Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
Status: Postprint (Auhtor's version)

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

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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 23 S 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 (*Eco*RV, *Hha*I, *HinH, Mse*I) 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.

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

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 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 taxonom-ic 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 re-latedness 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 $tRNA^{Ala}$).

Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
Status: Postprint (Auhtor's version)

Table 1 : *List of strains, origin, and morphology*

Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
Arthrospira maxima	CCAP 1475/9	Natron lake, Chad	H	D0873	CCAP	ATCC 53871; Lefevre 1963/ M132-1; SAG 84.79; UTEX 2342
Arthrospira maxima	SAG 84.79	Natron lake, Chad	H	D0879	SAG	
Arthrospira maxima	Lefevre 1963/M132-1	Natron lake, Chad	H	D0903	CCALA	
Arthrospira platensis	SAG 85.79	Natron lake, Chad	H	D0880	SAG	Laporte 1963/M132-2b; NIVA CYA 120; UTEX 2340
Arthrospira sp. ('*platensis*')	Laporte 1963/M 132-2b	Natron lake, Chad	H	D0906/H	CCALA	
Arthrospira sp. ('*platensis*')	Laporte 1963/M 132-2b	Natron lake, Chad	S	D0906/S	CCALA	
Arthrospira platensis	SP-4	Natron lake, Chad	S	D0887	Durham	
Arthrospira platensis	SAG 85.79 (Lill)	Natron lake, Chad	H		H. Lill	
Arthrospira platensis	SAG 86.79	Natron lake, Chad	S	D0882	SAG	Compère 86/79
Arthrospira platensis	SAG 86.79 (Lill)	Natron lake, Chad	S		H. Lill	
Arthrospira sp.	C1	Lake Bodou, Kanem, Chad	H (S)	D0918	A. Sanang-elantoni	
Arthrospira sp.	SP-8	Lake Simbi, Kenya	H	D0891	Durham	
Arthrospira maxima	CCAP 1406/2	Lake Naivasha, Kenya	H	D0867	CCAP	
Arthrospira fusiformis	CCAP 1475/8	Lake Chitu, Ethiopia	H	D0872/H	CCAP	
Arthrospira fusiformis	CCAP 1475/8	Lake Chitu, Ethiopia	S	D0872/S	CCAP	
Arthrospira 'Lonar'		Lonar Lake, Maharashtra, India	H	D0920	R. Fox	
Arthrospira Titicaca'		Lake Titicaca, Peru	H	D0922	R. Fox	
Arthrospira sp.	SP-14	Unknown	H	D0897	Durham	
Arthrospira sp.	SP-16	Unknown	H	D0899	SAC	
Arthrospira sp.	SP-17	Unknown	H	D0900	SAC	
Arthrospira sp.	Strain EF-18A	Unknown	H	D0925	Earthrise Farms	
Arthrospira sp.	PCC 9223	Lake Santa Olalla, Donana National Park, Spain	H	D0933	PCC	
Arthrospira sp.	PCC 7939	India, Kenya, Mexico or Peru	H	D0912	PCC	Records lost at PCC
Arthrospira sp.	PCC 7940	India, Kenya, Mexico or Peru	H	D0913	PCC	Records lost at PCC
Arthrospira sp.	PCC 8005	India, Kenya, Mexico or Peru	H	D0914	PCC	Records lost at PCC
Arthrospira indica	MCRC isolate straight	MCRC, Madras, India	S		N. Jeeji-Bai	

Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
Status: Postprint (Auhtor's version)

Table 1 continued

Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
Arthrospira sp. ('*platensis*')	Compere 86.79	Natron lake, Chad	H	D0905	CCALA	SAG 86.79
Arthrospira sp. ('*platensis*')	Compere 1968/3786	Lake Bodou, Kanem, Chad	H	D0904	CCALA	
Arthrospira sp. ('*platensis*')	Leonard and Wouters 1968	Lake Bodou, Kanem, Chad	H	D0907	CCALA	
Arthrospira platensis	SP-2	Lake Chad, Chad	H	D0885/H1	Durham	NIES-39; IAM M-135
Arthrospira platensis	SP-2	Lake Chad, Chad	H(S)	D0885/H2	Durham	
Arthrospira fusiformis	Hindak 1985/1	Lake Arenguade, Ethiopia	H	D0909	CCALA	
Arthrospira fusiformis	Hegewald 1976/83	Lake Nakuru, Kenya	S	D0910/S1	CCALA	
Arthrospira fusiformis	Hegewald 1976/83	Lake Nakuru, Kenya	S	D0910/S2	CCALA	
Arthrospira fusiformis	Hegewald 1976/83	Lake Nakuru, Kenya	H	D0910/H	CCALA	
Arthrospira platensis	UTEX 1926	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0875	UTEX	PCC 7345; UTEX 1928; ATCC 29408
Arthrospira platensis	UTEX 1928	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0876	UTEX	
Arthrospira platensis	PCC 7345	Saline marsh Del Mar Slough, San Diego Co., CA, USA	H	D0911	PCC	
Arthrospira platensis	SAG 257.80	Laguna Huacachina, lea, Peru	S	D0881	SAG	Hegewald 1977/229
Arthrospira platensis	SAG 257.80 (Lill)	Laguna Huacachina, lea, Peru	S		H. Lill	
Arthrospira 'crater'		Lake in volcano crater, Queretaro, Mexico	H	D0919	R. Fox	
Arthrospira 'Orovilca'		Lake Orovilca, lea, Peru	H	D0921	R. Fox	
Arthrospira platensis	SP-1	Lake Texcoco, Mexico	H	D0884	Durham	NIES-46; IAM M-185
Arthrospira sp.	SP-7	Lake Texcoco, Mexico	H	D0890	Durham	
Arthrospira sp	PCC 9108	Commercial culture facility, Chenghai, Yunnan, China	H	D0916	PCC	
Strain designation	Strain number	Origin	Morphology	Durham number	Source	Clones in other collections
Arthrospira platensis	Berhampur	Berhampur, India	H	D0930	N. Jeeji-Bai	
Arthrospira indica	MCRC isolate spiral	MCRC, Madras, India	H	D0929	N. Jeeji-Bai	
Arthrospira sp.	SP-13	Unknown	H	D0896	Durham	
Arthrospira sp.	SP-12	Unknown	H	D0895	Durham	

Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
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Arthrospira sp.	PCC 8006	India, Kenya, Mexico or Peru	H	D0915	PCC	Records lost at PCC
Arthrospira sp.	Strain EF-2	Unknown	H	D0923	Earthrise Farms	
Spirulina laxissima	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. Muhling and B.A. Whitton); S1/S2, differences in length of filament (unpublished results, M. Scott, M. Mühling 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 Universitât Gδttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

As many *Arthrospira* strains as possible were obtained from culture collections and other laboratories. Digestions by *EcoRN, Hinfl, Ndell,* and *Taql* 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 (AR-DRA) 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 (10-40 µE m^{-2} 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 (16000xg, 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 µg μl^{-1} proteinase K in 1 × 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 × *g,* 5 min) was performed prior to PCR or storing the samples at -20°C.

2.3. PCR of 16 S rRNA plus ITS

In a total volume of 50 µl, 0.5 µl of lysis mixture or DNA extraction was added to 1 × PCR buffer of Super Taq Plus, 0.2 mM dNTPs, 0.4 µM primer 16S5T, 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 1XTAE 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 minicol-umn 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.

Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
Status: Postprint (Auhtor's version)

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 16S5T 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 × reaction buffer and 5 U of the restriction enzymes *Eco*RV, *Hha*I, *Hinf*I or *Mse*I (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 µg 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//*Hae*III 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.med kem.gu.se/cutter/) and the two published sequences [6], we could only find four discriminative restriction enzymes giving usable patterns. Other enzymes chosen at random were tested in case the two published sequences were not representative of the overall diversity of the studied strains. *Nde*II and *Msp*I did not cut at all the ITS of 15 strains chosen at random, *Rsa*I, *BsiZ*I and *Ava*II cut only in conserved areas and did not differentiate the strains, *Taq*I gave too slight differences in band lengths to allow a reliable analysis, *Alu*I gave too small fragments (under 100 bases) to be visible, and *Hae*III gave partial digests which did not allow a good and reproducible analysis.

Published in: FEMS Microbiology Letters (1999), vol. 172, iss. 2, pp. 213-222
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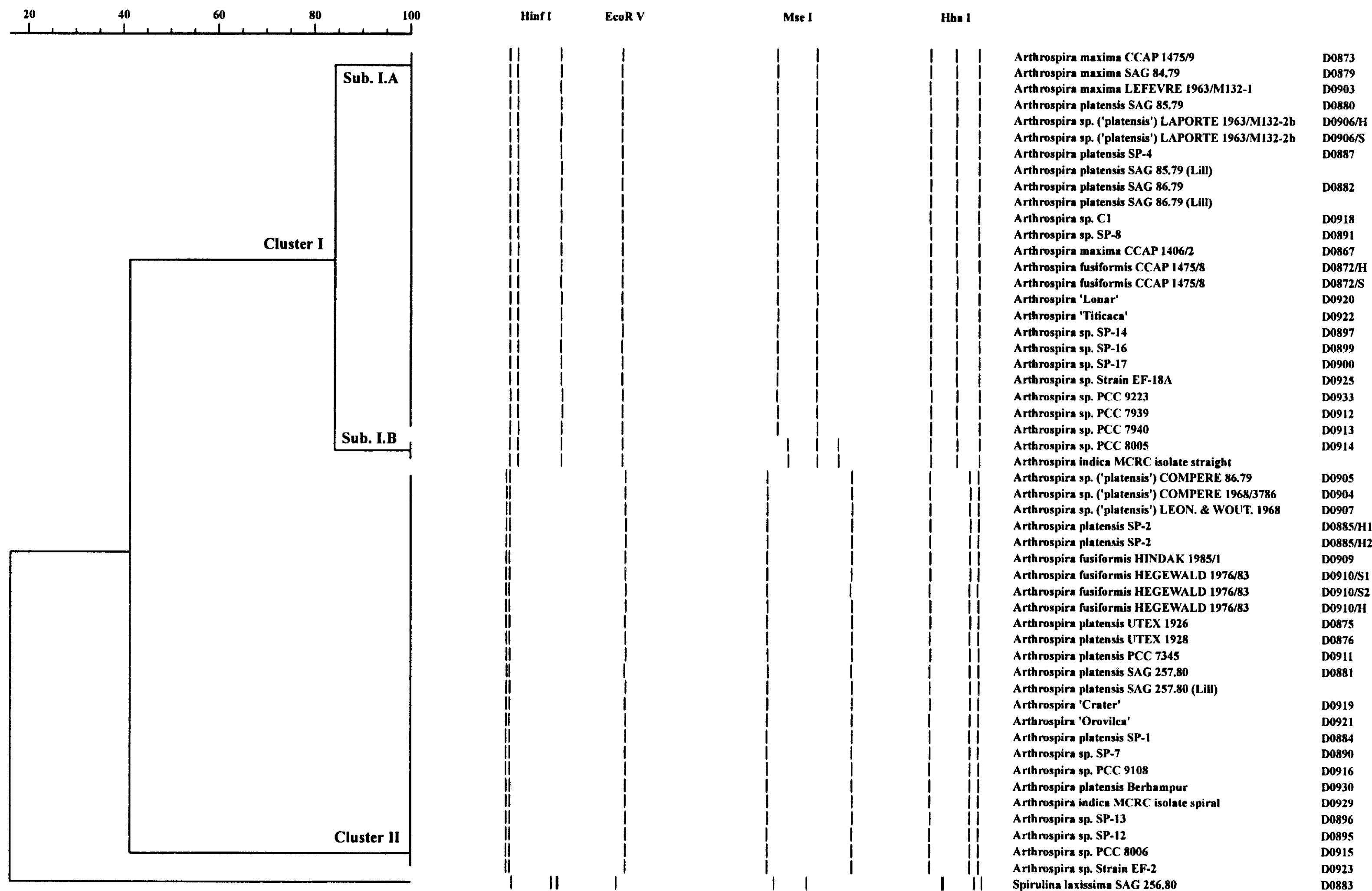
Fig. 1. *Dendrogram built by UPGMA clustering of Dice correlation coefficients of normalized combined ARDRA patterns of ITS for 51 Arthrospira and one Spirulina cultures obtained with the restriction enzyme combination Eco*RV, *Hha*I, *Hinf*I, *and Mse*I. The schematic representation of the four ARDRA 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 *Hinf*I-cluster I: 260, 214, 67; *Hinf*I-cluster II: 281, 260; *Eco*RV-cluster I: 499; *Eco*RV-cluster II: 540; *Hha*I-cluster I: 290, 162, 88; *Hha*I-cluster II: 290, 113, 88; *Mse*I-subcluster IA: 389, 151; *Mse*I-subcluster IB: 305, 151, 84; *Mse*I-cluster II: 490, 52 bases.



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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, DO 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 out-group. 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 Tex-coco 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 CI 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 *Msel* 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], *Micro-coleus 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 Osnabriick, 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.

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