Identification of a Blue-Green Alga *Arthrospira maxima* Using Internal Transcribed Spacer Gene Sequence

Wen-Ji Yeh[1,*] Gen-Yuan Chen[1] and Yi-Chun Chen[2]

ABSTRACT

An unknown helical blue green alga, cultured in the Tungkang Marine Laboratory (Biotechnology Division), Fisheries Research Institute, COA, was identified as *Arthrospira maxima*, using scanning and transmission electron microscopy and sequence comparison of ribosomal DNA between the regions of 16S-23S rRNA. Scanning electron microscopy shows that the end cells of the helical alga are round or calyptrate, which are mainly characters of *Arthrospira maxima*. Transmission electron microscopy shows that organelles and components in the cell of this specimen are all fundamental elements of *Arthrospira maxima*. Gene sequence between 16S and 23S rRNA containing both $tRNA^{Ile}$ and $tRNA^{Ala}$ in internal transcribed spacer region in the present specimen is identical to that of *Arthrospira maxima* registered in GenBank. Thus, we conclude that this algal specimen is *Arthrospira maxima*.

(Key words: Blue-green alga, *Arthrospira maxima*, Internal transcribed spacer (ITS), Gene sequence)

INTRODUCTION

Microalgae have been suggested for many years as a source of nutritive proteins with a very high production potential. In the 1960s, attention was paid to helical blue-green algae or cyanobacterial species: genus *Spirulina*, and *Arthrospira*, Oscillatoriaceae (Hedenskog and Hofsten, 1970). Due to its higher protein content (over 68%) and linolenic acid, *Arthrospira* demonstrated almost equal nutritive value to that of other conventional plants (Ciferri, 1983). The hypocholesterolaemic and antiherpes simplex virus function of *Arthrospira* indicated an added advantage of algal-protein diet for human consumption (Hernández-Corona et al., 2002). Since the increased awareness of the nutritional potential of *Arthrospira*, they are sold as a health food supplement under the name "Spirulina".

[1] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

[2] Department of Food Nutrition, Chung Hwa College of Medical Technology, Jen-Te Township, Tainan County 717, Taiwan

* Corresponding author. E-mail: cosmos@mail.npust.edu.tw

The cyanobacterial genera *Spirulina* and *Arthrospira*, members of the Oscillatoriaceae, are both characterized by helical trichomes but their taxonomy is controversial. Both of them form multicellular, coidal filaments. The distinction between *Spirulina* Turpin, 1827 and *Arthrospira* Stizenberger, 1852 has been traditionally based on the presence or absence of septa in spiral trichomes. Members of the Oscillatoriaceae with septa trichomes have been regularly placed in the genus *Arthrospira* and those with nonsepta spiral trichomes have been placed in *Spirulina*. However, such generic separation based on the presence or absence of septa has been questioned by numerous workers (Holmgren and Hostetter, 1971). Septa in many *Spirulina* species are demonstrable after prolonged staining with dyes or demonstrated in ultrastructural observation with transmission electron microscope (Holmgren and Hostetter, 1971). Under different environmental conditions, *Arthrospira* or *Spirulina* strains may show great variation in morphology, such as the degree of spiralisation and the spontaneous appearance of straight trichomes in a previously coiled strain (Mühling *et al.*, 2003). It was recommended the joining of *Arthrospira* with *Spirulina* (Nelissen et al., 1994). Therefore, the taxonomic situation of these genera was unsolved using traditional approaches to these cyanobacteria.

Molecular approaches, however, have revealed a useful tool to solve taxonomic problems within cyanobacterial organisms. A spacer DNA sequence between the 16S and 23S rRNA gene in the ribosomal operon is extremely variable and provides an excellent tool to simplify the prokaryotic diversity (Garcia-Martinez et al., 1999). Nelissen et al. (1994) distinguished *Arthrospira* from *Spirulina* by using 16S rRNA gene sequence comparison, which was also shown 100% similarity between *A. maxima* and *A. fusiformis*, resulting in the strong suggestion that *A. maxima* and *A. fusiformis* are the same species (Li et al., 2001). Scheldeman et al. (1999) resolved the 37 cultivated *Arthrospira* strains into only two clusters using rDNA restriction analysis of the internally transcribed spacer (ITS). Thus, the gene sequence between the 16S and the 23S rRNA regions in the ribosomal operon is a tool for studying molecular systematics and population genetics at different taxonomic levels in cyanobacteria (Iteman et al., 2000; Boyer et al., 2001). An unknown helical alga was cultured at Tungkang Marine Laboratory (Biotechnology Division), Fisheries Research Institute, COA, Taiwan. In this study we aimed to clarify the taxonomy of this specimen using electron microscopy and rRNA gene sequence comparison, including ITS sequence to confirm the taxonomic position and construct a phylogenetic relationship with other cyanobacteria species.

MATERIALS AND METHODS

Cell culture

The cells of helical filamentous alga were provided by Dr. Su Hui Mei of the Tungkang Marine Laboratory (Biotechnology Division), Fisheries Research Institute. The cells were cultured with BG-11 medium in 25-28℃ room temperature under 12 hours light and 12 hours dark cycles of fluorescent light. Live cells were examined under a Nikon microscope at 400× and images were made by a Nikon Coolpix 5000 digital camera.

Scanning and transmission electron microscopy (SEM and TEM)

One ml of cultured cells was placed in a 1.5 ml sterile Eppendorf tube and centrifuged at 5,000 rpm for 5 min to concentrate the cells. The pelleted cells were suspended in 2% glutaraldehyde and kept at 4℃ for 1 hr. They were then washed in 50 mM phosphate buffer (pH 7.5), containing 0.25 M sucrose. The fixed sample was dehydrated with a graded alcohol series (50-100%). For SEM observation, the dehydrated samples were immediately removed to be critical point dried (Rascio et al., 1980). The sample was coated with carbon and gold and observed by a Hitachi S-3000N at the Electron Microscope Center of National Pingtung University of Science and Technology.

For TEM observation, the dehydrated samples were transferred to acetone, postfixed with 1% osmium tetroxide before embedding in Spurr's resin. Sections were made on a Sorvall MT-2 ultramicrotome equipped with a diamond knife. Sections were transferred to copper grids and poststained with uranyl acetate and lead citrate before examination with a Hitachi H-7500 electron microscope, operating at 80 kV (Maruyama et al., 1986; Ikeda and Takeda, 1995).

Extraction of genomic DNA

The extraction of genomic DNA was modified from the CTAB DNA isolation protocol of plants, fungi, and algae (Hillis et al., 1996). One ml of cultured cells was placed in a 1.5 ml sterile Eppendorf tube and centrifuged at 5,000 rpm for 5 min to concentrate the cells. The supernatant was removed and precipitated cells were mixed with 1 ml of 2× CTAB extraction buffer with a final concentration 0.2% (v/v) β-mercaptoethanol. The cells were crushed with ultrasonic pressure until tissue pieces were uniformly dispersed. We heated the homogenate with extraction buffer in 60℃ for 50 min and inverted the tube several times to mix the contents. Five hundred μl of CI (chloroform:isoamyl alcohol, 24:1) was added and DNA was extracted by gently inverting the tube for 10 min. The sample was centrifuged for 5 min at 10,000 rpm in a microcentrifuge. The upper phase was transferred to a fresh tube and reextracted with an equal volume of CI. The sample was centrifuged as above and the aqueous phase was transferred to a fresh tube. One ml of absolute ethanol was added to a new aqueous phase tube and the DNA was allowed to precipitate at -70℃ for at least 30 min. The precipitated DNA was pelleted by centrifuging for 10 min at 13,000 rpm. We decanted the ethanol and washed the DNA twice with 70% ethanol and centrifuged again. Ethanol was decanted and pelleted DNA was dried in a vacuum evaporator centrifuge (Iwaki VEC-260) one min. Finally, the pelleted DNA was dissolved in 200 μl of distilled deionized water and stored in 4℃ for polymerase chain reaction amplification.

Polymerase chain reaction (PCR) amplification of 16S-23S rRNA genes

The rRNA gene sequences were amplified from the isolated genomic DNA by PCR using automatic thermocyclers (Amplitron II, Thermolyne or Perkin-Elmer 2400). To the final PCR volume of 25 μl, 2 μl of diluted genomic DNA, 0.7 unit of *Taq* polymerase (Biotools, Biotechnological & Medical Laboratories, S.A. Spain), 0.2 μM dATP, dCTP, dGTP, dTTP reaction buffer with BSA/2 mM $MgCl_2$ and 0.1 μM of forward primer A (5'- TGT GGC TGG ATC ACC TCC TTT -3'), and reverse primer B (5'- TAC ATT GGA ATT GTC TTT

ACG -3'), were added to amplify DNA fragments. Primer A and B sequences were deduced from the sequence comparison among the rRNA gene sequences of some blue-green algae and *Arthrospira* species deposited in GenBank.

For PCR amplification, 25 μl reaction solution was set up and acted in a thermal cycler. Initial denaturation was set at 95℃ for 1:30 min, then PCR was performed for 35 cycles. The reaction condition for denaturation was carried out at 94℃, 1 min; annealing at 51℃, 1: 20 min; extension at 72℃, 2 min; final extra extension at 72℃, 5 min. After the completion of amplification, the product solution was pipetted off the tube. To PCR product solution, 1 μl of 10× tracking dye was added and loaded in a well of a 1.2% agarose minigel for electrophoresis (Mupid 21, 100V, 30 min). After electrophoresis, a predicted profile of ca. 0.5 kb PCR amplified DNA product was visualized in UV light and cut for elution. The eluted DNA fragment was cloned into the bacterial plasmid sequencing vector pGEM-Teasy, using the TA cloning system provided by the Promega. The ligation and digestion of inserted fragment in vector were identified by following the protocol provided by manufacturer (Promega). The inserted fragment was sequenced by ABI PRISM 377 automated sequencer.

Similarity in rRNA gene sequence and phylogenetic analysis with other algae

Sequences were aligned and similarity was compared with other blue green algae and *Arthrospira/Spirulina* species (Table 1) retrieved from GenBank database using DNAstar software (DNASTAR Inc., 1997). Aligned sequences were transferred to a new file for the construction of a phylogenetic tree using PAUP program (Swofford, 2000). Phylogenetic analysis with aligned sequences was performed using maximum parsimony (MP) algorithms available in the computer program PAUP. In MP bootstrap analysis, gaps were treated as a "missing". Initial searches consisted of 100 random sequence additions. Support for nodes of parsimony tree was assessed by calculating 1000 bootstrap resamplings. The heuristic searches were based on random stepwise additions and tree bisection reconnection.

RESULTS

The observations of SEM, TEM, and identity of species

Under light microscopy, the specimen exhibited a spiral shape, similar to those of common *Spirulina* and *Arthrospira* (Fig. 1a). The length of the filaments as well as their shape is dependent on the conditions of cultivation (Hedenskog and Hofsten, 1970). Straight trichomes were observed, which are usually seen in the *A. maxima* (Fig. 1b). The filaments were usually 5-10 μm wide and 200-300 μm long. The surface of the filaments was seen in the SEM. The SEM pictures showed that the end cells of spiral trichomes examined were round or calyptrate, which are distinctive characters of *A. maxima* (Figs. 1c-f).

Under TEM examination, the trichomes were divided into many parts by the septa (Fig. 2). Photosynthetic thylakoids in cyanobacteria are dispersed through the cytoplasm, and are apparently distinct from the cytoplasmic membrane. In the present sample, thylakoids were dispersed through the cytoplasm (Fig. 2c). The phycobilisomes were situated on the surfaces of the thylakoid membranes, in the form of regular arrays of granules (Rippka et al., 1974;

Table 1. Taxa, sources, sequence lengths between 16S-23S rRNA genes and GenBank accession numbers of sequenced isolates of cyanobacteria species used in this study for sequence comparison and phylogenetic deduction. *Chlamydomonas eugametos*, Chlorophyta was used as a outgrout in the phylogenetic comparison. Taxa from other algae were retrieved from authors listed in the sources. Authors marked with asterisk mean that the sources were unpublished in the Journals and retrieved from GenBank.

Specimen number and Taxon	Source	Sequence length used for comparison	GenBank accession number
Cyanobacteria			
Oscillatoriales			
1. *Arthrospira* sp. TKF1	This study	496	AY101599
2. *Arthrospira fusiformis*	*Baurain et al., 2001	475	AJ292321
3. *Arthrospira fusiformis*	*Baurain et al., 2001	477	AJ292328
4. *Arthrospira indica*	*Baurain et al., 2001	477	AJ292334
5. *Arthrospira indica*	*Baurain et al., 2001	475	AJ292336
6. *Arthrospira platensis*	*Baurain et al., 2001	477	AJ292322
7. *Arthrospira platensis*	*Baurain et al., 2001	477	AJ292324
8. *Arthrospira platensis*	*Baurain et al., 2001	477	AJ292337
9. *Arthrospira maxima*	*Baurain et al., 2001	475	AJ292323
Nostocales			
10. *Anabaena cylindrica*	*Lu et al., 1998	461	AF105130
11. *Anabaena* cf. *lemmermannii* 262	Gugger et al., 2002	508	AJ293101
12. *Anabaena spiroides*	Gugger et al., 2002	503	AJ294540
13. *Nostoc ellipsosporum*	*Lu et al., 1998	416	AF105136
14. *Nostoc linckia*	*Lu et al., 1998	434	AF105138
15. *Nostoc muscorum*	*Lu et al., 1998	421	AF105137
16. *Nostoc* sp. PCC 7120	*Lu et al., 1998	416	AF105132
17. *Nostoc* sp. PCC 7120	Iteman et al., 2000	512	AF180968
Chlorophyta			
18. *Chlamydomonas eugametos*	Turmel et al., 1991	684	Z17234

Allen, 1984). The cell walls (Fig. 2) in the examined cells were congruent with that of *S. platensis* (Hedenskog and Hofsten, 1970). Blue green algae have a relatively diffuse nuclear region containing DNA instead of a true nucleus with a clear nuclear membrane such as higher plants have (Hedenskog and Hofsten, 1970). Figures 2b and 2c showed the nucleoplasm as a region of electron transparent areas filled with more dense DNA filaments. Planktonic cyanobacteria in culture always show the regulation of gas vacuolation in response to light irradiation. Our specimen in culture medium was buoyant, and showed a higher density of gas vacuoles (Figs. 2b and 2c).

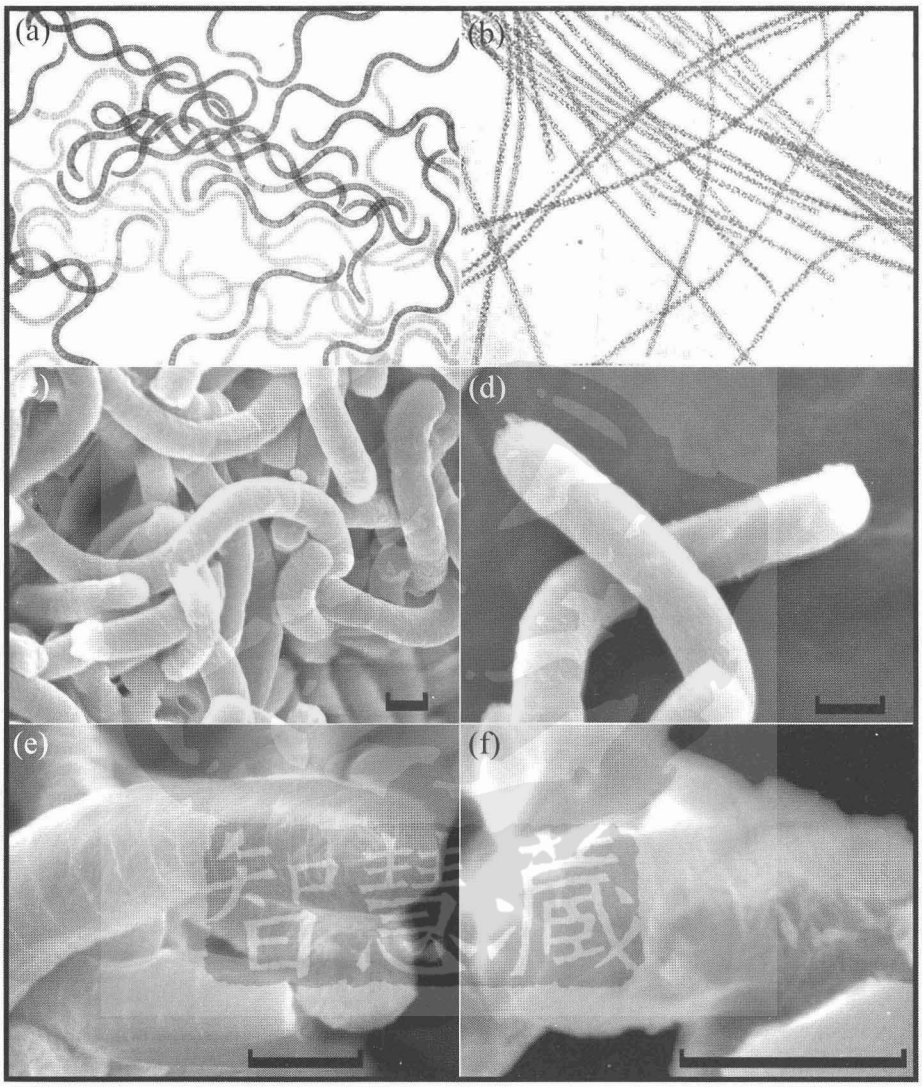

Fig. 1. Light microscope and scanning electron microscope pictures of the present *Arthrospira maxima*
(a). Spiral trichomes of the present *A. maxima* at 400× light microscope.
(b). Straight trichomes of the present *A. maxima* at 400× light microscope.
(c)-(f). The present *A. maxima* under SEM. The trichomes in (c) and (d) are shown at 1800× and 3000×, respectively.
(e). Rounded end cell of the present *A. maxima* (5000×).
(f). Calyptrate end cell of the present *A. maxima* (8000×).
The bar scales in the figure denote 5 μm.

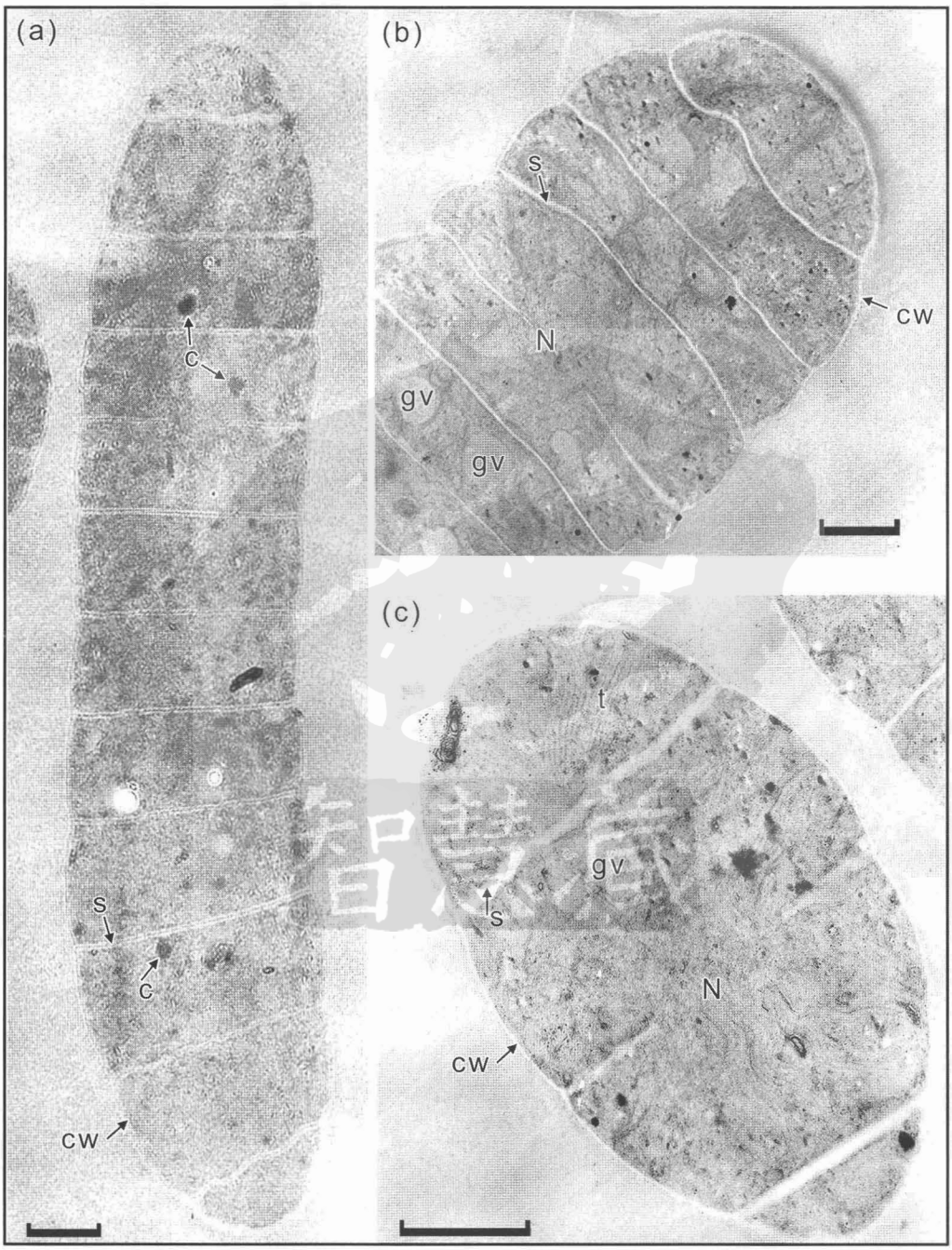

Fig. 2. TEM observation indicates fundamental elements of the present *Arthrospira maxima*. photosythetic thylakoids (t), septa (s), cell wall (cw), carboxysome (c), gas vacuoles (gv), and diffuse nucleoplasm (N) in the cell. The bar scales in the figure denote 1 μm.

Sequence property, similarity in 16S-23S rRNA genes and phylogenetic relationship with other algae

The ribosomal DNA sequence between 16S-23S region indicated that the amplified product with a primer pair A (forward) and B (reverse) was a fragment, size about 500 bp (Fig. 3a). This target fragment was finally sequenced as a length of 496 bp (Fig. 3b). Comparing the amplified 496 bp sequence with those found in GenBank (Mao et al., unpublished data: GenBank accession number AF329391-94; Baurani et al., unpublished data: AJ292321, AJ292323), the present total sequence includes partial 16S rRNA and ITS cotaining $tRNA^{Ile}$, $tRNA^{Ala}$ (Fig. 3b). The total sequence is available in GenBank (accession number AY101599). A 21 bp from 5' end of amplified fragment is a portion of 16S rRNA gene. The rest of 475 bp is demonstrated for the ITS region. The genes range for both $tRNA^{Ile}$ and $tRNA^{Ala}$ are 74 bp (136-209) and 73 bp (225-297), respectively (Fig. 3b). The 16S-23S rRNA gene sequence amplified from the present sample is identical to those of *A. maxima* and *A. fusiformis* for the same fragment (data not shown). The phylogenetic tree, constructed with MP algorithms, shows that they are closely related to each other (Fig. 4).

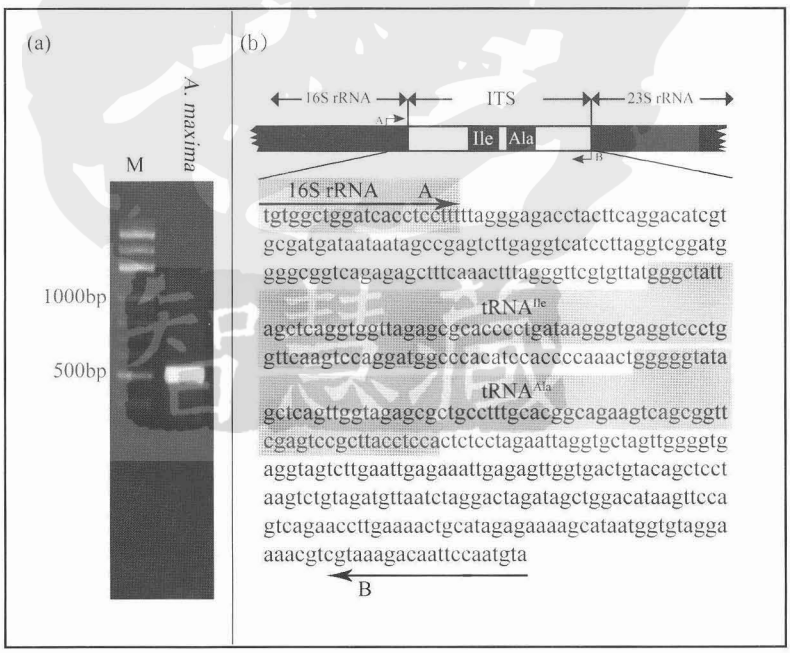

Fig. 3. The PCR product and its partial rRNA gene sequence of the present *Arthrospira maxima* genomic DNA amplified by a primer pair of A and B.
(a): DNA molecular weight standard marker (M) and PCR product of the present *A. maxima*. (b): The total length of 496 nucleotides DNA sequence between 16S and 23S rRNA region of the present *A. maxima*, including part of 16S rRNA, ITS region, containing $tRNA^{Ile}$ and $tRNA^{Ala}$. The arrows show the positions of forward primer A and reverse primer B. The sequence has been deposited in GenBank database (accession number AY101599).

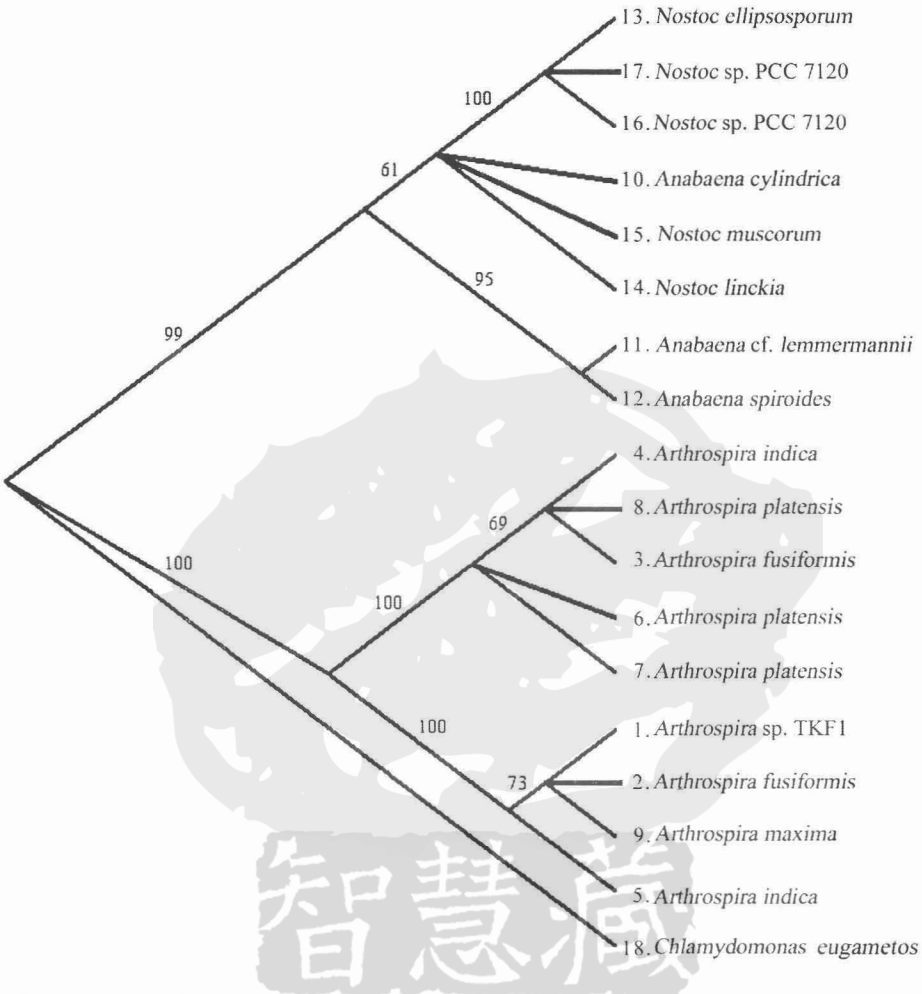

Fig. 4. Maximum parsimony tree showing the phylogenetic relationship of *Arthrospira maxima* and other species, based on aligned rRNA gene sequence ranging from the 3' end of 16S rRNA to 5' end of 23S rRNA, including internal transcribed spacer containing $tRNA^{Ile}$ and $tRNA^{Ala}$. The species *Chlamydomonas eugametos* (Chlorophyta) was used as a outgroup in phylogenetic analysis. Bootstrap values >50% are indicated above the internal branches for 1000 replicates analysis. See Table 1 for taxa information in the figure.

DISCUSSION

The trichome of *Arthrospira maxima* is arranged as an open helix with easily seen transverse walls under light microscopy (Tomaselli et al., 1993). The trichomes are shorter than wide. The apical cells are generally like a truncated cone. It has been suggested that the end cell morphology provides a very important, consistent and reliable feature recognized in the different *Arthrospira* groups (Desikachary and Jeeji Bai, 1996). The calyptrate and the briefly attenuated apices of morphological features in end cells present distinctive

taxonomic characteristics as an aid to distinguish the species *A. maxima* from *A. platensis* (Tomaselli et al., 1993). The straight and regular coil trichomes of *A. maxima* are characterized by rounded or calyptrate end cells. Reviewing the observations of the present specimen with light microscope and SEM, we conclude that the characters of the end cells are consistent with those listed for *A. maxima* by some investigators (Tomaselli et al., 1993; Desikachary and Jeeji Bai, 1996; Viti et al., 1997). Therefore, the morphological characters shown in light microscope and SEM pictures support the classification of the present specimen as *A. maxima* species.

Although *Spirulina* and *Arthrospira* are confused together in spiral morphology and difficult to identify morphologically under light microscopy, they are easily identified as prokaryotes in TEM examination. Both of them are readily separated from the eukaryotes by some key criteria: one is that the nucleoplasm is free of an envelope, the other is that the photosynthetic lamellae (thylakoids) are not enclosed by a limiting envelope characteristic of chloroplasts (Ris and Singh, 1961; Hedenskog and Hofsten, 1970; Beams and Kessel, 1977). These two criteria are clearly shown in the TEM examinations of the present sample. Cyanobacteria are recognized as diffuse nuclear in the cell. The nuclear region in our specimen was clearly located on the disrupted septa in TEM examination. Photosynthetic thylakoids in cyanobacteria are dispersed through the cytoplasm, and are apparently distinct from the cytoplasmic membrane. In the present sample, thylakoids were dispersed through the cytoplasm, and showed that the phycobilisomes were situated on the surface of the thylakoid membranes, in the form of regular arrays of granules (Rippka et al., 1974; Allen, 1984). *Arthrospira*, established by Stizenberger (1852), was characterized by nonheterocystous filaments with regular coils and visible septa. Our specimen clearly showed septa between the cells in both light microscopy and TEM examination. Many planktonic cyanobacteria possess gas vacuoles, made up of assemblages of hollow structures. The density of the gas vacuoles is such that the cells can become buoyant (Allen, 1984). The present specimen was buoyant when cultured in the medium, showing larger gas vacuoles in the cells. Polyphosphate granules in blue green algae appear to be bordered by dark lines, which have been interpreted as membranes, and often occupy the central part of the vegetative cells of *Spirulina* (Hedenskog and Hofsten, 1970). But in our work, no such structure has been observed in the specimen examined.

rRNA genes in bacteria are commonly organized in an operon in the order 16S rRNA -23S rRNA -5S rRNA, each rRNA gene being separated by ITS region. Two types of ITS region have been suggested that the spacer sequence could reflect intra-species phylogeny in bacteria. These types of heterogeneities in various regions of the ITS contain major genes coding for $tRNA^{Glu}$ and $tRNA^{Ile}$ and $tRNA^{Ala}$ (Boyer et al., 2001). The ITS regions of cyanobacteria vary in length from 283 to 545 nucleotides and contain both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, only the $tRNA^{Ile}$ gene, or neither (Iteman et al., 2000). The present specimen contains 475 bp for the ITS region, including both $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. The sequence composition of this fragment is identical to those of *A. maxima* (AJ292323) and *A. fusiformis* (AJ292321), shown by Baurain et al. (Table 1, unpublished data). Nelissen et al. (1994) determined and compared the sequence of the 16S rRNA gene and the spacer between the 16S and 23S rRNA genes for filamentous coiled cyanobacteria: *Spirulina* and *Arthrospira* and concluded that the two *Arthrospira* strains were not closely related to the

Spirulina strain but belonged to a cluster of strains assigned to the genera *Oscillatoria*, *Lyngbya*, and *Microcoleus*. For the two *Arthrospira* strains, the sequenced ITS region contain the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, whereas the spacer region of *Spirulina* strain contains only the $tRNA^{Ile}$ gene. The fragment sequenced in the present specimen contains $tRNA^{Ile}$, $tRNA^{Ala}$ in the region between 16S rRNA and 23S rRNA genes. This result strongly supports identifying this specimen as *Arthrospira* species.

The rRNA gene sequence comparison, containing the ITS region between 16S rRNA and 23S rRNA gene, indicated that the present specimen was identical to those of *A. maxima* (AJ292323) and *A. fusiformis* (AJ292328), respectively. Phylogentic tree also placed these three specimens: *A. maxima* (AJ292323), *A. fusiformis* (AJ292328) and our specimen into the same group. The trichomes of *A. maxima* was considered to be freely coiled and the *A. fusiformis* was to be tightly coiled. However, both types of coiling populations were produced in subsequent subcultures. In addition, identical 16S rRNA sequences were found in both of them (Li et al., 2001). Thus, based on morphological study in culture and 16S rRNA gene sequences comparison, Li et al. (2001) suggested that *A. maxima* and *A. fusiformis* appeared identical. Based on the results of morphological characteristics shown in the present SEM and TEM, and DNA sequence comparison in ITS region among *Arthrospira* species and others, we conclude that the specimen used in this study is a species of *A. maxima* and reconfirm *A. maxima* and *A. fusiformis* are identical.

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利用轉錄區間去氧核醣核酸序列鑑定藍綠菌種類 *Arthrospira maxima*

葉文吉[1*] 陳金源[1] 陳怡君[2]

摘 要

一種未確定學名的螺旋狀藍綠色菌種，經光學顯微鏡、掃描式電子顯微鏡觀察外部型態，及穿透式電子顯微鏡內部細胞胞器等構造觀察後，併同 16S-23S rRNA 轉錄區間去氧核醣核酸序列比對，結果認為此一藍綠菌種類之學名為 *Arthrospira maxima*。此一藍綠菌種在光學顯微鏡下呈螺旋狀，偶有直線型者。掃描式電子顯微鏡下觀查，菌體表面平滑，絲狀體(trichome)末端細胞圓形或囊蓋狀，此為 *Arthrospira maxima* 外部形態主要特徵。而穿透式電子顯微鏡顯示的內部細胞胞器等也具備 *Arthrospira maxima* 之基本構造。再利用此菌種 16S-23S rRNA 轉錄區間去氧核醣核酸序列和 GenBank 資料庫的其它種比對，結果顯示和 *Arthrospira maxima* 100%相同。綜合上述各種方法所得結果，推論此一藍綠菌種為 *Arthrospira maxima*。

（關鍵詞：藍綠菌，轉錄區間，基因序列）

[1]國立屏東科技大學 水產養殖系
[2]中華醫事學院 食品營養系
*通訊作者