

Arthrospira ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer

Patsy Scheldeman^{1,a}, Denis Baurain^a, Rachel Bouhy^a, Mark Scott^b,
Martin Mühlhling^b, Brian A. Whitton^b, Amha Belay^c, Annick Wilmotte^{a,*}

^a *Laboratory of Algology, Mycology, and Experimental Systematics, Department of Botany B22, University of Liège, B4000 Liège, Belgium*

^b *Department of Biological Sciences, University of Durham, Durham DH1 3LE, UK*

^c *Earthise Farms, Calipatria, CA 92233, USA*

Received 14 September 1998; received in revised form 3 January 1999; accepted 6 January 1999

Abstract

We present the results of a phylogenetic study, based on amplified ribosomal DNA restriction analysis of the rDNA operon, of 37 *Arthrospira* ('*Spirulina*') cultivated clonal strains from four continents. In addition, duplicates from different culture collections or markedly different morphotypes of particular strains established as clonal cultures were treated as separate entries, resulting in a total of 51 tested cultures. The strain *Spirulina laxissima* SAG 256.80 was included as outgroup. The 16S rRNA genes appeared too conserved for discrimination of the strains by amplified ribosomal DNA restriction analysis, and thus the internally transcribed spacer was selected as molecular taxonomic marker. The internally transcribed spacer sequences situated between the 16S and the 23S rRNA were amplified by polymerase chain reaction and yielded amplicons of about 540 bp. Direct use of cells for polymerase chain reaction seemed to inhibit the amplification reaction. This was overcome by the design of a crude lysis protocol and addition of bovine serum albumin in the polymerase chain reaction mix. The amplicons were digested with four restriction enzymes (*EcoRV*, *HhaI*, *HinfI*, *MseI*) and the banding patterns obtained were analyzed. Cluster analysis showed the separation of all the strains into two main clusters. No clear relationships could be observed between this division into two clusters and the geographic origin of the strains, or their designation in the culture collections, or their morphology. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: rRNA; Internally transcribed spacer; *Arthrospira*; *Spirulina*; Genotype; Taxonomy

* Corresponding author. Tel.: +32 (4) 366-3856;
Fax: +32 (4) 366-2853; E-mail: awilmotte@ulg.ac.be

¹ Present address: Center for Agricultural Research-Ghent (CLO), Department for Animal Product Quality and Transformation Technology, Brusselsesteenweg 370, B9090 Melle, Belgium.

1. Introduction

The main characteristics of the genus *Arthrospira* (wrongly merged with the genus *Spirulina* by Geitler in 1932 [1]) defined in the Bergey's Manual of Systematic Bacteriology [2] are the loosely coiled tri-

Table 1
List of strains, origin, and morphology

Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
<i>Arthrospira maxima</i>	CCAP 1475/9	Natron lake, Chad	H	D0873	CCAP	ATCC 53871; Lefevre 1963/MI32-1; SAG 84.79; UTEX 2342
<i>Arthrospira maxima</i>	SAG 84.79	Natron lake, Chad	H	D0879	SAG	
<i>Arthrospira maxima</i>	Lefevre 1963/MI32-1	Natron lake, Chad	H	D0903	CCALA	
<i>Arthrospira platensis</i>	SAG 85.79	Natron lake, Chad	H	D0880	SAG	Laporte 1963/MI32-2b; NIVA CYA 120; UTEX 2340
<i>Arthrospira</i> sp. (' <i>platensis</i> ')	Laporte 1963/MI32-2b	Natron lake, Chad	H	D0906/H	CCALA	
<i>Arthrospira</i> sp. (' <i>platensis</i> ')	Laporte 1963/MI32-2b	Natron lake, Chad	S	D0906/S	CCALA	
<i>Arthrospira platensis</i>	SP-4	Natron lake, Chad	S	D0887	Durham	
<i>Arthrospira platensis</i>	SAG 85.79 (Lill)	Natron lake, Chad	H	H. Lill	H. Lill	
<i>Arthrospira platensis</i>	SAG 86.79	Natron lake, Chad	S	D0882	SAG	Compère 86/79
<i>Arthrospira platensis</i>	SAG 86.79 (Lill)	Natron lake, Chad	S	H. Lill	H. Lill	
<i>Arthrospira</i> sp.	C1	Lake Bodou, Kanem, Chad	H (S)	D0918	A. Sanang-elantoni	
<i>Arthrospira</i> sp.	SP-8	Lake Simbi, Kenya	H	D0891	Durham	
<i>Arthrospira maxima</i>	CCAP 1406/2	Lake Naivasha, Kenya	H	D0867	CCAP	
<i>Arthrospira fusiformis</i>	CCAP 1475/8	Lake Chitu, Ethiopia	H	D0872/H	CCAP	
<i>Arthrospira fusiformis</i>	CCAP 1475/8	Lake Chitu, Ethiopia	S	D0872/S	CCAP	
<i>Arthrospira</i> 'Lonar'		Lonar Lake, Maharashtra, India	H	D0920	R. Fox	
<i>Arthrospira</i> 'Titicaca'		Lake Titicaca, Peru	H	D0922	R. Fox	
<i>Arthrospira</i> sp.	SP-14	Unknown	H	D0897	Durham	
<i>Arthrospira</i> sp.	SP-16	Unknown	H	D0899	SAC	
<i>Arthrospira</i> sp.	SP-17	Unknown	H	D0900	SAC	
<i>Arthrospira</i> sp.	Strain EF-18A	Unknown	H	D0925	Earthrise Farms	
<i>Arthrospira</i> sp.	PCC 9223	Lake Santa Olalla, Donana National Park, Spain	H	D0933	PCC	
<i>Arthrospira</i> sp.	PCC 7939	India, Kenya, Mexico or Peru	H	D0912	PCC	Records lost at PCC
<i>Arthrospira</i> sp.	PCC 7940	India, Kenya, Mexico or Peru	H	D0913	PCC	Records lost at PCC
<i>Arthrospira</i> sp.	PCC 8005	India, Kenya, Mexico or Peru	H	D0914	PCC	Records lost at PCC
<i>Arthrospira indica</i>	MCRC isolate straight	MCRC, Madras, India	S		N. Jeeji-Bai	

Table 1 (Continued).
List of strains, origin, and morphology

Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
<i>Arthrospira</i> sp. ('platensis')	Compere 86.79	Natron lake, Chad	H	D0905	CCALA	SAG 86.79
<i>Arthrospira</i> sp. ('platensis')	Compere 1968/3786	Lake Bodou, Kanem, Chad	H	D0904	CCALA	
<i>Arthrospira</i> sp. ('platensis')	Leonard and Wouters 1968	Lake Bodou, Kanem, Chad	H	D0907	CCALA	
<i>Arthrospira platensis</i>	SP-2	Lake Chad, Chad	H	D0885/H1	Durham	NIES-39; IAM M-135
<i>Arthrospira platensis</i>	SP-2	Lake Chad, Chad	H(S)	D0885/H2	Durham	
<i>Arthrospira fusiformis</i>	Hindak 1985/1	Lake Arenguade, Ethiopia	H	D0909	CCALA	
<i>Arthrospira fusiformis</i>	Hegewald 1976/83	Lake Nakuru, Kenya	S	D0910/S1	CCALA	
<i>Arthrospira fusiformis</i>	Hegewald 1976/83	Lake Nakuru, Kenya	S	D0910/S2	CCALA	
<i>Arthrospira fusiformis</i>	Hegewald 1976/83	Lake Nakuru, Kenya	H	D0910/H	CCALA	
<i>Arthrospira platensis</i>	UTEX 1926	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0875	UTEX	PCC 7345; UTEX 1928; ATCC 29408
<i>Arthrospira platensis</i>	UTEX 1928	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0876	UTEX	
<i>Arthrospira platensis</i>	PCC 7345	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0911	PCC	
<i>Arthrospira platensis</i>	SAG 257.80	Laguna Huacachina, Ica, Peru	S	D0881	SAG	Hegewald 1977/229
<i>Arthrospira platensis</i>	SAG 257.80 (Lill)	Laguna Huacachina, Ica, Peru	S		H. Lill	
<i>Arthrospira</i> 'crater'		Lake in volcano crater, Queretaro, Mexico	H	D0919	R. Fox	
<i>Arthrospira</i> 'Orovilca'		Lake Orovilca, Ica, Peru	H	D0921	R. Fox	
<i>Arthrospira platensis</i>	SP-1	Lake Texcoco, Mexico	H	D0884	Durham	NIES-46; IAM M-185
<i>Arthrospira</i> sp.	SP-7	Lake Texcoco, Mexico	H	D0890	Durham	
<i>Arthrospira</i> sp	PCC 9108	Commercial culture facility, Cheng-hai, Yunnan, China	H	D0916	PCC	

Table 1 (Continued).
List of strains, origin, and morphology

Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
<i>Arthrospira platensis</i>	Berhampur	Berhampur, India	H	D0930	N. Jeeji-Bai	
<i>Arthrospira indica</i>	MCRC isolate spiral	MCRC, Madras, India	H	D0929	N. Jeeji-Bai	
<i>Arthrospira</i> sp.	SP-13	Unknown	H	D0896	Durham	
<i>Arthrospira</i> sp.	SP-12	Unknown	H	D0895	Durham	
<i>Arthrospira</i> sp.	PCC 8006	India, Kenya, Mexico or Peru	H	D0915	PCC	Records lost at PCC
<i>Arthrospira</i> sp.	Strain EF-2	Unknown	H	D0923	Earthrise Farms	
<i>Spirulina laxissima</i>	SAG 256.80	Lake Nakuru, Kenya		D0883	SAG	Hegewald 1976/75

No horizontal lines are drawn between duplicates and/or subcultures of one strain. Line spacings separate strains from clusters I and II within *Arthrospira*, and the strains from genera *Arthrospira* and *Spirulina*. H, helical filaments; S, straight filaments; H(S), straight filaments appearing among the helical filaments; H1/H2, differences in length of the helix pitch, helix diameter, and trichome diameter (unpublished results, M. Scott, M. Mühlhling and B.A. Whitton); S1/S2, differences in length of filament (unpublished results, M. Scott, M. Mühlhling and B.A. Whitton). ATCC, American Type Culture Collection, Rockville, MD, USA; CCALA, Culture Collection of Autotrophic Organisms, Trebon, Czech Republic; CCAP, Culture Collection of Algae and Protozoa, Ambleside, Cumbria, UK; Durham, Culture Collection of Durham University, Durham, UK; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; NIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; NIVA, Norwegian Institute for Water Research, Oslo, Norway; PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; SAC, SIAM ALGAE, Samutprakarn, Thailand; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

chomes of width varying between 3 and 12 μm with cross-walls visible in light microscopy. They are generally found in tropical and subtropical regions in warm water bodies with high carbonate and bicarbonate content, and elevated pH and salinity [3]. Due to their richness in amino acids and γ -linolenic acid, they are currently sold as a food supplement and health food under the name 'Spirulina' [4,5], though the 16S rRNA sequences of these two genera show that they are not related [6]. Since the increased awareness of the nutritional potential of *Arthrospira* in the 1960s, many strains have been deposited in culture collections and used in laboratories and mass cultivation plants [7,8]. However, the taxonomic situation of this genus, with at least six currently recognized binomials (*A. fusiformis*, *A. geitleri*, *A. indica*, *A. jenneri*, *A. maxima*, *A. platensis*), is confused and conflicting hypotheses have been published [3,9,10]. A major problem is the morphological variability of the strains under different environmental conditions. For example, the degree of spiralisation may show great variation and the spontaneous appearance of straight trichomes in a previously coiled strain is a well-documented phenomenon [3].

There is clearly a need to study the genotypic relatedness of many *Arthrospira* strains to give a firmer basis for future taxonomic revisions. Two sequences of the 16S rRNA and internally transcribed spacer (ITS) from *Arthrospira* PCC 7345 and PCC8005 have already been published [6]. They show a 16S rRNA sequence similarity of 99.7%. On the other hand, the ITS sequences were less similar, having 83.6% similarity in the non-coding areas (excluding tRNA^{Ile} and tRNA^{Ala}).

As many *Arthrospira* strains as possible were obtained from culture collections and other laboratories. Digestions by *EcoRV*, *HinfI*, *NdeII*, and *TaqI* of the 16S rRNA gene of 16 *Arthrospira* strains taken at random yielded identical restriction patterns and thus, we concluded that the 16S rRNA could not be used to resolve the *Arthrospira* strains (data not shown). Instead, the ITS appeared to present a variability suitable for discriminating the strains. Four restriction enzymes were selected and used for the amplified ribosomal DNA restriction analysis (ARDRA) of the ITS. This technique has already been used for screening of large numbers of bacterial strains in the framework of polyphasic taxonomic

studies [11] and for identification of cyanobacterial strains of very diverse phylogenetic affiliations [12], but the present study describes its first use for a detailed study of a single cyanobacterial genus. Because amplification from a few cells was not feasible, a crude lysis protocol had to be designed and polymerase chain reaction conditions had to be modified by the addition of bovine serum albumin (BSA).

2. Materials and methods

2.1. Cyanobacterial strains

Fifty-one *Arthrospira* cultures were obtained from culture collections, representing theoretically 37 unique genotypes. Several were duplicates of the same strain, present in different culture collections or obtained from different laboratories. They were treated as different entries, because the possibility of mistakes having occurred in the various collections could not be ruled out. These duplicates could also be considered as internal controls for reproducibility of the data. In addition, four strains (D0872, D0885, D0906, D0910) included different morphotypes and these were separated into axenic clonal subcultures by successive transfers and washings in drops of sterile culture medium.

As only the strains from the Pasteur Culture Collection were guaranteed to be axenic, all strains were purified and established as axenic clonal cultures as explained above.

Spirulina medium [13] was used for cultivation. Stock cultures of 5 ml were kept under low light ($10\text{--}40 \mu\text{E m}^{-2} \text{s}^{-1}$) and at a constant temperature of 25°C at the University of Liège and 30°C at the University of Durham. Table 1 lists the origins and morphology of the strains.

2.2. Lysis with proteinase K

About 1 ml of a dense culture was harvested by centrifugation in a microcentrifuge tube ($16\,000 \times g$, 15 min) and cells were washed three times with RS buffer (0.15 M NaCl, 0.01 M EDTA, pH 8.0). Twenty μl of a solution of $0.05 \mu\text{g} \mu\text{l}^{-1}$ proteinase K in $1 \times$ PCR buffer of Super Taq Plus (HT Biotechnology, UK) was added and incubated at 37°C for

1 h, followed by 5 min in a boiling water bath. Five μl 5 M NaCl was added and gently mixed. A final centrifugation ($8000\times g$, 5 min) was performed prior to PCR or storing the samples at -20°C .

2.3. PCR of 16S rRNA plus ITS

In a total volume of 50 μl , 0.5 μl of lysis mixture or DNA extraction was added to $1\times$ PCR buffer of Super Taq Plus, 0.2 mM dNTPs, 0.4 μM primer 16S5'F, 0.4 μM primer 23S5'R, 1 mg ml^{-1} BSA, and 0.8 U Super Taq Plus polymerase with a proof-reading activity (HT Biotechnology, UK). The primer sequences, derived from [14] were 'AGAGTTTGATCCTGGCTCAG' and 'TCTGTG-TGCCTAGGTATCC' respectively. The thin-wall tubes were submitted to thermal cycling in the Gene Cycler (Bio-Rad, USA): 180 s at 94°C ; 10 cycles of 45 s at 94°C , 45 s at 57°C , 120 s at 68°C ; 25 cycles of 45 s at 90°C , 45 s at 57°C , 120 s at 68°C , and a final elongation step of 7 min at 68°C . The PCR products were visualized after 1% (w/v) agarose gel electrophoresis and stored at -20°C .

2.4. Purification of the PCR products

The PCR products were subjected to electrophoresis in 1% (w/v) agarose gel in $1\times$ TAE buffer (40 mM Tris acetate, 2 mM EDTA). The bands of expected length were excised from the gels and the DNA was extracted by centrifugation on a minicolumn with glasswool [15]. Only two PCR products were loaded at opposite sides of a minigel and care was taken to avoid any cross-contamination. The DNA was precipitated with ethanol and resuspended in 20 μl of buffer TE^{-4} (10 mM Tris pH 7.4, 0.1 mM EDTA pH 8.0) and stored at -20°C .

2.5. PCR of ITS

This PCR was performed like the first PCR (Section 2.3), except that 1 μl of the purified PCR product (Section 2.4) was generally used instead of the lysis material and primer 16S5'F was replaced by primer 16S3'F (TGYGGCTGGATCACCTCCTT). The same cycling conditions were used except that the annealing temperature was 53°C instead of 57°C ,

40 s was used instead of 45 s, and 75 s instead of 120 s.

2.6. Digests

Ten μl of the PCR product was added to 2 μl of the $10\times$ reaction buffer and 5 U of the restriction enzymes *EcoRV*, *HhaI*, *HinfI* or *MseI* (Gibco Life Sciences, USA) in a total volume of 20 μl . Incubation was carried out for 2 h in the optimum temperature of the enzyme (manufacturer's conditions). The reaction was stopped by addition of 0.4 μl 0.5 M EDTA, pH 8.0.

2.7. Electrophoresis

A standardized electrophoresis protocol using LSI MP agarose (Life Sciences International) at a concentration of 2% was used to maximize the reproducibility between different gels. The agarose was slowly sprinkled into TBE buffer at room temperature and stirred with a magnetic bar. The flask was weighted before heating until the agarose was completely molten. The flask was returned to its original weight by addition of distilled water. When the solution had reached 60°C , it was poured into a 15×15 cm tray and left to polymerize for 20 min. Prior to loading the gel, it was placed at 4°C for 30 min. Electrophoresis proceeded at a constant voltage of 3.8 V cm^{-1} during 130 min. The gel was colored in an ethidium bromide bath ($1\text{ }\mu\text{g ml}^{-1}$ in TBE) for 20 min and rinsed before visualization on an UV-transilluminator.

2.8. Analysis with GelCompar

Gel images were saved as TIF files using a gel imaging device (Vilber Lourmat) and the included software Bio Profil v.6.0. They were loaded in the software package GelCompar 4.0 (Applied Maths, Kortrijk, Belgium), cut into gel tracks and normalized using the digest of pBR322/*HaeIII* as the standard. After assigning logical bands to the normalized patterns, the four gels were combined for analysis. The similarity between the banding patterns was calculated using the Dice coefficient and the UPGMA method was used to draw a dendrogram based on the matrix of similarity coefficients. A band matching

tolerance of 0.4% was selected. The bands above 50 bp were visible and well resolved, and thus were used for the analysis. The addition of the band lengths to reach about 540 bp was performed to rule out the presence of non-specific bands and partial digests.

3. Results and discussion

3.1. Amplifications by PCR of the rRNA genes

Despite numerous trials, the cells of *Arthrospira* strains could not be used directly as template in the PCR reaction, probably due to inhibitory compounds. It was necessary to design a slightly longer

lysis protocol involving proteinase K. The addition of BSA to the PCR mix was mandatory for the success of the reaction.

The PCR reactions gave products of identical lengths for all the *Arthrospira* strains, corresponding to the ca. 2000 and 540 bp found in published sequences of 16S plus ITS, and ITS alone, respectively [6]. Based on this length conservation, it is probable that all the ITS contained tRNA^{Ile} and tRNA^{Ala}.

3.2. ARDRA of ITS

Using the software CUTTER (<http://www.medkem.gu.se/cutter/>) and the two published sequences [6], we could only find four discriminative restriction enzymes giving usable patterns. Other enzymes chos-

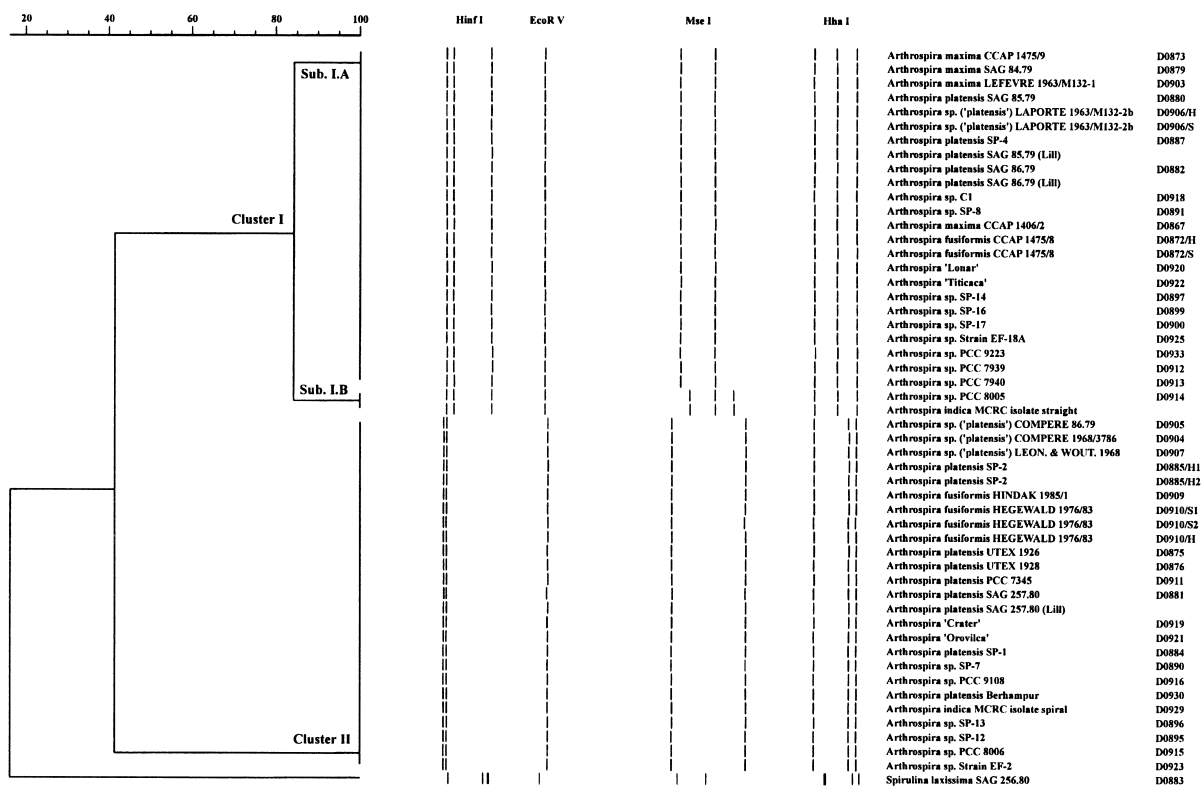


Fig. 1. Dendrogram built by UPGMA clustering of Dice correlation coefficients of normalized combined ARDRA patterns of ITS for 51 *Arthrospira* and one *Spirulina* cultures obtained with the restriction enzyme combination *EcoRV*, *HhaI*, *HinfI*, and *MseI*. The schematic representation of the four ARDRA patterns is given in front of the strain designations and of the numbers in the Durham Collection. The lengths of fragments longer than 50 bases are for *HinfI*-cluster I: 260, 214, 67; *HinfI*-cluster II: 281, 260; *EcoRV*-cluster I: 499; *EcoRV*-cluster II: 540; *HhaI*-cluster I: 290, 162, 88; *HhaI*-cluster II: 290, 113, 88; *MseI*-subcluster IA: 389, 151; *MseI*-subcluster IB: 305, 151, 84; *MseI*-cluster II: 490, 52 bases.

en at random were tested in case the two published sequences were not representative of the overall diversity of the studied strains. *NdeII* and *MspI* did not cut at all the ITS of 15 strains chosen at random, *RsaI*, *BsiZI* and *AvaII* cut only in conserved areas and did not differentiate the strains, *TaqI* gave too slight differences in band lengths to allow a reliable analysis, *AluI* gave too small fragments (under 100 bases) to be visible, and *HaeIII* gave partial digests which did not allow a good and reproducible analysis.

For 21 cultures, the banding patterns were obtained twice in independent experiments, starting from new cultures, and they were identical. In addition, duplicate strains from different culture collections or laboratories gave the same results, except SAG 86.79 from SAG (D0882) and from Lill's laboratory which were different from their putative 'duplicate' Compère 86.79 (D0905) from CCALA at the level of the ARDRA of ITS and the morphology. This distinctiveness at the genotypic and phenotypic levels implies that either SAG or CCALA does not hold the original strain. In the cases where different morphotypes were isolated from the same culture (D0872, D0 885, D0906, D0910), the ARDRA results were identical.

Cluster analysis resolved the 51 *Arthrospira* cultures into only two main genotypic clusters, designated I and II in Fig. 1. The *Arthrospira* cultures were clearly separated from the *Spirulina laxissima* SAG 256.80 strain (D0883) which serves as outgroup. It is interesting to note that Viti et al. [16] also found two clusters when 10 *Arthrospira* strains were studied by total DNA restriction profile analysis. However, only strain C1 (D0918) is common to both studies. It is possible that their Texcoco strains (Sosa 6, Sosa 18, 6 Mx, 3 Mx) are related to our strains D0884 and D0890, which have the same origin (Table 1). In this case, their separation of Mexican strains and Chad strain C1 into two genotypes would coincide with our observations.

The two sequenced strains which were used to choose the restriction enzymes, PCC 7345 (D0911) and PCC 8005 (D0914), belong to different clusters and appear thus representative of the whole diversity of the studied *Arthrospira* strains. Thus, the 16S rRNA gene and ITS sequence similarities between

the clusters I and II are probably close to the values of 99.7 and 83.6%, respectively [6].

The cultures from SAC and Earthrise Farms (D0899, D0900, D0923 and D0925) fall into different clusters, but have ARDRA patterns identical to those of other culture collection strains. This is to be expected since culture collection facilities are both sources and recipients of these commercial grown strains.

Inside cluster I, a subgroup of two cultures could be distinguished and is designated as 'subcluster IB'. Strains PCC8005 (D0914) and *A. indica* MCRC isolate straight had *MseI* patterns, which were different from the other members of cluster I ('subcluster IA'). The precise origin of strain PCC8005 has been lost, though it was known that it was included in a group of four strains given by N. Jeeji-Bai to the PCC (<http://www.pasteur.fr/Bio/PCC>). It seems possible that both these strains are in fact cryptic duplicates.

The ITS clusters do not appear to have any well-defined geographical distribution and overlap each other in a rather intriguing way. Cluster I contains strains from Chad, Ethiopia, Kenya, Madagascar, India, and Peru, whereas cluster II includes other strains from Chad, Ethiopia, Kenya, India, California, Mexico, Peru, China, and Spain. Concerning the taxonomic assignment of the strains, strains assigned to the species *A. platensis*, *A. maxima*, *A. indica* and *A. fusiformis* can be found in both clusters. In addition, morphological observations revealed that straight and helical morphotypes can be found in any of the two clusters. To a certain extent, the wide geographic distribution of the ITS clusters can be explained by migration of water birds (flamingoes, pelicans, ibis, ducks...) between lakes. However, the geographic overlaps and the apparently quite random distribution of both clusters are difficult to explain.

The conservation of the 16S rRNA sequences in the *Arthrospira* strains from four continents is remarkable. Such cosmopolitan distribution has been found for marine planktic cyanobacteria [17], *Microcoleus chthonoplastes* growing in the microbial mats of intertidal zones or pools and lagunas close to the sea [18], and marine filamentous strains with narrow trichomes (*Phormidium* or *Leptolyngbya*) [19]. However, these cyanobacteria were oceanic or their habitats could be linked by circulation of seawater. In

the present case, *Arthrospira* strains have very specialized habitats with a patchy distribution.

The ARDRA data from the 16S rRNA genes and the ITS suggest either a very recent divergence or a great genotypic conservatism, as well as complex patterns of geographic spreading. The ARDRA technique does not allow to conclude on the taxonomic status [20] of the studied *Arthrospira* strains, but it seems likely that they belong to one species (based on 16S rRNA gene) or two species (based on the ITS). The use of additional enzymes might allow to find small ITS variations inside the clusters, but would not destroy the observed dichotomy in two clusters. However, more detailed genotypic information (e.g. sequences) would be needed to make firm taxonomic conclusions.

Acknowledgments

Dr. N. Jeeji-Bai (Parry Agro Industries, Madras, India), Dr. A. Sanangelantoni (University of Pavia, Italy), Dr. R. Fox (ACMA, St. Bauzille-de-Putois, France), Dr. H. Lill (University of Osnabrück, Germany) are all thanked for their kind gift of strains and P. Compère (Belgian Botanical Garden, Meise, Belgium) for information on the origin of the strains from Chad. We thank Prof. Dr. R. Matagne (University of Liège) for use of the imaging system, and an anonymous referee for asking for useful clarifications.

References

- [1] Geitler, L. (1932): Cyanophyceae. In: Kryptogamen-Flora von Deutschland, Österreich und der Schweiz, Vol. 14 (Rabenhorst, L., Ed.), pp. 1–1196. Akad. Verlagsgesellschaft, Leipzig.
- [2] Castenholz, R.W. (1989) Subsection III. Order Oscillatoriales. In: Bergey's Manual of Systematic Bacteriology, Vol. 3 (Staley, J.T., Bryant, M.P., Pfennig, N., Holt, J.G., Eds.), pp. 1710–1806. William and Wilkins, Baltimore, MD.
- [3] Tomaselli, L. (1997) Morphology, ultrastructure and taxonomy. In: *Spirulina platensis (Arthrospira)*: Physiology, Cell-Biology and Biotechnology (Vonshak, A., Ed.), pp. 1–15. Taylor and Francis, London, UK.
- [4] Ciferri, O. and Tiboni, O. (1985) The biochemistry and industrial potential of *Spirulina*. *Annu. Rev. Microbiol.* 39, 503–526.
- [5] Belay, A., Ota, Y., Miyakawa, K. and Shimamatsu, H. (1993) Current knowledge on potential health benefits of *Spirulina*. *J. Appl. Phycol.* 5, 235–241.
- [6] Nelissen, B., Wilmotte, A., Neefs, J.-M. and De Wachter, R. (1994) Phylogenetic relationships among filamentous helical cyanobacteria investigated on the basis of 16S ribosomal RNA gene sequence analysis. *Syst. Appl. Microbiol.* 17, 206–210.
- [7] Belay, A. (1997) Mass culture of *Spirulina* outdoors – the Earthrise Farms experience. In: *Spirulina platensis (Arthrospira)*: Physiology, Cell-Biology and Biotechnology (Vonshak, A., Ed.), pp. 131–158. Taylor and Francis, London, UK.
- [8] Vonshak, A. (1997) Outdoor mass production of *Spirulina*: the basic concept. In: *Spirulina platensis (Arthrospira)*: Physiology, Cell-Biology and Biotechnology (Vonshak, A., Ed.), pp. 79–99. Taylor and Francis, London, UK.
- [9] Komárek, J. and Lund, J.W.G. (1990) What is '*Spirulina platensis*' in fact? *Arch. Hydrobiol., Algol. Stud.* 58 (Suppl. 85), 1–13.
- [10] Desikachary, T.V. and Jeeji-Bai, N. (1996) Taxonomic studies in *Spirulina* II. The identification of *Arthrospira* ('*Spirulina*') strains and natural samples of different geographical origins. *Algol. Stud.* 83, 163–178.
- [11] Vinuesa, P., Rademaker, J.L.W., De Bruijn, F.J. and Werner, D. (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S–23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Appl. Environ. Microbiol.* 64, 2096–2104.
- [12] Lu, W., Evans, H., McColl, S.M. and Saunders, V. (1997) Identification of cyanobacteria by polymorphisms of PCR-amplified ribosomal DNA spacer region. *FEMS Microbiol. Lett.* 153, 141–149.
- [13] Schlösser, U.G. (1994) SAG-Sammlung von Algenkulturen at the University of Göttingen. Catalogue of Strains 1994. *Bot. Acta* 107, 113–186.
- [14] Wilmotte, A., Van der Auwera, G., De Wachter, R. (1993) Structure of the 16S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF ('*Mastigocladus laminosus* HTF') strain PCC7518, and phylogenetic analysis. *FEBS Lett.* 317, 96–100.
- [15] Heery, D.M., Gannon, F. and Powell, R. (1990) A simple method for subcloning DNA fragments from gel slices. *Trends Genet.* 6, 173.
- [16] Viti, C., Ventura, S., Lotti, F., Capolino, E., Tomaselli, L. and Giovannetti, L. (1997) Genotypic diversity and typing of cyanobacterial strains of the genus *Arthrospira* by very sensitive total DNA restriction profile analysis. *Res. Microbiol.* 148, 605–611.
- [17] Mullins, T., Britschgi, T.B., Krest, R.L. and Giovannoni, S.J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol. Oceanogr.* 40, 148–158.
- [18] Garcia-Pichel, F., Prufert-Bebout and Muyzer, G. (1996) Phenotypic and phylogenetic analyses show *Microcoleus chthono-*

- plastes* to be a cosmopolitan cyanobacterium. *Appl. Environ. Microbiol.* 62, 3284–3291.
- [19] Wilmotte, A., Stam, W. and Demoulin, V. (1997) Taxonomic study of marine oscillatoriacean strains (Cyanophyceae, Cyanobacteria) with narrow trichomes. III. DNA–DNA hybridization studies and taxonomic conclusions. *Algol. Stud.* 87, 11–28.
- [20] Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K. and De Vos, P. (1996) Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J. Microbiol. Methods* 26, 247–259.