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REMARKABLE CONSERVATION OF INTERNALLY TRANSCRIBED SPACER SEQUENCES OF *ARTHROSPIRA* ("*SPIRULINA*") (CYANOPHYCEAE, CYANOBACTERIA) STRAINS FROM FOUR CONTINENTS AND OF RECENT AND 30-YEAR-OLD DRIED SAMPLES FROM AFRICA[1]

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The internally transcribed spacer (ITS) sequences of 21 *Arthrospira* clonal strains from four continents and assigned to four different species (*A. platensis*, *A. maxima*, *A. fusiformis*, *A. indica*) in the culture collections were determined. Two main clusters, I and II, were differentiated by 49 positions out of 475 nt or 477 nt, respectively. Each cluster was further subdivided into two subclusters. Subclusters I.A and I.B were separated by two substitutions, whereas subclusters II.A and II.B were distinguished by four substitutions. After direct sequencing of the PCR products, three dried samples from Chad aged between 3 months and 35 years yielded a sequence belonging to subcluster I.A, as did a recent commercial product. The strains grown in production plants belonged to the same (sub)clusters as strains from culture collections, mainly I.A and II. PCR primers specific for each cluster and subcluster were designed and tested with crude cell lysates of *Arthrospira* strains. One dried sample ("dihé" 1) and a herbarium sample from Lake Sonachi (Kenya) only contained I.A sequences, whereas the commercial product was a mixture of the four genotypes and the other two dried samples contained minor polymorphisms characteristic of different clusters. Five clonal *Arthrospira* strains, thought to be duplicates, showed the simultaneous presence of the two forms of the four diagnostic positions that distinguish subclusters genotype II.A and genotype II.B. This is likely to be caused by multiple copies of the rDNA operon, in a intermediate stage of homogenization between subcluster II.A and subcluster II.B. The high conservation of ITS sequences is in contrast with the assignment to four different species, the great morphological variability of the strains, and their wide geographic distribution.

Key index words:* *Arthrospira*; "dihé"; evolution; ITS; molecular taxonomy; rRNA; sequences; *Spirulina

The genus *Arthrospira* Stizenberg includes filamentous cyanobacteria characterized by loosely coiled trichomes of 3 to 12 μm width with cross-walls visible in light microscopy (Castenholz et al. 2001). The species studied in this article grow in tropical and subtropical water bodies with high carbonate and bicarbonate content and elevated pH (Tomaselli 1997) but can also be found in marine habitats. Members of this genus were often identified as "*Spirulina*," a concept that dates back to the merging of *Arthrospira* and *Spirulina* Turpin carried out by Geitler in 1932. In the last 20 years, it has been shown that both genera were very distinct at the ultrastructural (Guglielmi and Cohen-Bazire 1984) and genetic level: percentage of GC (Herdman et al. 1979) and 16S rDNA sequences (Nelissen et al. 1994).

Arthrospira spp. were shown to be particularly rich in amino acids, gamma-linolenic acid, and pigments (Ciferri and Tiboni 1985, Belay et al. 1993). Their nutritional potential is used by local populations in the vicinity of Lake Chad. A number of small lakes in this region support dense seasonal development of *Arthrospira*. Local Chad women harvest and sun-dry the *Arthrospira* masses to make "dihé," which is sold at markets and used in cooking (sauces). In the 1960s, the potential of *Arthrospira* species as a food supplement was rediscovered and scientifically supported (Léonard and Compère 1967, Iltis 1971, Doumenge et al. 1993). Presently, many production plants produce *Arthrospira* on a large scale for animal and human diet under the name *Spirulina* (Belay 1997, Vonshak 1997).

The taxonomy of the genus *Arthrospira* is quite confused, and at least 12 binomials are currently recognized: *A. funiformis*, *A. fusiformis*, *A. geitleri*, *A. gomontiana*, *A. indica*, *A. jenneri*, *A. khannae*, *A. massartii*, *A. maxima*, *A. miniata*, *A. platensis*, and *A. tenuis* (Jeeji-Bai 1999). However, different interpretations were given to their de-

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scriptions, and these species are difficult to distinguish (Komárek and Lund 1990, Desikatchary and Jeeji-Bai 1996, Tomaselli 1997). The origin of these problems is the morphological variability shown in nature and in culture by many taxa. This plasticity is especially striking in the degree of spiralization and the arrangement of the spirals. In particular, the spontaneous appearance of straight trichomes in a previously helical strain culture is a well-documented phenomenon (Jeeji-Bai 1985, Tomaselli 1997).

Because of nutritional interest, a number of strains from four continents have been deposited in culture collections and used in laboratories or cultivation plants throughout the world. We were interested in determining their degree of genotypic relatedness and in comparing these data with their geographical origin and their taxonomic assignment in the collections. A morphological study was separately performed at the University of Durham (Mühling et al., unpublished data).

An ARDRA (Amplified Ribosomal DNA Restriction Analysis) study was previously carried out on the internally transcribed spacer (ITS) of 37 cultivated clonal strains from four continents (Scheldeman et al. 1999). The data showed that all these strains were closely related. Only two different major restriction patterns were found, defining two clusters I and II. Inside cluster I, two strains composed a small subcluster labeled I.B as opposed to the other cluster I strains, named I.A (Scheldeman et al. 1999). In the present study, we amplified and determined the full ITS sequence of 21 strains, representative of different ARDRA clusters, habitats, and identifications. In addition, ITS sequences were obtained for one commercial product and three Chad dried samples, including one dihé bought 30 years ago at a local market (dihé 1) and a 35-year-old sun-dried sample (3832).

Using the new sequence data we designed and tested PCR primers to specifically detect each cluster and subcluster, starting from crude cell lysates or genomic DNA. These primers should be useful to rapidly assess the affiliation of new strains to the two clusters and/or four subclusters and for purity controls of commercial powders.

MATERIALS AND METHODS

List of strains and dried samples and culture conditions of strains. Forty *Arthrospira* clonal strains used in this work are listed in Table 1. The five dried samples are listed in Table 2. The origin and characteristics of the dried sample 3832 have been described in detail by Léonard and Compère (1967): it corresponds to an almost pure sample of *A. platensis*. The specimen of *Cyperus laevigatus* L. was covered by a bloom of *Arthrospira* at the time of harvest, and the green powder was scratched from the stems. Strains were cultivated in Spirulina medium (Schlösser 1994) as 5-mL stock cultures kept under low light (10 to 40 $\mu E \cdot m^{-2} \cdot s^{-1}$) at a constant temperature of 25° C.

DNA isolation. Crude cell lysates of living strains were prepared as previously described (Scheldeman et al. 1999). A genomic DNA extraction method modified from Pitcher et al. (1989) was used for the dried samples and commercial product. About 25 mg of dried material was washed twice with ethanol to remove lipophilic pigments. The pellet was washed twice with 1 mL of RS buffer (0.15 M NaCl and 0.01 M EDTA, pH 8.0). After a 30-min incubation at 37° C with 100 μL of lysozyme ($50 mg \cdot mL^{-1}$), 2.5 μL of a proteinase K solution ($10 mg \cdot mL^{-1}$) was added to the mix, before a new incubation at 37° C for 1 h. Cell lysis was achieved by a last incubation at 37° C for 15 min in the presence of 500 μL GES solution (60% wt/vol guanidium thiocyanate, 0.1 M EDTA, pH 8.0, and 1% wt/vol sarkosyl). This mix was then kept on ice for 10 min, before sequential addition of 150 μL 5 M NaCl solution and of 250 μL of a cold 7.5 M ammonium acetate solution and further incubation on ice for 10 min. A first extraction with 500 μL of phenol:chloroform:isoamylalcohol (25:24:1) solution was followed by an extraction with chloroform:isoamylalcohol (24:1). The nucleic acids were precipitated with ethanol (Sambrook et al. 1989) and redissolved overnight at 4° C in 100 μL of a TE solution (10 mM Tris-HCl pH 7.4, 0.1 mM EDTA pH 8.0). The sample was incubated for 1 h at 37° C in the presence of 1 μL of RNase ($10 mg \cdot mL^{-1}$) and then stored at −20° C.

PCR conditions for direct sequencing and for PCR detection with specific primers. For sequence determination, the ITS was amplified from crude cell lysates of living strains as previously described (Scheldeman et al. 1999). The forward primer 16S3′F was situated at positions 1522–1541 of the 16S rDNA and the reverse primer 23S5′R (primer 18 of Wilmotte et al. 1993 without the *Not* I site) was located at positions 26–44 of the 23S rDNA (*Escherichia coli* numbering; Table 3). In the case of the dried samples and commercial product, the template was purified genomic DNA instead of cell lysates.

For the PCR detections with specific primers, 1 μL of cell lysate was used as template for the strains and 1 μL of genomic DNA for the dried samples and product. The reaction mixture contained 0.2 mM dNTPs, 0.4 μM cluster- or subcluster-specific primer (Table 3), 0.4 μM 16S3′F or Long 23S5′R primer (*E. coli* positions 20–44), $1 mg \cdot mL^{-1}$ BSA, 1× Super Taq Plus buffer, and 1 U Super Taq Plus Polymerase (HT Biotechnology, Cambridge, UK) in a total volume of 50 μL. For the cluster PCR detections, the following program was used in a GeneCycler (Bio-Rad, Melville, NY, USA): 3 min at 94° C for 1 cycle; 10 cycles of 45 s at 94° C, and 2.5 min at 68° C, followed by 20 cycles of 45 s at 90° C, 45 s at 62° C, and 2 min at 68° C. A final elongation step of 7 min at 68° C was performed. In the case of the subcluster PCR detections, the cycling program was identical, except for an annealing temperature of 65° C in the 20-cycle-long round. Primer positions and product sizes are shown in Figure 1. PCR products were visualized on a UV transilluminator after standard TBE agarose (2%) electrophoresis and ethidium bromide staining (Sambrook et al. 1989).

Sequence determination and analysis. Each PCR product was sequenced on both strands by the primers 16S3′F and 23S5′R on an ABI PRISM system 377 (PE Applied Biosystems, Foster City, CA, USA) by Genome Express (Paris, France). In the case of dried samples and commercial product, each sample was independently processed and both strands were sequenced twice. In addition, to test the cluster-specific primers, the samples were used again for a second DNA extraction 3 years later, and a negative control was used to monitor the absence of cross-contamination during extractions.

Whenever dried samples and the commercial product gave PCR products with specific primers that did not match the cluster expected on the basis of direct sequencing, these products were further sequenced with the primers 16S3′F or Long 23S5′R. In the case of 3832, a reamplification was necessary before sequencing because the band was very faint.

Nucleotide sequences were introduced in the GCG (Genetics Computer Group) software package (available at the Belgian EMBL Node, ULB/VUB) and assembled in consensus sequences using their overlapping segments. Final consensus ITS sequences were aligned with the MAP alignment tool available online at the Human Genome Center (Baylor College of Medicine, Houston, TX, USA) and processed by the TREECON for Windows v1.2 software package (Van de Peer and De Wachter 1994). The distance matrix was computed using the Jukes and Cantor (1969) correction for dissimilarity values, followed by the construction of a tree with the neighbor-joining method (Saitou and Nei 1987). These analyses were performed on full sequences, including $tRNA^{Ile}$ and $tRNA^{Ala}$, but insertions and deletions were not used. The sequences were given the accession numbers AJ292321 to AJ292341 in the EMBL Nucleotide Sequence Database.

TABLE 1. List of strains, origin, number in the Durham collection, and results obtained by the ARDRA method (Scheldeman et al 1999) sequence analysis of the ITS, and cluster-specific PCR detection

Strain name	Strain[a]	Origin	Durham number	ARDRA	ITS sequencing	PCR detection
Arthrospira sp.	EF-18A	EF, CA, USA	D0925	I.A	I.A	I.A
Arthrospira sp.	SP-16	SAC, Thailand	D0899	I.A	I.A	nd[b]
Arthrospira sp.	'Titicaca'	Lake Titicaca, Peru	D0922	I.A	I.A	I.A
Arthrospira sp.	PCC 9223	Lake Santa Olalla, Donana National Park, Spain	D0933	I.A	I.A	nd
Arthrospira sp.	C1	Lake Bodou, Kanem, Chad	D0918	I.A	I.A	I.A
Arthrospira sp.	SP-10	Madagascar	D0893	I.A[c]	I.A	nd
Arthrospira maxima	SAG 84.79	Natron Lake, Chad	D0879	I.A	I.A	nd
Arthrospira maxima	LEFEVRE 1963/M132-1	Natron Lake, Chad	D0903	I.A	nd	I[d]
Arthrospira fusiformis	CCAP 1475/8	Lake Chitu, Ethiopia	D0872/H[e]	I.A	I.A	nd
Arthrospira fusiformis	CCAP 1475/8	Lake Chitu, Ethiopia	D0872/S[f]	I.A	I.A	nd
Arthrospira sp.	SP-17	SAC, Thailand	D0900	I.A	nd	I.A
Arthrospira sp.	PCC 7939	India, Kenya, Mexico or Peru	D0912	I.A	nd	I.A
Arthrospira sp.	SP-15	unknown		nd	nd	I.A
Arthrospira sp.	SP-18	SAC, Thailand		nd	nd	I.A
Arthrospira sp.	SP-19	SAC, Thailand		nd	nd	I.A
Arthrospira indica	MCRC isolate straight	MCRC, Madras, India		I.B	I.B	I.B
Arthrospira sp.	PCC 8005	India, Kenya, Mexico or Peru	D0914	I.B	I.B	I.B
Arthrospira platensis	COMPERE 86.79	Natron lake, Chad	D0905	II	II.A	II.A
Arthrospira platensis	SP-1	Lake Texcoco, Mexico	D0884	II	II.A	II.A
Arthrospira sp.	PCC 9108	Commercial culture facility, Cheng-hai, Yunnan, China	D0916	II	II.A	II.A
Arthrospira platensis	SP-2	Lake Chad, Chad	D0885/H1[g]	II	nd	II.A
Arthrospira platensis	SP-2	Lake Chad, Chad	D0885/H2[g]	II	nd	II.A
Arthrospira sp.	SP-7	Lake Texcoco, Mexico	D0890	II	nd	II.A
Arthrospira platensis	COMPERE 1968/3786	Lake Bodou, Kanem, Chad	D0904	II	nd	II.A
Arthrospira platensis	LEONARD & WOUTERS 1968	Lake Bodou, Kanem, Chad	D0907	II	nd	II.A
Arthrospira fusiformis	HINDAK 1985/1	Lake Arenguade, Ethiopia	D0909	II	nd	II.A
Arthrospira sp.	'crater'	Lake in volcano crater, Queretaro, Mexico	D0919	II	nd	II.A
Arthrospira sp.	'Orovilca'	Lake Orovilca, Ica, Peru	D0921	II	nd	II.A
Arthrospira platensis	'Berhampur'	Berhampur, India	D0930	II	nd	II.A
Arthrospira platensis	*SAG 257.80 (Lill)*	*Laguna Huacachina, Ica, Peru*		*II*	*II.B*	*II.A/II.B*
Arthrospira platensis	SAG 257.80	Laguna Huacachina, Ica, Peru	D0881	II	nd	II.A/II.B
Arthrospira indica	MCRC isolate spiral	MCRC, Madras, India	D0929	II	II.B	II.B
Arthrospira fusiformis	HEGEWALD 1976/83	Lake Nakuru, Kenya	D0910/S1[h]	II	II.B	II.B
Arthrospira fusiformis	HEGEWALD 1976/83	Lake Nakuru, Kenya	D0910/S2[h]	II	II.B	II.B
Arthrospira sp.	PCC 8006	India, Kenya, Mexico or Peru	D0915	II	nd	II.B
Arthrospira platensis	UTEX 1926	Saline marsh Del Mar Slough, San Diego Co., CA, USA	D0875	II	nd	II.A/II.B
Arthrospira platensis	UTEX 1928	Saline marsh Del Mar Slough, San Diego Co., CA, USA	D0876	II	II.A/II.B	II.A/II.B
Arthrospira platensis	PCC 7345	Saline marsh Del Mar Slough, San Diego Co., CA, USA	D0911	II	II.A/II.B	II.A/II.B
Arthrospira sp.	SP-12	Proteus, CA, USA	D0895	II	nd	II.A/II.B
Arthrospira sp.	EF-2	EF, CA, USA	D0923	II	II.A/II.B	II.A/II.B

A solid line separates the clusters I and II. Dash lines separate known duplicates, which are strains having the same origin but kept separately in culture collections or laboratories. Morphotypes (H, S, H1, H2, S1, S2) of the same strain are not separated by horizontal lines.

[a] CCAP, Culture Collection of Algae and Protozoa, Ambleside, Cumbria, England, UK; EF, Earthrise Farms, Calipatria, CA, USA; MCRC, Shri Amm Murugappa Chettiar Research Centre, Madras, India; PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; Proteus, Previous company on the site of Earthrise Farms; SAC, Siam Algae Co., LTD, Samutprakarn, Thailand; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, Texas, USA.

[b] nd, not done.

[c] Data not shown.

[d] PCR for subcluster affiliation not performed.

[e] H, helical filaments; [f] S, straight filaments.

[g] H1/H2, differences in length of the helix pitch, helix diameter, and trichome diameter (M. Scott, M. Mühling and B. A. Whitton, unpublished results).

[h] S1/S2, differences in length of filament (M. Scott, M. Mühling and B. A. Whitton, unpublished results).

TABLE 2. List of dried samples (including one commercial product), origin, and results obtained by the ARDRA method (data not shown), sequence analysis of the ITS, and cluster-specific PCR detection.

Sample designation and origin	ARDRA	ITS sequencing	PCR detection
Commercial product (pill) (Marcus Rohrer, the Netherlands)	I.A	I.A	I.A, I.B, II.A, II.B
dihé Q+ (N′garangou market, originating from Latir, 19/03/1998; gift of Dr. Brouers)	nd[a]	I.A	I.A, I.B[b]
Dried sample 3832 (Lake Yoan, Ounianga Kébir, Northern Chad, 12/1964; Léonard & Compère, 1967)	I.A	I.A	I.A, I.B[b]
dihé 1 (Bol market, Chad, 1970; gift of Dr. Fox)	I.A	I.A	I.A
Cyperus laevigatus L., Herbarium Universitatis Gandavensis, CC 443 (near the shore of Lake Sonachi, Kenya, 13/04/1999; gift of the collector, Dr. C. Cocquyt)	nd	nd	I.A

[a] nd, not done.
[b] Faint band.

RESULTS AND DISCUSSION

ITS sequences from 21 culture collection strains. Our alignment of the ITS sequences from 21 strains (including two different morphotypes of D0872 and D0910) was 477 nucleotides long (Fig. 2). There were two tRNA in the ITS: $tRNA^{Ile}$ (115–187) and $tRNA^{Ala}$ (204–276). The dendrogram inferred from this alignment is shown in Figure 3.

All the ITS sequences fell into two clusters, exactly matching clusters I and II found by ARDRA (Scheldeman et al. 1999). There were 49 base differences between the clusters. Moreover, the division of cluster I into two subclusters (I.A and I.B) obtained by ARDRA was confirmed by the sequences, whereas cluster II was further split into two new subclusters (II.A and II.B). Previously published ITS sequences of PCC 7345 (D0911) and PCC 8005 (D0914) made by manual sequencing (Nelissen et al. 1994) were updated in EMBL using the new data. For PCC 8005 (D0914; X70769), seven nucleotide differences and one insertion/deletion in each sequence were observed and four ambiguities were resolved by the new sequence. For PCC 7345 (D0911; X75044), one nucleotide was added and four positions were deleted in the new sequence. Moreover, four ambiguities were detected (see below) at positions where a cluster II.B sequence had been read by Nelissen et al. (1994).

The dissimilarity between clusters I and II was 10.3% and the polymorphic sites were grouped in only four regions: 18–47, 284–290, 309–342, and 359–398. These regions are underlined in Figure 2. Subclusters I.A and I.B were separated by two consecutive nucleotides: A284 G285 in I.A and T284 T285 in I.B. These nucleotides were part of an *Mse* I restriction site, and this explains why these subclusters had been detected by ARDRA (Scheldeman et al. 1999). Subclusters II.A and II.B were distinguished by four nucleotides situated in two regions: A319 G321 G369 G373 in II.A and G319 T321 A369 C373 in II.B. These subclusters were only revealed by sequence data.

Iteman et al. (2000) inferred the secondary structure of the ITS of several cyanobacteria. The polymorphisms that we detected did not have any impact on the conserved features found by Iteman et al. (2000). The first discriminative region was situated between boxes D1 and D1′, the second and third ones were between $tRNA^{Ala}$ and box B, and the fourth one was located inside box B but the two polymorphic positions (369 and 373) are in the loop of box B that is variable.

Three strains from cluster II (D0876, D0911, and D0923) showed an ITS sequence with four residual ambiguities: R319 K321 R369 S373. These ambiguities exactly matched the four discriminative nucleotides between subclusters II.A and II.B. Thus, it seemed likely that these cultures contained at least two different ITS: one from subcluster II.A and another from subcluster II.B. Such coexistence could not be explained by the mix of different strains because each one had been made clonal before ITS amplification. Hence, this observation probably reflected the presence of several rRNA operons exhibiting a few microheterogeneities. These mutations could either be lost or spread to all

TABLE 3. List of PCR primers, orientation, position relative to the ITS (Fig. 1), targeted discriminative region, and nucleotide sequences (5′ to 3′ direction).

Primer	Orientation	Position	Discriminative region	Sequence
CL I	Reverse	320–346	3	CAG-TCA-CCA-ACT-CTC-AAT-TTC-TCA-A
CL II	Reverse	284–308	2	CTC-ACC-CCA-ACT-AGC-ACC-ATC-ACA-A
CL I SUB A	Reverse	284–308	2	CTC-ACC-CCA-ACT-AGC-ACC-TAA-TTC-T
CL I SUB B	Reverse	284–308	2	CTC-ACC-CCA-ACT-AGC-ACC-TAA-TTA-A
CL II SUB A	Forward	297–321	3	AGT-TGG-GGT-GAG-ATG-AGA-TGA-GAT-G
CL II SUB B	Forward	297–321	3	AGT-TGG-GGT-GAG-ATG-AGA-TGA-GGT-T
16S3′F	Forward	na[a]	na	TGY-GGC-TGG-ATC-ACC-TCC-TT
Long 23S5′R	Reverse	na	na	TCT-GTG-TGC-CTA-GGT-ATC-CAC-CGT-T

[a] na, not applicable (see text).

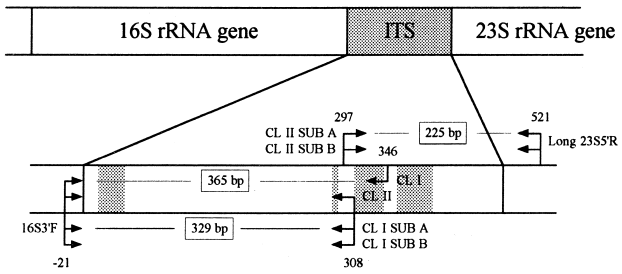

FIG. 1. Scale drawing of a partial *Arthrospira* rRNA operon with an enlarged view of the ITS. PCR primer positions are shown relative to the consensus alignment shown in Figure 2, whereas PCR product sizes (in a box) were calculated for each particular ITS sequence. The gray shaded areas are the four discriminative regions.

operon copies because of gene conversions and homogenizations (Petes and Hill 1988, Hillis et al. 1991). The rRNA operon number is unknown in *Arthrospira*, but 35 operons were found in a five times coverage during genomic sequencing (C.-C. Zhang, personal communication) and strains with up to six rRNA operons have been reported in several cyanobacterial genera (Nichols et al. 1982). Two of the three concerned strains were publicly available duplicates sampled in Californian salt marshes (D0876, D0911), whereas the third one (D0923) came from the Earthrise Farms strain collection (Earthrise Nutritionals Inc., Petaluma, CA, USA). The three strains might be identical, considering that Californian strains could have been easily obtained by Earthrise Farms.

Two strains (D0899 and D0925) shared a transversion near the 3′ end of their ITS: C460 instead of the A460 usually found. This feature has been thoroughly checked by multiple amplifications and sequencing reactions. Because these strains were obtained from related companies, they could be duplicates of each other. It is interesting to speculate that this peculiarity in the ITS could coincide with other divergences elsewhere in the genome, resulting from industrial selection pressure and which might be of advantage in particular mass cultivation conditions. An ambiguity, close to the 3′ end of the $tRNA^{Ile}$, was observed in two strains (D0922 and D0933): R183 instead of the G183 usually found.

Three ITS sequences of *Arthrospira* found in GenBank (Mao et al., unpublished data) belonged to subcluster II.A without any modifications (AF329391), presented the insertion of T between positions 228 and 229 (AF329393), or showed a deletion at position 193 and A instead of T at position 283 (AF329392).

PCR detections of clusters and subclusters with 34 culture collection strains. Using the ITS variations characterizing each cluster and subcluster, we designed six PCR primers specific for each cluster (I and II) and subcluster (I.A, I.B, II.A, and II.B). Each cluster- and subcluster-specific primer was combined with a nonspecific primer (Table 3 and Fig. 1) to obtain a specific primer pair.

The PCR detection conditions for each cluster and subcluster were tested and optimized for crude cell lysates of five sequenced and three unsequenced strains: D0912 and D0918 (I.A), D0914 and *A. indica* MCRC isolate straight (I.B), D0905 and D0930 (II.A), and D0910/S1 and D0915 (II.B). The results were clear-cut, as shown in Figure 4. Then, 23 strains, for which molecular data were available, were tested. These additional results, as well as those of three new subcluster I.A strains for which no molecular data were available (SP-15, SP-18, SP-19), are listed in Table 1.

It was noted that five strains displayed equal amplification products with both specific primers for subclusters II.A and II.B: D0875, D0876, D0895, D0911, and D0923 (data not shown). This could be anticipated for strains D0876, D0911, and D0923, because their ITS sequences showed a mix of both subclusters sequences (see above), and for strain D0875, which is a known duplicate of D0876 (Starr and Zeikus 1993). In the case of D0895, a commercial strain from Proteus, it seems highly probable that this strain was a duplicate of strain D0923 from Earthrise Farms, considering that Proteus was the former name of this company. These five strains could thus correspond to a single genome, with two different ITS types in at least two operons. Whether this peculiarity corresponds to other mutations in the whole genome would be interesting to investigate.

One strain, D0881, unexpectedly showed a double response to primers II.A and II.B. The ITS sequence of this strain clearly belonged to subcluster II.B and gave a strong signal with the PCR primers for this subcluster. However, a faint amplification product was also detected with the primer pair specific for subcluster II.A (data not shown). This result has been repeatedly obtained on multiple cultures from several sources (SAG and Prof. Lill). It could be explained if one rDNA operon belonging to subcluster II.A was present but remained hidden by more abundant copies of subcluster II.B operons during PCR with the nonspecific primers used for sequencing.

The observation of the appearance of straight filaments in spiral cultures has led to discussions about the origin of this phenomenon: contaminations or morphological plasticity (Jeeji-Bai 1985). In our study, the strain D0872, for which two variants, "straight" and "helical," were isolated in clonal culture (M. Scott, M. Mühling, and B. A. Whitton, personal communication), gave identical ITS sequences. This supports the hypothesis that the straight morphotypes arise from the helical one, but not that straight contaminants have invaded the original culture. In the case of the two strains of *A. indica*, MCRC isolate helical and MCRC isolate straight, they were given as separate strains by N. Jeeji-Bai and they displayed ITS sequences characteristic of two different clusters.

ITS sequences from dried samples and the commercial product. When the PCR product was obtained by direct sequencing after PCR with primers 16S3′F and 23S5′R (Figs. 2 and 3), the commercial product and three

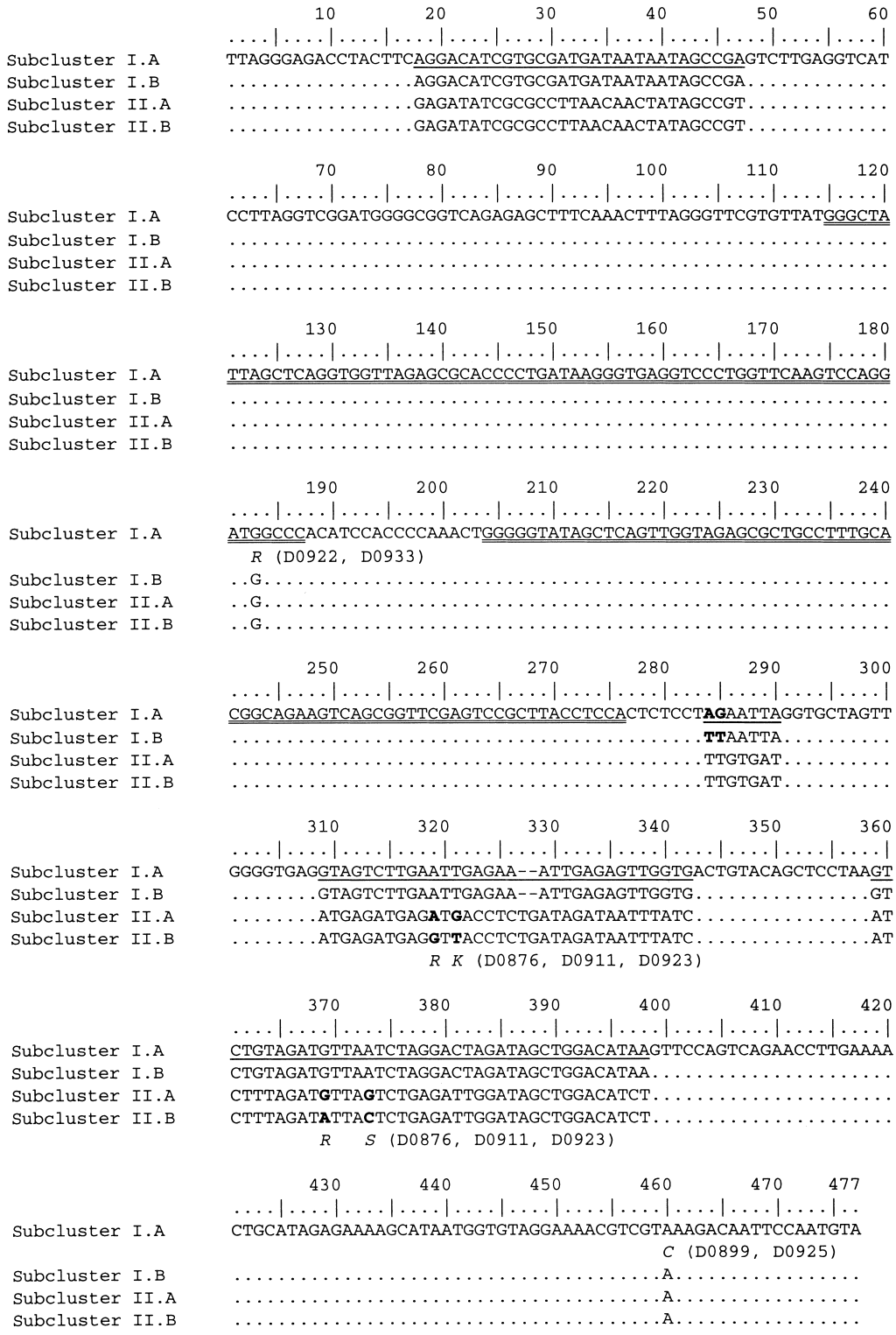

FIG. 2. Consensus alignment of the four subcluster ITS sequences. Identical nucleotides are indicated by one point. $tRNA^{Ile}$ and $tRNA^{Ala}$ are indicated by a double underline, whereas cluster-discriminative segments are simply underlined and subcluster diagnostic nucleotides shown in bold. Strain-specific punctual mutations are shown in italic, just below the closest subcluster sequence, and are followed by the clone (Durham) numbers in brackets.

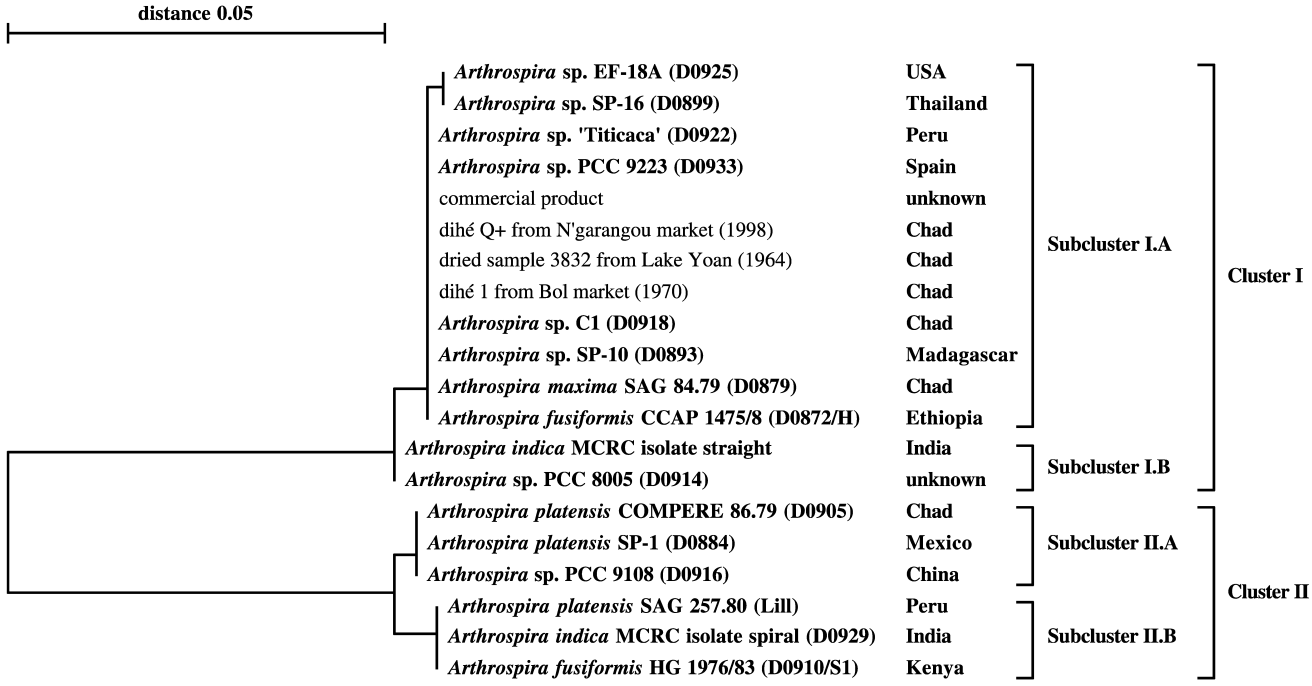

FIG. 3. Dendrogram built by the neighbor-joining method, applied to a distance matrix calculated with the Jukes and Cantor (1969) correction and based on the ITS sequences from 16 living strains, 3 dihés, and 1 commercial product (pill). Strains D0876, D0911, and D0923, which display ambiguities for the positions diagnostic of subclusters II.A and II.B, are not included in the tree. For strains D0872 and D0910, where identical sequences were obtained for two morphotypes, only one is used.

dried samples from Chad showed sequences belonging to subcluster I.A. However, because PCR is a competitive process, we could not rule out the possibility that other minor ITS sequences were outcompeted during amplification. Therefore, additional PCR detections on these genomic DNAs were performed.

PCR detections of clusters and subclusters with dried samples and the commercial product. Results shown in Figure 5 demonstrated that, in addition to a sequence belonging to subcluster I.A, the commercial product contained sequences related to subclusters I.B, II.A, and II.B. The sequence of these bands was in conformity with the sequences expected (data not shown). This is likely to reflect the fact that commercial products could be mixtures of different strains, pooled from several cultivation ponds and plants.

In addition, for the dried samples 3832 and dihé Q+, a weaker band was visible with the primer specific for subcluster I.B (Fig. 5). After sequencing with 16S3′F, it appeared to contain a sequence belonging to cluster II in the portion of the first discriminative region that could be sequenced (positions 28–47), although no product had been detected with primers specifically targeted to cluster II or its subclusters (positions 284–308 and 297–321). Thus, complex samples like our dihés may yield less clear patterns than strains, due to genetic variation in natural populations or to the generation of chimeras during the PCR process. Chimeras might be expected when a mixture of very similar sequences is present in a PCR reaction (Wang and Wang 1997), as in our case.

For complex samples, the formation of chimeras and the genetic heterogeneity limit the precision of the PCR detection method because only the presence of the annealing sites can be ascertained but not the identity of the sequence between the primers. However, the additional polymorphisms detected appeared as weaker bands on the agarose gel and correspond thus to minor components.

Genetic and geographical considerations. The sequence data have given a more detailed characterization of the conspicuous ITS conservation of *Arthrospira* reported by Scheldeman et al. (1999). Such conservation is rather remarkable, because the ITS is a variable evolutionary marker often used to discriminate species or strains (Jensen et al. 1993, Gürtler and Stanisich 1996). Another observation is that no clear relationships could be drawn between our ITS clusters and the strain denominations, their morphologies, or their geographical origins. If our strain collection was representative of the natural diversity of the four *Arthrospira* species studied, we could hypothesize that they correspond to only one or two cosmopolitan species, based on the similarities of the 16S rDNA (Nelissen et al. 1994) and the ITS, respectively. On the basis of the 99.7% similarity of the 16S rDNA of PCC 8005 (D0914) and PCC 7345 (D0911), which belong to subclusters I.B and II.A./II.B, respectively, Castenholz et al. (2001) considered it likely that

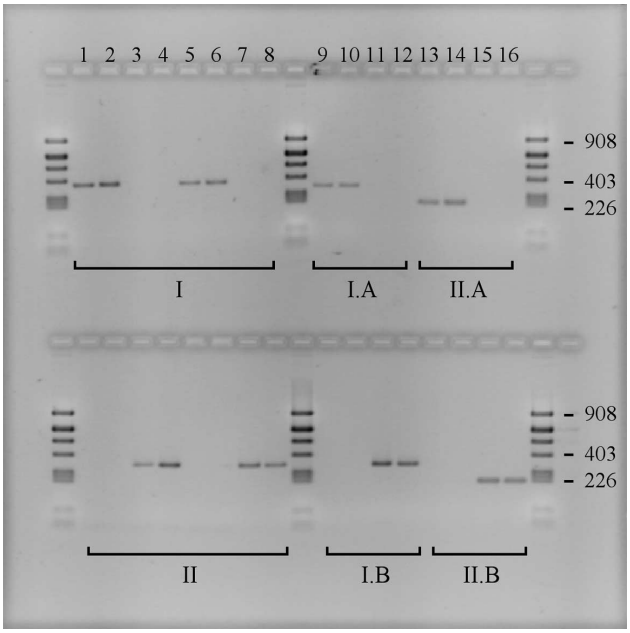

FIG. 4. Agarose gel electrophoresis (2%) of 8 μL of the PCR products obtained by PCR detection of the various clusters and subclusters on the lysates from eight strains. Lanes 1–8, upper half: cluster I primer pair; lanes 1–8, lower half: cluster II primer pair; lanes 9–12, upper half: subcluster I.A primer pair; lanes 9–12, lower half: subcluster I.B primer pair; lanes 13–16, upper half: subcluster II.A primer pair; lanes 13–16, lower half: subcluster II.B primer pair. Lanes 1 and 9: D0918 (I.A); 2 and 11: D0914 (I.B); 3 and 13: D0905 (II.A); 4 and 15: D0910/S1 (II.B); 5 and 10: D0912 (I.A); 6 and 12: *A. indica* MCRC isolate straight (I.B); 7 and 14: D0930 (II.A); 8 and 16: D0915 (II.B). Length marker was pBR322/*Alu* I.

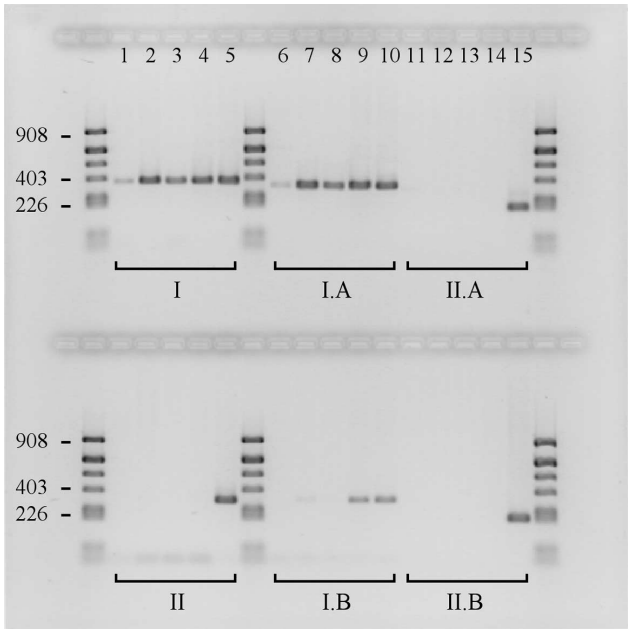

FIG. 5. Agarose gel electrophoresis obtained in the same conditions as Figure 4 on the genomic DNA from four dried samples and a commercial product. Lanes 1–5, upper half: cluster I primer pair; lanes 1–5, lower half: cluster II primer pair; lanes 6–10, upper half: subcluster I.A primer pair; lanes 6–10, lower half: subcluster I.B primer pair; lanes 11–15, upper half: subcluster II.A primer pair; lanes 11–15, lower half: subcluster II.B primer pair. Lanes 1, 6, and 11: dried herbarium specimen from Lake Sonachi (Kenya); 2, 7, and 12: dried sample 3832 (Chad); 3, 8, and 13: dihé 1 (Chad); 4, 9, and 14: dihé Q+ (Chad); 5, 10, and 15: commercial product.

all *Arthrospira* strains represent a unique nomen species. Of course, a DNA/DNA hybridization study would be useful to decide on this taxonomic issue. Note that we also searched without success for strains of Australian origin, although *Arthrospira* species were recorded in natural biotopes on this continent (http://plantnet.rbgsyd.gov.au/PlantNet/fwalgae/; P. Baker, personal communication).

When we combine data from the ARDRA study (Scheldeman et al. 1999) and from the ITS sequence determination, we observe that genotypes I and II were found in strains from the same countries (Chad, Ethiopia, Kenya, India, and Peru) but from different lakes. This could reflect the fact that there was indeed only one genotype in the original lake or that, by chance, only one genotype was isolated from a mixture. The unambiguous ITS sequences from Chad dried samples (natural populations) indicate a dominant or unique genotype I.A in their lakes of origin. This observation was confirmed by PCR detections for Chad dihé 1 and for a herbarium specimen from Lake Sonachi, Kenya. However, in the cases of dried sample 3832 and dihé Q+, a mosaic of both clusters was identified, although only as minor polymorphisms. Thus, we can conclude that the studied natural populations either contained only one detectable genotype or a dominant genotype accompanied by minor ones.

The combination of genetic conservation at the level of 16S rDNA and ITS and cosmopolitan distribution is easier to understand in the case of marine cyanobacteria, like *Microcoleus chthonoplastes*, *Phormidium*, or *Leptolyngbya* spp., which can be transported over long distances by seawater circulation (Mullins et al. 1995, Garcia-Pichel et al. 1996, Wilmotte et al. 1997). In contrast, *Arthrospira* are limited to very specialized habitats, showing a patchy distribution on continents.

Two possible explanations for this result are that, aside from particular characteristics of the speciation process in cyanobacteria (Castenholz 1992), the populations are constantly mixed and homogenized and their habitats are recent, temporary, and fluctuating. Thus, they are never able to establish a population in stable and isolated conditions for very long periods of time.

Concerning the mixing of strains, migratory birds are good candidates to transport them between habitats. Though not typical migrants, flamingo populations tend to move a lot between their habitats, saline or alkaline lakes, lagoons, and coastal zones. They could connect Africa to Europe (Spain) or Asia (India), although transatlantic flight is hazardous and is probably very rare (Cabot et al. 1992). In Chile and Peru, differ-

ent species of flamingos are found in the Altiplano lakes. Furthermore, in Africa, mass movements of flamingos from lake to lake are quite common when there is drought. However, the seemingly random distribution of ITS genotypes I and II throughout the world and the dominance of each one in different lakes of the same countries remain difficult to explain: the replacement of an adapted local genotype by another bird-imported genotype would be theoretically possible but not highly credible (R. Fox, personal communication).

Based on the available geological data, the habitats of origin of our strains appear to be recent, such as the Ethiopian crater lakes (Mohr 1961) or Rift Valley lakes in Kenya (Tindall 1988). Many lakes show fluctuations in salinity and densities of *Arthrospira* populations as the Huacachina Lake in Peru (Hegewald et al. 1976) and Lake Sonachi in Kenya (which is the origin of the herbarium sample studied here). In this soda lake, a decline of the *Arthrospira platensis* populations between the 1930s and 1970s was recorded (Verschueren et al. 1999). A number of the soda lakes are subjected to periods of dryness or may evaporate completely. This was the case of Lonar Lake (India), a meteor crater lake, from where strain D0920 (Scheldeman et al. 1999) was isolated in 1984. In 1985, a dry period caused a disappearance of *Arthrospira* populations in the water column. They reappeared in 1986 after sufficient rain (Fox 1996). In Chad, the small lakes around Lake Chad undergo important fluctuations due to the variable groundwater level. For example, most lakes studied in the 1960s by Léonard and Compère (1967) and Iltis (1970) are now dry (M. Brouers, personal communication). This seems to be quite general for the African soda lakes, subject to many environmental variations. This instability might be adverse to a genetic differentiation that would require millions of years in a stable environment.

In some cases, the origin given for the strains is even doubtful. For example, no extensive populations of *Arthrospira* have been documented in Lake Titicaca (Hegewald et al. 1976), which is moderately saline (Mourgiart et al. 1997), and the culture of D0922 grew as a contaminant in a culture of a filamentous alga (R. Fox, personal communication). The Navaisha Lake (Kenya) was described as a large, shallow, and wind-exposed freshwater lake, with conductivities about 500 $\mu S \cdot cm^{-1}$ (Verschueren 1999), and it is not likely that strain D0867 (Scheldeman et al. 1999) was growing in the main water body. However, a more precise record of its isolation is not available (A. Walsby, personal communication). Lake Chad is also cited by some culture collections as the origin of *Arthrospira* strains, like D0885, whereas its salinity is too low and only the small soda lakes in its vicinity could harbor this organism (P. Compère, personal communication).

Future biological investigations will be facilitated by the availability of specific primers, allowing the fast detection of each cluster and subcluster in three short steps: cell lysis, PCR amplification, and detection by electrophoresis. These primers should also be useful for purity control purposes in companies growing commercial strains. They appear to give less straightforward results with complex samples, as in natural populations.

No subcluster I.B strains were obtained from companies, but this subcluster might be reduced to one strain if D0914 and *A. indica* MCRC isolate straight are indeed identical, as hypothesized in Scheldeman et al. (1999). Strains originating from companies (D0899, D0900, D0925, SP-18, SP-19, D0916, D0895, and D0923) belong to subclusters I.A or II.A or to a mix of subclusters II.A and II.B. Thus, their ITS sequences do not appear different from those of other strains in culture collections or dried environmental samples, except for one substitution common to D0899 and D0925. The commercial product is a blend of sequences from the four subclusters.

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