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SYNECHOCOCCUS SALSUS SP. NOV. (CYANOBACTERIA): A NEW UNICELLULAR, COCCOID SPECIES FROM YUNCHENG SALT LAKE, NORTH CHINA

HONG-RUI LV, JIE WANG, JIA FENG, JUN-PING LV, QI LIU AND SHU-LIAN XIE[1]

School of Life Science, Shanxi University, Taiyuan 030006, China

Keywords: New species; China; *Synechococcus salsus*; DNA barcodes; Taxonomy.

Abstract

A new species of the genus *Synechococcus* C. Nägeli was described from extreme environment (high salinity) of the Yuncheng salt lake, North China. Morphological characteristics observed by light microscopy (LM) and transmission electron microscopy (TEM) were described. DNA barcodes (16S rRNA+ITS-1, *cpcBA*-IGS) were used to evaluate its taxonomic status. This species was identified as *Synechococcus salsus* H. Lv *et* S. Xie. It is characterized by unicellular, without common mucilage, cells with several dispersed or solitary polyhedral bodies, widely coccoid, sometimes curved or sigmoid, rounded at the ends, thylakoids localized along cells walls. Molecular analyses further support its systematic position as an independent branch. The new species *Synechococcus salsus* is closely allied to *S. elongatus,* C. Nägeli, but differs from it by having shorter cell with length 1.0–1.5 times of width.

Introduction

Synechococcus C. Nägeli (Synechococcaceae, Cyanobacteria) was first discovered in 1849 and is a botanical form-genus comprising rod-shaped to coccoid cyanobacteria with the diameter of 0.6–2.1 μm that divide in one plane. It is a group of ultra-structural photosynthetic prokaryote and has the close genetic relationship with *Prochlorococcus* (Johnson and Sieburth, 1979), and both of them are the most abundant phytoplankton in the world's oceans (Huang *et al*., 2012). The genus is a taxon of unicellular cyanobacteria with an ongoing debate concerning its revision into several genera (Rippka *et al*., 1979).

The genus *Synechococcus* is surely heterogeneous and its taxonomy needs to be solved by ultrastructural features and methods of molecular taxonomy (Komárek *et al*., 2014). The genetic diversity and phylogeny of *Synechococcus* in freshwater were determined by nucleotide sequence such as 16S rRNA gene, 16S-23S rRNA gene internal transcribed spacer (ITS-1) and *cpcBA*-IGS (phycocyanin operon) (Jasser *et al*., 2011). 16S rRNA gene is the most widely used gene sequence in the classification of cyanobacteria. Due to its highly conserved structure, it is only appropriate for evolutionary studies at the level of genera and above (Long and Chen, 2006). With the high variability and the rapid rate of change in molecular evolution, the internal transcribed spacer is often used to study the phylogenetic relationships at the taxa of genus, species, subspecies and so on (García-Martínez *et al*., 1996). In addition, *cpc*BA-IGS, the phycocyanin operon, can be used as the classification basis in identifying the level of species and the following (Bolch *et al*., 1996).

Yuncheng salt lake is located in southern suburbs of Yuncheng city where the algal distribution has its particularity. On the north of beach of Yuncheng salt lake, there were nearly 2×10^5 m^2

[1] Corresponding author: Email: xiesl@sxu.edu.cn

waters extensively appeared rosy during June, 2015. In order to find the reasons why the water turns red, we specifically took water samples and a coccoid strain with flickering red light of *Synechococcus* species was isolated.

In the present paper, we describe *Synechococcus* species isolated from Yuncheng salt lake as a new species *Synechococcus salsus* sp. nov. and discuss its taxonomic position based on morphological property observed by light microscopic and transmission electron microscopic, molecular data of 16S rRNA gene with the adjacent ITS, *cpc*BA-IGS and flanking regions were also used to support the entity as a new species.

Materials and Methods

Sample preparation and culture-dependent method

The unicellular, coccoid strain in this study was collected from Yuncheng salt lake, Shanxi Province, North China, in June 2015. It was preliminarily identified belonging to the genus *Synechococcus* through light microscopic examination (Olympus BX-51, Tokyo, Japan). We prepared BG-11 medium and then added it to a 24-well cell culture plate, each hole with 2 ml. Pasteur Micropipette was used to pick individual cells (Rippka, 1988). Cyanobacteria were isolated into unialgal culture and they were inoculated on the medium in the 24-well cell culture plate after washing eight times by sterile water. Then, it was cultured in a light incubator (BSG-300, Shanghai, China) at 25°C under a 12 h/12 h light/dark photoperiod. The light intensity was 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Three weeks later, they reached exponential phase, then we examined them with microscope. In the case of growing well and axenic culture, the homogenates were transferred to conical flasks with 200 ml BG-11 liquid medium. Similarly, they were placed in the same culture condition for large scale culture. To make them fully absorb nutrients and equally distribute, we manually shook the conical flasks every two days. The unialgal strain was deposited in Algae Culture Collection at Shanxi University.

Morphological observations

Morphological characteristics were examined under a light microscope when the cyanobacteria in large scale culture reached stationary phase. Photographs of main characters were taken with a digital camera (CAMEDIA C5060WZ, Olympus) and a CCD camera (DP72, Olympus) mounted on the microscope. Moreover, transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan) was used to observe the ultrastructure through the process of centrifuging, fixation, dehydration, embedding, sectioning and dye (Lim *et al*., 2012).

Molecular analyses

Genomic DNA was extracted from 10 ml axenically exponential phase cultures according to the modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle, 1987).PCR was carried out in 20 µl reaction volume containing 12.3 µl of double-distilled water, 2 µl of 10×Taq polymerase reaction buffer (Takara, Dalian, China), 0.2 µl of *EasyTaq* DNA polymerase (5 U/µl, Takara, China), 1.5 µl of each primer (10 µM), 2 µl dNTP mix (2.5 mM each; Takara) and 0.5 µl of undiluted genomic DNA. Part of the 16S ribosomal RNA gene (16S rRNA) and all of the 16S-23S rRNA internal transcribed spacer region (16S-23S ITS) were amplified using the primers P1 (5'-CTC TGT GTG CCT AGG TAT CC-3') and P2 (5'-GGG GAA TTT TCC GCA ATG GG-3') as described by Boyer *et al*. (2001). The *cpc*BA-IGS region of the phycocyanin operon were amplified with the cyanobacterium-specific primer pairs (Crosbie *et al*., 2003a) *cpc*BF (5'-TAG TGT AAA ACG ACG GCC AGT TGY YTK CGC GAC ATG GA-3') and *cpc*AR (5'-TAG CAG GAA ACA GCT ATG ACG TGG TGT ARG GGA AYT T-3'). Polymerase chain reaction (PCR) was performed in a My Cycler thermal cycler (Bio-Rad, Hercules, CA, USA). The program consisted of the following condition: 5 min at 95 °C, 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1

min at 72 °C, and a final extensionstep of 10 min at 72 °C. Sequencing was carried out from purified PCR products by BGI (Beijing, China). Sequences generated from the research were deposited in GenBank (accessions KU925869 for 16S rRNA gene+ITS-1, KU925870 for phycocyanin operon).

Sequences were aligned using Bioedit and then manually adjusted. Phylogenetic trees were constructed from the aligned gene sequences using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian (BI) methods. NJ, ML and BI analyses were performed through MEGA 5.0 (Tamura *et al*., 2011), PhyML 3.0 (Guindon and Gascuel, 2003) and MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003), respectively. Best fit models were selected under the Akaike information criterion (AIC) using Modeltest 3.7 (Posada and Crandall, 1998). For NJ analyses, evolutionary distances were computed using the Kimura 2-parameter method with 1000 bootstrap replicates. Treeview was used to view the phylogenetic trees. Final graphic refinement of all trees was done in Adobe Illustrator CS5 (Adobe Systems, San Jose, CA, USA).

Results and Discussion

The information and GenBank accession numbers for *Synechococcus salsus* sp. nov. and the other eighty-nine strains analyzed in this study were listed in Table 1. For the 16S rRNA+ITS-1 sequences, 44 strains of cyanobacteria were used and the aligned sequence dataset comprised 1321 nucleotides, of which 515 (38.99%) were variable sites and 455 (34.44%) were parsimony-informative sites. The aligned phycocyanin operon (*cpc*BA-IGS) dataset was 449 bp long. Forty-eight aligned *cpc*BA-IGS sequences had 307 (68.37%) variable sites and 281 (62.58%) parsimony-informative sites. The best fit model used for each locus was presented in Table 2.

For both the 16S rRNA+ITS-1 and the *cpc*BA-IGS data, tree topologies resulting from three methods had some similarities and differences. Based on 16S rRNA+ITS-1 sequences, the tree derived from the BI analysis is presented in Figure 1 to explain the relationships between *Synechococcus salsus* sp. nov. and the other accessions. *Leptolyngbya* sp. KIOST-1 was chosen as out group. *S. salsus* sp. nov. is grouped together with many *Synechococcus* strains. They are in a well-supported clade. Nevertheless, the bootstrap value by NJ method is less than 50%. The phylogenetic tree recovered by BI analysis of *cpc*BA-IGS is shown in Figure 2. *Cyanidium caldarium* was chosen as outgroup. In this topology, *S. salsus* sp. nov. and many other *Synechococcus* strains also constitute one cluster. The strain *Synechococcus* PCC9005 AF223465 was confirmed as closely related to *S. salsus* sp. nov., but the support value was low.

Synechococcus salsus H. R. Lv *et* S. L. Xie, **sp. nov.** **(Fig 3).**

Diagnosis: LM observations-Unicellular, cells solitary or agglomerated in groups (Figs A, B), but without common mucilage. Cells widely coccoid, sometimes curved or sigmoid, rounded at the ends, with a mean diameter of 2.0–3.5 μm and a length of 3–5 μm, usually pale blue-green, rarely olive-green, bright blue-green or pinkish, division always by binary fission, perpendicular to the longer axis of the cell, reproduction by solitary cells (Figs C, D). TEM observations-Cells with several dispersed or solitary polyhedral bodies, thylakoids localized along cells walls, photosynthetic pigments attached to it (Figs E, F).The new species is closely allied to *S. elongatus* C. Nägeli, but differs from it by having shorter cell with length 1.0–1.5 times of width, and cell length of *S. elongatus*is 1.5–3.0 times of width.

Type: China. Shanxi: Yuncheng City, Yuncheng salt lake, 21 June 2015, *Jie Wang & Chaoyan Gong*, SAS15yc (*Holotype*: SXU).

Table 1. Strains information and GenBank accession numbers for *Synechococcus salsus* H. Lv *et* S. Xie. and other taxa analyzed in phylogenetic comparison based on 16S rRNA+ITS-1 and Phycocyanin Operon (*cpc*BA-IGS).

Taxon	Strain/Isolate	16S rRNA + ITS-1	Phycocyanin Operon
Arthrospirafusiformis (Voronikhin) Komárek&J.W.G. Lund	AB2002/11	AY575929	
Arthrospiraindica Desikachary & N. Jeeji Bai	PD1997/ram	AY575931	
Arthrospira maxima Setchell & N.L. Gardner	FACHB-438	FJ826622	
Arthrospira platensis Gomont	FACHB-834	FJ826623	
Cyanidium caldarium (Tilden) Geitler			S77125
Cyanobium sp.	NS01		KF528824
	Sai001		GU935387
	Sai004		GU935390
	Sai005		GU935391
Leptolyngbya boryana (Gomont) Anagnostidis & Komárek	UTEX 'B 488'	EF429295	
Leptolyngbya corticola J.R. Johansen, Kovácik, Cassamata, Fucikova & Kastovsky	CCALA 085	EF429299	
Leptolyngbya saxicola (N.L. Gardner) Anagnostidis	BDU 91391		DQ829688
Leptolyngbya tenerrima (Hansgirg) Komárek	UTCC 77	EF429288	
Leptolyngbya valderiana (Gomont) Anagnostidis & Komárek	BDU 30501		AJ973264
	BDU 20041		AJ973263
	BDU 40231		DQ829696
	BDU 41001		DQ829697
	BDU 80221		DQ829698
	BDU 91712		DQ829699
	BDU 140441		AJ973265
Leptolyngbya sp.	KIOST-1	JX401929	
	HA4237-MV6	KJ939031	
Merismopedia sp.	AICB1014	KJ746509	
	AICB1015	KJ746510	
Microcoleus chthonoplastes Thuret *ex* Gomont	SAG 2209	EF654055	
Microcystis aeruginosa (Kützing) Kützing	2009-A		JN226767
	H107		KF840319
	PCC7806		AF195177
	UAM-MarB5		EU643824
	UAM-VMA-12		EU643811
Microcystis botrys Teiling	H179a		KF840323
Microcystis sp.	CYN06	EF634465	
	CYN10	EF634466	
	KLL-C018	KP726243	
	KLL-C005	KP726246	
	KLL-C019	KP726247	
	UWOCC Q		AF195179
Nostoc calcicola Brébisson *ex* Bornet & Flahault	Ind30		JF923546
Nostoc commune Vaucher *ex* Bornet et Flahault	NC3-K1	EU586723	
	NC5	EU586728	
	WY1KK1	EU586733	
Nostoc linckia Bornet ex Bornet & Flahault	PACC 5085		AY466120

Taxon	Strain/Isolate	16S rRNA + ITS-1	Phycocyanin Operon
Nostoc muscorum C. Agardh *ex* Bornet & Flahault	Ind33		JN402387
Nostoc punctiforme Hariot	NC6	EU586731	
Nostoc spongiaeforme C. Agardh *ex* Bornet & Flahault	Ind42		JF923547
Nostoc sp.	CAVN2		KJ511230
	CAVN10		KJ511236
	HA4355-MV2	HQ847576	
	HA4356-MV1	HQ847577	
	HK-01		JF740675
	KNUA003		JF740672
	MCC2741		KT166439
	Os-1	KP001508	
	PCC 6720		JF740673
	UAM 307	HM623782	
Pannus brasiliensis C.F.da Silva Malone *et al*.	CCIBt3594	KF668649	
Phormidium cf. *terebriformis*	AB2002/07	AY575933	
Spirulina subsalsa Oersted *ex* Gomont	FACHB-351	FJ826621	
	PD2002/gca	AY575935	
Synechococcus salsus H. Lv *et* S. Xie.	SAS15yc	KU925869	KU925870
Synechococcus sp.	ACT 0613		HQ859449
	ACT 0616		HQ859452
	ACT9701		GQ888569
	BE0807F	FJ763770	
	BE0807G	FJ763772	
	BE0807H		FJ763791
	BE0807L	FJ763778	
	CCY9201		EF513488
	EC-LC05PC		EU413956
	EC-LM05PC		EU420180
	MA0607E		FJ763807
	MI0608F		FJ763799
	MW97C4		AY151223
	NIBB 1026	AB610894	
	NIBB 1070	AB610895	
	NIBB 1071	AB610896	
	PCC 7918		AF223462
	PCC9005		AF223465
	PS673		AF223434
	Suigetsu-CG2	AB610891	
	WH8101		KF528825
Synechocystis sp.	AICB51	KJ746512	
	LSCB 01		KJ018112
	PUPCCC 62		KF573457
	Sai001	GU935367	
	Sai002	GU935368	GU935393
Uncultured *Synechococcus* sp.	MB11E09	AY033308	
Uncultured *Synechocystis* sp.	clone AF10-37-C9	JQ410261	
Uncultured marine bacterium	SPOTSOCT005m10	DQ009323	
	SPOTSOCT005m14	DQ009324	

Table 2. Model selection results for each phylogenetic dataset. Best fit models were selected under the Akaike information criterion (AIC) using Modeltest 3.7.

Gene	Model selected	Base frequencies	Substitution model
16S rRNA+ITS-1	GTR + I + G		R(a) [A-C] = 0.8415
	- lnL = 10568.6123	freqA = 0.2750	R(b) [A-G] = 2.0325
	K = 10	freqC = 0.2136	R(c) [A-T] = 1.1733
	AIC = 21157.2246	freqG = 0.2855	R(d) [C-G] = 0.5105
	(I) = 0.5199	freqT = 0.2259	R(e) [C-T] = 2.9532
	(G) = 0.7675		R(f) [G-T] = 1.0000
Phycocyanin Operon (*cpcBA*-IGS)	TIM+I+G		R(a) [A-C] = 1.0000
	- lnL = 5649.5356	freqA = 0.2653	R(b) [A-G] = 2.5283
	K = 8	freqC = 0.2977	R(c) [A-T] = 1.6029
	AIC = 11315.0713	freqG = 0.1944	R(d) [C-G] = 1.6029
	(I) = 0.2250	freqT = 0.2426	R(e) [C-T] = 3.8615
	(G) = 1.2670		R(f) [G-T] = 1.0000

Etymology: The specific epithet refers to its living environment with the high salinity.

Habitat and distribution: *Synechococcus salsus* was discovered in Yuncheng salt lake in Shanxi Province, North China, which is located at 34°48′ to 35°30′ N and 110°12′ to 111°41′ E. The lake is about 1.3×10^8 m^2 in area, with a salinity of 6.8%and pH 7.54 in June.

Note: The genus *Synechococcus* belongs to Cyanobacteria, Cyanophyceae, Synechococcophycideae, Synechococcales, Synechococcaceae. *Synechococcus* is polyphyletic and currently recognized as a distinct genus. The *Synechococcus* species are unicellular and rod-shaped to coccoid. Such tiny cells can be identified through the fluorescence of the main pigments phycoerythrin (PE) they contain, which is the evidence with respect to the phenomenon that Yuncheng salt lake appears rosy during June, 2015. According to Algae Base (http://www.algaebase.org/), 38 species have been flagged as currently accepted taxonomically. Zhao *et al*. (2010) studied spatial and temporal patterns of plankton assemblage structure of the saline lake Namuka Co in Northern Tibet and found *Synechococcus* strain. In the study of taxonomic and functional diversity in the saline Qinghai Lake, Huang *et al*. (2014) pointed out that *Synechococcus* was the dominant genus. Among the reports about cyanobacteria in Yuncheng Salt Lake region, only one *Synechococcus* species (*S. aeruginosus* Nägeli) was reported (Li and Xie, 2006). However, it is generally accepted belonging to the genus *Cyanothece* Komárek, namely *C. aeruginosa* (Nägeli) Komárek. *Synechococcus salsus* sp. nov. has no homology with it.

Synechococcus is an important type genus and species of this genus grow well in many different mediums. Most are freshwater species, and some grow within mats and colonies of other algae, or form fine colonies on wet substrates including mud, wood, stones, etc. A group of species is known from the metaphyton and periphyton of thermal and mineral springs (Dor, 1967), and few are described from aerophytic sites (Skuja, 1964). There are also some marine species (Jao, 1948; Komárek, 1956). Interesting picoplanktonic or planktonic species were found in oceans as well as in freshwater reservoirs (lakes) (Komárek and Anagnostidis, 1995).

In the past, the classification of cyanobacteria only relied on traditional morphological criteria. However, it is difficult to distinguish the members of *Synechococcus* because of their small differences, and its taxonomy should be resolved by help of ultrastructural procedures and

molecular approaches. With the development of electron microscope technique and molecular phylogeny, the accuracy of describing the phylogenetic relationship about cyanobacteria has been greatly improved (Komárek, 2010).

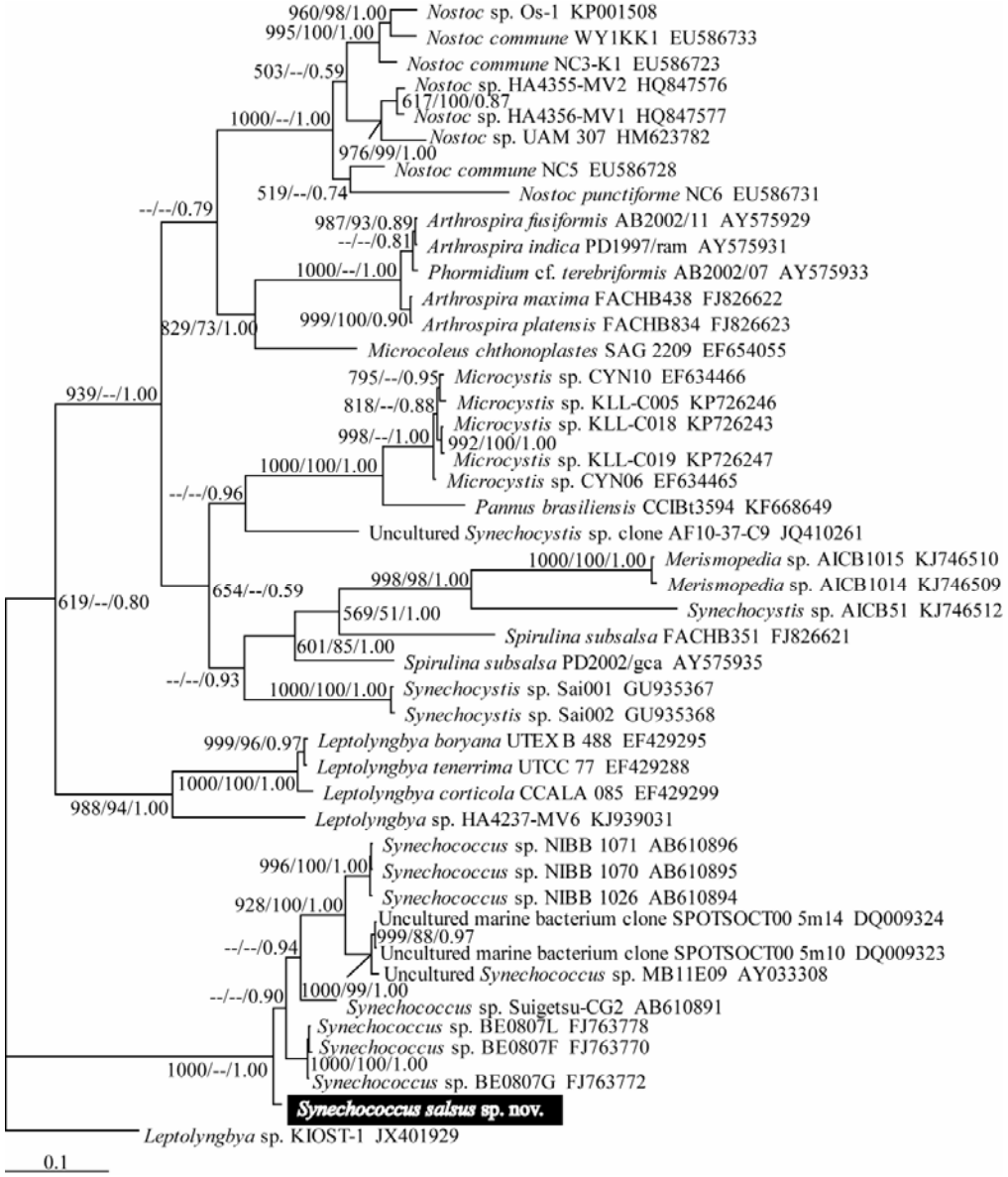

Fig. 1. Phylogenetic tree reconstructed using Bayesian inference from the aligned 16S rRNA+ITS-1 sequence. Numbers at nodes represent maximum likelihood bootstrap/neighbor-joining bootstrap/Bayesian posterior probabilities values. Support values less than 50% are not shown. Scale bar represents 0.1 substitutions per site.

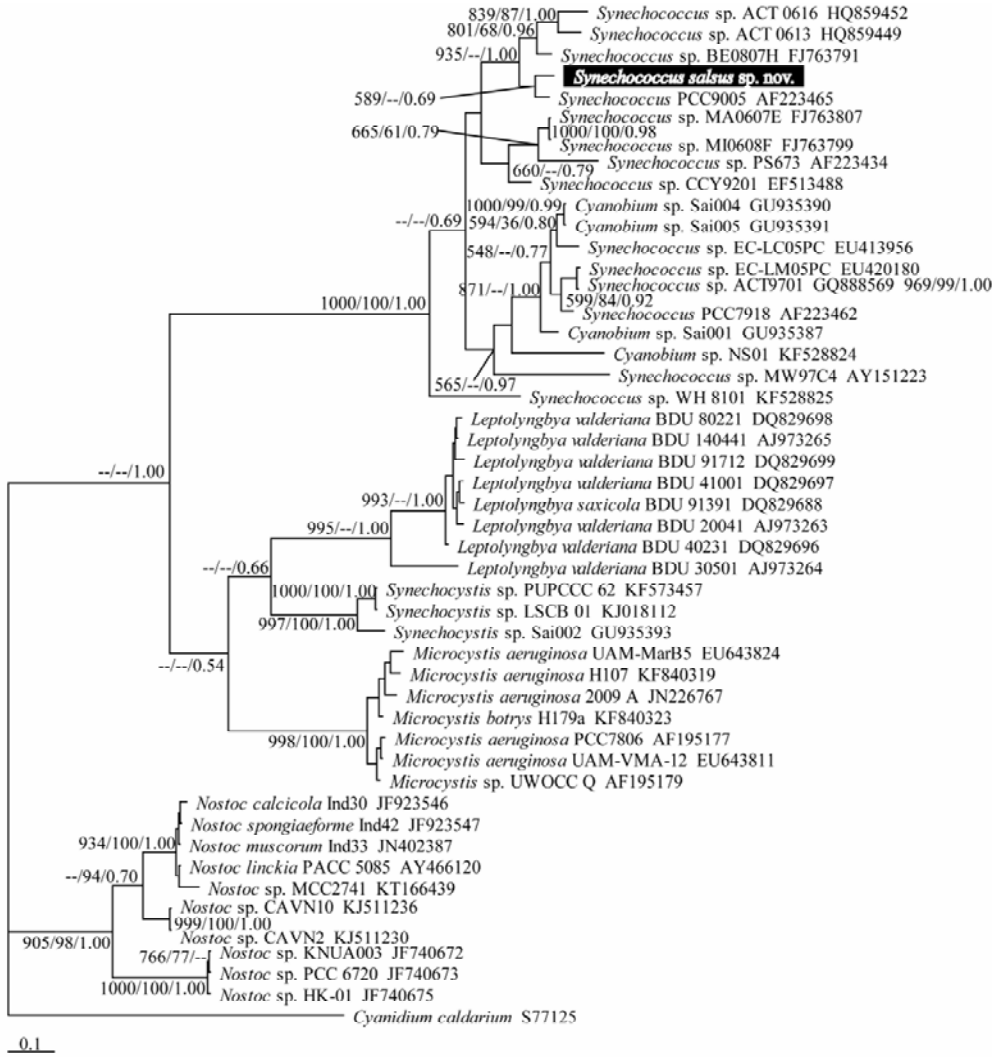

Fig. 2. Phylogenetic tree reconstructed using Bayesian inference from the aligned *cpc*BA-IGS sequence. Numbers at nodes represent maximum likelihood bootstrap/neighbor-joining bootstrap/Bayesian posterior probabilities values. Support values less than 50% are not shown. Scale bar represents 0.1 substitutions per site.

16S rRNA gene encodes the small subunit ribosomal in prokaryote. Because of its high conservation and the largest database available for comparison, it has been widely chosen as one of the molecular markers for phylogeny (Gupta, 2009). However, even for species with distinct physiological differences, 16S rRNA does not have enough distinguishing ability in solving the relationship problems (Jaspers and Overmann, 2004). ITS (internal transcribed sequences) with relatively high mutation rate, when it comes to some closely related species, is quite effective. Despite three pure cultures of *Synechococcus* strains and a *Prochlorococcus* strain was close to each other in the phylogenetic tree based on the 16S rRNA, but the length of their ITS sequences differ greatly (Laloui *et al.,* 2002). Rocap *et al*. (2002) also indicated that the length of the ITS

sequences and G+C content vary greatly among different strains. It is always used to distinguish *Synechococcus* strains with highly similar sequences but different in physiological characteristics (e.g. different pigment composition, of light and nutrients of different growth response, etc.). The phycocyanin operon is composed of the two genes (*cpc*B and *cpc*A) that code for phycocyanin, a short and quite variable intergenic region (IGS) and three linker polypeptides (Belknap and Haselkorn, 1987). It can avoid many non-specific amplified products when used to study the diversity of *Synechococcus.*

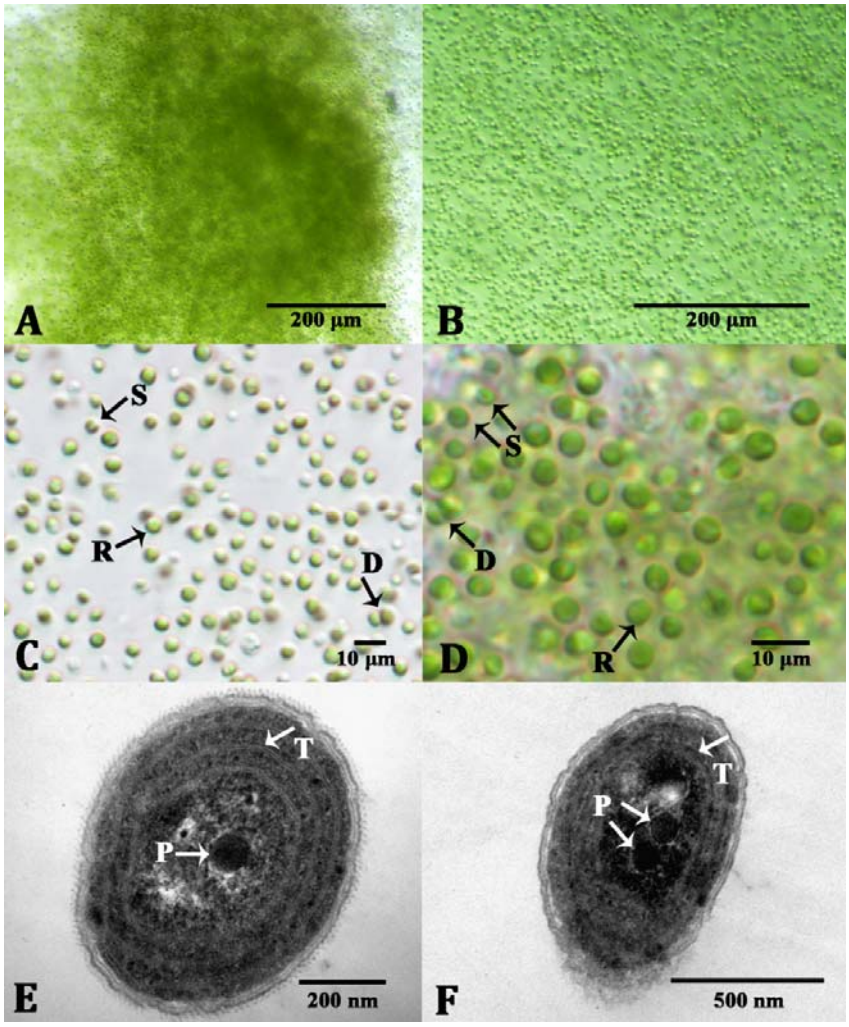

Fig. 3. LM and TEM of *Synechococcus salsus* H.R. L*vet*S.L. Xie. A. Cells agglomerated in groups. B. Cells solitary. C-D. Cells usually pale blue-green, rarely olive-green, bright blue-green or pinkish, widely oval or coccoid, sometimes curved or sigmoid (S, arrowhead), rounded at the ends (R, arrowhead). Cell division (pinching or cleavage) always by binary fission (D, arrowhead). E-F. Thylakoids localized along cells walls (T, arrowhead). Photosynthetic pigments attached to it. Cells with dispersed or solitary polyhedral bodies (P, arrowhead).

This study relatively accurately evaluated the taxonomic status of *S. salsus* sp. nov. based on the 16S rRNA+ITS-1 gene sequence and phycocyanin operon (*cpc*BA-IGS). Based on 16S rRNA+ITS-1 sequences, the tree showed the relationships between *Synechococcus salsus* sp. nov. and the other accessions. The members of *Synechococcus* are gathered together into one cluster, which is in a well-supported rate. But in the *Synechococcus*-clade, it is quite obvious that *S. salsus* sp. nov. is separated from the other *Synechococcus* strains and alone in a small clade. On the phylogenetic tree recovered by BI analysis of *cpc*BA-IGS, the genus *Synechococcus* is not a monophyletic group. The strain *Synechococcus* PCC9005 AF223465 was confirmed as closely related to *S. salsus* sp. nov., but the support values were weak. From the above, it is reasonable to make it as a new *Synechococcus* species. Finally, salt lake (salinity > 0.5 g/L) is a special habitat. Although the salinity cannot be a criterion to justify a new species (Anagnostidis and Komarek, 2005), it may indeed contain many special organisms.

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