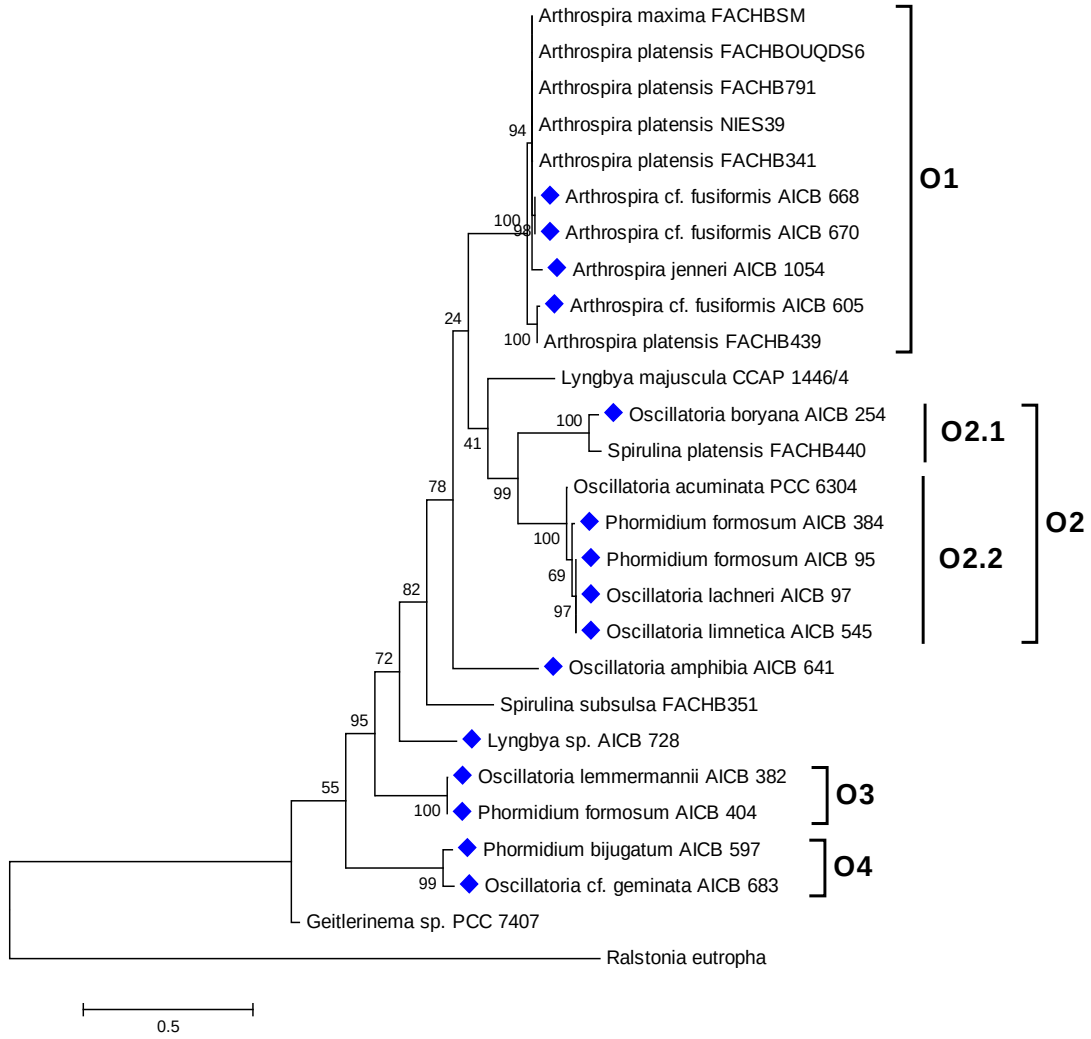

Fig. 9 Maximum likelihood tree of the AICB strains and strains downloaded from GenBank for the Oscillatoriales based on the *hox*H sequences. AICB sequences are marked with blue rhombes. Proposed clusters are indicated on the right . Bootstrap values (500 replicates) are indicated at the nodes. The outgroup was the *hox*H sequence from *Ralstonia eutropha*.

Table no. 4

The 18 cyanobacterial strains from the Oscillatoriales studied, the presence/absence of *hox*H and the number of *hox*H base pairs sequenced

No.	Strain	Species	Presence/absence of *hox*H	No. of base pairs
1	AICB 95	*Phormidium formosum*	+	901
2	AICB 97	*Oscillatoria lachneri*	+	903
3	AICB 254	*Oscillatoria boryana*	+	907
4	AICB 343	*Phormidium fragile*	-	-

5	AICB 382	*Oscillatoria lemmermannii*	+	895
6	AICB 384	*Phormidium formosum*	+	902
7	AICB 404	*Phormidium formosum*	+	1006
8	AICB 450	*Arthrospira jennerii*	-	-
9	AICB 545	*Oscillatoria limnetica*	+	891
10	AICB 597	*Phormidium bijugatum*	+	1196
11	AICB 605	*Arthrospira fusiformis*	+	1207
12	AICB 627	*Arthrospira fusiformis*	+	1190
13	AICB 668	*Arthrospira fusiformis*	+	1201
14	AICB 641	*Oscillatoria amphibia*	+	1188
15	AICB 670	*Arthrospira fusiformis*	+	1201
16	AICB 683	*Oscillatoria geminata*	+	1202
17	AICB 728	*Lyngbya* sp.	+	880
18	AICB 1054	*Arthrospira jennerii*	+	1285

VII THE ULTRASTRUCTURE, TAXONOMY AND MOLECULAR PHYLOGENY OF THE STRAIN AICB 1012 *Coelomoron pusillum* (Van Goor) Komárek

The *Coelomoron pusillum* AICB 1012 strain has a colonial organization; the colonies are formed out of small cell aggregates (fig. 10 a-b, d-e). The examination of samples coloured with China Ink (fig.10 d-f) revealed the presence of a mucilaginous matrix/mass apparently unstructured, in which the colonies are embedded.

In tab. 5 we show the the main diagnostic features identified for *Coelomoron pusillum* AICB 1012 compared with the ones presented in other two papers.

The transmission electron microscopy (TEM) observations (through the negative staining method) have shown that the AICB 1012 cells are surrounded by cyanobactreria type pili (fig. 11 a-c).

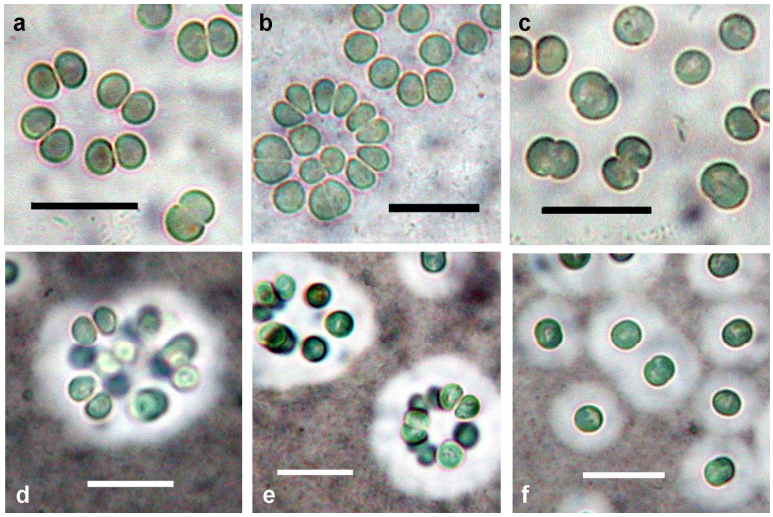

Fig. 10 (a-f). *Coelomoron pusillum* AICB 1012 – light microscopy. The mucilaginous envelope of the colonies was highlighted with China Ink (d-f). Colonies have a small number of cells, sometimes only two (f). Single cells were frequently observed (c, f), each with its mucilaginous envelope (f). The mucilaginous envelope is barely visible without China Ink (a-c). In colonies, cells are radially arranged at the periphery (a, e). Bar = 10 μm.

Table no. 5

Morphological characters of *C. pusillum*, in light microscopy, in comparison with the ones presented by other authors.

Morphological character	**Komárek & Anagnostidis, 1999**	**McGregor et al., 2007**	**AICB 1012**
Colony shape	± spherical, irregulary oval	± spherical, irregulary oval	± spherical, irregulary oval
Simple or composed colonies	simple, sometimes composed	often composed	simple
Colony diameter (μm)	15-20-(30)	12,0 – 30,0	14,6 – 27,7 (incl. mucilage) 10,1-20,9 (without mucilage)
Cell shape	Oval, obovoid, pale blue-green	widely oval, obovoid, pale blue-green	sphaerical or sub sphaerical, oval, young cells obovoid; blue-grey, pale, slightly brown
Cell dimensions (μm)	2,2-4,5 x 1,8-4	2,2-4,5(-5,5) × 1,8-4,0	3,1-5,4;
Gas vesicles	not present	not present	not present

Cell arrangement in the colony	radially arranged in the periphery, slightly distant and displaced from one another	radially arranged in the periphery, slightly distant and displaced from one another	ordered, arranged in the periphery; cells slightly from one another
No. of cells/colony	10-30, sometimes 2-4	10-30, sometimes 2-4	4-15, frequent 8-9, many solitary cells
Mucilage morphology	colourless, diffluent, recognizable only afther staining; poorly visible protuberances that radiate from the centre of the colony	colourless, diffluent; poorly visible mucilage strings that radiate from the centre of the colony.	colourless, recognizable only afther staining, apparently unstructured
Mucilage size (µm)	cca. 2,5, exceding beyond the cell	1,2-2,5	2,25 – 3,4

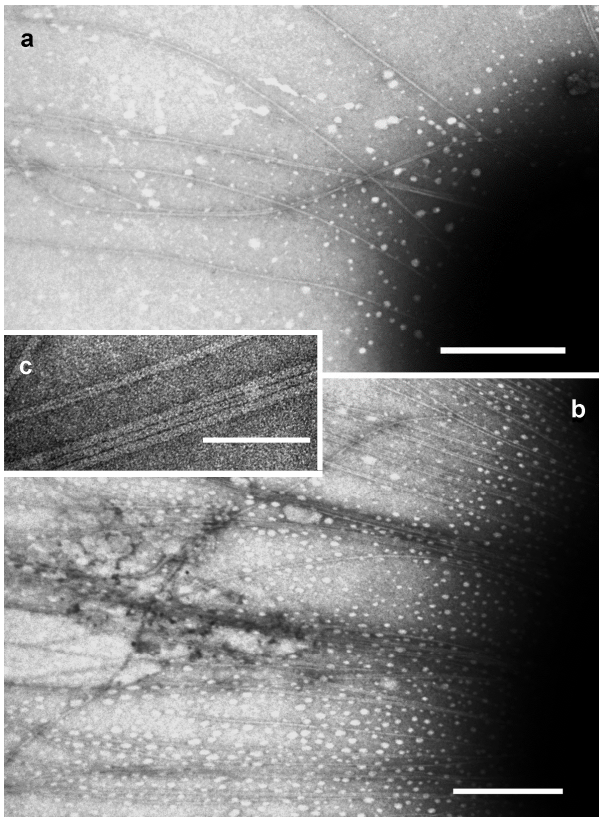

Fig. 11 (a-c). *Coelomorom pusillum* AICB 1012. Microphographs of cyanobacterial fimbriae observed in transmission electron microscopy (TEM) through negative staining of unfixed cell suspensions. The pili radiate from the cell surface and are several times longer than the cell. They can form bundles by association (c). Bar = 500 nm (fig. 5 a-b), and 100 nm (fig. 5 c).

The ultrastructure of a *C. pusillum* cell is shown in fig. 12 and 13.

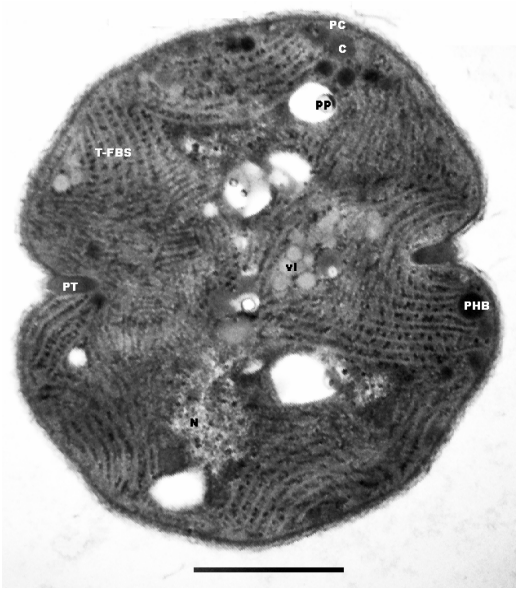

Fig. 12. Longitudinal section through a *Coelomoron pusillum* AICB 1012 cell in division. Tylacoides are arranged peripherycally, with phycobilisomes attached (T-FBS). In the central region is the nucleoid (N). The cross-wall (PT) forming was simetrically sectioned. The following structures were identified in the cytoplasm: parental cell wall (PC), polihydroxibutirate granules (PHB), carboxisomes (C) and vesicle-like – vl structures. Bar=1μm.

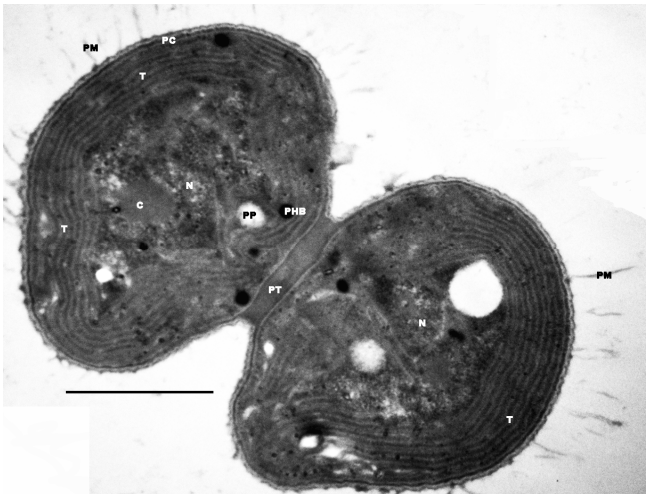

Fig. 13. . Longitudinal section through a *Coelomoron pusillum* AICB 1012 cell in division. Same structures as in fig. 7 can be observed. The differences from the previous picture are the numerous mucilaginous extensions (PM) dispersed more or less evenly on the cell surface, the parallelism between the tylacoides and the cell wall (T) and the structure of the cell wall with the peptidoglycan layer very thick at the cross wall (PT) which separates the daughter cells. Bar=1μm.

In our analyses we could observe inside the colonies the presence of some peduncular mucilaginous formations (fig.14, 15) which keep the cells together in the colonies, and also many unstructured, probably mucilaginous extensions, which are dispersed more or less evenly on the cell surface (fig.16 b). A closer examination of the thin sections allowed us to show also the presence of pores which pass through the cell wall (fig.17). The presence of these pores and their distribution, uniform on the cell surface (fig. 17 a) or localized close to the cross wall which separates the daughter cells (fig.17 b), gives us an explanation on how the peduncular mucilaginous formations and the mucilaginous extensions were formed.

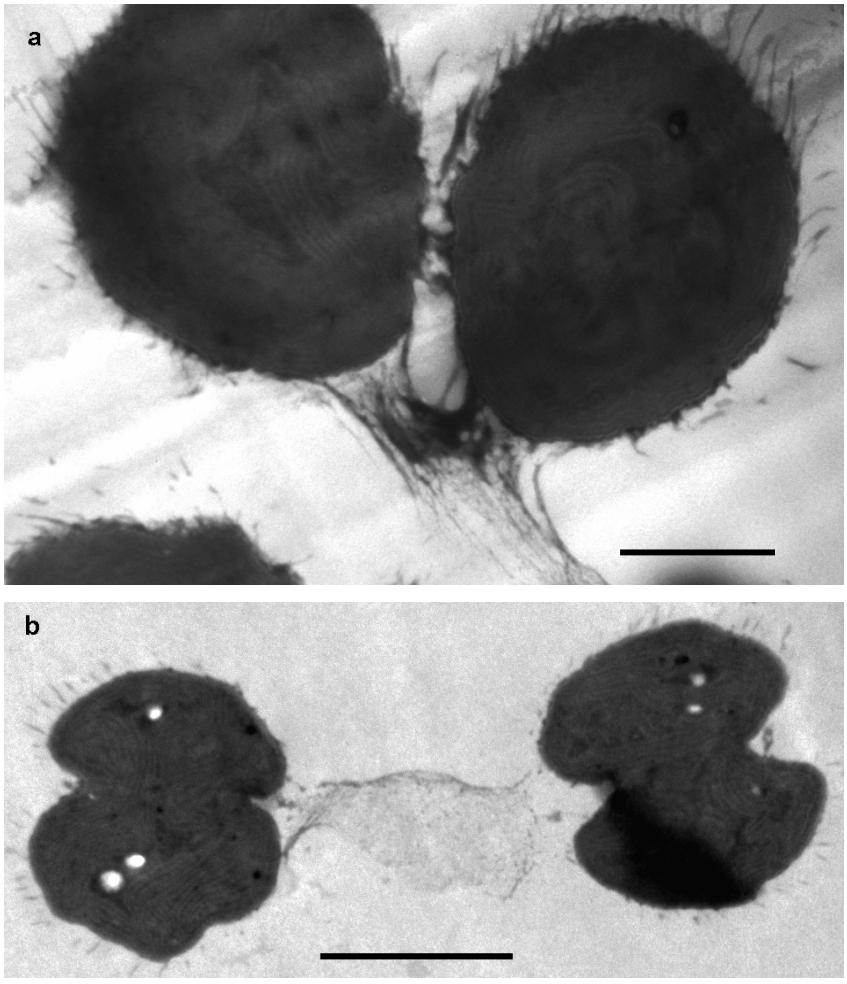

Fig. 14 (a-b). *C. pusillum* AICB 1012 in TEM. Both images show the mucilaginous stalks which hold the cells together and arrange them in colonies. The mucilage is unstructured (a, b) and apparently dicotomic (a), each cell being held by a peduncular ramification (a). The plane of the binary fision of the cell is paralell with the peduncular plane (b). Bars: a=1μm, b=2 μm.

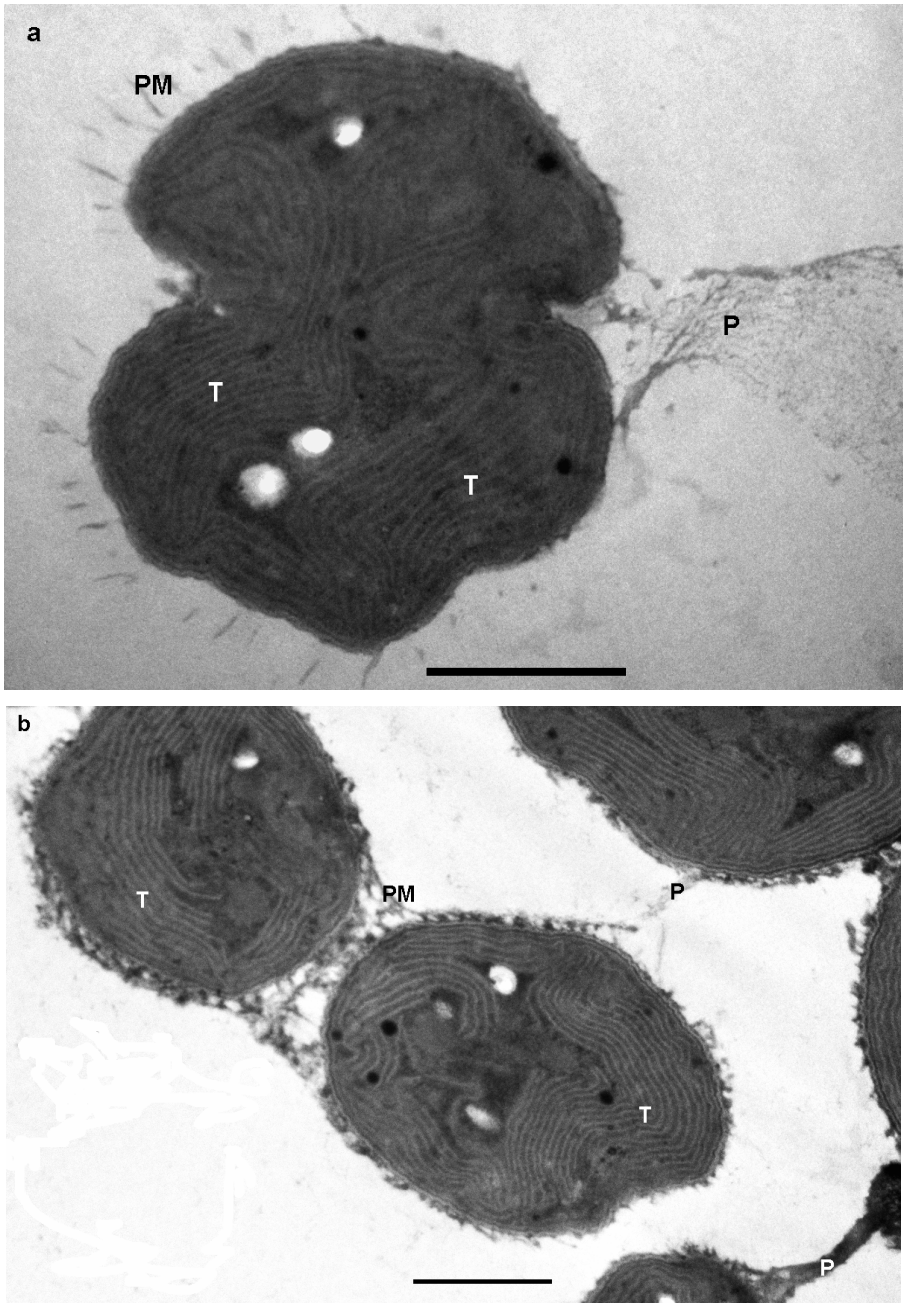

Fig. 15 (a-b). *C. pusillum* AICB 1012 in TEM. Fig. 11a shows a cell in division, with muculaginous filaments aroud the cell and with a mucilaginous stalk anchored close to the area where the cross wall is formed. Fig. 11b shows the bridge formed by the mucilaginous filaments between the daughter cells afther binary fision. Bars=1 μm.

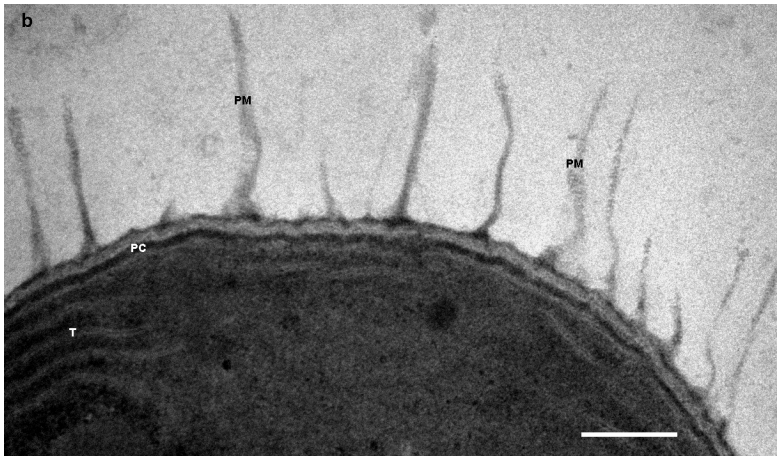

Fig. 16. *C. pusillum* AICB 1012 in TEM. Detail of the mucilaginous extensions (PM). Bars: a=500nm, b=200nm

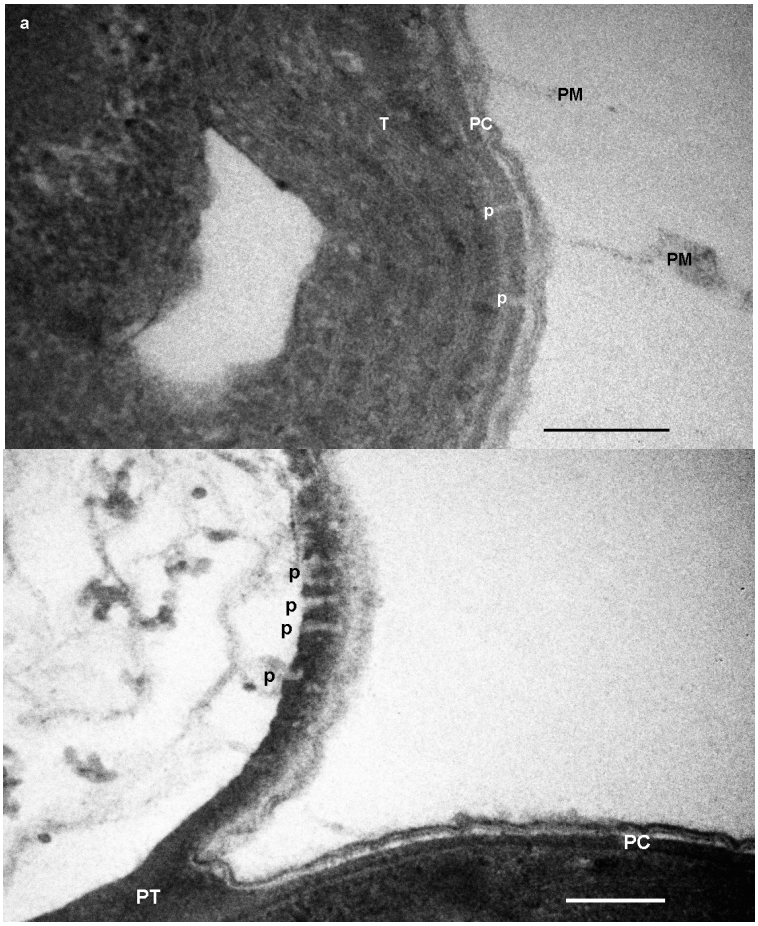

Fig. 17 (a-b). *C. pusillum* AICB 1012 in TEM. Fig.12a – The cyanobacterial cell wall shows pores (p) which, apparently, seem to be involved in the externalisation/secretion of mucilage. Pores are clearly visible in the peptidoglycan layer. Pore frequency seems to be higher close to the area where the cross wall is formed (fig.12b). Bars = 200 nm.

The trees obtained through different methods were wery similar, which suggests the fact that the topology of the tree (fig. 18) shows the real phylogenetic relations between *Coelomoron pusillum* AICB1012 and the other cyanobacterial strains. The clustering of AICB 1012 with strains from the genera *Woronichinia* and *Snowella* is in agreement with the classification according to the Botanical Code made by Komarek & Anagnostidis (1999), in which all these strains belong to the subfamily Gomphosphaerioideae (fig.18 group II). According to this data we can state that the classification based on motphological traits of *Coelomoron pusillum* AICB1012 is strongly supported by its molecular phylogeny.

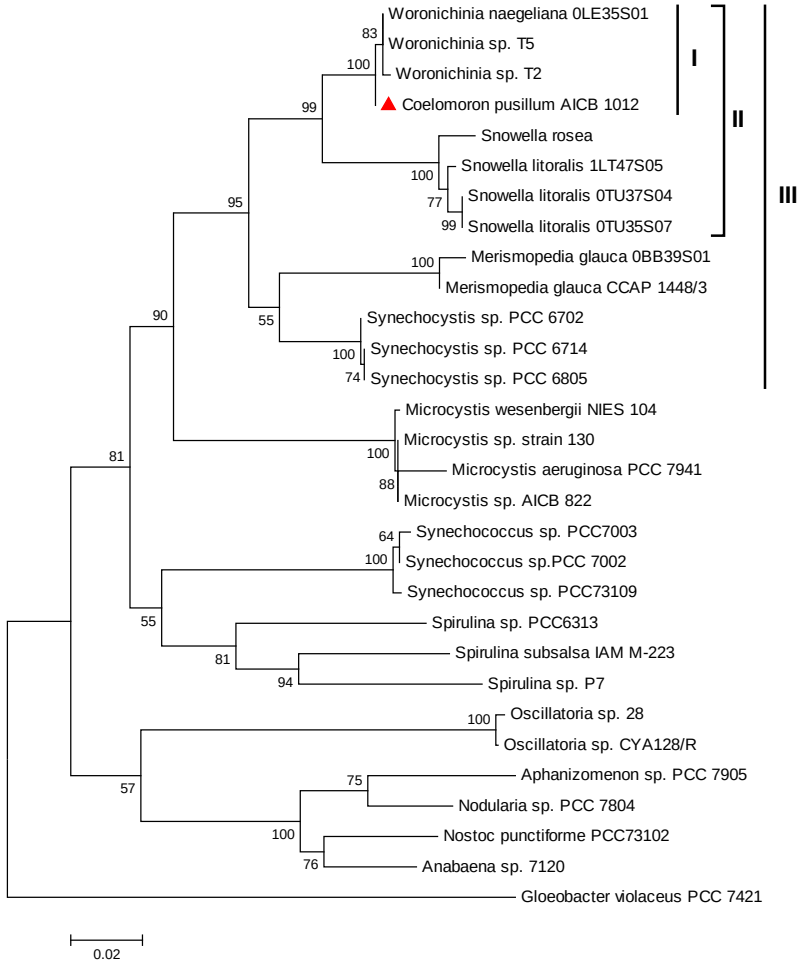

Fig. 18 Maximum Likelihood (ML) tree based on 16S sequences which shows the phylogenetic relations between *Coelomoron pusillum* AICB 1012 and strains from Chroococcales, Nostocales and Oscillaoriales. Bootstrap values for 1000 replicates are presented at the nodes. The outgroup was the 16S sequence from *Gloeobacter violaceus* PCC 7421.

VIII. TRANSCRIPTIONAL REGULATION OF THE BIDIRECTIONAL HYDROGENASE BY OXYGEN AND LIGHT IN CYANOBACTERIA FROM THE ORDER NOSTOCALES

➢ Effect of light on *hox* gene expression under low oxygen tension

The *hox* genes showed the highest relative induction at the first time point of the anaerobic incubation, which was taken 30 minutes after the dissolved oxygen content of the media dropped below 1 µM: *hox*E 70±25 fold in *Anabaena variabilis* ATCC 29413 and *hox*Y and *hox*H showed 400±100 and 200±80 fold induction in *Anabaena* sp. PCC7120 (fig.19 A and C).

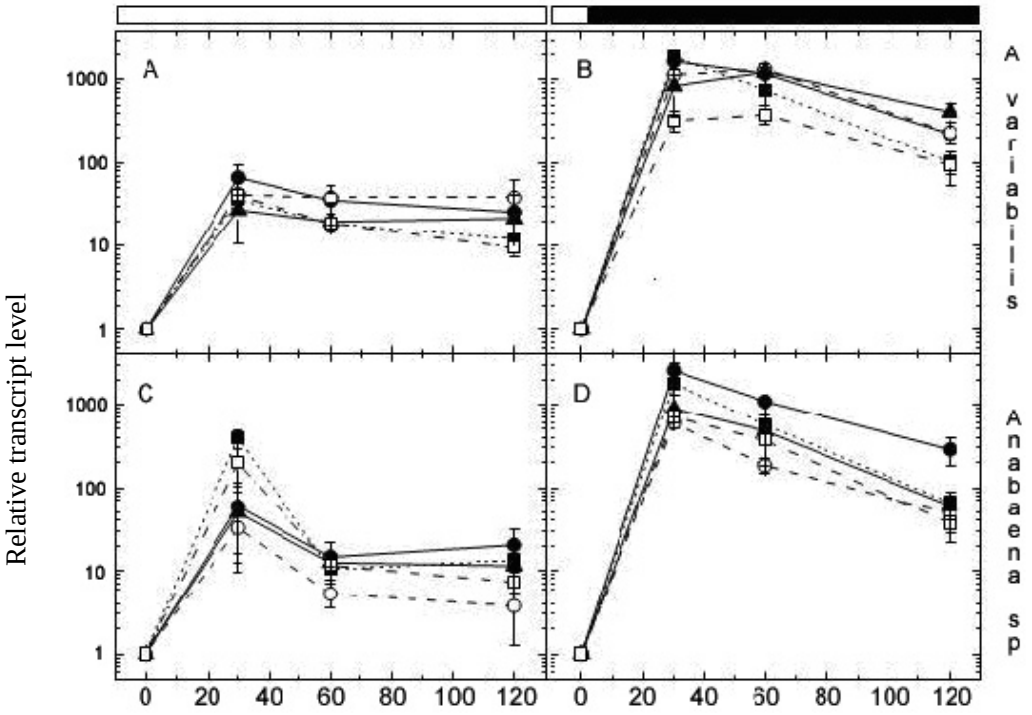

Fig. 19 Effect of light on the induction of *hox* genes under oxygen deprivation. The oxygen content of the medium of the *A. variabilis* (A and B) and *Anabaena* (C and D) cells were kept below 1 LM during 2 hours of illumination (A and C) or darkness (B and D). The relative expression levels of the *hox*E (thin line, solid circle), *hox*F (dashed line, open circle), *hox*U (thick line, solid triangle), *hox*Y (short dashed line, solid square), and *hox*H (dash dotted line, open square) genes are shown after normalization to their respective initial values represented by 1. Error bars correspond to standard deviation derived from three independent experiments. The white and black bars indicate the light and dark periods.

Transferring the oxygen deprived cells into darkness for 30 minutes caused three orders of magnitude higher transcript accumulation compared to the growth conditions (fig.19 B și D).

➢ Reversible effect of light on the transcriptional regulation of the bidirectional hydrogenase

In *Anabaena variabilis* during the 2 hours of dark incubation the *hox*E, *hox*F and *hox*U genes encoding the diaphorase moiety remained at significantly high relative transcript levels, while the *hox*Y and *hox*H genes encoding the catalytic subunit of the hydrogenase moiety showed a considerably lower level of induction (fig.20 A).

In the case of *Anabaena* sp. PCC 7120 the *hox*F, *hox*U, *hox*Y and *hox*H genes were moderately induced compared to *hox*E. The reversible effect of darkness was more apparent in the case of *hox*E and *hox*U compared to the other *hox* genes of *Anabaena* sp. PCC 7120 (fig.20 B).

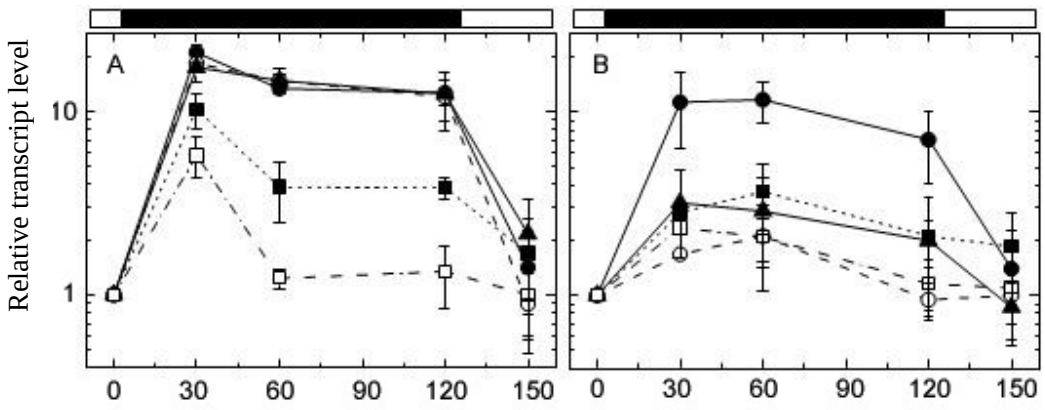

Fig. 20 Dark induction of *hox* genes in aerobic cultures. During the dark incubation A. variabilis (A) and Anabaena (B) cells were kept under atmospheric oxygen supplemented with 2 % CO2 and transferred from light to dark for 2 hours followed by 30 minutes recovery period in light. The relative expression levels of the *hox*E (thin line, solid circle), *hox*F (dashed line, open circle), *hox*U (thick line, solid triangle), *hox*Y (short dashed line, solid square), and *hox*H (dash dotted line, open square) genes are shown after normalization to their respective initial values represented by 1. Error bars correspond to standard deviation derived from three independent experiments. The white and black bars indicate the light and dark periods.

FINAL CONCLUSIONS

- Trees generated based on the 16S-ITS and cpcBA-IGS sequences showed a very similar topology, which supports the validity of the proposed phylogenetic clusters and confirms the high phylogenetic resolution of these markers to strain level and their usefulness in phylogenetic studies for cyanobacteria.
- The bidirectional hydrogenase was detected in 60 (85.7%) of the 70 AICB strains used in this study. The lack of amplification in the 10 (14.3%) AICB strains suggest the absence of the *hox*H gene which is in agreement with the previous studies on the distribution of *hox*H in cyanobacteria.
- The topology of the phylogenetic trees obtained based on the *hox*H sequences was wery similar to that of the 16S-ITS and cpcBA-IGS trees, which shows that the phylogeny of the *hox*H gene is very similar with the phylogeny of the organisms where it is present.
- Using molecular phylogeny we show for the first time the presence of the horizontal gene transfer (HGT) in cyanobacterial bidirectional hydrogenases. This process was observed in 4 cases between the AICB strains: *Anabaena oscillatorioides* AICB 563 and *Nostoc*; *Merismopedia* sp. AICB 1014, 1015 and *Microcystis* and between *Spirulina platensis* FACHB 440 and *Oscillatoria* and also between strains belonging to different groups: *Coelomoron pusillum* AICB 1012 (Chroococcales) and *Oscillatoria amphibia* AICB 641 (Oscillatoriales).
- An advanced ultrastructural and phylogenetic study was undertaken for the first time for the genus *Coelomoron* (strain AICB 1012), which showed the presence of cyanobacterial pili, mucilaginous filaments and mucilaginous stalks.
- The formation of the mucialginous stalks and their ramification in AICB 1012 was explained also for the first time, by showing the preferential and simetric arrangement of pores in the cross wall which separates the daughter cells during binary fision.
- The dark-aerobic induction of the *hox* genes in *Anabaena* sp. PCC7120 and *Anabaena variabilis* ATCC29413 strongly indicates that the bidirectional hydrogenase enzyme can take part in aerobic respiration in these cyanobacteria.

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Key words

Cyanobacteria; AICB Culture Collection; bidirectional hydrogenase; ARNr 16S-ITS; phycocyanin locus; molecular phylogeny; real-time PCR.

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