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Identification of Microalgae Isolated from Green Water of Tilapia Culture Ponds in the Philippines

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Abstract

Four microalgal strains, NN-G, TB-G, CC-B, and SC-B isolated from green water of tilapia culture ponds in the Philippines were cultivated in 10 ml of ESS liquid medium at 23℃ with a 12 h light-12 h dark cycle. The cell forms of NN-G and TB-G were green small round and green large oval, respectively, while both CC-B and SC-B had brown cubic or quadrangular cells. Predominant bacteria isolated from the four microalgal cultures were commonly restricted to *Moraxella-Acinetobacter* group, *Pseudomonas* and *Flavobacterium*. Strains NN-G and TB-G contained chlorophyll *b*, chlorophyll *a*, violaxanthin and zeaxanthin, while strains CC-B and SC-B possessed chlorophyll *c*, chlorophyll *a*, and fucoxanthin as major pigments. Phylogenetic analysis based on SSU rDNA sequences demonstrated that strains NN-G, TB-G, CC-B and SC-B have close taxonomic relationships with *Chlorella* spp., *Tetraselmis* spp., *Chaetoceros* spp., and *Chaetocertos* spp., respectively.

Massive mortalities due to luminous vibrios, including *Vibrio harveyi*, has been a major problem in the culture of black tiger shrimp (*Penaeus monodon*) in Southeast Asia.[1] In intensive aquaculture ponds, various adverse conditions such as excess animal population and unconsumed feeds stimulate the growth of and infection by pathogenic vibrios.[2), 3] In order to protect the shrimps from infectious diseases and maintain the productivity, a high water exchange coupled with the massive use of antibiotics has been employed. This, however, has led to the spread of antibiotic resistant pathogenic strains.[4] More recently, the application of probiotics and microalgae have been considered as a more viable alternative. The use of "green water", the microalgae rich culture water originating from tilapia culture, especially, is seen as a more natural and less costly alternative in addressing the problem of disease control.

It is hypothesized that the extracellular substances produced by microalgal cells promote the growth of coexisting bacteria which inhibits the growth of pathogenic vibrios by a process of competitive exclusion. There is also a possibility that the extracellular metabolites and/or the cellular protoplasts of microalgal cells themselves act di-

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rectly to inhibit the growth of the bacterial pathogens. Many researchers[4-7] have shown that several microalgae produce substances that inhibit bacterial growth. Sieburth[8), 9)], in particular, described the antibacterial activity of corals which is due to their endosymbiotic zooxanthellae (dinoflagellates) and various diatoms.

Tilapia green water, as used in the culture of black tiger shrimps in the Philippines, contains several microalgae, the growth of which may be stimulated by the presence of organic or inorganic substances in the tilapia culture water. However, the specific components that stimulate microalgal growth, the mechanism of action wherein the microalgae suppress the growth of pathogenic vibrios, and the microalgal composition of tilapia green water are still unknown. This paper aims to identify microalgal strains isolated from tilapia green water in the Philippines mainly based on the analysis of pigment composition and SSU rDNA sequences.

Materials and Methods

Microalgal strains and culture

Four microalgal strains, NN-G, TB-G, CC-B and SC-B were isolated from so-called "green water" (greenish water) of tilapia (*Oreochromis niloticus*) culture ponds in Panay island of the Philippines in 2000. The microalgal strains were cultured in 10 ml of ESS liquid medium (modified Provasoli's enrichment seawater medium) at 23℃ under illumination condition (12 h light and 12 h dark cycle) as described in a previous paper[10]. Aliquots of the liquid cultures were put on a Thoma counting chamber and microalgal cells were counted.

Isolation and identification of bacteria from microalgal cultures

The microalgal cultures were diluted in 1/10 dilution series with ESS liquid medium and 0.1 ml of appropriate dilution solutions was spread on Z-CII agar plates. The agar plates were incubated at 25℃ for 6 days and then counted for bacterial colonies. Representative bacterial isolates (50 strains for each algal culture) were selected at random and examined for general morphological and physiological properties. The bacterial strains were identified at the genus level according to a brief identification scheme proposed by Ezura *et al*[11].

Extraction and identification of algal pigments

Microalgal cells cultivated in 100 ml of ESS medium were filtered through Whatman GF/F glass fiber filter. Pigments were extracted from the algal cells on the filter with 10 ml of cold 90% acetone by griding in a mortar as described by Akase *et al.*[12] Extracted solutions were immediately applied to high performance liquid chromatography (HPLC). Fifty microliter of the extracts was injected to an HPLC system (Waters Alliance System) equipped with a Model 2690 programmable solvent pump module and a Model 996 photodiode array detector module (Waters, USA) according to the procedure by Garrido and Zapata[12]. The column used was a Symmetry C18 monomeric octadecylsilane column (Waters, USA; the column size, 4.6 x 250 mm). A mixture of eluents A and B was used for elution; eluent A contained methanol and 1 M ammonium acetate (80:20 v/v), and eluent B contained acetonitrile and acetone (60:40, v/v). Elution was programmed as a linear gradient from 5% to 100% of eluent B against eluent A for 28 min, followed by an isocratic hold at 100% eluent B for 32 min. The

flow rate was 0.5 ml/min. Pigments were detected by absorption at 430 nm, and absorption spectrum from 200 to 800 nm was also determined for each pigment peak.

Extraction of DNA from microalgal cells

The microalgal cells were collected by centrifugation at 1,000 x g for 20 min after cultivation in 200 ml ESS medium for 10 days at 23℃ under the illumination (12 h light: 12 h dark). The total DNA was extracted using the nucleic acid purification kit SepaGene (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions.

Phylogenetic analysis of PCR-amplified SSU rDNA

Polymerase chain reaction (PCR) amplification of SSU rDNA of microalgal isolates was carried out using universal primers, 18SF (5'-AACCTGGTTG ATYCTGCCAG-3') and 18SR (5'-TGATCCTTC YGCAGGTTCACCTAC-3') specific for eukaryotic small subunit ribosomal RNA genes (SSU rDNA). Polymerase chain reaction solutions were prepared according to the instruction manual of AmpliTaq DNA polymerase (Applied Biosystems, USA) and thermal cycling was programmed as follows: 1 min at 95℃ for initial denaturation of template DNA, followed by 35 cycles of 1 min at 95℃, 2 min at 50 ℃, and 3 min at 72℃, with a final extension step for 8 min at 72℃. The nucleotide sequences of SSU rDNAs were determined for the PCR products cloned into a plasmid vector pT7Blue(R) (Novagen, USA) by using an ABI PRISM 310 genetic analyzer (Applied Biosystems, USA). The SSU rDNA nucleotide sequences of microalgal strains were aligned by the Clustal X multiple sequence alignment program version 1.8 (Thompson *et al.*, 1997)[13] with those of the representative eukaryotes from DNA Databank of Japan and Ribosome Database Project II. The multiple alignments obtained were used for bootstrap resamplings of 1,000 replications (Felsenstein, 1985)[14], and the construction of a phylogenetic tree by the neighbor-joining method using the PHYLIP program package version 3.572c (Felsenstein, 1989).[15]

Results

Coexistent bacteria in microalgal culture

The cell morphology of each microalgal strain is

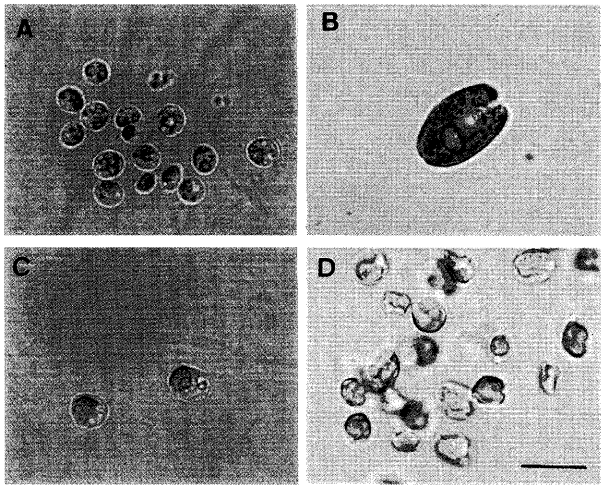

Fig. 1 Cell morphology of microalgal strains isolated from green water in tilapia culture ponds. Microalgal cells were cultivated in ESS liquid medium at 23℃ for 5 days under illumination (12 h light and 12 h dark). A, NN-G strain; B, TB-G; C, CC-B; D, SC-B. A scale bar indicates 10 μm.

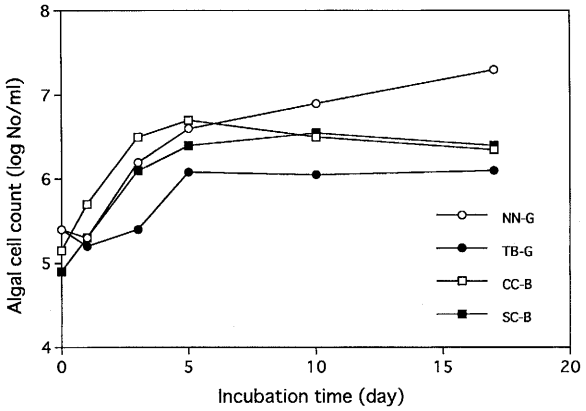

Fig. 2 Growth curves of microalgal strains grown in ESS liquid medium. Algal cells were counted on a Thoma counting chamber.

Table 1 Generic compositions of coexistent bacteria in microalgal cultures

Algal strains	Bacterial Genera			
	Total	*Mor.-Aci.*	*Pse.*	*Fla.*
NN-G	50	26	20	4
TB-G	50	22	28	0
CC-B	50	37	13	0
SC-B	50	21	21	8
Total number	200	106	82	12

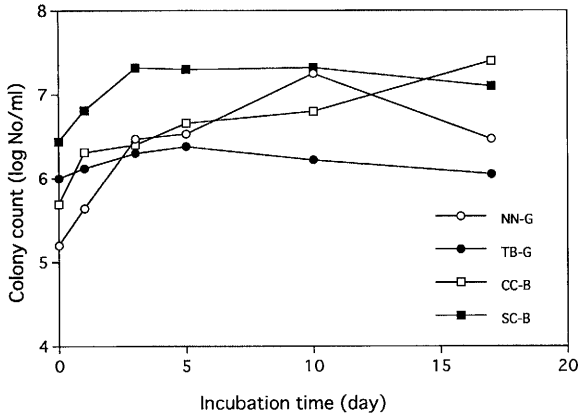

Fig. 3 Growth curves of bacteria grown in microalgal cultures. Bacterial colonies were counted after aliquots of microalgal culture were spread on Z-CII agar plates and incubated at 25℃ for 6 days.

demonstrated in Fig. 1. The cells of strain NN-G are green small round (3~5 μm in diameter) and surrounded with mucus materials. The cells of strain TB-G are green large oval (15~20 μm in long axis) and motile with two flagella. The cells of CC-B and SC-B are brown cubic or quadrangular (7~10 μm) and occasionally in short chains. As shown in Fig. 2, all strains grew fastly for 5 days and SC-B and NN-G continued to grow gradually after 5th day until 10th or 17th day. The strain TB-G was largest in cell size but lowest in cell number at maximum growth. Bacterial growth curves in the microalgal cultures are shown in Fig. 3. Viable count of bacteria was highest in SC-B culture and lowest in TB-G culture. As shown in Table 1, generic compositions of coexistent bacteria in the microalgal cultures were limited to 3 gram negative bacterial groups, *Moraxella-Acinetobacter*, *Pseudomonas* and *Flavobacterium*. *Moraxella-Acinetobacter* group was

Table 2 Retention times on HPLC and absorption maxima of pigments extracted from microalgal cells isolated from tilapia culture ponds

	Retention time (min) and absorption maxima (nm)					
	Standard pigments	NN-G	TB-G	CC-B	SC-B	C-16
Chlorophyll *c*				17.28	17.28	17.29
				(447, 582, 632)	(447, 584, 632)	(447, 582, 632)
Fucoxanthin	22.13			22.13	22.13	22.12
	(449)			(449)	(449)	(449)
Unknown a			22.40			
			(446, 475)			
Unknown b		22.83	22.82			
		(413, 438, 466)	(413, 437, 466)			
Violaxanthin	23.89	23.98	23.97			
	(417, 441, 470)	(417, 440, 470)	(417, 441, 470)			
Unknown c				26.09	26.10	26.09
				(448, 478)	(448, 478)	(448, 478)
Unknown d				26.48	26.48	26.46
				(442)	(442)	(443)
Zeaxanthin	29.9	29.38	29.36			
	(454, 481)	(447, 474)	(446, 475)			
Unknown e			33.63			
			(448, 476)			
Chlorophyll *b*	36.81	36.89	36.89			
	(457, 645)	(457, 646)	(457, 645)			
Unknown f		39.72	39.72	39.64	39.57	
Chlorophyll *a*	40.73	40.99	41.00	41.04	41.05	41.02
	(430, 613, 662)	(430, 617, 662)	(430, 616, 661)	(431, 617, 662)	(431, 617, 662)	(431, 617, 662)

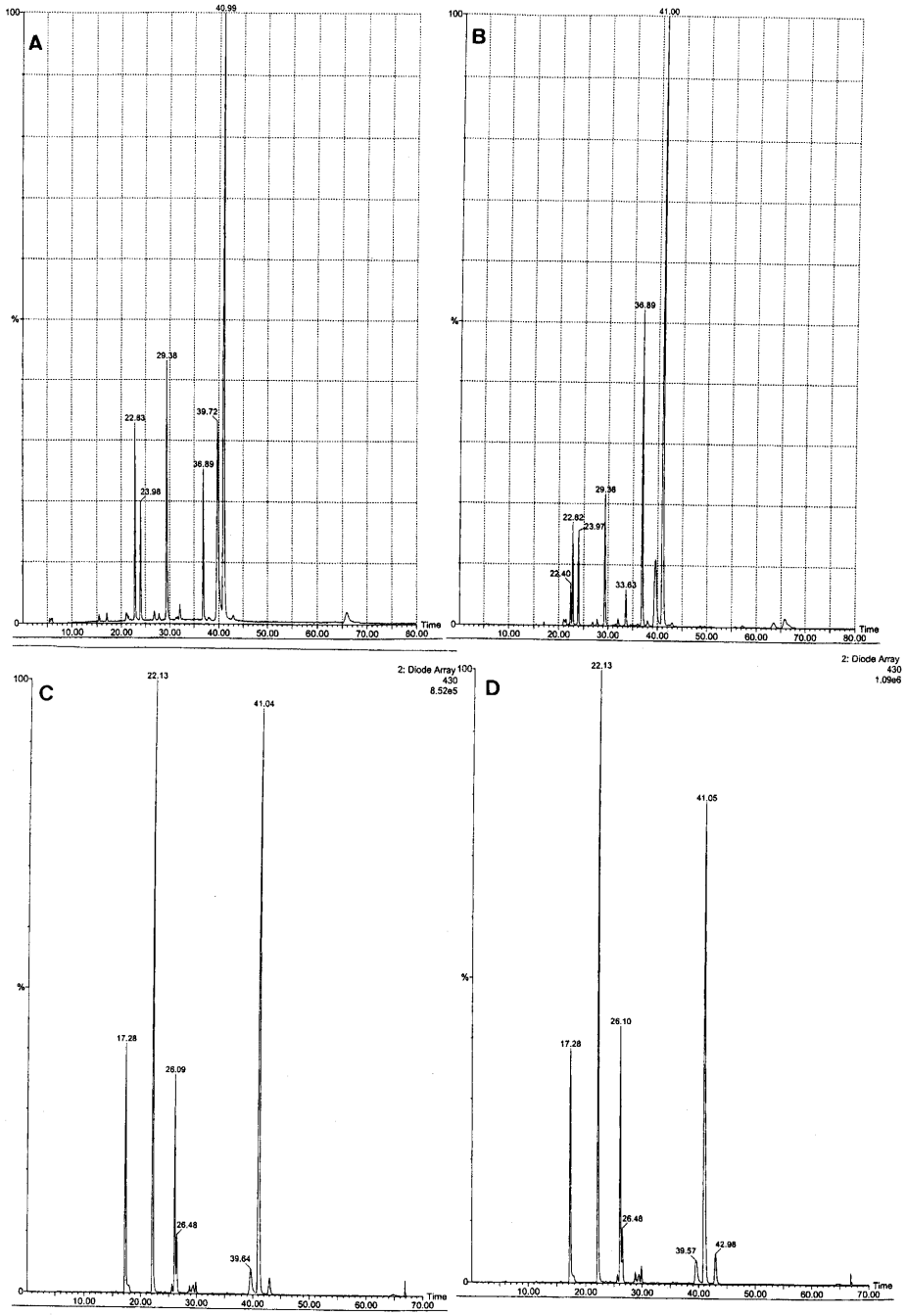

Fig. 4 HPLC chromatograms of pigments extracted from microalgal cells grown in 300 ml of ESS medium for 10 days. A, NN-G strain; B, TB-G; C, CC-B; D, SC-B.

isolated at 53% and the genera *Pseudomonas* and *Flavobacterium* were found at 41% and 6%, respectively.

Pigment compositions of microalgal cells

Chromatograms of pigments extracted from microalgal cells on HPLC using C18-ODS column are shown in Figs. 4A, B, C, and D. Fig. 5 and Table 2 demonstrate identification of main pigments based on their absorption spectra. Strains NN-G and TB-G contained chlorophyll *b*, chlorophyll *a*, violaxanthin, and zeaxanthin as main pigments. On the other hand, strains CC-B and SC-B possessed chlorophyll *c*, chlorophyll *a*, and fucoxanthin

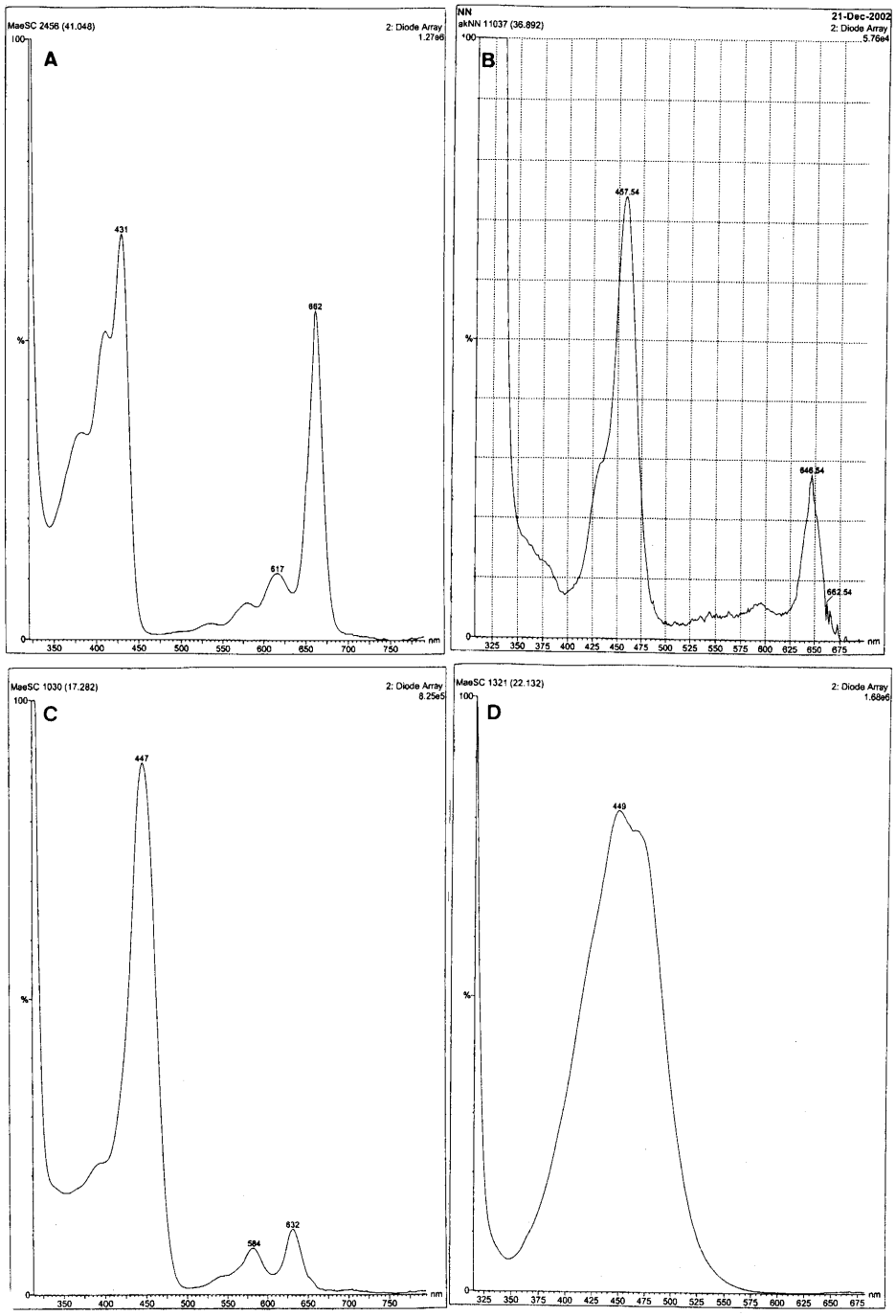

Fig. 5 Absorption spectra of pigment peaks on HPLC chromatograms. A, chlorophyll *a* from SC-B strain; B, chlorophyll *b* from NN-G strain; C, chlorophyll *c* from SC-B strain; D, fucoxanthin from SC-B strain.

similarly with *Chaetoceros* C-16 strain.

Phylogenetic analysis of microalgal strains

A phylogenetic tree based on SSU rDNA sequences is demonstrated in Fig. 6. CC-B and SC-B are clustered with *Chaetoceros* spp. On the other hand, NN-G is clustered with *Chlorella* sp. and TB-G is clustered with *Tetraselmis striata*. Table 3 shows the results of homology search on SSU rDNA sequences of the Database. These results also indicated that NN-G, TB-G, CC-B and SC-B have close taxonomic relationships with *Chlorella* spp.,

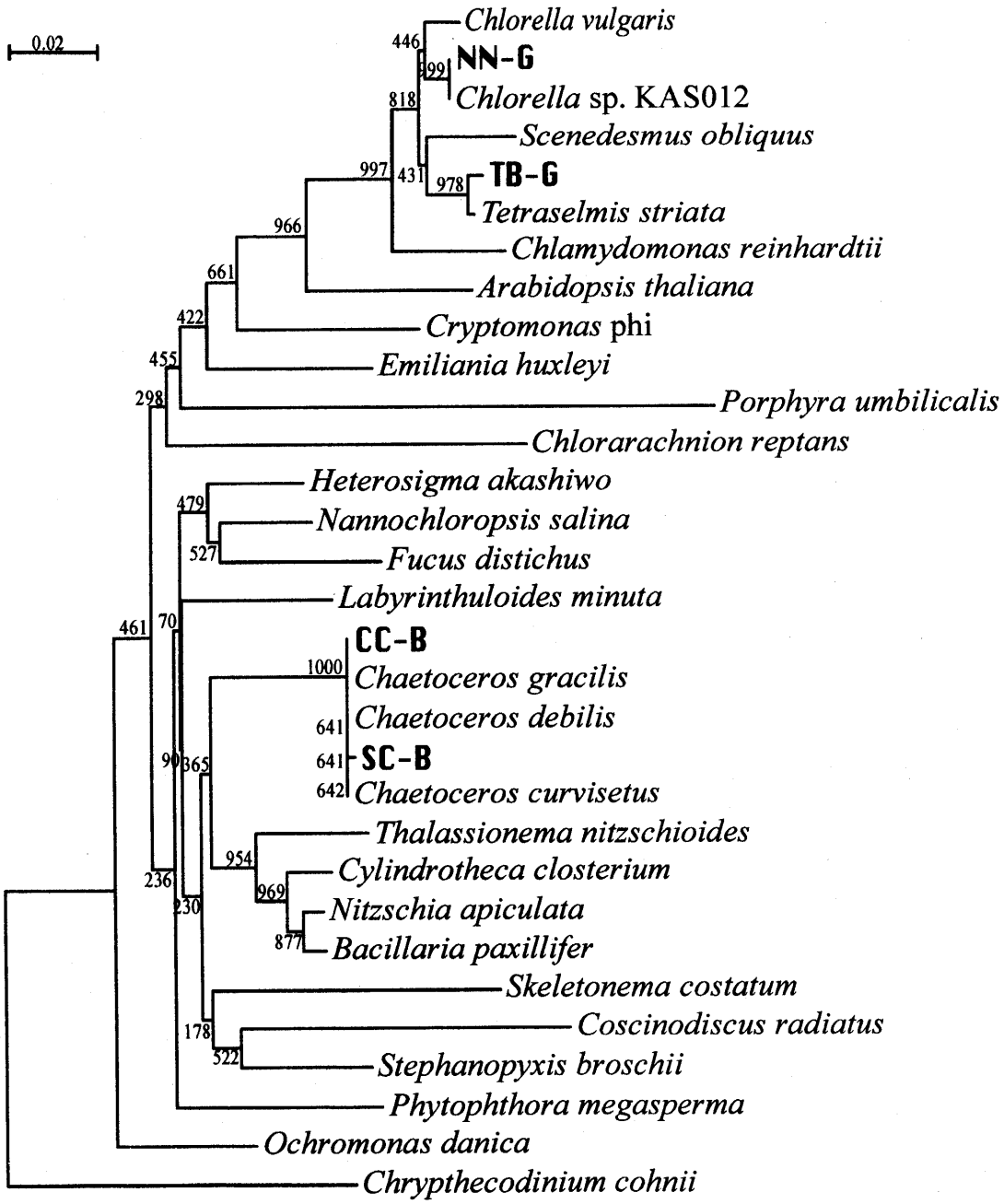

Fig. 6 Phylogenetic tree inferred from SSU rDNA sequences of microbial strains and eukaryote representatives. The tree was constructed by the neighbor-joining method. A scale bar indicates genetic distance. Numbers at the branching points indicate bootstrap values of 1,000 replicates.

Tetraselmis spp., *Chaetoceros* spp., and *Chaetocertos* spp., respectively.

Discussion

Luminous vibriosis caused by *Vibrio harveyi* is a serious problem to shrimp industry in Southeast Asia countries. Various measures have been attempted to protect or to decrease the damage in shrimp aquacultures. Among them, green water occurred in tilapia culture ponds is utilized for black tiger prawn in the Philippines. Green water from finfish culture ponds is empirically thought to suppress the growth of luminous vibrios due to

Table 3 Homology among SSU rDNA sequences of microalgal isolates and Database strains

Test strains	DNA length (bp)	Database strains and homology (%)		
NN-G	384 (5'terminus)	*Chlorella* sp. KAS012 97	*Chlorella* sp. KAS005 97	*Chlorella* sp. KAS001 97
	821 (3'terminus)	*Chlorella* sp. MBIC10088 98	*Chlorella* sp. KAS012 98	*Chlorella* sp. KAS001 98
TR-G	614 (5'terminus)	*Tetraselmis striata* 99	*Tetraselmis* sp. RG-07 98	*Tetraselmis convolutae* 98
	559 (3'terminus)	*Tetraselmis* sp. RG-07 99	*Tetraselmis striata* 99	*Tetraselmis convolutae* 98
CC-B	1634	*Chaetoceros gracilis* 99	*Chaetoceros debilis* 99	*Chaetoceros curvisetus* 99
SC-B	875 (5'terminus)	*Chaetoceros gracilis* 99	*Chaetoceros debilis* 99	*Chaetoceros curvisetus* 99
	288 (3'terminus)	*Chaetoceros gracilis* 95	*Chaetoceros debilis* 95	*Chaetoceros muelleri* 95

inhibitory substances produced by some microalgal components. It is not clear what kinds of microalgae are most effective and what materials inhibit the growth of pathogenic vibrios. In this study, the authors identified microalgal strains isolated from green water of aquaculture ponds of tilapia in the Philippines, being based mainly on the analysis of pigment compositions and SSU rDNA sequences.

Firstly, coexistent bacteria isolated from the culture of each microalgal strain were characterized and identified at the genus level. Composition of bacterial colonies with characteristic appearance from each microalgal strain was significantly different but the generic composition of dominant bacteria was very similar for each microalgal strain. They were composed of 53% *Moraxella-Acinetobacter* groups, 41% the genus *Pseudomonas*, and 6% *Flavobacterium*. *Vibrio* was not at all detected. It is not known whether extracellular products from microalgal cells or coexistent bacteria suppressed the growth of *Vibrio* spp. These coexistent bacteria have been selected in each microalgal culture for long transfer process and the bacterial composition of microalgal cultures should be different with that of original green water. However, the selected bacteria are considered to be closely associated with microalgal cells. The addition of antibiotics suppresses the growth of coexistent bacteria in microalgal culture, and the growth rate and level of microalgal strains without bacteria significantly decreased compared to those with bacteria (data not shown). These results indicate the mutual relationship between microalgal cells and coexistent bacteria in the culture.

Analysis of intracellular pigments of each microalgal cells demonstrated that NN-G and TB-G strains contained chlorophyll *a* and chlorophyll *b* as photosynthetic pigments which are characteristic to green algae, while CC-B and SC-B contained chlorophyll *a*, chlorophyll *c*, and fucoxanthin specific to diatoms. Furthermore, taxonomic positions of four microalgal strains were determined by phylogenetic analysis based on SSU rDNA sequences; NN-G, TB-G, CC-B, and SC-B strains have very close relationship with *Chlorella* spp., *Tetraselmis* spp., *Chaetoceros* spp., and *Chaetoceros* spp., respectively.

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