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GENETIC VARIATION OF *ARTHROSPIRA* (*SPIRULINA*) STRAINS ASSESSED BY PCR TECHNIQUE BASED ON GENOMIC REPETITIVE SEQUENCES

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Abstract: Genetic variation of *Arthrospira* (*Spirulina*) *fusiformis* strains isolated from an alkaline pond near Cluj-Napoca city was assessed by PCR fingerprinting based on primers designed for two families of repetitive sequences: STRR (short tandemly repeated repetitive) and HIP 1 (highly iterated palindromic sequence) present in many species of cyanobacteria. The HIP 1 and STRR sequences generated electrophoretic profiles with a wide range of molecular weight that were able to group the strains in two clusters. The genetic groupings were in accordance with the morphological characters that distinguish two types of strains: N - with the normal coiling of the trichomes for *A. fusiformis* species and S - with more tightly coiled filaments. To our knowledge this is the first report of the presence of HIP 1 and STRR in *Arthrospira* strains.

Introduction

Arthrospira is a group of cyanobacteria that includes species with high nutritional quality, cultivated at industrial scale [22]. Nonheterocystous filaments with regular coils and visible septa characterize the genus *Arthrospira*, established by Stizenberger (1852). Cyanobacteria within this genus usually grow in warm water bodies with high carbonate content and high pH.

The taxonomy of the genus *Arthrospira* is quite confused because of the morphological variability shown in nature and culture by many taxa. Molecular approaches have been used to precisely distinguish cyanobacteria at the species and strain level and to solve taxonomic problems within several genera [8,9,13,15]. Studies using 16S rRNA gene sequences and RFLP analysis of the internal transcribed spacer (ITS) between 16S rRNA and 23S rRNA have revealed phylogenetic differences between *Spirulina* and *Arthrospira* [19,21,23]. In other cyanobateria, repeated sequences have been used to distinguish and identify species or strains. Two types of repeated sequences, both specific for cyanobacteria, have been described: short tandemly repeated repetitive (STRR), a heptanucleotide sequence well represented in the genome [12] and long tandemly repeated repetitive (LTRR), a 37-bp sequence [11]. Also, highly iterated palindromic sequences of 8 bp have been shown to be present in the cyanobacterial genome, but they have a wider specificity including other eubacteria as well [6,18,20]. The presence of all three types of repeated sequences in the

cyanobacterial genome has opened the possibility of distinguishing cyanobacteria by PCR fingerprinting [5,14,16,20].

A persistent large cyanobacterial blooms have been recorded during several years in the warm season in a small alkaline pond near Cluj-Napoca city. Around twenty strains of *Arthrospira* were isolated from field samples originating from this alkaline pond. The light microscopy investigation of field material assigned the strains to *A. fusiformis* species. Significant morphological differences were observed between isolates, especially regarding the degree of coiling of the trichomes. Two kinds of strains with stable characters have been described: N - with the normal coiling trichomes of *A. fusiformis* species and S - with more tightly coiled filaments [1]. In a previous paper strains were characterized by SDS-PAGE of whole cell proteins in order to establish if other phenotypic features also show the morphological differences [1]. Although minor differences were detected, they were sufficient to distinguish the two types of strains, N and S, by cluster analysis.

The main purpose of this study was to investigate if phenotypic differences between the isolates have a genetic support. We used a PCR fingerprinting technique based on primers designed for STRR and HIP sequences. Both types of sequences generated patterns that allowed an accurate distinction between the two types of *A. fusiformis* strains, N and S.

Materials and methods

Biological material. Twenty strains of *A. fusiformis* deposited in the Culture Collection of Algae at the Institute of Biological Research, Cluj-Napoca were investigated (Tab. 1). The nonaxenic strains were isolated by micromanipulation starting from single trichomes and were grown in Zarrouk medium [4].

Table 1: List of cyanobacterial strains

Strain	Origin	Morphology
Arthrospira fusiformis		
AICB 605	Apahida, Cluj	N
AICB 606	Apahida, Cluj	N
AICB 607	Apahida, Cluj	S
AICB 608	Apahida, Cluj	S
AICB 609	Apahida, Cluj	S
AICB 611	Apahida, Cluj	S
AICB 613	Apahida, Cluj	N
AICB 616	Apahida, Cluj	N
AICB 660	Apahida, Cluj	N
AICB 663	Apahida, Cluj	N
AICB 664	Apahida, Cluj	N
AICB 665	Apahida, Cluj	N
AICB 666	Apahida, Cluj	N
AICB 667	Apahida, Cluj	N
AICB 668	Apahida, Cluj	S
AICB 669	Apahida, Cluj	S
AICB 670	Apahida, Cluj	S
AICB 671	Apahida, Cluj	S
Arthrospira platensis		
AICB 49	Egipt	normal trichomes
AICB 49-1	Isolated from AICB 49	straight trichomes

DNA isolation. Exponential or early stationary phase cultures were harvested by centrifugation (8000g, 10 min). The pellet was resuspended in buffer A (100 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8) and kept at room temperature for 10 minutes for cell disruption. After a 90 minute incubation with 0.2% sarkosyl at 4°C, filaments were collected by centrifugation (8000g, 10 min). The pellet was washed several times with TES (50 mM Tris, 5 mM EDTA, NaCl 50 mM, pH 8) and resuspended in the same buffer. The release of phycocyanin pigments allowed cell breakage to be monitored by eye. Lysozyme was added to a final concentration of 0.5 mg/ml and the sample was incubated 30 minutes at 37°C in the presence of proteinase K (50 μg/ml). The sample containing the lysed cells was subjected to sequencial extractions with an equal volume of phenol and an equal volume of cloroform:isoamylic alcohol mixture (24:1) and centrifuged at 6000g, 3 minutes for a better separation of the phases. The top aqueous phase was transferred to a fresh tube and precipitated with 2/3V of 5M NaCl and 1V isopropanol at room temperature. DNA was recovered by centrifugation (21.000g, 15 min) and washed with 70% ethanol prior to air drying of the sample at room temperature and resuspended in TE buffer (10mM Tris, pH 7.5, 1mM EDTA). Genomic DNA quality was also tested by 1.5% agarose gel electrophoresis.

PCR and electrophoresis of the products. PCR reactions were run as 25μl volumes in 250μl thin walled PCR tubes (Gilson). Reaction mixture contained: STRR - 50 pmol primer, 0.4 mM dNTP, 50 ng template DNA, 1U Taq polymerase (Invitrogen), 1/10 10x reaction buffer, 2 mM $MgCl_2$ and HIP - 30 pmol primer, 0.2 mM dNTP, 15 ng template, 1U Taq polymerase, 1/10 10x reaction buffer and 1.5 mM $MgCl_2$. Primers were synthesized by Oligold, Belgium. Reactions were cycled on a Biometra Temperature Gradient Thermocycler using a temperature profile different for each type of sequence: **STRR** - one cycle 95°C, 6 min; 30 cycles of 94°C, 1 min; 56°C, 5 min; 65°C, 5 min; one cycle 65°C, 16 min and **HIP** - one cycle of 95°C, 5 min; 30 cycles of 95°C, 30s; 30°C, 30s; 72°C, 1min ; one cycle of 72°C, 5 min. Three types of STRR primers and four of the 16 possible variants of 3' 2- bp extended primers based on the HIP 1 sequence 3'-GCGATCGC-5' were used (Tab. 2). PCR products were separated by agarose gel electrophoresis in TAE buffer according to standard protocols by loading 10 μl of reaction mixture with loading buffer onto an agarose gel 1.5% (w/v), stained with 0.5 μg/ml etidium bromide [2]. All amplification reactions were repeated at least three times.

Banding profile analysis. Gel images were captured using a gel imaging device (VilberLourmat) and the included software BioProfil. The similarity between the banding patterns was calculated using Dice coefficient and unweight pair group method using aritmethic averages (UPGMA) was used to draw a dendrogram with the Bio1D++ software.

Results and discussions

HIP fingerprinting. The sequence of the oligonucleotide primers used for PCR fingerprinting were based on the HIP 1 consensus sequence 5'-GCGATCGC-3' with a 2-bp extension at the 3' end as described by Smith et al. [20] to avoid generation of smearing banding patterns[14].

Table 2: Primers used for PCR

Primers	Sequence	Reference
STRR		
STRR 1A	3' CCCCT(G/A)ACCCCT(A/G)ACC 5'	Rasmussen and Svenning, 1998
STRR 1B	3' GGT(T/C)AGGGGT(C/T)GGGG 5'	
STRR 2F	3' ACTGGTTACTGGTT 5'	
HIP		Smith et al., 1998
HIP-AT	3' TACGCTAGCG 5	
HIP-TG	3' GTCGCTAGCG 5'	
HIP-GC	3' CGCGCTAGCG 5'	
HIP-CA	3' ACCGCTAGCG 5'	

Each of the four HIP1 extended primers was tested for its ability to distinguish between cyanobacterial strains of *A. fusiformis* (Tab. 2). All electrophoretical profiles of the HIP-PCR products consisted in 4-5 bands ranging in size from approximately 300 to 1.232 bp and distinguished two clusters : N- AICB 605, AICB 606, AICB 613, AICB 616, AICB 660, AICB 663, AICB 664, AICB 665, AICB 666, AICB 667 and S-AICB 607, AICB 608, AICB 609, AICB 611, AICB 668, AICB 669, AICB 670, AICB 671 and AICB 49-1. (Fig. 1, Fig. 2). The most specific primer was HIP-AT which generated 4-5 bands with a major band at 600 bp, specific for N type isolates. Two strains of *A. platensis* were also included in the study as reference species: AICB 49 with normal coiling of the trichomes and AICB 49-1, a mutant strain with straight trichomes. The fingerprints obtained with all four primers included AICB 49 in the cluster containing the N type of strains and HIP-CA grouped AICB 49-1, occasionally tested, within the cluster S.

STRR fingerprinting. The PCR-STRR amplifications were performed with three types of STRR primers (Tab. 1) and yielded multiple distinct DNA bands ranging in size from 40-2.888 bp.

Each primer generated two different banding profiles that clustered the isolates in two groups (Fig. 3, Fig. 4). STRR 1A generated the most complex profiles comprising up to 11 bands ranging from 40-2.888 bp.

The two types of fingerprints (N and S) shared bands within the 40-1.277 bp domain and the 2.888 bp band was present only in the N fingerprint (Fig. 3A). However, the fingerprints were obviously different. STRR 1B generated profiles composed of 6-7 bands (Fig. 3B). A major drawback is the low specificity of this primer, most bands were common for both profiles. A single band at 40 bp distinguishes the fingerprints.

STRR 2F was the most specific primer generating very distinct profiles for the two types of isolates (Fig. 3C). For the N isolates two major bands at 466 bp and

767 bp made up the profile, while for S strains only a 983 bp band was separated. Clustering of DNA profiles show different similarity: STRR 1B fingerprint gave the highest similarity of 75% and STRR 2F the lowest, close to 0% (Fig. 4).

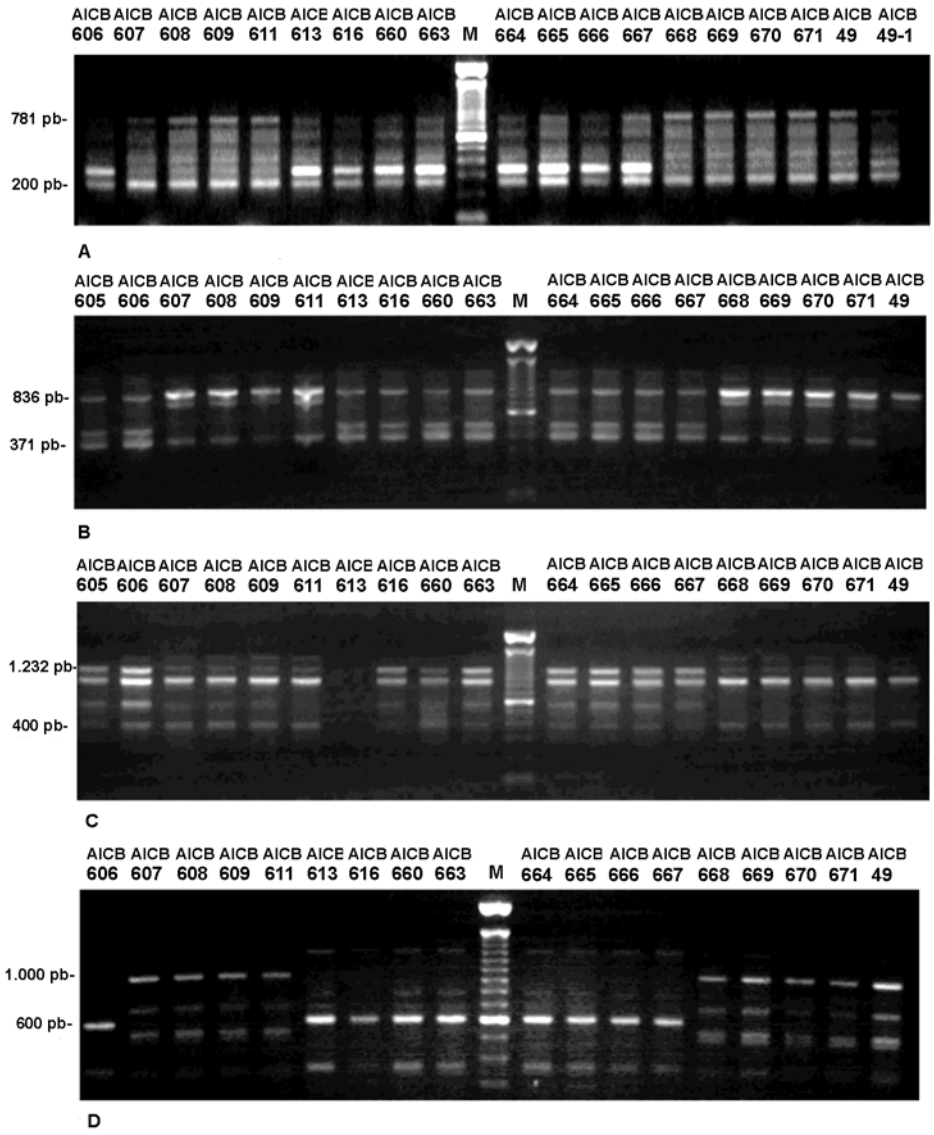

Fig. 1: HIP-PCR fingerprints of *A. fusiformis* and *A. platensis* (AICB 49 and AICB 49-1) strains were obtained with four extended primers: HIP-CA (A), HIP-TG (B), HIP-GC (C), and HIP-AT (D). Lane M contained DNA molecular weight standards.

The function of STRR and HIP sequences are still unknown. It has been suggested that STRR sequences might be the target of specific DNA-binding proteins responsible for chromosome condensation or might be involved in the control of chromosome replication and/or partitioning. The conserved status of these repetitive sequences makes them important tools for diversity studies among related microorganisms [5, 16, 20]. Rasmussen and Svenning [16] used primers corresponding to STRR and LTRR sequences in PCR, resulted in a method that generates specific fingerprints for individual cyanobacterial isolates. HIP 1

sequences are unique among repetitive sequences in that they are abundant in protein-encoding regions of the genome [18]. They are present in many, though not all cyanobacteria and are supposed to be involved in adaptive response [14]. HIP-PCR fingerprinting performed on nonaxenic isolates are not as reliable as fingerprinting based on STRR because HIP sequences are not restricted to cyanobacteria, they are present in other eubacteria as well [20].

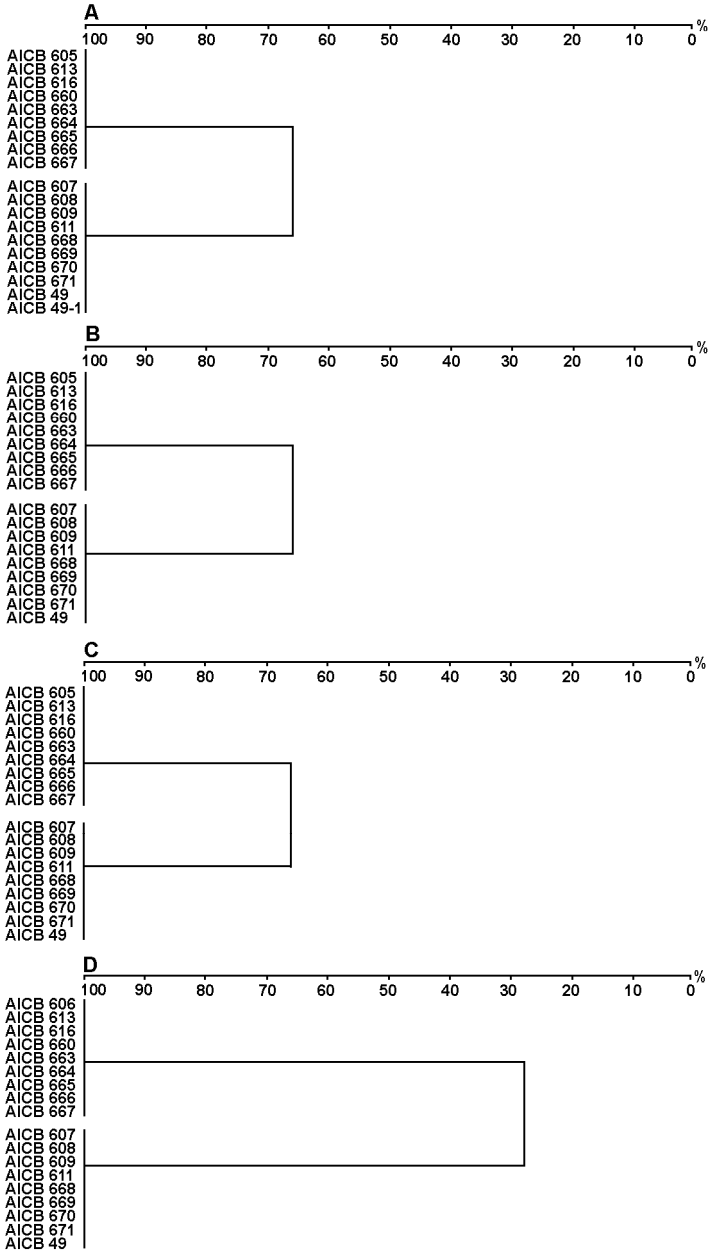

Fig. 2: UPGMA dendrogram constructed for *A. fusiformis* and *A. platensis* strains based on HIP-PCR fingerprints: HIP-CA (A), HIP-TG (B), HIP-GC (C), and HIP-AT (D).

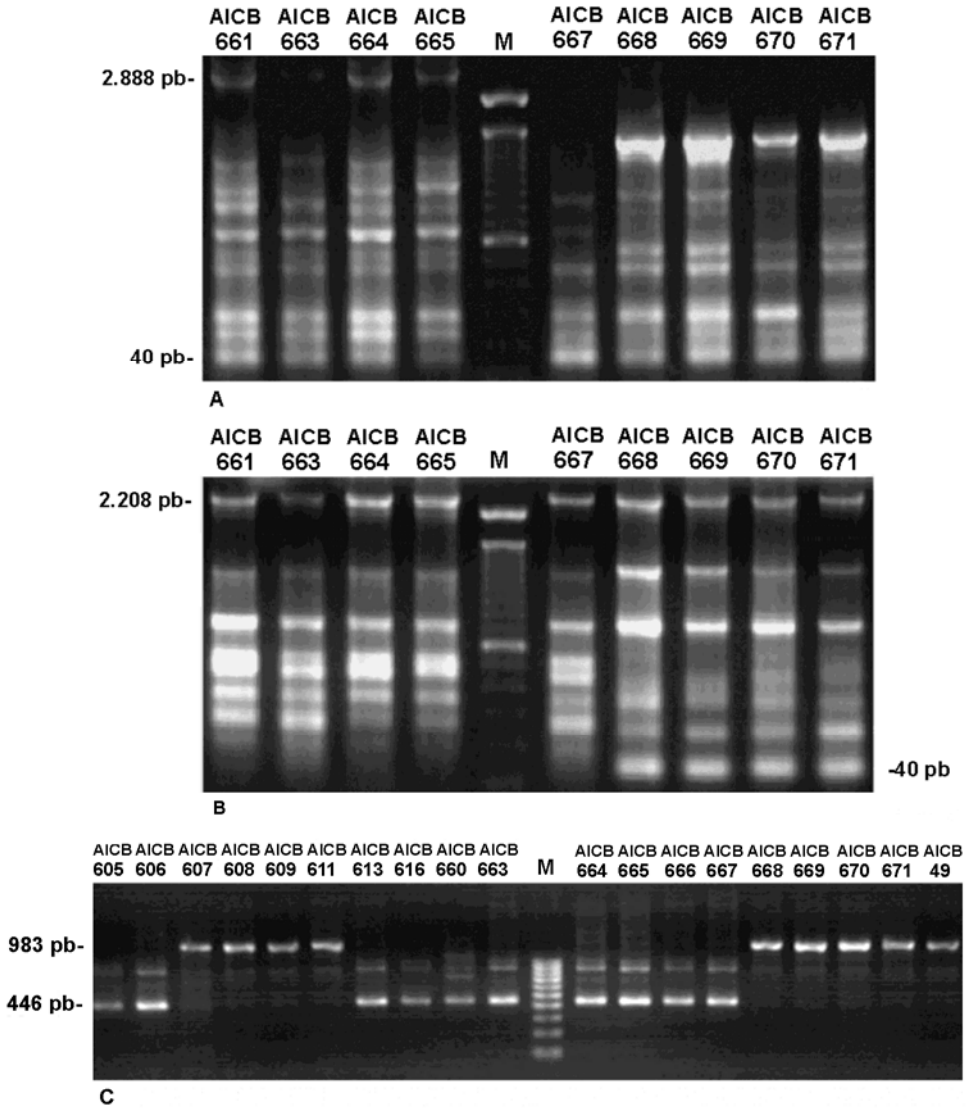

Fig. 3: STRR-PCR fingerprints of *A. fusiformis* and *A. platensis* (AICB 49 and AICB 49-1) strains obtained with three types of primers: STRR 1A (A), STRR 1B (B), and STRR 2F (C). Lane M contained DNA molecular weight standards.

The results obtained with STRR and HIP primers offer different patterns of genomic structure. Nevertheless, both are indicating that there is a genetic difference between the isolates. All primers have been able to cluster the strains in two groups based on the banding profile, according to the morphological distinction made by light microscopy. The molecular characterization of the isolates strongly suggests a genetic distance, however more molecular data are needed to make inferences on the taxonomical relevance of these findings.

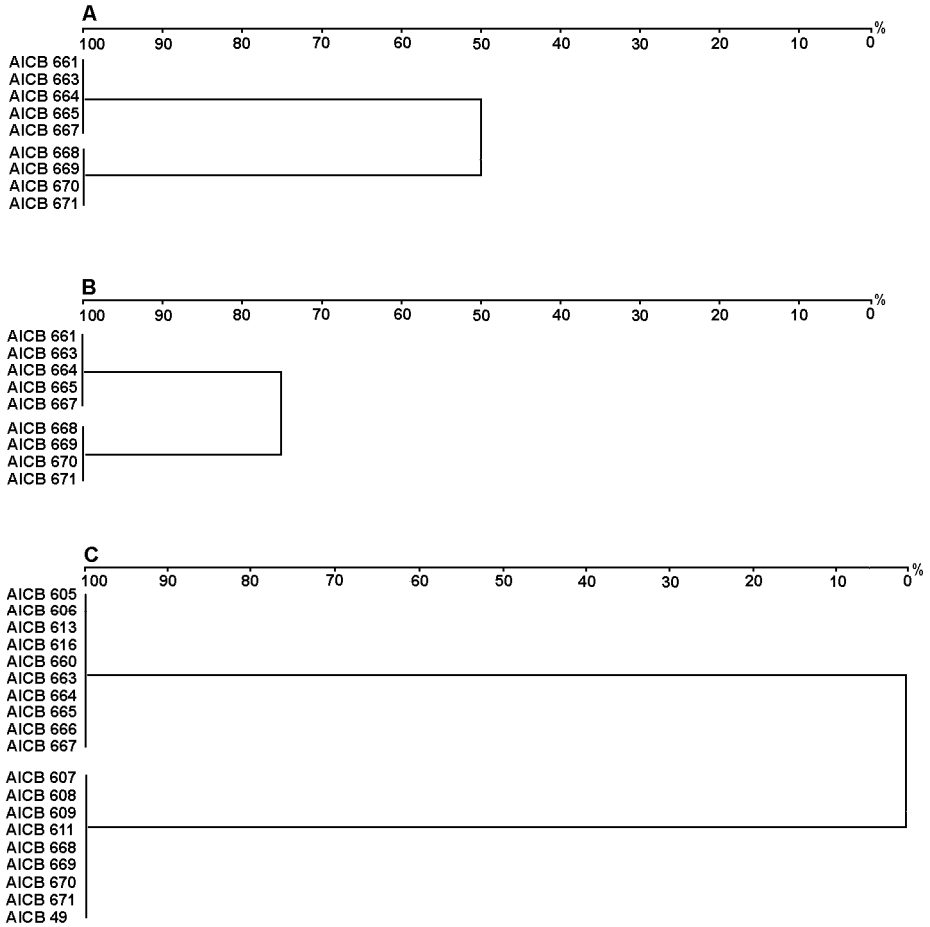

Fig. 4: UPGMA dendrogram constructed for *A. fusiformis* and *A. platensis* strains based on STRR-PCR fingerprints: STRR 1A (A), STRR 1B (B), and STRR 2F (C). *A. fusiformis* strains, N form: AICB 605, AICB 606, AICB 613, AICB 616, AICB 660, AICB 661, AICB 663, AICB 664, AICB 665, AICB 666, AICB 667. *A fusiformis* strains, S form: AICB 607, AICB 608, AICB 609, AICB 611, AICB 668, AICB 669, AICB 670, AICB 671. *A. platensis*: AICB 49 with normal trichomes.

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REFERENCES

1. Aldea, R., Guțu, A., Nicoară, A., Dragoş, N., 2001, Un nou taxon de *Spirulina* ? Discriminarea este posibilă utilizînd pattern-ul electroforetic al proteinelor şi amprenterea STRR/LTRR-PCR. În:"A. Ardelean, C. Crăciun, G. Ardelean (eds), *Probleme curente de biologie celulară şi moleculară* VI", Ed. Risoprint Cluj-Napoca: 421-433.
2. Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1992, *Short Protocols in Molecular Biology*, 3rd ed., John Wiley & Sons, Inc.

3. Baurain, D., Renquin, L., Grubisic, S., Scheldeman, P., 2002, Remarcable conservation of internally transcribed spacer sequences of *Arthrospira* ("*Spirulina*") (Cyanophyceae, Cyanobacteria) strains from four continents of recent and 30-year old dried samples from Africa, *J. Phycol*., **38**: 384-393.
4. Dragoş, N., Peterfi, S.L., Momeu, L., Popescu, C., 1997, *An Introduction to the Algae and the Culture Collection of Algae at the Institute of Biological Research, Cluj-Napoca*, Cluj Univ. Press: 129-133.
5. Guevaral, R., Armestol, J.J., and Caru, M., 2002, Genetic Diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STRR fingerprinting, *Microbial Ecology*, in press.
6. Gupta, A., Morby, A.P., Turner, J.S., Whitton, B.A., Robinson, N.J., 1993, Deletion within the metallothionein locus of cadmium-tolerant *Synechococcus* PCC 6301 involving a highly iterated palindrome (Hip1), *Mol. Microbiol.*, **7**: 189-195.
7. Iteman, I., Rippka, R., Tandeau de Marsac, N., Herdman, M., 2000, Comparison of conserved structural and regulatory domains within divergent 16S rRNA-23SrRNA spacer sequences of cyanobacteria, *Microbiology*, **146**: 1275-1286.
8. Lehtimaki, J., Lyra, C., Suomalainen, S., Sundman, P., Rouhiainen, Paulin, L., Salkinija-Salonen, M., Sivonen, K., 2000, Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotyping and phenotyping methods, *Int. J. Syst.Evol. Microbiol.*, **50**: 1043-1053.
9. Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L., Sivonen, K., 2001, Molecular characterization of planktic cyanobacteria of *Anabaena, Aphanizomenon, Microcystis* and *Planktothrix* genera, *Int. J. Syst .Evol. Microbiol*, **51**: 513-526.
10. Manen, J-F. and Falquet, J., 2002, The cpcB-cpcA as a tool for the genetic characterization of the genus *Arthrospira* (Cyanobacteria): evidence for horizontal transfer, *Int .J. Syst. Evol Microbiol.*, **52**: 861-867.
11. Masepohl, B., Gorlitz, K., Bohme, H., 1996, Long tandemly repeated repetitive (LTRR) sequences in the filamentous cyanobacterium *Anabaena* sp PCC 7120, *Biochem. Biophys. Acta*, **1307**: 26-30.
12. Mazel, D., Houmard, J., Castets, A.M., Tandeau de Marsac, N., 1990, Highly repetitive DNA sequences in cyanobacterial genomes, *J. Bacteriol.*, **172**: 2755-2761.
13. Neilan, B., Stuart, J.,L., Goodman, A.E., Cox, P.T., Hawkins, P.R., 1997, Specific amplification and restriction polymorphisms of the cyanobacterial rRNA operon spacer region, *Syst. Appl. Microbiol.*, **20**: 693-697.
14. Orcutt, K., M., Rasmussen, U., Webb, E., A., Waterbury, J., B., Gundersen, K., Bergman, B., 2002, Characterization of *Trichodesmium* spp. by genetic techniques, *Appl. Environ. Microbiol.*, **68**: 2236-2245.
15. Otsuka, S., Suda, S., Li, R., Watanabe, M., Oyaizu, H., Matsumoto, S., Watanabe, M.M., 1999, Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence, *FEMS Microbiol. Lett.*, **172**: 15-21.
16. Rasmussen, U., Svenning, M.M., 1998, Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences, *Appl. Environ. Microbiol.*, **64**: 265-272.
17. Renhui, Li, Debella, J., Habte, Carmichael, W., Wayne, 2001, Isolates identifiable as *Arthrospira maxima* and *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) appear to be identical on the basis of a morphological study in culture and 16S rRNA gene sequences, *Phycologia*, **40**, (4): 367-371.
18. Robinson, N. J., Robinson, P.J., Gupta, A., Bleasby, A.J., Whitton, B.A., Morby, A.P., 1995, Singular over-representation of an octameric palindrome, in DNA from many cyanobacteria, *Nucleic Acids Res*., **23**:729-735.
19. Scheldeman, P., Baurain, D., Bauhy, R., Scott, M., Muhling, M., Whitton, B.A., Belay, A.,

Wilmotte, A., 1999, *Arthrospira* (‘*Spirulina*’) strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer, *FEMS Microbiol. Lett*, **172**: 213-222.
20. Smith, J.K., Parry, J.D., Day, J.G., Smith, R.J.,1998, A PCR technique based on the Hip 1 interspersed repetitive sequence distinguishes cyanobacterial species and strains, *Microbiology*, **144**: 2791-2801.
21. Viti, C., Ventura, S., Lotti, F., Capolino, E., Tomaselli, L., Giovannetti, L.,1997, Genotyping diversity and typing of cyanobacterial strains of the genus *Arthrospira* by very sensitive total DNA restriction profile analysis, *Res. Microbiol.*, **148**: 605-611.
22. Vonshak, A., Boussiba, S., Abeliovici, A., Richmond, A., 1983, Production of *Spirulina* biomass: maintenance of monoalgal culture outdoors, *Biotechnol. Bioeng.*, **25**: 341-349.
23. Wilmotte, A., Turner, S., van De Peer, Y., Pace, N.R., 1992, Taxonomic study of marine oscillatoriacean strains (cyanobacteria) with straight narrow trichomes. II Nucleotide sequence analyses of the 16S ribosomal RNA, *J. Phycol.*, **28**: 828-838.

VARIABILITATEA GENETICĂ A UNOR TULPINI DE *ARTHROSPIRA* (*SPIRULINA*) EVALUATĂ PRIN TEHNICA PCR PE BAZA SECVENȚELOR GENOMICE REPETITIVE

(Rezumat)

S-a analizat variabilitatea genetică a unor tulpini de *Arthrospira* (*Spirulina*) *fusiformis* izolate dintr-o baltă alcalină situată lângă localitatea Apahida (jud. Cluj), unde această cianobacterie produce înfloriri masive în perioada mai-septembrie. În acest scop s-a utilizat amplificarea PCR cu amorse sintetizate pe baza secvențelor de tip repetitiv, STRR ("short tandemly repeated repetitive") şi HIP1 ("highly iterated palindromic sequences") specifice cianobacteriilor, ambele fiind puternici caracterizanți genomici. Profilurile electroforetice obținute prin migrarea produşilor de amplificare în gel de agaroză au permis diferențierea a două grupuri de tulpini care corespund grupărilor morfologice. Dintre cele trei tipuri de amorse STRR (STRR 1A, STRR 1B şi STRR 2F), STRR 2F s-a dovedit a fi cea mai specifică, conform dendrogramei construite pe baza similarității profilurilor de amplificare, iar STRR 1A a generat cel mai mare număr de ampliconi. Din familia HIP 1 s-au utilizat patru amorse cu o extensie de două nucleotide la capătul 3' obținându-se un profil electroforetic relaxat, favorabil amprentării. Rezultatele obținute evidențiază capacitatea discriminativă a amprentării PCR a cianobacteriilor pe baza secvențelor repetate.