Molecular taxonomic evaluation of *Anabaena* and *Nostoc* strains from the Mosonmagyaróvár Algal Culture Collection

N. Makra a, G. Gell a, A. Juhász a, V. Soós a, T. Kiss a, Z. Molnár b, V. Ördög b, d, L. Vörös c, E. Balázs a, d, e

a Department of Applied Genomics, Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár H-2462, Brunszvik u. 2., Hungary
b Department of Plant Sciences, Faculty of Agricultural and Food Sciences, Széchenyi István University, Mosonmagyaróvár H-9200, Kolbasz u. 8., Hungary
c Department of Hydrobiology, Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Tihany H-8237, Klebesberg Kuno u. 3., Hungary
d Research Centre for Plant Growth and Development, University of KwaZulu-Natal, Pietermaritzburg, South Africa
e E-mail address: balazs ervin@agrar.mta.hu (E. Balázs).

**Abstract**

The taxonomy of genera *Anabaena* and *Nostoc* is very controversial. They are typically paraphyletic within phylogenetic trees and show similar morphological characters. The present study aimed to determine the taxonomic relationships among *Anabaena* and *Nostoc* strains of the Mosonmagyaróvár Algal Culture Collection (MACC) using 16S rRNA and rbcLX gene sequences. We concluded on the basis of the number of unsuccessful amplifications that more of the examined MACC *Nostoc* cultures are axenic than the *Anabaena* cultures. In agreement with previous studies we noticed that the applied phylogenetic algorithms gave congruent results in phylogenetic analyses. However, the genus *Nostoc* clearly was found not monophyletic in the present study and this finding differed from many of the previous studies. Molecular results contradicted the previous morphology-based classification of some MACC cyanobacteria strains, therefore polyphasic taxonomic approaches are required for the reliable identification of cyanobacterial species. Some strains seemed to be identical based on the alignment of 16S rRNA or rbcLX sequences.

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1. Introduction

Cyanobacteria species represent an ancient lineage of Gram-negative photosynthetic prokaryotes. They are monophyletic but morphologically diverse. *Nostoc* and *Anabaena* cyanobacterial genera have been traditionally differentiated on the basis of morphological and life cycle characteristics. Identification of cyanobacteria strains in culture by a morphological based system usually leads to ambiguities. Loss of phenotypic attributes during serial inoculations has been observed in numerous microalgal cultures (Day et al., 2005; Lehtimäki et al., 2000; Gugger et al., 2002). According to Komárek and Anagnostidis (1989), the features of more than 50% of strains in collections do not correspond to the characteristics of the taxa to which they are assigned. Additionally, relatively few species grow under axenic culture conditions, which makes the identification even more difficult (Casamatta et al., 2005). To address the above challenges, it was essential to introduce a multidimensional classification system. Polyphasic taxonomy utilises all available data: (i) phenotypic information, such as chemotaxonomic features, morphology, staining behaviour, and culture characteristics, and (ii) genetic properties, such as G + C content, DDH value, and highly-conserved gene sequences. Numerous studies have demonstrated that genetic relationships sometimes conflict with the morphological classification (Lyra et al., 2001; Itehan et al., 2002). The data from the molecular taxonomic separation of *Anabaena* and *Nostoc* genus are also incongruent with the morphological analyses. Based on 16S rRNA gene sequence analysis, Svenning et al. (2005) divided the examined microalgal strains in four clades. Whereas clades II and III contained only *Nostoc* strains, clades I and IV included both *Nostoc* and other (e.g. *Anabaena, Aphanizomenon, Nodularia*) strains, thus suggesting paraphyletic origin. Within the genus *Anabaena* it is difficult to separate species and strains as they often disperse among other species, or even different genera, with a high similarity (Gugger et al., 2002; Lyra et al., 2001; Rajaniemi et al., 2005; Willame et al., 2006). Based on molecular markers, genus *Nostoc* forms a monophyletic group with high genetic diversity, and the different strains may represent individual species (Rajaniemi et al., 2005; Rasmussen and Svenning, 2001; Wilmutte and Herdman, 2001). However, Rajaniemi et al. (2005) also noted that in certain situations the opposite may be true. In these cases, the high similarities of the 16S rRNA sequence suggested that previously distinct morphospecies belong to a single species.

Although the application of 16S rRNA sequence for taxonomy is wide-spread, the low variability of this region does not allow discrimination among species or strains (Bosshard et al., 2006; Mignard and...
16S rRNA gene sequence was done by using the cyanobacteria specific primer. The performed in a Veriti Thermal Cycler (Applied Biosystems).

Consequently, application of multigene phylogeny is recommended. This approach has been used to study the evolution of various groups of algae (Hoef-Emden et al., 2002). In addition to the 16S rRNA, the Rubisco large subunit gene sequence (rbcLX) has also been used as a phylogenetic marker in the taxonomy of these microorganisms.

This study focused on the Nostoc and Anabaena strains of the Mosonmagyaróvár Algal Culture Collection (MACC). The MACC collection has 580 strains isolated from soil samples and altogether 270 cyanobacteria and 500 eukaryotic microalgae strains. The strains serve as subjects to investigations related to plant hormone production; efficacy against plant pathogenic fungi; volatile organic compounds and lipid production used for biofuel production (Ördög et al., 2013). MACC strains were previously classified based on the morphological attributes by the staff of the Centre for Ecological Research Balaton Limnological Institute (Hungarian Academy of Sciences). In this study, we characterised them by molecular taxonomic methods using both 16S rRNA and rbcLX gene sequences.

2. Materials and methods

2.1. Cultivation

Samples of 40 Nostoc and 40 Anabaena strains, obtained from the MACC were examined in this study. Stock cultures of the selected cyanobacterial strains were inoculated into 500 ml Erlenmeyer flasks containing 250 ml Zehnder-8 nutrient medium and incubated for a week in a culture apparatus described earlier by Ördög (1982). Afterwards, the culture suspensions were re-inoculated into new flasks to get an initial dry matter (DM) content of 10 mg/l. All culture suspensions were aerated with 20 l/h air, which was supplemented with 1.5% CO₂ during the light period. The culture suspensions were re-inoculated into new flasks every week in a culture apparatus described earlier by Ördög (1982). Afterward, the culture suspensions were re-inoculated into new flasks to get an initial dry matter (DM) content of 10 mg/l. All culture suspensions were aerated with 20 l/h air, which was supplemented with 1.5% CO₂ during the light period. The culture suspensions were re-inoculated into new flasks every week in a culture apparatus described earlier by Ördög (1982). Afterward, the culture suspensions were re-inoculated into new flasks to get an initial dry matter (DM) content of 10 mg/l. All culture suspensions were aerated with 20 l/h air, which was supplemented with 1.5% CO₂ during the light period.

2.2. DNA extraction

Two microlitre cyanobacteria suspension was added to 100 μl 10% Chelex 100 solution from BioRad. The samples were incubated at 100 °C for 20 min followed by centrifugation at 12,000g for 1 min. The supernatant, which contained the DNA, was aliquoted and kept at −20 °C.

2.3. PCR amplification

Extracted DNA was amplified by PCR, separated by 1.5% (wt/vol) agarose gel electrophoresis, and visualised using ethidium bromide staining. 16S rRNA amplification was carried out in two steps resulting in two overlapping sequences. The first sequence was amplified by the 27F (Lane, 1991) universal and CYA718R (Nübel et al., 1997) cyanobacteria specific primers. Amplification of the second part of the 16S rRNA gene sequence was done by using the cyanobacteria specific CYA359F (Nübel et al., 1997) and universal 1492R (Lane, 1991) primer pair. The rbcLX gene sequences were amplified using the primer sequences CX-f and CW-r by Rudi et al. (1998). PCR amplifications were performed in a Veriti Thermal Cycler (Applied Biosystems).

The PCR cycling conditions were as follows: 98 °C for 5 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; 72 °C for 7 min, and a final cooling to 4 °C. Each PCR was carried out in 40 μl volume containing 0.5 μM of each primer, 0.25 mM dNTPs, 1.875 mM MgCl₂, 4 μl 10x Taq Buffer with KCl (Thermo Fisher Scientific Inc.) and 5 U of the mixture of Taq and Pfu polymerases (40:1) (Thermo Fisher Scientific Inc.). PCR products were excised by UV irradiation in a transilluminator, and well-separated bands were carefully excised from the gels using a sterile surgical scalpel. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) and were sequenced by an external service (Macrogen Europe). Two biological (ie. from the harvesting of algal cells) and two technical replicates were used to determine the exact gene sequences.

2.4. Bioinformatic analysis of the amplified sequence

The obtained 16S rRNA and rbcLX sequences were deposited in GenBank, their accession numbers are listed in Table 1. Sequence similarity searches were done on the NCBI databases with a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment, editing and phylogenetic tree constructions were carried out using the CLC Genomic Workbench software package, version 7.8.1 (CLC Bio, Aarhus, Denmark). Trees were created with CLC Genomic Workbench using the maximum-likelihood, UPGMA (Michener and Sokal, 1957) and Neighbour-joining (Saitou and Nei, 1987) algorithms. The significance was assessed using 500 bootstrap replicates. Average diversities and genetic distances (p-distance) were calculated using the Kimura two-parameter method (Kimura, 1980).

3. Results

The Neighbour-joining, UPGMA and maximum likelihood algorithms were used in the phylogenetic reconstruction. Since the three methods gave congruent results for the major branching patterns of the trees, only the UPGMA tree (cladogram) with Kimura 80 distance measure are presented in the figures.

The sequencing of some samples failed: they resulted in too short reads or noisy peaks after multiple repeats, or the resulting sequences unequivocally proved to be of non-cyanobacterial origin. These cultures were excluded from the phylogenetic analyses.

3.1. Anabaena strains

Twelve different 16S rRNA sequences and 28 rbcLX sequences were identified.

3.1.1. Alignment

We found over 99% sequence similarity between strains MACC-177 and -146 (marked as group II/B in Fig. 1), strains MACC-103 and -206 (marked as group II/C in Fig. 1) and strains MACC-187 and -189 (marked as group II/D in Fig. 1) based on the 16S rRNA sequencing results. The rbcLX sequences were identical in strains MACC-113, -127, -128, -110, -255, -259, -798, -201, -797 and -57 (marked as group I in Fig. 2), strains MACC-103 and -206 (marked as group II/C in Fig. 2), strains MACC-269, -177, -146, -183, -174 and -270 (marked as group II/B in Fig. 2) and strains MACC-247, -229, -133, -238, -134, -124 and -304 (marked as group II/A in Fig. 2).

3.1.2. Nucleotide BLAST

Morphological taxons, provenance and habitat information and the BLAST results of the amplified 16S rRNA sequences are shown in Fig. 1, next to the branches. Four strains belonged to the Trichormus species, five strains represented Nostoc species, and three strains resulted in uncertain classification based on the 16S rRNA sequences.
Table 1
GenBank accession numbers of 16S rRNA and rbcLX gene sequences used in the phylogenetic analyses.

| Strain   | Species name       | GenBank accession number of 16S rRNA | GenBank accession number of rbcLX |
|----------|--------------------|-------------------------------------|----------------------------------|
| MACC-77  | Nostoc sp.         | MH702220                           | MH713672                         |
| MACC-112 | Nostoc commune     | MH702221                           | MH713673                         |
| MACC-125 | Nostoc sp.         | MH702222                           | MH713674                         |
| MACC-132 | Nostoc sphaerium   | MH702223                           | MH713675                         |
| MACC-139 | Nostoc sp.         | MH702224                           | MH713676                         |
| MACC-148 | Nostoc ellipsosporum | MH702225                      | MH713677                         |
| MACC-150 | Nostoc sp.         | MH702226                           | MH713678                         |
| MACC-154 | Nostoc commune     | MH702227                           | MH713679                         |
| MACC-172 | Nostoc linckya     | –                                  | MH713680                         |
| MACC-173 | Nostoc sp.         | –                                  | –                                |
| MACC-175 | Nostoc muscorum    | –                                  | MH713681                         |
| MACC-178 | Nostoc commune     | MH702228                           | MH713682                         |
| MACC-181 | Nostoc paludosum   | –                                  | –                                |
| MACC-185 | Nostoc punctiforme | –                                  | MH713683                         |
| MACC-193 | Nostoc punctiforme | –                                  | MH713684                         |
| MACC-198 | Nostoc punctiforme | MH702229                           | MH713685                         |
| MACC-208 | Nostoc sp.         | MH702230                           | MH713686                         |
| MACC-210 | Nostoc punctiforme | MH702231                           | MH713687                         |
| MACC-218 | Nostoc sp.         | MH702232                           | MH713688                         |
| MACC-231 | Nostoc sp.         | MH702233                           | MH713689                         |
| MACC-286 | Nostoc punctiforme | MH702234                           | –                                |
| MACC-287 | Nostoc punctiforme | MH702235                           | –                                |
| MACC-291 | Nostoc sp.         | –                                  | MH713690                         |
| MACC-294 | Nostoc sp.         | MH702236                           | MH713691                         |
| MACC-420 | Nostoc muscorum    | –                                  | MH713692                         |
| MACC-427 | Nostoc sp.         | –                                  | –                                |
| MACC-461 | Nostoc sp.         | MH702237                           | MH713693                         |
| MACC-462 | Nostoc sp.         | MH702238                           | –                                |
| MACC-484 | Nostoc sp.         | –                                  | MH713694                         |
| MACC-498 | Nostoc sp.         | MH702239                           | MH713695                         |
| MACC-513 | Nostoc sp.         | MH702240                           | MH713696                         |
| MACC-605 | Nostoc sp.         | –                                  | –                                |
| MACC-612 | Nostoc enophymum   | MH702241                           | –                                |
| MACC-627 | Nostoc sp.         | MH702242                           | MH713696                         |
| MACC-633 | Nostoc sp.         | MH702243                           | –                                |
| MACC-634 | Nostoc sp.         | –                                  | MH713697                         |
| MACC-661 | Nostoc sp.         | MH702244                           | MH713698                         |
| MACC-668 | Nostoc sp.         | –                                  | MH713699                         |
| MACC-683 | Nostoc sp.         | MH702245                           | MH713700                         |
| MACC-707 | Nostoc sp.         | –                                  | MH713701                         |

Two strains were Scytonema crispum, eight strains belong to Trichomos variabilis, one strain was Nostoc calcicola, four strains fell under the Nostoc genus, seven strains represented Anabaena variabilis and six strains resulted in uncertain classification based on the rbcLX sequences (Fig. 2).

3.1.3. Phylogenetic trees

The main topology of the trees was similar for the 16S rRNA and rbcLX genes. Three clusters and four subclusters within cluster II were formed in the 16S rRNA and the rbcLX tree. Trichomos variabilis strains were grouped in cluster I. Nostoc species were represented by cluster III and subcluster II/C and II/D. Some strains were grouped into subcluster II/B. According to the results of the rbcLX sequence analysis, we grouped two Scytonema crispum species in cluster I.
Fig. 1. Phylogenetic tree of the Anabaena isolates based on the nucleotide sequence of the 16S rRNA gene. All data obtained from the Genbank are indicated by accession numbers. Bootstrap values ≥70 are indicated at the branch nodes (70–79 values are grey, 80–89 values are blue and 90–100 values are red). Morphological classification is indicated on the right side of the tree with grey font colour. Most relevant BLAST results are on the right with black letters (A. = Anabaena, D. = Dolichospermum, N. = Nostoc and T. = Trichormus). Habitat information is labelled with circles and stars.

Fig. 2. Phylogenetic tree of the Anabaena isolates based on the nucleotide sequence of the rbcLX gene. All data obtained from the Genbank are indicated by accession numbers. Bootstrap values ≥70 are indicated at the branch nodes (70–79 values are labelled in grey, 80–89 values in blue and 90–100 values are labelled in red). Morphological classification is indicated on the right side of the tree with grey font colour. Most relevant BLAST results are on the right with black letters (A. = Anabaena, N. = Nostoc, S. = Syctonema and T. = Trichormus). Habitat information is marked with circles and stars.

Fig. 3. Phylogenetic tree of the Nostoc isolates based on the nucleotide sequence of the 16S rRNA gene. All data obtained from the Genbank are indicated by accession numbers. Bootstrap values ≥70 are indicated at the branch nodes (70–79 values are labelled in grey, 80–89 values are in blue and 90–100 values are in red). Morphological classification is indicated on the right side of the tree with grey font colour. Most relevant BLAST results are on the right with black letters (N. = Nostoc and R. = Roholtella). Habitat information is marked with circles and stars.
3.2. Nostoc strains

Eighteen strains gave the expected 16S rRNA sequences, and 25 resulted in correct rbcLX sequences.

3.2.1. Alignment

Only MACC-231 and MACC-208 strains seemed to be identical based on the alignment of 16S rRNA sequences (Fig. 3). rbcLX alignment resulted in more conformity. Sequences were identical within the group I/A and I/L in the 16S rRNA tree and were absent in 16S rRNA tree. Trichormus genus was represented by the sub-cluster I/L. Nostoc species were classified into the sub-clusters I/A-I/L in the 16S rRNA tree and the sub-clusters I/A-I/L in the rbcLX tree. Only one strain from Calothrix genus appeared in the sub-cluster I/L in the 16S rRNA tree and one strain from Trichormus azollae species appeared in the sub-cluster I/B in the rbcLX tree.

3.2.2. Nucleotide BLAST

Sixteen strains fell under the Nostoc genus, one of them was Nostoc insulare, one was Nostoc punctiforme. One strain belonged to Rohohlliella fluviatilis, another one to Chalothrix species for the BLAST of 16S rRNA sequence (Fig. 4).

rbcLX sequences of 20 strains represented Nostoc species. Five of them were identified as Nostoc commune, two as N. flagelliforme, two as N. linckia. We identified one N. calcicola, one N. punctiforme, one N. carmen and one N. piscinale strain, too. Three strains belong to the Trichormus variabilis and one the Trichormus azollae taxa. Besides, there was a Rohohlliella mojavensis strain (Fig. 4).

3.2.3. Phylogenetic trees

The overall topology of the trees of the Nostoc strains was slightly different for the 16S rRNA and the rbcLX genes. One cluster and nine sub-clusters within cluster I were represented in the 16S rRNA tree. All of sub-clusters present in the 16S rRNA tree could also be recognised in the rbcLX tree. Four sub-clusters (I/J, I/K, I/L, I/M) were only identified in the rbcLX tree. The isolates MACC-154, MACC-150, MACC-661 and MACC-125 were classified into different genera based on the two gene sequences. The sub-cluster I/F of the 16S rRNA tree was separated into two polyphyletic groups based on the rbcLX gene sequences (I/F and I/L). The Trichormus variabilis strains were contained by the clusters I/H and I/L in the rbcLX analysis and were absent in 16S rRNA tree. Rohohlliella genus was represented by the sub-cluster I/L. Nostoc species were classified into the sub-clusters I/A-I/H in the 16S rRNA tree and the sub-clusters I/A-I/L in the rbcLX tree. Only one strain from Calothrix genus appeared in the sub-cluster I/L in the 16S rRNA tree and one strain from Trichormus azollae species appeared in the sub-cluster I/B in the rbcLX tree.

4. Discussion

4.1. Full-length 16S rRNA amplification

In the present study mixtures of universal and cyanobacteria specific primers were used to investigate a nearly full-length 16S rRNA gene sequence, avoiding the uncertainty and inaccuracy caused by the short sequences. In case of some non-axenic cultures, the universal components of primer pairs resulted in amplicon mixtures, thereby interfering with the further sequence analysis.

4.2. Comparison of BLAST results with morphological identification

Prior to this examination, there were no molecular taxonomic studies carried out using MACC strains. The classification of strains was done exclusively by morphological characteristics although numerous studies have demonstrated that morphological determination in itself is not always sufficient (Giovannoni et al., 1988; Wilmotte et al., 1994). Furthermore, morphological determinations of strains were not up to date, and in some cases there has been a change in of official names since AlgaeBase (Guiry and Guiry, 2018). The most significant difference was the re-classification of Anabaena variabilis and Anabaena azollae taxa into Trichormus variabilis and Trichormus azollae.

4.2.1. Anabaena strains

The 16S rRNA-based taxonomical analysis of Anabaena strains provided a clear species-level match with the morphological-based assay in case of two strains (MACC-57 and MACC-251). Of these, only the result of the MACC-251 was corroborated by the result obtained from the rbcLX sequence. Further four strains exhibited strong similarity with...
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Trichormus variabilis sequences. The 16S rRNA sequences of five strains were more closely related to Nostoc species. MACC-247 strain was likely to be A. variabilis according to the rbcLX results.

For the rbcLX sequences, three strains matches with the morphological classification at the species level (MACC-127, -133, -251). However, we could not confirm this match using the 16S rRNA data of MACC-127 and -133 strains. The BLAST results of further 13 strains showed a close relationship with the Trichormus (or Anabaena variabilis) taxon, at least at the genus level, confirming the prior morphological classifications. However, five strains showed a closer relationship with Nostoc taxa. Two groups showed to Scytonema crispum based on the rbcLX sequences.

The information about the natural habitat of the strains (Figs. 1 and 2) was consistent with the general environment information available in AlgaeBase at species level to the Anabaena strains.

The strains with uncertain classification and Anabaena variabilis strains (cluster II/A and II/B) sharply separated from Trichormus variabilis strains (cluster I) in both trees. This fact and the close clustering with Nostoc strains suggest that they belong to a Nostoc or an authentic (not Trichormus variabilis) Anabaena species.

4.2.2. Nostoc strains

BLAST analysis of the morphological Nostoc strains resulted mostly in Nostoc hits. However, the results of the two gene sequences were controversial in some cases. Also, the habitat information of the strains (Figs. 1 and 2) was not always consistent with the general environment information available in AlgaeBase at a species level.

4.3. Strains with identical genotype

4.3.1. Anabaena strains

Some strains were found with identical genotype for the examined gene sequences. MACC-103 and -206 Anabaena strains were identical to both gene sequences. Morphological identification and information about their origin supported this finding. MACC-146 and -177 had identical sequences for the examined genes and they could be the same according to morphology and origin information too. The present BLAST analysis results suggest that these strains belong to the same species, which is concordant with the previous morphological results. The rest of the conformity was not verified by both genes and just partially supported by morphological and origin data. Strains with identical genotype may belong to the same species. This is not confuted by the morphological classification of the strains of II/D clade and members of II/B. At the same time, morphological identification contradicts with the genotype results of yellow strains in II/A clade.

4.3.2. Nostoc strains

MACC-208 and -231 Nostoc strains were identical according to both gene sequences. The morphology confirmed this result only at the genus level, but they were collected from similar habitats. There were some strains identical to the previous ones, but morphology and 16S rRNA sequences did not support this. Only MACC-210 showed strong similarity (99.93%) with their 16S rRNA sequences. The identity of rbcLX sequences of MACC-150 and -154 was not confirmed by 16S rRNA sequences, morphology or origin. Although they originated from similar habitats, neither morphology nor the 16S rRNA sequences (which were missing) confirmed the concordance of MACC-172 and MACC-193 strains. The situation was comparable to MACC-178, -218 and -686 strains, except that in this case the origin only partially met. The morphological differences used in the original classification contradict many of the molecular difference detected in the present study.

4.4. Tree topologies

The tree building algorithms we used gave congruent results for the major branching patterns as can be seen in many other studies (Rajaniemi et al., 2005; Willame et al., 2006). The genera Anabaena and Nostoc seemed to be paraphyletic in the obtained topologies. This confirmed the observation of many other authors (Gugger et al., 2002; Itteman et al., 2002; Lyra et al., 2001; Rajaniemi et al., 2005; Svenning et al., 2005; Tamas et al., 2000; Willame et al., 2006). Nostoc strains were intermixed within the main clusters and not monophyletic as previously described by many authors (Rajaniemi et al., 2005; Rasmussen and Svenning, 2001; Wilmotte and Herdman, 2001).

5. Conclusion

We managed to complement the existing morphology-based taxonomical system of MACC with a new, molecular taxonomy results. In some cases, we got contradictory results. Regarding the morphological classification, it should be updated by also considering the nomenclature changes. In particular for Nostoc strains, 16S rRNA-based BLAST results show fewer contradictions with the habitat information, so the 16S rRNA sequences seem to be more reliable.

It is challenging to produce perfectly axenic cyanobacteria cultures. In the absence of certain symbionts, the cyanobacteria cells became unviable which led to sequencing difficulties. We could conclude from the inaccuracy of the sequencing that more of the examined MACC Nostoc cultures are axenic than the Anabaena (Trichormus) cultures. We noticed that the three phylogenetic algorithms (Neighbour-joining, UPGMA, maximum likelihood) resulted in congruent outcomes.

The alignment of gene sequences revealed that there are some strains which seem to be identical. These strains could be suitable for similar biotechnological applications. Examination of similarities between the biological activities of these strains should also give interesting results.

Acknowledgements

We wish to thank all participants assisted in the implementation of this study or helped us with recommendations. The authors wish to thank Zoltán Divík, PhD for the critical editing of the manuscript. This work was funded by the TÁMOP-4.2.2.A-11/1/KONV-2012-0003 project.

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