



Phylogenetic relationships of *Arthrospira* strains inferred from 16S rRNA gene and *cpcBA*-IGS sequences

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Arthrospira platensis and *Arthrospira maxima* are species of cyanobacteria used in health foods, animal feed, food additives, and fine chemicals. This study conducted a comparison of the 16S rRNA gene and *cpcBA*-intergenic spacer (*cpcBA*-IGS) sequences in *Arthrospira* strains from culture collections around the world. A cluster analysis divided the 10 *Arthrospira* strains into two main genotypic clusters, designated I and II, where Group I contained *A. platensis* SAG 86.79, UTEX 2340, *A. maxima* KCTC AG30054, and SAG 49.88, while Group II contained *A. platensis* PCC 9108, NIES 39, NIES 46, and SAG 257.80. However, although *A. platensis* PCC 9223 belonged to Group II-2 based on its *cpcBA*-IGS sequence, this strain also belonged to Group I based on its 16S rRNA gene sequence. Phylogenetic analyses based on the 16S rRNA gene and *cpcBA*-IGS sequences showed no division between *A. platensis* and *A. maxima*, plus the 16S rRNA gene and *cpcBA*-IGS sequence clusters did not indicate any well-defined geographical distribution, instead overlapping in a rather interesting way. Therefore, the current study supports some previous conclusions based on 16S rRNA gene and *cpcBA*-IGS sequences, which found that *Arthrospira* taxa are monophyletic. However, when compared with 16S rRNA sequences, *cpcBA*-IGS sequences may be better suited to resolve close relationships and intraspecies variability.

Key Words: *Arthrospira* (*Spirulina*); *cpcBA*-IGS; phylogenetic analysis; 16S rRNA gene

INTRODUCTION

Arthrospira is a commercially important filamentous cyanobacterium with an annual production estimated at over 3,000 tons per year, the largest among microalgae (Pulz and Gross 2004). Found in tropical and subtropical regions in warm lakes with a high carbonate and bicarbonate content, and high pH and salinity (Tomaselli 1997), *Arthrospira* is a rich source of proteins, minerals, vitamin B₁₂, β-carotene, and essential fatty acids, such as γ-linolenic acid. Owing to its high protein content of up to approximately 60-70% on a dry weight basis, the amino acids present in *Arthrospira* match the proportions recommended by the Food and Agriculture Organi-

zation (FAO) (Dillon et al. 1995). Plus, it does not require any chemical or physical processing in order to become digestible due to the absence of a cellulose cell wall (Vonshak 1997b). Indeed, it can be used as feed for fish, poultry, and farm animals (Belay 1997, Vonshak 1997a). Originating from the taxonomic studies of cyanobacteria by Geitler (1932), some confusion has arisen between the genera *Spirulina* and *Arthrospira* (Desikachary and Jeeji-Bai 1996). While the 16S rRNA gene sequences of these two genera indicate that they are only distantly related (Nelissen et al. 1994, Scheldeman et al. 1999), putative species of *Arthrospira* have been recognized in the

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so-called 'Spirulina' commercial industrial field.

Microscopy identification of cyanobacteria is rapid and sensitive. However, even with skilled and experienced operators, identification is sometimes uncertain. Thus, molecular approaches are particularly useful in the detection and identification of specific strains, especially those that are morphologically identical at the species level. Plus, genetic identification has been used to discriminate nuisance species in cyanobacterial genera, including *Microcystis*, *Anabaena*, *Nodularia*, and *Cylindrospermopsis* (Wilmotte and Golubic 1991, Neilan et al. 1995).

Phylogenetic analyses based on the 16S rRNA gene and *cpcBA*-intergenic spacer (*cpcBA*-IGS) take considerable time and effort, yet they provide certainty in the identification of genera using both the length and the sequence of the *cpcBA*-IGS. In addition to 16S rRNA gene sequence analyses (Otsuka et al. 1998, Honda et al. 1999, Turner et al. 1999), the genes encoding the major light-harvesting accessory pigment proteins, particularly the phycocyanin operon (*cpc*), including the IGS between *cpcB* and *cpcA* and the corresponding flanking regions (*cpcBA*-IGS), have also been targeted for phylogenetic studies of cyanobacteria (Neilan et al. 1995, Barker et al. 1999). Furthermore, to determine the phyletic relationships between *Arthrospira* strains, a region of the phycocyanin locus, namely *cpcBA*-IGS, has been sequenced, as its nucleotide substitution rate is potentially higher than

that of the widely used 16S rRNA gene sequence (Lyra et al. 2001).

Accordingly, the aim of this study is to clarify the diversity of cultivated strains of *Arthrospira* using sequence data from a highly variable DNA fragment, including a comparison of the phylogeny of *Arthrospira* strains based on the 16S rRNA gene and *cpcBA*-IGS. Plus, to define and delimit the genus *Arthrospira*, this study also includes other cyanobacteria. Finally, this study attempts to identify a specific molecular marker to fingerprint different strains of *Arthrospira* and develop tools for species and strain identification.

MATERIALS AND METHODS

Strains and growth conditions

Ten *Arthrospira* cultures and one *Spirulina* culture were obtained from different culture collections across the world. Table 1 shows a list of the 10 *Arthrospira* strains, along with their origin, culture history, and the corresponding DNA database accession numbers for the 16S rRNA gene and *cpcBA*-IGS. The cultures were maintained in an SOT medium (Choi et al. 2003), containing per liter 16.8 g of NaHCO₃, 0.5 g of K₂HPO₄, 2.5 g of NaNO₃, 1 g of K₂SO₄, 1 g of NaCl, 0.2 g of MgSO₄·7H₂O, 0.04 g of

Table 1. Origins and accession numbers for *Arthrospira* and *Spirulina* strains

Strain designation	Strain number ^a	Origin	Accession no.	
			16S rRNA gene	<i>cpcBA</i> -IGS
<i>Arthrospira platensis</i>	NIES 39	Chad Lake, Chad	DQ393279	DQ393287
	NIES 46	Texcoco Lake, Mexico	- ^b	DQ393288
	UTEX 2340	Natron Lake, Chad	DQ393280	DQ393289
	SAG 86.79	Natron Lake, Chad	-	DQ393291
	SAG 257.80	Laguna Huacachina, Ica, Peru	DQ393282	DQ393292
	SAG 21.99	Walfishbay, Lagune, Namibia	DQ393283	DQ393293
	PCC 9108	Commericla Culture Facility, Cheng-hai, Yunnan, China	DQ393284	DQ393295
	PCC 9223	Lake Santa Olalla, Donana National Park, Spain	DQ393285	DQ393296
<i>Arthrospira maxima</i>	KCTC AG30054	Bangkok, Thailand	DQ393281	DQ393290
	SAG 49.88	Italy	-	DQ393294
<i>Spirulina laxissima</i>	SAG 256.80	Lake Nakuru, Kenya	DQ393278	DQ393286

cpcBA-IGS, *cpcBA*-intergenic spacer.

^aNIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; KCTC, Korean Collection for Type Cultures, Daejeon, Korea.

^b:- not amplified.

CaCl₂·2H₂O, 0.01 g of FeSO₄·7H₂O, 0.08 g of Na₂EDTA, 0.03 mg of H₃BO₃, 0.025 mg of MnSO₄·7H₂O, 0.002 mg of ZnSO₄·7H₂O, 0.0079 mg of CuSO₄·5H₂O, and 0.0021 mg of Na₂MoO₄·2H₂O. Stock cultures of 10 mL were kept under low light (20 μmol photons m⁻² s⁻¹) and at a constant temperature of 25 ± 1°C.

DNA extraction, amplification, and sequencing

The cultured strains were harvested using centrifugation, and the genomic DNA extracted following the method of Neilan et al. (1995) using lysozyme and proteinase K. The 16S rRNA gene was then amplified using the universal primers 27F (5'-AGAGTTGATCMTGCTGGCTCAG-3') and 1512R (5'-GGYTACCTTGTTACGACTT-3') (Cho and Giovannoni 2003). The PCR products were purified using a Qiagen QIAquick PCR purification column and cloned using a pDrive vector (Qiagen, Hilden, Germany). The *cpcBA*-IGS was amplified using primers located within the coding region of *cpcB* and *cpcA*, respectively, where the forward primer was CPC1F (5'-GGC KGC YTG YYT GCG YGA CAT GGA-3') and the reverse primer was CPC1R (5'-AAR CGN CCT TGR GWA TCD GC-3') (Kim et al. 2006). The amplified fragments (annealing temperature 55°C) were purified using a Qiagen QIAquick PCR purification column and cloned using a pDrive vector (Qiagen). The PCR products were then sequenced using the SP6 and T7 primers with the chain-termination method on an ABI377 automated sequencer (Solgent Ltd., Daejeon, Korea). The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers DQ393278-DQ393296.

Phylogenetic analysis

The 16S rRNA gene and *cpcBA*-IGS sequences obtained from the *Arthrospira* strains were initially compared with sequences available in the National Center for Biotechnology Information database using BLAST network services (<http://www.ncbi.nlm.nih.gov/BLAST>) to determine their approximate phylogenetic affiliations (Altschul et al. 1997). The sequences were aligned using PHYDIC 3.0, and unambiguously aligned nucleotide positions then used for phylogenetic analyses using PHYDIC 3.0 (Chun and Goodfellow 1995). The similarity values between the sequences were calculated from distance matrices by reversing the Jukes-Cantor distance formula (Jukes and Cantor 1969). Phylogenetic trees were then inferred by neighbour joining (NJ) (Saitou and Nei 1987) using the Kimura two-parameter model. The resulting NJ

tree was evaluated by bootstrap analyses based on 1,000 resamplings. Due to the spacer variability, a phylogenetic analysis of the matrix was also performed using just the two coding regions. Finally, an overview of the phylogenetic position of *Arthrospira* in cyanobacteria was created by comparing the 16S rRNA gene and *cpcBA*-IGS sequences to corresponding cyanobacterial sequences available in databases and the sequences obtained in this study for *Spirulina laxissima* SAG 256.80 and *Oscillatoria sancta* NIER 10027.

RESULTS AND DISCUSSION

Phylogenetic tree of 16S rRNA gene

Near-complete 16S rRNA gene sequences were determined for 7 *Arthrospira* strains and 1 *Spirulina* strain received from culture collections across the world. A phylogenetic tree was then reconstructed using a NJ analysis based on aligning the 8 sequences with *Escherichia coli* K-12 and *Bacillus subtilis* as the outgroup (Fig. 1). The corrected sequence alignment, providing the basis of the phylogenetic analyses, corresponded to positions 8-1512 according to the *E. coli* numbering system and was 1,383 nucleotides (nt) in length after removing all gaps and ambiguous positions.

The cluster analysis resolved the 7 *Arthrospira* strains into two main genotypic clusters, designated Group I and II. Group I contained *A. platensis* SAG 21.99, PCC 9223, UTEX 2340, and *A. maxima* KCTC AG30054, while Group II contained *A. platensis* NIES 39, SAG 257.80, and PCC 9108. The bootstrap value between the Group I and Group II clusters was 78.4% in the phylogenetic tree, and the 16S rRNA gene similarity was 99.5%. Thus, the clusters were poorly supported by the bootstrap analysis. However, in the 16S rRNA gene sequences for the *Arthrospira* strains, the number of different nucleotides was less than 7 out of a total of 1,420 nt. In the geographical analysis, the strains in Group I originated from Chad, Namibia, and Kenya in Africa, while the strains in Group II originated from Chad in Africa and Peru in South America. In a previous report, a complete analysis of the dendrogram structure grouped the strains into two well-separated genotypic groups (Viti et al. 1997). The genotypic diversity of several strains attributed to these two species was also previously investigated on the basis of morphological criteria using a very sensitive total DNA restriction profile analysis (Scheldeman et al. 1999). In this case, the strains were also divided into two well-separated genotypic groups.

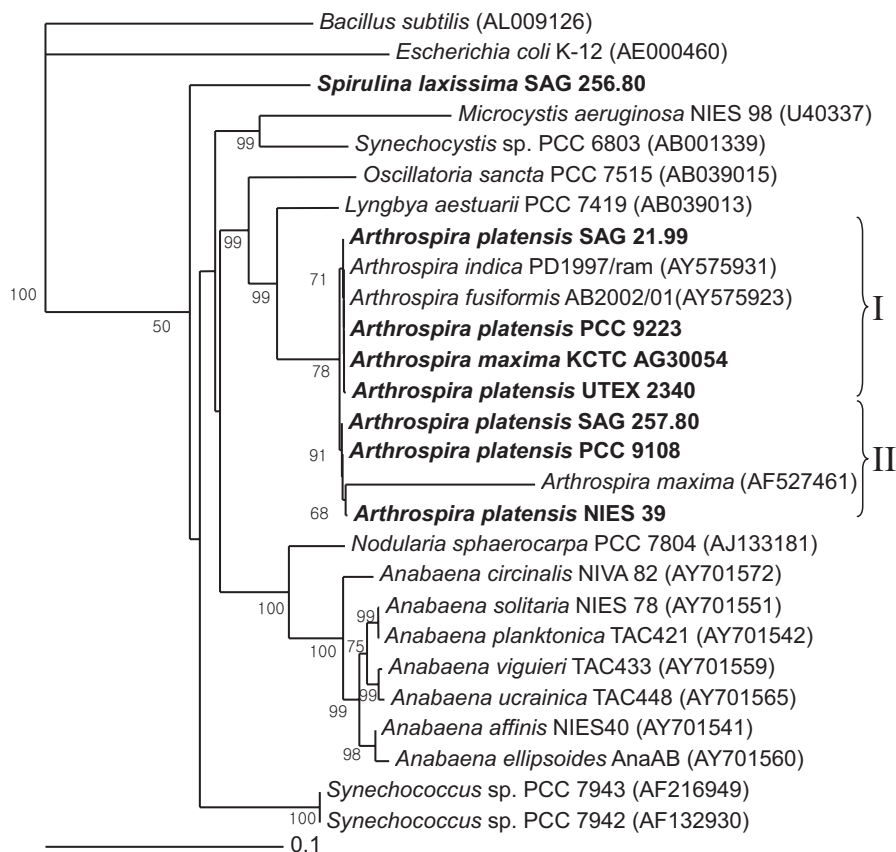


Fig. 1. Neighbour-joining tree showing relationships among *Arthrospira* strains and other cyanobacteria, inferred from 16S rRNA gene sequence analyses. Bootstrap values >50% are shown. *Bacillus subtilis* (AL009126) and *Escherichia coli* (AE000452) were used as an outgroup to define the root of the tree. Bar, 0.1 substitutions per nucleotide position. SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; NIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; KCTC, Korean Collection for Type Cultures, Daejeon, Korea; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

The similarity of the 16S rRNA genes between the *Arthrospira* strains and *Lyngbya aestuarii* PCC 7419 was about 95%. However, the similarity of the 16S rRNA genes between the *Arthrospira* strains and *S. laxissima* SAG 256.80 was about 89%. Thus, as shown by the 16S rRNA gene sequences (Nelissen et al. 1996, Ishida et al. 1997), it would seem that *L. aestuarii* PCC 7419 is also closely related to *Arthrospira* and a sister to the clade *Planktothrix/Arthrospira*.

Phylogenetic tree of *cpcBA*-IGS

The *Arthrospira* strain analyses were conducted using both the coding sequences and the spacer. Plus, the outgroup was the *cpcBA*-IGS from the chloroplast of *Cyanidium caldarium*. The NJ tree derived from the translated *cpcBA*-IGS sequences (Fig. 2) clustered the *Arthrospira* species into two groups, where Group I clustered with the

Group II clusters in 100% of the bootstrap trees and the *cpcBA*-IGS similarity was more than 95.5%. Group I contained *A. platensis* SAG 86.79, SAG 21.99, UTEX 2340, *A. maxima* SAG 49.88, and KCTC AG30054, whereas Group II was subdivided into two lineages: one clade containing *A. platensis* PCC 9108, NIES 39, and NIES 46 supported by 96.6% of the bootstrap replications, and the other clade consisting of *A. platensis* PCC 9223 and SAG 257.80. The similarity of the strains belonging to Group I was 100%. However, while *A. platensis* PCC 9223 belonged to Group II-2 based on its *cpcBA*-IGS sequence, it also belonged to Group I based on its 16S rRNA gene.

With respect to the terminal branching patterns, the results suggested a high degree of congruence between the 16S rRNA genes and the *cpcBA*-IGS-based phylogenies of the nonmarine members of the picophytoplankton clade, yet with less well delineated subgroups in the more conserved 16S rRNA gene phylogeny (Robertson et al. 2001,

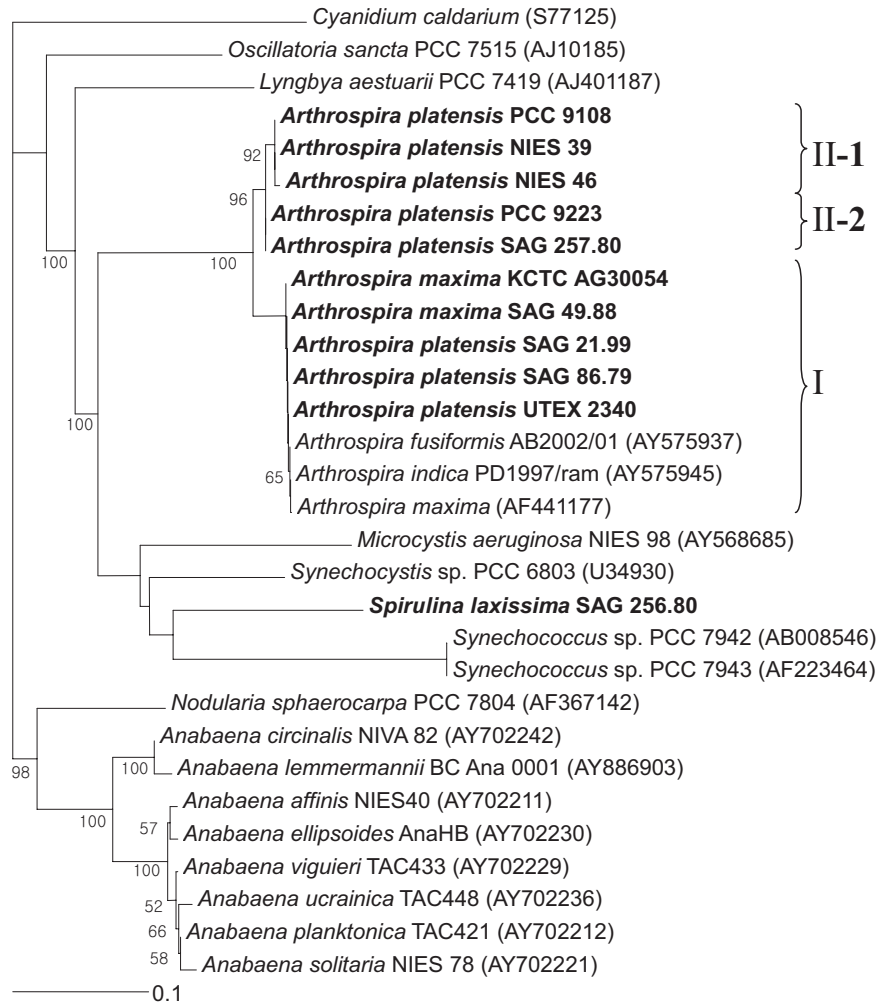


Fig. 2. Neighbour-joining tree showing relationships among *Arthrospira* strains and other cyanobacteria, inferred from *cpcBA*-intergenic spacer sequence analyses. Bootstrap values >50% are shown. The chloroplast of *Cyanidium caldarium* was used as an outgroup to define the root of the tree. Bar, 0.1 substitutions per nucleotide position. PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; NIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; KCTC, Korean Collection for Type Cultures, Daejeon, Korea; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

Crosbie et al. 2003). In a previous report, 16S rRNA gene data and *cpcBA*-IGS data also indicated that several taxa were genetically heterogeneous and that the taxonomy of cyanobacteria needed to be reconsidered. Manen and Falquet (2002) suggested that 16S rRNA gene data and *cpcBA*-IGS data were often in agreement. Furthermore, another study investigated the use of the *cpcBA*-IGS region of the phycocyanin operon, flanked by *cpcB* and *cpcA*, as a marker for the subgenus characterization of cyanobacteria (Neilan et al. 1995, Bolch et al. 1996).

The phylogenetic analyses based on the 16S rRNA gene and *cpcBA*-IGS sequences showed no division between *A. platensis* and *A. maxima*, plus the 16S rRNA gene and *cpcBA*-IGS clusters did not exhibit any well-defined geo-

graphical distributions, and instead overlapped in a rather interesting way. Thus, it is now widely accepted that *S. laxissima* SAG 256.80 is distinct and relatively distant from *Arthrospira* strains.

Structure and characterization of *cpcBA*-IGS

The sequences of the amplified fragments of the genus *Arthrospira* comprised the last 259 nt of *cpcB*, a 111 nt spacer, and the first 33 nt of *cpcA*. These regions were identified based on sequence comparisons using the BLASTN Sequence (<http://www.ncbi.nlm.nih.gov/BLAST>). For comparison, the *Spirulina* (Oscillatoriales) sequence comprised 259 nt of *cpcB*, a 100 nt spacer, and

Taxon	Group	<i>cpcB</i> 3' end										Spacer								
		1	1	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3		
PCC 9223	II-2	3	1	3	4	1	9	2	3	4	4	4	4	6	8	8	1	1	1	2
SAG 257.80		C A T T C T G T T G C T	T C C T T T C																	
PCC 9108	II-1	C A T T T T G T T G C A	C A G G C G G																	
NIES 39		C A T T T T G T T G C A	C A G G C G G																	
NIES 46		C A T T T T G T T G C A	C A G G C G G																	
KCTC AG30054	I	T T C C C C A A A T G T	C A G G C G G																	
SAG 49.88		T T C C C C A A A T G T	C A G G C G G																	
SAG 21.99		T T C C C C A A A T G T	C A G G C G G																	
SAG 86.79		T T C C C C A A A T G T	C A G G C G G																	
UTEX 2340		T T C C C C A A A T G T	C A G G C G G																	

Fig. 3. Mosaic distribution of variable informative sites in *cpcBA*-intergenic spacer sequence of *Arthrospira* strains. For easier observation, the strains are classified according to the clusters in Fig. 2. The numbers above the alignment indicate the nucleotide position of the informative sites. Blocks of orthologous sequences are boxed. PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; NIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; KCTC, Korean Collection for Type Cultures, Daejeon, Korea; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

the first 33 nt of *cpcA*. For *Phormidium autumnale* (Oscillatoriales), the *cpcB-cpcA* locus comprised 224 nt of *cpcB*, a 89 to 106 nt spacer, and 281 nt of *cpcA* (Teneva et al., 2005). The sequence analyses of *Nodularia* isolates (Nostocales) resulted in 283 nt of *cpcB*, a spacer of 78-79 nt, and 306 nt of *cpcA* (Barker et al. 1999), whereas for the different *Aphanizomenon* colonies (Nostocales), this region comprised 227 nt of *cpcB*, a spacer of 77-103 nt, and 47 nt of *cpcA* (Barker et al. 2000). For *Synechococcus* (Chroococcales), the sequence comprised 274 nt of *cpcB*, a 43 to 60 nt spacer, and 195 nt of *cpcA* (Robertson et al. 2001). Interestingly, the length of the *cpcBA*-IGS spacer in Chroococcales was shorter than that in the other cyanobacteria (Nostocales and Oscillatoriales).

The information sites of the *cpcB-cpcA* DNA matrix for *Arthrospira* (with the taxa arranged according to the results in Fig. 2) are shown in Fig. 3. The mosaic distribution of the substitutions suggests recombination events at these phycocyanin loci. The first block comprises positions 1-259 (3' end of *cpcB* translated sequences), while the second block comprises positions 260-403 (*cpcB-cpcA* spacer and 5' end of *cpcA* translated sequences). Fig. 3 shows the trees obtained using positions 1-259 and 260-403, respectively. While the first block clearly differentiates Groups I and II, as defined in Fig. 3, the substitutions in the second block do not separate Clade 1 and 2, but rather differentiate Group II-2 from all the other strains. In Group II-1 and II-2, the nucleotide substitution was T to C and A to T at 115 and 247, respectively. No amino acid changes were found in any of the groups. However, in

Group I and II, the substitutions at 224 and 242 involved a change from Ser to Gly and Cys to Ala, respectively.

The LARD maximum-likelihood method (Holmes et al. 1999) was applied to find the breakpoint in the alignment that gave the highest likelihood under an evolutionary model incorporating recombination. This point was located after position 259, in agreement with Fig. 3. Thus, the *Arthrospira* tree topology based on the *cpcBA*-IGS fragment did not reflect the true cladogenesis due to horizontal transfers. Also, there was no correlation between the DNA matrix and the geographical origin. These results were the same as those reported by Manen and Falquet (2002), and also reminiscent of the mosaic distribution of informative sites previously observed in the *rbcLX* gene of *Nostoc* (Rudi et al. 1998). In the genus *Arthrospira*, the phycocyanin locus also indicates intragenic recombination and suggests exchanges of genetic material between strains. Genetic exchanges have also been postulated in *Nodularia* (Barker et al. 2000) and *Phormidium* (Teneva et al. 2005).

In summary, the current study supports some previous conclusions based on 16S rRNA gene and *cpcBA*-IGS sequences that *Arthrospira* taxa are monophyletic. Thus, the combination of morphological features with molecular markers, such as 16S rRNA gene sequences and *cpcBA*-IGS sequences, as used in this study, can be useful tools for identification and / or phylogenetic studies at the species level or higher taxonomic ranks in the order Oscillatoriales. When compared with 16S rRNA sequences, *cpcBA*-IGS sequences may be better suited to resolve

close relationships and intraspecies variability. The probe based on *cpcBA*-IGS that showed a high specificity for cyanobacteria enabled the amplification and sequencing to be simple and fast.

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