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Nuclear ITS and LSU sequence analysis of *Skeletonema costatum* (Bacillariophyta)-like species from the China Sea

Guo F. CHEN[1,2], Guang C. WANG[*1,3], Bao Y. ZHANG[1], Bai C. ZHOU[1] and Xiao L. FAN[1,2]
(1) *Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, 7# Nanhai Road, Qingdao 266071, P.R. China;*
(2) *Graduate School, Chinese Academy of Sciences, Beijing 100039, P.R. China*
(3) *College of Oceanology, Tianjin University of Science Technologys, Tianjing 100222, P.R. China*
**E-mail: gcwang@ms.qdio.ac.cn. Phone: +86-532-82898574; Fax: +86-532-82880645.*

Abstract: Five strains of *Skeletonema costatum*-like species were isolated from different coastal regions of the China Sea, where red tides occurred. The internal transcribed spacer regions (ITS-1, 5.8S rDNA and ITS-2) and the partial large subunit rDNA (D1-D2 hyper-variable domain) regions of these strains were sequenced and aligned with sequences obtained from other *Skeletonema* strains. The five strains showed considerable sequence variation and also length variation among their ITS regions, whereas their partial LSU regions exhibited less variation. Their 5.8S rDNA were identical. The LSU and ITS trees provided different topologies and phylogenetic inferences suggested that the five isolates belonged to at least three different species. The strain from Yellow Sea might be *S. tropicum*; the strains from East Sea and Xiamen were grouped in the same clade in both trees; the other two strains clustered in the LSU tree, but belonged to different clades in the ITS tree. Results reveal considerable diversity in the genus *Skeletonema* from the Chinese coast, especially strains causing red tides.

Résumé : *Analyse des séquences LSU et ITS nucléaires d'espèces proches de* Skeletonema costatum *(Bacillariophyta) provenant de la Mer de Chine.* Cinq souches d'espèces proches de *Skeletonema costatum* ont été isolées de différentes régions côtières de la Mer de Chine où des marées rouges se produisent. Les régions ITS-1, 5,8S et ITS-2 de l'ADNr et une séquence partielle de la grande sous-unité (LSU) ribosomique (domaine hypervariable D1-D2) de ces souches ont été séquencées et alignées avec les séquences obtenues pour d'autres souches de *Skeletonema*. Les cinq souches montrent une grande variation de séquences ainsi qu'une variation de longueur de leurs régions ITS, alors que les régions LSU étudiées présentent moins de variations. Leurs séquences 5,8S sont identiques. Les topologies des arbres obtenues à partir des séquences LSU et ITS sont différentes et les inférences phylogénétiques suggèrent que les cinq isolats appartiennent au moins à trois espèces différentes. La souche de Mer Jaune pourrait être *S. tropicum* ; les souches de la Mer orientale et de Xiamen forment un clade dans chaque arbre ; les deux autres souches sont regroupées dans l'arbre obtenu à partir des séquences LSU mais appartiennent à des clades différents dans l'arbre obtenu à partir des séquences ITS. Le genre *Skeletonema* est donc diversifié le long des côtes chinoises, notamment les espèces à l'origine des marées rouges.

Keywords: HABs • *Skeletonema* • ITS • LSU rDNA • Phylogeny • Diversity

Abbreviations: HABs, harmful algal blooms; ITS, internal transcribed spacer; LSU, large subunit; NJ, neighbor joining; SSU, small subunit

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Introduction

In recent years, considerable attention has been focused on harmful algal blooms (HABs) because of the severe economic losses they have caused in aquaculture, fisheries and tourism industries as well as their negative impact on the environment and human health along temperate coasts throughout the world (Hallegraeff, 2003). Along the Chinese coast, HABs occur more and more frequently and over larger areas (Guo, 2004). To understand and give warning of the occurrence of HABs, it is essential to ascertain the causative species.

Skeletonema spp. usually give rise to HABs (Karentz & Smayda, 1984; Hong, 1994; Zoppini et al., 1995; Totti et al., 2002; Wei et al., 2003; Bernardi Aubry et al., 2004). The genus *Skeletonema* is cosmopolitan and one of the most abundant diatoms in the coastal marine phytoplankton (Hasle, 1973). Losses caused by members of this genus were often underestimated, because the species are less harmful than other toxic species, such as *Alexandrium* spp. Unfortunately, HABs caused by *Skeletonema* occur more and more frequently, and damage is attributed to these species (Huo et al., 2001; Qi, 2003; Wei et al., 2003).

Skeletonema features prominently in research ranging from biochemistry, ecophysiology and population genetics to ecology, oceanography and aquaculture, due to its easy cultivation and fast growth. *Skeletonema costatum* (Greville) Cleve 1873 and some allied species have many remarkable features, such as cylindrical cells with long tubular processes (rimoportulae) associated with a peripheral ring of strutted processes (fultoportulae), and colonies of variable length and cell size. Since the establishment of *Skeletonema,* i.e., the transference from *Melosira costata* (Greville, 1866) to *S. costatum* (Cleve, 1873), five more species were described in the genus, exclusively based on morphological data, including *S. tropicum* Cleve 1900, *S. subsalsum* (Cleve) Bethge 1975, *S. potamos* (Weber) Hasle 1976, *S. cylindraceum* Proshkina-Lavrenko & Makarova 1964 and *S. menzelii* Guillard & Reimann 1974. Subsequently, Gallagher (1980, 1982) and Gallagher et al. (1984) demonstrated high diversity in allozyme patterns among strains of *S. costatum* and suggested that several species might be hidden within this taxon. Medlin et al. (1991) recognized another new species – *S. pseudocostatum* Medlin & Sogin 1991. Recently, Zingone et al. (2005) reexamined the type material of *S. costatum* and emended description of it based on EM observations. They also described an additional species present in the type material, namely *S. grevillei* Sarno & Zingone. Sarno et al. (2005) reported four additional *S. costatum*-like species, including *S. dohrnii* Sarno & Kooistra 2005, *S. grethae* Zingone & Sarno 2005, *S. japonicum* Zingone & Sarno 2005, and *S. marinoi* Sarno & Zingone, bringing the number of described species in the genus to eleven. Another new species known as *S. ardens* Sarno & Zingone, 2007 will be described in an accompanying paper by Sarno et al. (2007). Most important of all, in this study the authors will present molecular data of *S. costatum sensu stricto*, *S. ardens* and *S. grevillei* (Sarno et al., 2007). These new results also put into question all previous identifications associated to GenBank sequences of *Skeletonema* published before 2005.

In the present study, five strains of *S. costatum*-like species were isolated from different marine sites where HABs occurred. To explore their taxonomic identity, we obtained the internal transcribed spacer region (ITS-1, 5.8S rDNA, and ITS-2) and the partial large subunit D1-D2 LSU rDNA region from these five isolates and aligned these sequences with published ones from known *Skeletonema* species.

Materials and Methods

Isolation of strains and culture conditions

Seawater samples were collected from five different sites along the coast of the China Sea, including Bohai Sea Bay, Qingdao Fishery and Jiaozhou Bay in the Yellow Sea, East Sea, and Xiamen in the Strait of Taiwan (Table 1). Samples were collected between summer 2003 and spring 2004, when HABs occurred. Correspondingly, clonal cultures of *Skeletonema*, which were established from these samples

Table 1. Isolates used in this study and corresponding GenBank accession numbers.
Tableau 1. Isolats utilisés dans cette étude et numéros GenBank correspondants.

Strains	Isolation site	Isolation date	GenBank accession number (LSU)	GenBank accession number (ITS)
SK-BH	Bohai Sea Bay, East Pacific Ocean	4 Apr. 2005	DQ234262	DQ280328
SK-FQ	Qingdao Fishery, Yellow Sea, East Pacific Ocean	10 May. 2004	DQ234259	DQ280325
SK-HH	Jiaozhou Bay, Yellow Sea, East Pacific Ocean	10 Oct. 2003	DQ234261	DQ280327
SK-DH	Donghai Sea, East Pacific Ocean	5 Jul. 2003	DQ234258	DQ280324
SK-XM	Xiamen, Taiwan Strait, East Pacific Ocean	22 Sep. 2004	DQ234260	DQ280326

by micropipette isolation, were marked with SK-BH (Jiaozhou Bay), SK-FQ (Qingdao Fishery), SK-HH (Yellow Sea), SK-DH (East Chinese Sea) and SK-XM (Xiamen), respectively. All isolates were assigned to the genus *Skeletonema* because they resembled LM and TEM illustrations in Zingone et al. (2005) and Sarno et al. (2005). All isolates were grown in f/2 + Si medium (Guillard, 1975) with a salinity of 36. Cultures were kept in 250 mL flasks containing 100 mL medium, at 20-22°C and a 12:12-h light:dark cycle at an irradiance of 50-100 µmol photons·m^{-2}·s^{-1} provided by cool white fluorescent tubes. The cultures were stirred manually daily and medium was transferred on a weekly basis.

Isolation of DNA

Cells were harvested during log phase by centrifugation, rinsed with bi-distilled water and resuspended in extraction buffer (50 mM Tris-Cl, pH 8.0; 100 mM NaCl, 100 mM EDTA), then incubated for 1-2 h at 50-52°C with SDS and proteinase-K added to a final concentration of 1.5% and 300 respectively. Total nucleic acids were extracted first with an equal volume of phenol, then with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and subsequently with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). All extractions were carried out by centrifugation at 12000 g for 5 min. Nucleic acids were concentrated by centrifugation at 12000 g for 10 min with two times the volume of ethanol and 1/10 volume of sodium acetate (3 M, pH 5.2). The pellet was rinsed by centrifugation at 12000 g for 10 min with 1 mL of 70% ethanol. To reduce the contaminating RNA and polysaccharides, the resulting DNA pellet was digested with RNase (20 ng·mL^{-1}), subsequently resuspended in high salt buffer (2 M NaCl; 10 mM Tris pH 8) and cleared by centrifugation at 12000 g for 10 min. The supernatant was extracted first with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1, v/v/v), then with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), and subsequently precipitated using twice volume of ethanol. Finally, DNA was dissolved in TE (10 mM Tris-Cl, pH 7.6; EDTA 0.05 mM), stored at 4°C or frozen at -16°C before being used as templates for PCR reaction. DNA quality and quantity was assessed by agarose gel electrophoresis analysis and determining the OD_{260}/OD_{280} ratio using a spectrophotometer.

DNA amplification

Primers that anneal to the 3' end of the 18S rDNA (TW81: 5'-GGGATCCGTTTCCGTAGGTGAACCTGC-3') and the 5' end of the 28S rDNA (AB28: 5'-GGGATCCATATGCTTAAGTTCAGCGGGT-3') were used to amplify the region including ITS-1, 5.8S rDNA, and ITS-2 (White et al., 1990; Steane et al., 1991). The forwards primer D1: 5'-ACCCGCTGAATTTAAGCATA-3', and the reverse primer D2: 5'-CCTTGGTCCGTCTTTCAAGA-3' (Scholin et al., 1994) were used to amplify the LSU D1-D2 rDNA. PCR-reactions were carried out using a Mastercycler Gradient (Eppendorf, Hamburg, Germany). Both DNA regions were PCR amplified in a reaction mixture (final volume, 20 µL) containing 5-10 ng genomic DNA, 1xPCR buffer, 2.5 mM $MgCl_2$, 50 µM each of dATP, dTTP, dCTP, and dGTP, 0.2 µM of each primer, and 0.5 U (5U·μL^{-1}) of T*aq* DNA polymerase (Tarkara, Tokyo, Japan). PCR conditions involved one DNA denaturing cycle at 94°C for 4 min, followed by 29 cycles of: 94°C for 1 min, 50 or 55°C for 50 s, 72°C for 50 s, and a final extension at 72°C for 7 min. Quality and length of the products were examined by agarose gel electrophoresis against known standards.

DNA purification

Amplified rDNA fragments were separated by means of agarose gel electrophoresis; DNA was stained with ethidium bromide, and visualized and excised under weak ultraviolet light. The excised gel was cut into pieces and put onto siliconized fiberglass in a 500 µL Eppendorf tube with a small hole pinched in the bottom. This tube was placed in a 1.5 mL Eppendorf tube and the whole contraption was spun at 2000 g for 2 min, 5000 g for 4 min and 10000 g for 5 min. The collected liquid was pooled, and the DNA was purified and precipitated following the methods described as above. Finally, the purified DNA was reexamined by agarose gel electrophoresis to establish the final concentration for cloning.

Cloning and sequencing

To obtain sequences of a single copy of the target DNA region, purified PCR products were ligated with pMD 18-T Vector and transformed into competent *Escherichia coli* DH-5. The positive colonies containing the objective DNA fragments were identified to sequence using the Vector primer M13 as sequencing primer. Sequencing was performed by Shanghai Invitrogen Biotechnology Co., Ltd.

Sequences analysis

The approximate boundaries of the rDNA and ITS regions were predicted by alignment of sequences from other diatoms in GenBank database with Blast search. Twenty eight nucleotide sequences of ITS from three *Skeletonema* species (labeled as *S. costatum*, *S. marinoi* and *S. pseudocostatum* in GenBank), five *S. costatum*-like strains in this study, and two species belonging to thalassiosiroid diatoms *Cyclotella meneghiniana* Kütz, 1844 and *Thalassiosira weissflogii* (Grunow) Fryxell & Hasle, 1977 (Tables 1, 2), were aligned utilizing the ClustalW multiple alignment

Table 2. List of *Skeletonema* and *Thalassiosira* introduced into phylogenetic analysis, with GenBank accession numbers of their partial LSU rDNA sequences or ITS.
Tableau 2. Liste des *Skeletonema* et *Thalassiosira* utilisés dans l'analyse phylogénétique, numéros GenBank de leurs séquences ADNr LSU ou ITS.

Species	GenBank accession number (LSU)	References	GenBank accession number (ITS)	References
Skeletonema. costatum			AY660001	Zhang et al. (2006)
S. costatum sensu stricto	DQ396491, DQ396489	Sarno et al. (2007)		
S. dohrni	AJ633537, AJ633538	Sarno et al. (2005)		
S. grethae	AJ633521–AJ633523	Sarno et al. (2005)		
S. japonicum	AJ633524	Sarno et al. (2005)		
S. marinoi	AJ633530–AJ633536	Sarno et al. (2005)	AY748217–AY748221, AY748226, AY748230,	
	AY699024–AY699048	Godhe et al. (2006)	AY748232, AY748233, AY748236, AY954681	Godhe et al. (2006)
			AY954682, AY954684	
S. menzelii	AJ633525–AJ633528	Sarno et al. (2005)		
S. pseudocostatum	AJ633507–AJ633514	Sarno et al. (2005)	Y11511	Van der Auwera & De Wachter (1998)
			AY748235	Unpublished
S. subsalsum	AJ633539	Sarno et al. (2005)		
S. tropicum	AJ633515–AJ633520	Sarno et al. (2005)		
Cyclotella meneghiniana			U03073	Zechman et al. (1994)
Thalassiosira weissflogii			DQ469927	Von Dassow et al. (2006)
T. sp. B101	AJ633506	Sarno et al. (2005)		
T. rotula	AJ633505	Sarno et al.(2005)		

method in BioEdit. The analysis of genetic distances based on Kimura's two-parameter model was carried out with Mega2.1 (Kumar et al., 2001). The molecular phylogenetic tree was constructed with neighbor-joining (NJ) method, implemented with Mega2.1. Nodal support was estimated by bootstrap analyses using 1000 replicates in the NJ analyses. Similarly, sixty-seven nucleotide sequences of LSU from eight *Skeletonema* species including *S. costatum*, *S. dohrni*, *S. grethae*, *S. japonicum*, *S. marinoi*, *S. menzelii*, *S. pseudocostatum*, *S. subsalsum*, *S. tropicum*, the five isolates in this study, and two species, *Thalassiosira* sp. B101 and *T. rotula* (Meunier, 1910), belonging to the Thalassiosirales were analysed phylogenetically as above.

Results

DNA isolation and amplification

The extraction procedure resulted in high quality DNA as shown in the electrophoresis patterns (Fig. 1). The OD260/OD280 ratios of the DNA solutions were ca. 1.8 demonstrating the purity of the obtained DNA. Moreover the procedure provided about 60 ng DNA per gram wet weight microalgae.

Both the amplified ITS and LSU rDNA from the five isolates exhibited a single band in the electrophoresis pattern (Figs 2 & 3) without apparent size differences within each of the two regions. According to the standard molecular marker, both sizes of ITS and LSU rDNA including the primers were close to 800 bp.

DNA sequencing and sequence analysis

The nucleotide composition of ITS is shown in Table 3. The sizes of ITS from the five isolates were different from each other. The nucleotide number excluding the primers was 690, 684, 691, 689 and 692 for the isolates SK-BH, SK-FQ, SK-HH, SK-DH, and SK-XM, respectively. All isolates had nearly identical sizes of 5.8S rDNA, with ITS1 containing 238 or 239 nucleotides and ITS2 containing nucleotide number ranging from 271 to 279. The nuclear-encoded partial LSU rDNA sequences (D1-D2) were 599 nucleotides for the SK-DH and SK-XM isolates and 600 for the SK-BH, SK-FQ and SK-HH isolates, excluding the primer sequence.

The five ITS and LSU sequences were aligned with corresponding sequences available from GenBank, respectively. Regions outside those obtained in this study were trimmed from the alignment. The final alignments, including introduced gaps, contained 713 and 595 positions, respectively.

With *C. meneghiniana* and *T. weissflogii* as outgroups, molecular phylogenetic analyses were performed with the

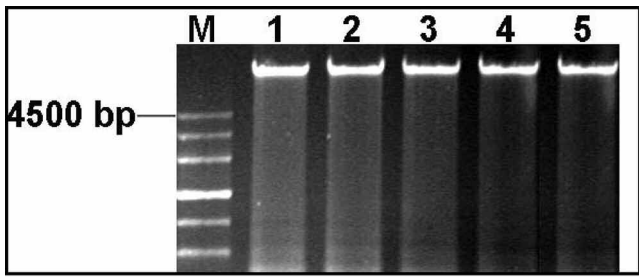

Figure 1. Electrophoresis patterns of the genomic DNA from *Skeletonema* isolates in 1% agarose gel. Lane M, marker; Lane 1, the SK-BH isolate; Lane 2, the SK-FQ isolate; Lane 3, the SK-HH isolate; Lane 4, the SK-DH isolate; Lane 5, the SK-XM isolate.

Figure 1. Electrophorèse de l'ADN des isolats de *Skeletonema* sur gel d'agarose 1%. Bande M, marqueur ; bande 1, isolat SK-BH ; bande 2, isolat SK-FQ ; bande 3, isolat SK-HH ; bande 4, isolat SK-DH ; bande 5, isolat SK-XM.

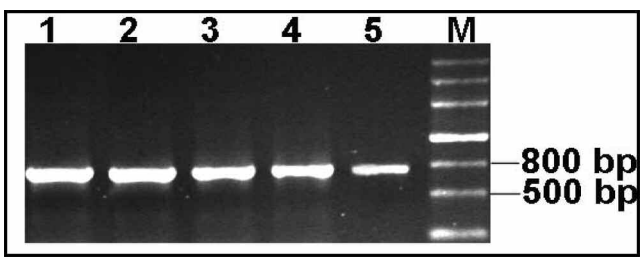

Figure 2. Electrophoresis patterns of the PCR-amplified ITS product in 1% agarose gel. Lane M, marker; Lane 1, the SK-BH isolate; Lane 2, the SK-FQ isolate; Lane 3, the SK-HH isolate; Lane 4, the SK-DH isolate; Lane 5, the SK-XM isolate.

Figure 2. Electrophorèse des ITS amplifiés par PCR sur gel d'agarose 1%. Bande M, marqueur ; bande 1, isolat SK-BH ; bande 2, isolat SK-FQ ; bande 3, isolat SK-HH ; bande 4, isolat SK-DH ; bande 5, isolat SK-XM.

Table 3. List of taxa and length of ITS regions in base pairs (bp).

Tableau 3. Liste des taxons et longueur des régions ITS en paires de bases (bp).

Isolates	ITS 1	5.8S	2	Total
SK-BH	238	174	278	690
SK-FQ	239	174	271	684
SK-HH	238	174	279	691
SK-DH	238	174	277	689
SK-XM	239	174	279	692

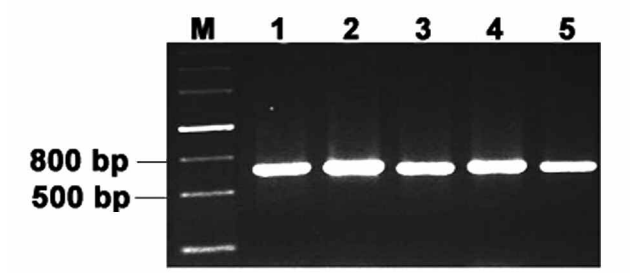

Figure 3. Electrophoresis patterns of the PCR-amplified LSU rDNA product in 1% agarose gel. Lane M, marker; Lane 1, the SK-BH isolate; Lane 2, the SK-FQ isolate; Lane 3, the SK-HH isolate; Lane 4, the SK-DH isolate; Lane 5, the SK-XM isolate.

Figure 3. Electrophorèse des ADNr LSU amplifies par PCR sur gel d'agarose 1%. Bande M, marqueur ; bande 1, isolat SK-BH ; bande 2, isolat SK-FQ ; bande 3, isolat SK-HH ; bande 4, isolat SK-DH ; bande 5, isolat SK-XM.

NJ method in Mega2.1. The NJ analysis using all ITS sequences resulted in a single NJ tree (Fig. 4). Analyses with or without outgroup resulted in the same NJ topology within *Skeletonema*. The NJ tree estimated from ITS sequences showed that all our five isolates clustered together with other *Skeletonema* spp. whose sequences were downloaded from GenBank with a 100% bootstrap value. The *Skeletonema* ingroup was divided into two lineages, lineage I and lineage II, strongly supported by a high confidence level (bootstrap value was 100%). In the lineage I, isolate SK-FQ, *S. marinoi* (Godhe et al., 2006) and *S. costatum* from Genbank were recovered. Lineage II contained two clades (A and B). Clade A was composed of isolates SK-DH and SK-XM, and their sister-group relationship was well supported by a bootstrap value of 97%. SK-HH, SK-BH and *S. pseudocostatum* from GenBank clustered and formed as clade B, supported by a low bootstrap value (52%). Additionally, SK-BH has a closer relative relationship to *S. pseudocostatum* than to SK-HH.

The NJ analysis with all LSU D1-D2 sequences resulted in a single NJ tree (Fig. 5). The topology of the tree corresponded partially to that reported by Sarno et al. (2005). The tree revealed four lineages within the genus *Skeletonema*, and most of them were well resolved. Lineage I contained *S. tropicum*, *S. pseudocostatum*, *S. grethae*, and *S. japonicum* and the LSU-sequence of isolate SK-HH was clustered together with those of all *S. tropicum* (Sarno et al., 2005). *S. menzelii* (Sarno et al., 2005) was recovered in lineage II, the sister clade to lineage I. Within lineage III, the sequences of SK-FQ and SK-BH grouped together, and as sister to those of *S. dohrnii* and *S. marinoi* in Sarno et al. (2005). Sequences of strains SK-DH and SK-XM grouped together, with *S. subsalsum* (Sarno et al.,

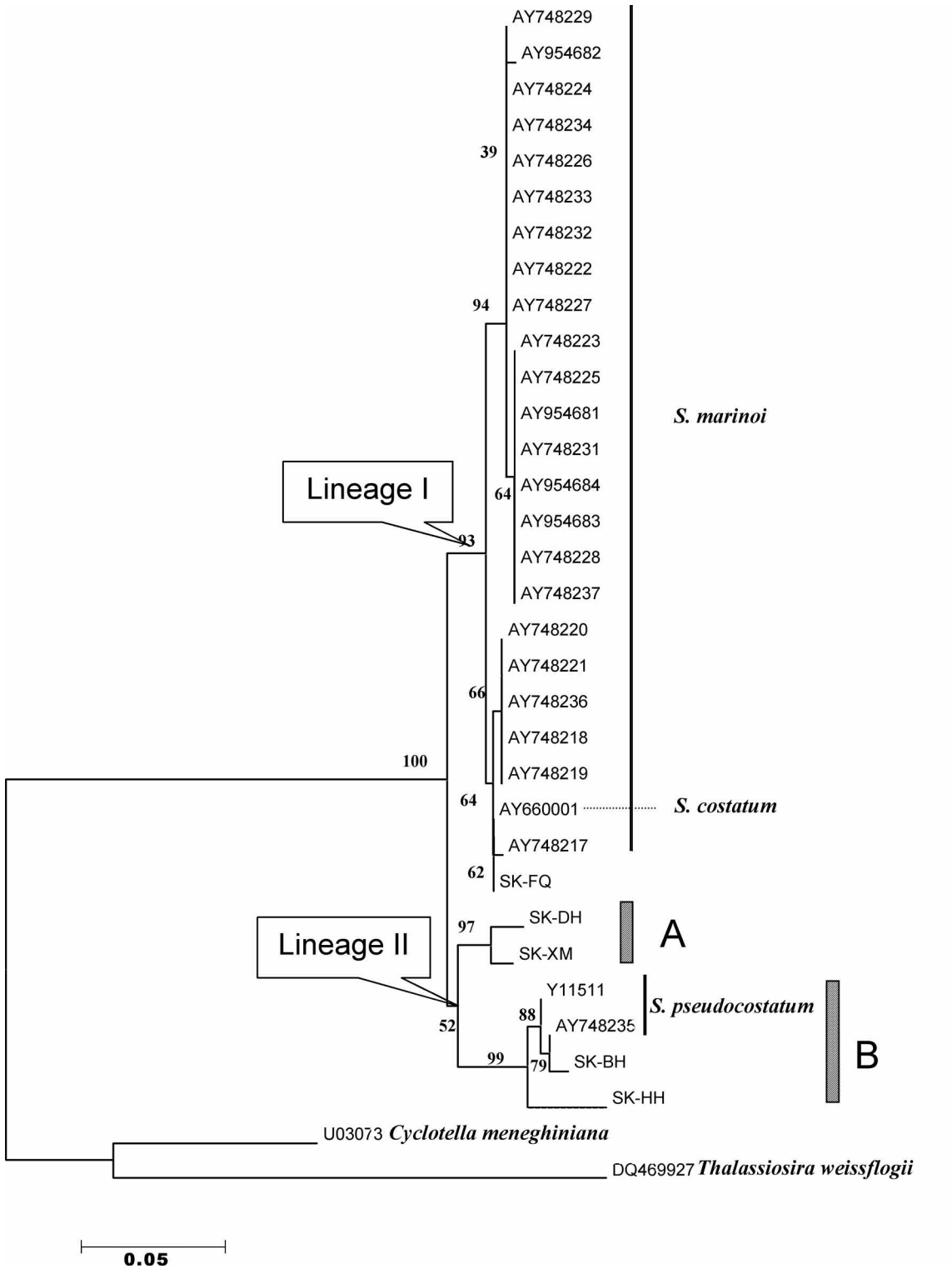

Figure 4. NJ tree inferred from the ITS sequences of *Skeletonema*; *Cyclotella meneghiniana* and *Thalassiosira weissflogii* have been chosen as outgroup. The outgroup sequences are downloaded from Genbank.

Figure 4. Arbre NJ construit d'après les séquences ITS de *Skeletonema*; *Cyclotella meneghiniana* et *Thalassiosira weissflogii* ont été choisis comme groupes externes. Les séquences des groupes externes ont été obtenus par GenBank.

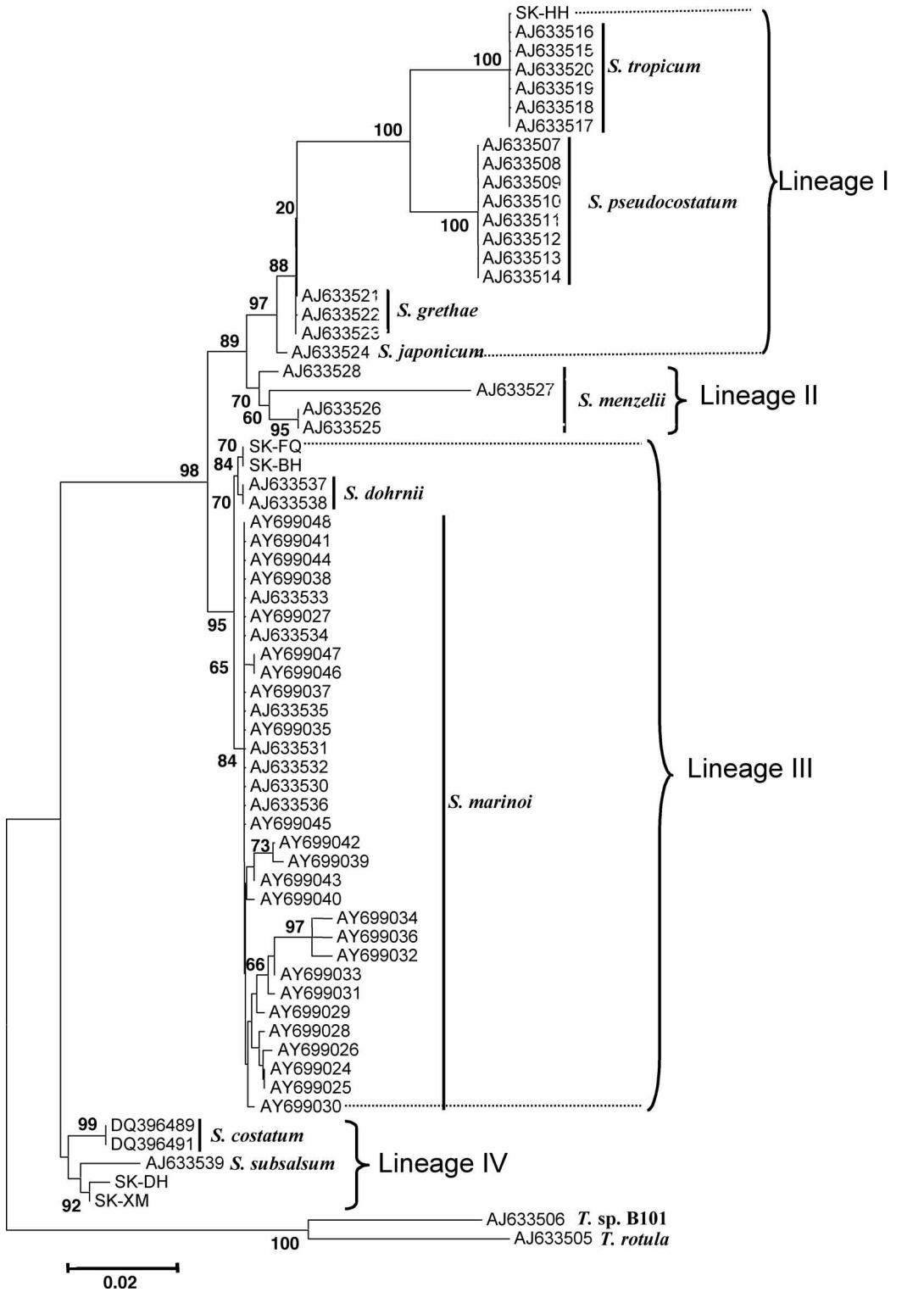

Figure 5. NJ tree inferred from the LSU rDNA sequences of *Skeletonema*; *Thalassiosira* sp. B101s and *T. rotula* have been chosen as outgroup. The outgroup sequences are downloaded from Genbank.

Figure 5. Arbre NJ construit d'après les séquences ADNr LSU de *Skeletonema*; *Thalassiosira* sp. B101s et *T. rotula* ont été choisis comme groupes externes. Les séquences des groupes externes ont été obtenus par GenBank.

2005) as sister clade in lineage IV, which contained *S. costatum* (Sarno et al., 2007) and was basal to the lineages I, II, and III.

Discussion

The nuclear ribosomal DNA (rDNA), including small subunit (SSU), LSU and ITS sequences have been used extensively to infer relationships among algal taxa at or below the species level (e.g. Medlin et al., 1991; Connell, 2001; Sarno et al., 2005). The SSU was originally used to explore molecular relationships within algae (Gunderson et al., 1987; Medlin et al., 1988). Because of its conservation, comparison of the SSU sequences is useful at higher taxonomic levels. The partial or whole LSU rDNA sequences are less conserved and therefore useful at lower taxonomic levels (Hillis & Dixon, 1991; Daugbjerg et al., 2000). In fact, the 5'- end of the large subunit rRNAs has been used widely to infer phylogenies of closely related taxa in protozoa, and alga (Qu et al., 1988; Gill et al., 1988; Baroin et al., 1988; Nanney et al., 1989). The internal transcribed spacers evolve even faster because these sequences do not participate in the post-processing of transcribed rDNA. Unfortunately, however, large insertions or deletions have resulted in considerable size variation among spacers of different organisms (Appels & Honeycutt, 1986). The characteristic of relatively rapid divergence rates (Schlötterer et al., 1994) makes the ITS region potentially useful as a tool for geographical and taxonomic studies at the population level within species (Steane et al., 1991; Adachi et al., 1996; Connel, 2001).

However, recently, more and more studies seemingly showed the perils of using rDNA at low taxonomic levels. Namely, many have found that high level of intragenomic polymorphism exists among cistrons (eg. Armbrust et al., 2004; Behnke et al., 2004; Orsini et al., 2004; Alverson & Kolnick, 2005), which maybe confound identification of species boundaries.

Despite that, Medlin et al. (1991) recognized a new species, by coupling the SSU sequences comparison and morphological analysis of four geographically separate *S. costatum*-like isolates. Sarno et al. (2005) performed a relatively comprehensive investigation of the phylogenetic relationships within all *Skeletonema* species excluding *S. costatum*, *S. potamos* and *S. cylindraceum* by comparing the SSU and partial LSU sequences, in spite of failing to obtain the SSU sequences of *S. tropicum* and *S. japonicum*. In this study, the phylogenetic relationships inferred from the SSU sequences correspond well to that inferred from the LSU sequences. Recently, Godhe et al. (2006) showed the utility of sequence variation along the variable domains (D1–D3) of the nuclear LSU rRNA gene and the ITS regions for distinguishing among clones of *S. marinoi* from various geographic regions. Specially, Sarno et al. (2007) introduced molecular sequences of *S. costatum sensu stricto*, *S. grevillei*, and *S. ardens* into phylogenetic analysis of genus *Skeletonema.* All these suggested the validity of applying rDNA sequences to phylogenetic analysis for *Skeletonema* strains from different locations.

In the light of studies performed by Zingone et al. (2005) and Sarno et al. (2005), we speculate on what is the real identity of our five red tide causative strains. Again, we introduce rDNA including LSU and ITS sequences into phylogenetic analysis, mainly performing comparisons with their sequence results. We did not consider SSU in our study, because SSU sequences may be difficult to amplify (Sarno et al., 2005; Sarno et al., 2007).

Unfortunately, Sarno et al. (2005) did not publish any ITS data, and we failed to obtain the ITS sequences of all present *Skeletonema* species but *S. marinoi*, *S. costatum* and *S. pseudocostatum* in GenBank, which holds back the full sequences comparison of the five isolates and other species. However, we could compare our LSU sequences with those of identified specimens in Sarno et al. (2005) and Godhe et al. (2006), and *S. costatum sensu stricto* in Sarno et al. (2007), facilitating the inference of relationships between the five isolates and the species presented in Sarno et al. (2005) and Godhe et al. (2006), and *S. costatum sensu stricto* in Sarno et al. (2007).

The ITS and LSU tree did not completely corroborate each other topologically in this study, partially due to the incompleteness of rDNA sequences of *Skeletonema* or presumable intragenomic polymorphism among cistrons (Alverson & Kolnick, 2005). However, in the LSU tree, all reported species were monophyletic, and most of them were well resolved, though *S. dohrnii* obtained low support (70%) and *S. grethae* obtained no support at all. Therefore, the phylogeny of the five strains could be arguably deduced by the LSU tree rather than ITS tree.

SK-HH grouped with other *S. tropicum* individuals in the LSU tree, and then this clade formed a sister-group with *S. pseudocostatum.* The genetic distance between SK-HH and all *S. tropicum* individuals were 0.000, and 0.032 between SK-HH and all *S. pseudocostatum* individuals. In the ITS tree, because *S. tropicum* samples were absent, SK-HH clustered with two individuals of *S. pseudocostatum*, with genetic distances value of 0.048 and 0.031 (data not shown), respectively. Therefore, SK-HH could be thought to be *S. tropicum* rather than *S. pseudocostatum*, despite it could not be corroborated by ITS tree, because of lacking the ITS sequences of *S. tropicum*.

Two topologies yielded from sequences of ITS and LSU consistently showed that SK-DH and SK-XM clustered in the same clade. They were both well resolved with a high bootstrap support of 97% for ITS and 92% for LSU, and reciprocally have a genetic distance of 0.004 for LSU and

0.021 for ITS (data not shown). The topology and genetic distance suggested that both strains might be a single species, which might be *S. potamos* or *S. cylindraceum* excluded in the present phylogenetic analysis. Possibly, it provides an evidence for a new species in the genus *Skeletonema*, which required further morphological observations and further comparisons to ITS and LSU sequences of other *Skeletonema* species.

In the analysis of LSU sequences, SK-BH and SK-FQ were recovered in a clade with a low bootstrap value of 66% in the NJ tree and a genetic distance of 0.000, much closer to *S. dohrnii* than to *S. marinoi*. However, the analysis of ITS sequences blurred this relationship. In the NJ tree of ITS, SK-BH was much closer to *S. pseudocostatum*, and SK-FQ was much closer to *S. marinoi* and *S. costatum* (AY66001). Additionally, a genetic distance of 0.089 exists between SK-BH and SK-FQ. We can presume that both strains are a single species with two ecotypes (LSU tree); alternatively, they are two distinct species (ITS tree).

Most publications about the occurrence of *Skeletonema* red tide in China refer to *S. costatum* or *Skeletonema* complex (Qi, 2003), but studies on what is the real identity of the causative species are imperative. Han et al. (2004) firstly demonstrated the RAPD polymorphisms and growth rate difference between two different strains *S. costatum* from China Sea, indicating that they may be different species. Recently, two additional species were reported, namely *S. grevillei* (Zingone et al., 2005) and *S. marinoi* (Sarno et al., 2005), in Hong Kong, South China Sea. The five *Skeletonema* species in this study are well separated with a long distance in geography along the coast of China Sea, and they maybe at least three distinct species, despite that they have not been finally defined. Repeated experiments and EM observations and more information on variability are needed before species can be identified reliably based on only morphology. However, the preliminary results not only means its diversity of red tide causative *Skeletonema*, but denotes more new species could be recognized in the China Sea coast, with more close and continuous water sampling.

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