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Genetic Relationships among Multiple Strains of the Genus *Tetraselmis* Based on Partial 18S rDNA Sequences

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Molecular genetic tools are widely used to learn more about the identical characterization of obscure microalgal strains. At the Korea Marine Microalgae Culture Center (KMMCC), the authors deduced the genetic relationship of 41 strains of the genus *Tetraselmis* by analysing a small subunit ribosomal DNA (18S rDNA) sequences. Forty-one strains were seperated into five groups, which showed over a 98-99% similarity to *Tetraselmis striata* or *Tetraselmis* sp. Tsbre. Also, 13 strains among them had an identical genotype to *Tetraselmis striata* while 5 strains had with *Tetraselmis* sp. Tsbre, respectively. The mean size of each strain generally showed the tendency of different variation according to the groups.

Key Words: genotyping, microalgae, Prasinophyceae, *Tetraselmis*, 18S rDNA

INTRODUCTION

Species of *Tetraselmis* in Prasinophyceae are well known as the basic food organisms in aquaculture (Kim and Hur 1998; Park and Hur 2000; Cabrera *et al.* 2005), as an important source for antioxidative substances in pharmacological studies (Laguna *et al.* 1993; Kim *et al.* 2002), and for their inportance in marine ecotoxicological testing (Park *et al.* 2005).

Since the first description of *Tetraselmis* by F. Stein in 1878 (Norris *et al.* 1980), many taxonomical studies on this green motile unicellular algae have been reported. The genus of *Tetraselmis*, first formalized according to the Christensen Criterion (Christensen 1962) has been identified by not only characteristics such as scale, flagellar hair, and basal bodies (Moestrup and Throndsen 1988; Marin and Melkonian 1994; Throndsen 1997), but also by types of pigments (Egeland *et al.* 1997; Latasa *et al.* 2004).

Although, by using light and electron microscope, morphological classification is possible at the species level, taxonomic decisions within the *Tetraselmis* are difficult to make because of the complex process of cellular characterization.

Genetic data based upon the amplification and sequencing of genes are also accumulated to use as a powerful tools for analysis of diverse microalgae. Thus, it is necessary to collect molecular data on *Tetraselmis* to determine whether they are identical strains or not. However, it is still difficult to find molecular studies on *Tetraselmis*.

In this study, 18S rDNA sequence of 41 strains of *Tetraselmis* was analysed to discriminate the genomic variations. The strains were constructed as a phylogenetic tree by comparing them with other known sequences from the National Center for Biotechnology Information (NCBI) database. These variations were also compared with the differences in the mean size of each strain of *Tetraselmis*.

MATERIALS AND METHODS

Microalgae culture condition

Forty-one strains of *Tetraselmis* were received from the Korea Marine Microalgae Culture Center (KMMCC) (Hur 2008). Information about the strains is given in Table 1. The strains were grown in 100 mL of f/2 culture medium (Guillard and Ryther 1962) at 22°C with continuous light with 60 μmol photons m^{-2} s^{-1} for 10 days.

The Mean length of 30 specimens of the *Tetraselmis* strain was measured using a light microscope (×400). Significant differences in mean size of the strains were analysed using ANOVA and Ducan tests (SPSS software version 10.1; SPSS Inc., Chicago, IL, USA) at the level of 5%.

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Table 1. Culture history of forty-one strains of *Tetraselmis* and their GenBank accession numbers for the 18S rDNA sequences

KMMCC Strain no.	Species	Sampling area and date	Accession number	Length of sequences (base pairs)
P01	*Tetraselmis hazennii*	U.S.A. (UTEX 171)	FJ517748	1673
P02	*Tetraselmis tetrathele*	U.S.A. (The Oceanic Institute)	FJ517749	1674
P03	*Tetraselmis* sp.	Haeundae (1991-06-01)	FJ559376	1641
P04	*Tetraselmis suecica*	England (CCAP 66/22A)	FJ559377	1674
P05	*Tetraselmis striata*	Incheon: Oido (1986-06-01)	FJ559378	1653
P06	*Tetraselmis subcordiformis*	China (Qingdao institute)	FJ559380	1641
P08	*Tetraselmis* sp.	Deukryang Bay (1995-05-01)	FJ559379	1653
P09	*Tetraselmis suecica*	Deukryang Bay (1995-05-01)	FJ559381	1674
P10	*Tetraselmis* sp.	Deukryang Bay (1995-05-01)	FJ559382	1643
P11	*Tetraselmis striata*	Deukryang Bay (1995-05-01)	FJ559383	1653
P12	*Tetraselmis carteriiformis*	Nakdong river (1995-05-01)	FJ559384	1641
P13	*Tetraselmis carteriiformis*	Busan coast (1995-01-25)	FJ559385	1653
P17	*Tetraselmis* sp.	Anmyundo (1995-07-01)	FJ559393	1641
P18	*Tetraselmis striata*	Deukryang Bay (1995-07-01)	FJ559392	1641
P20	*Tetraselmis* sp.	Incheon: Oido (1986-05-01)	FJ559391	1674
P21	*Tetraselmis* sp.	Deukryang Bay (1996-01-23)	FJ559390	1674
P22	*Tetraselmis carteriiformis*	Deukryang Bay (1996-04-23)	FJ559389	1641
P24	*Tetraselmis carteriiformis*	Incheon: Oido (1986-04-01)	FJ559388	1641
P27	*Tetraselmis striata*	Chungmu (1996-07-12)	FJ559387	1632
P31	*Tetraselmis* sp.	Mokdo (2000-10-24)	FJ559394	1653
P32	*Tetraselmis striata*	Yangyang (2000-11-06)	FJ559398	1641
P33	*Tetraselmis* sp.	Buan (2001-03-29)	FJ559397	1641
P34	*Tetraselmis* sp.	Buan (2001-03-29)	FJ559396	1641
P35	*Tetraselmis striata*	Ulrungdo (2000-09-25)	FJ559395	1641
P36	*Tetraselmis striata*	Haeundae (1998-07-01)	FJ559399	1641
P37	*Tetraselmis striata*	Buan (1999-10-01)	FJ559402	1674
P39	*Tetraselmis chuii*	Scotland (CCAP 8/6)	FJ559401	1642
P42	*Tetraselmis* sp.	Haeundae (2004-03-05)	FJ559400	1641
P43	*Tetraselmis striata*	Haeundae (2004-03-05)	FJ559403	1620
P47	*Tetraselmis* sp.	Haeundae (2003-05-01)	FJ559404	1653
P48	*Tetraselmis* sp.	Haeundae (2004-08)	FJ559405	1641
P49	*Tetraselmis* sp.	Haeundae (2004-08)	FJ559406	1653
P52	*Tetraselmis* sp.	Yeosu (2005-12)	FJ559407	1641
P55	*Tetraselmis* sp.	Namhae (1990-05-14)	GQ917214	1674
P56	*Tetraselmis striata*	Buan (1999-08-14)	GQ917218	1674
P57	*Tetraselmis* sp.	Uljin (2001-03-01)	GQ917211	1674
P58	*Tetraselmis striata*	East sea (1999-10-15)	GQ917210	1674
P59	*Tetraselmis* sp.	Deukryang Bay (1995-05-26)	GQ917215	1674
P60	*Tetraselmis* sp.	Deukryang Bay (1995-05-06)	GQ917216	1674
P62	*Tetraselmis* sp.	South sea (1996-05-17)	GQ917217	1675
P63	*Tetraselmis* sp.	Hongdo (1999-07-22)	GQ917219	1674

KMMCC, Korea Marine Microalgae Culture Center; UTEX, University of Texas; CCAP, Culture Collection of Algae and Protozoa.

Genomic DNA extraction and polymerase chain reactions

The LiCl method was used to extract the genomic DNA of the *Tetraselmis* (Hong *et al.* 1995). The quality and quantity of the extracted genomic DNA were measured by electrophoresis (Mupid^{TM}; Advance, Tokyo, Japan) and spectrometry (NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA), respectively.

Polymerase chain reactions (PCR) (Mullis and Faloona 1987) performed using 10-100 ng of genomic DNA as a template and 0.5 μM of degenerated primers (Table 2) derived from a conserved region of 10 species of other

Table 2. List of sequences of oligonucleotides used for the polymerase chain reactions

Primer name	Sequence (5′→3′)
P1_SSU_F	5′- GCA TGT CTA AGT ATA AAC TGC -3′
P1_SSU_R	5′- GGT TTG GAG (A/G)AC TTC TCA GC -3′
P2_SSU_F	5′- GGC TCA TTA AAT CAG TTA TAG -3′
P2_SSU_R	5′- CCT TGT TAC GA(C/T) TTC TCC TTC -3′

Table 3. The strains used for the construction of a phylogenetic tree and their GenBank accession numbers of 18S rDNA sequences

Species	Strain name (when available)	Accession number
Tetraselmis sp.	Tsbre	EF473736
Tetraselmis chuii	Ifremer-Argenton	DQ207405
Tetraselmis kochiensis		AJ431370
Tetraselmis sp.	MBIC11125	AB058392
Tetraselmis sp.	RG-07	U41900
Tetraselmis convolutae	208	U05039
Tetraselmis sp.	NT18	AY954899
Tetraselmis sp.	TEQL01	AY954898
Tetraselmis sp.	RCC 500	AY425299
Tetraselmis striata	PLY443	X70802
Chlorella vulgaris	SAG 211-11b	X13688

known microalgae, listed in Table 3. Amplification conditions consisted of one cycle of denaturation at 95°C for 5 min, 30-35 cycles of denaturation at 95°C for 30 s, annealing at 50-55°C for 30 s and extension at 72°C for 1 min 45 s, followed by the final extension at 72°C for 7 min, and then stored at 4°C. About a 1.7 Kb size of PCR product was confirmed at 1% gel electrophoresis and extracted using the AccuPrep® Gel Purification Kit (Bioneer, Daejeon, Korea). Purified PCR products were ligated with the pGEM®-T Easy Vector Systems (Promega, San Luis Obisco, CA, USA) and then were transformed into the *Eschrichia coli* XL-1 blue. The recombinant plasmids were purified using the AccuPrep® Plasmid Extraction Kit (Bioneer, Korea). Selected clones were confirmed by colony PCR and EcoR restriction enzyme digestion (Fermentas, Burlington, Ontario, Canada) followed by DNA sequencing (Genotech, Daejeon, Korea).

Sequence alignments and phylogenetic analysis

The identities of the acquired partial 18S rDNA sequence from *Tetraselmis* strains were confirmed by using the Blast N program (http://blast.ncbi.nlm.nih.gov) and were aligned with the other known microalgae 18S rDNA sequences using ClustalW2 (Thompson *et al.* 1994). The accession numbers of the microalgae sequence found in the NCBI GenBank are given in Table 3.

To confirm the phylogenetic relationships, a total of 1615 positions in the final dataset were subjected to neighbor-joining (NJ) and maximum-parsimony (MP) analysis using the MEGA v.4.0 (MEGA, Tempe, AZ, USA) (Tamura *et al.* 2007). The evolutionary distance method to construct the NJ tree was calculated with the Kimura 2-parameter (Kimura 1980). The rate variation among sites was modeled with a gamma distribution (0.25-parameter) and all positions containing gaps and missing data were eliminated from the dataset. The MP tree was obtained using the close-neighbor-interchange algorithm (Nei and Kumar 2000) in which the initial trees were obtained with the random additional 100 replications of sequences. All positions containing gaps and missing data were eliminated from the dataset. The reliability of both trees was tested by 2000 replication bootstraps. The 18S rDNA sequence of *Chlorella vulgaris* was used as the outgroup sequence.

RESULTS AND DISCUSSION

To test their generic similarity, the almost complete 18S rDNA sequences were determined for the 36 strains collected from Korean coastal water and 5 strains from foreign areas. Their accession numbers registered at GenBank are given in Table 1 and the lengths of sequence were varied on the sense and antisense primer used in gene amplification (Table 2). Forty-one strains showed high levels of identity (98-99%) to the correspoding regions of known 18S rDNA sequences such as *Tetraselmis* striata PLY 443 or *Tetraselmis* sp. Tsbre using the Blast N program. No differences were found between 13 strains (KMMCC P-5, 11, 18, 22, 24, 27, 32, 35, 36, 37, 43, 56 and 58: group A) and *Tetraselmis striata* PLY 443 or among 5 strains (KMMCC P-2, 3, 8, 9 and 47: group C) and *Tetraselmis* sp. Tsbre in their acquired rDNA sequences. Thirteen sequence positions were different between group A and C (Fig. 1). Four strains in group C were collected in May and June from the southern coast of Korea, and 13 strains in group A were gathered from diverse Korean coastal waters during all four seasons. In terms of the algal length, those of group A ranged from 8.75 μm to 13.75 μm were shorter than those of group C, which ranged from 12.5 μm to 17.5 μm.

The sequences of the other 35 strains were seperated

		G	C	T	A	C	G	G	T	C	T	C	G	T	A	A	G	C	A	C	C	T	G	C	C	C	T	G	T	C	G	C	G	C	A	A	G	A
A																																						
B	P13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
	P31	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
	P33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P48	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P52	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P55	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
	P57	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P59	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P60	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
	P62	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P63	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C		.	.	C	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
D	P1	.	.	C	.	.	A	.	.	.	C	T	A	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
	P4	.	.	C	.	.	.	.	.	.	C	T	A	.	.	.	.	.	.	T	.	.	T	G	T	C	.	.	T	.	T	.	.	.	.	.	.	.
	P10	.	.	C	.	.	.	.	.	.	C	T	A	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
	P20	.	T	C	.	.	.	.	.	G	C	T	A	.	.	T	.	.	.	T	.	.	.	.	.	C	.	C	T	.	T	.	.	.	.	A	G	
	P34	.	.	C	.	.	.	.	.	.	C	T	A	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
	P39	.	.	C	.	.	.	.	.	.	C	T	A	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
	P49	.	.	C	.	.	.	.	.	.	C	T	A	.	.	.	.	.	G	T	.	.	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	.
E	P6	A	.	.	C	T	.	A	.	.	.	.	A	G	G	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	A	.	.	.	.
	P12	A	.	.	C	T	.	A	.	.	.	.	A	G	G	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	A	.	.	.	.

Fig. 1. Variable positions of 18S rDNA sequence from multiple strains of the genus *Tetraselmis* with five different groups (A-E). Dot corresponds to nucleotide of group A which has identical sequence with *Tetraselmis striata* and different sequence indicates nucleotide. Dash denotes a missing nucleotide.

A		C	T	A	C	T	G	-	C	G	T	-	T	A	-	A	T	T	A	A	G	A	A	C	T	C	A	G	T	T	G	G	T	C	T	G	A
B	P13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.
	P48	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
	P52	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
	P55	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P57	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
	P59	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
	P60	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P62	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P63	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C		.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	.	G
D	P1	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	.	G
	P4	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	.	.	.	A	.	.	T	.	G
	P10	.	.	.	.	.	A	.	.	.	.	A	.	.	G	.	.	.	G	.	.	.	.	T	.	.	.	A	.	.	.	A	.	.	T	.	G
	P20	T	.	.	T	.	A	.	.	.	.	.	.	T	.	.	C	.	.	.	T	.	.	.	.	.	.	A	.	.	.	A	.	.	T	.	G
	P34	.	.	.	.	.	A	.	.	A	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	A	.	.	T	.	G
	P39	.	.	.	.	.	A	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	A	A	.	.	T	.	G
	P49	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	T	T	.	G
E	P6	.	A	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	T	.	G
	P12	.	A	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	A	.	.	.	A	.	.	T	.	G

Fig. 1. Continued.

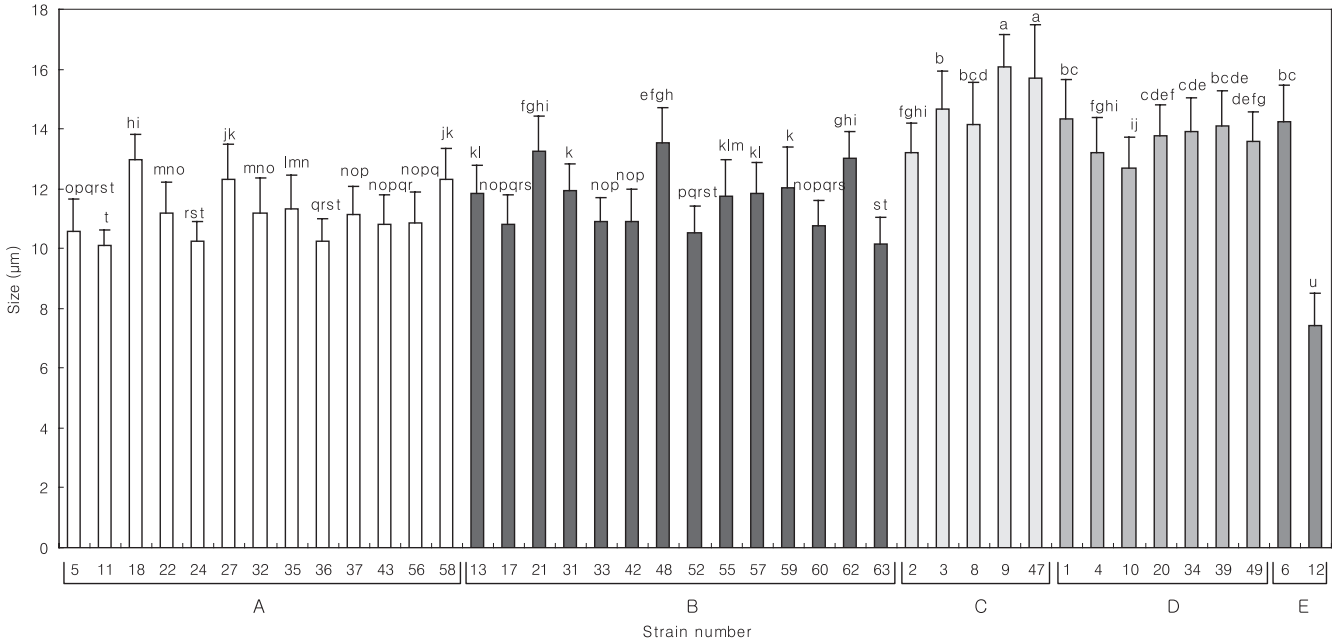

Fig. 2. Mean size in length of each *Tetraselmis* strain. Forty-one strains were divided into 5 groups (A-E). Data is expressed as mean ± SD. Bars with dissimilar superscripts indicate different significance at the level of 5%.

into 3 groups; B, D and E. Of these, fourteen strains belonged to group B, which was very close to group A. Only 1-2 sequence positions differentiated between group A and B, excepting KMMCC P-57. Group D contained seven strains which were close to group C but the level of similarity between groups C and D was less so than between groups A and B. Different sequence positions of six strains of group D were 1-4 base pairs compared with group C, whereas KMMCC P-20 was the furtherest from it with 14 sequence differences. The different sequence position of KMMCC P-6 and P-12 in group E showed only one base pair. But group E differed slightly from both of group A and group C. It had 17 different sequence positions in KMMCC P-6 or 18 differences in KMMCC P-12 compare with group A. Eighteen or 19 base pairs differed between group C and KMMCC P-6 or P-12, respectively.

Comparing the algal size with the 18S rDNA sequence analysis in each group, the relationship exhibited more clearly in the algal length than in the width (data not shown). The distribution of mean length is divided into; groups A and B, and groups C and D. The length of the strains from group B was similar to that of group A, *Tetraselmis striata*, but KMMCC P-21, P-48 and P-62 were larger than the others (Fig. 2.). These three strains were closer to group C than group A. Although KMMCC P-6 and P-12 were considered as the same group E at the point of the gene sequence, their size was significantly different. Among the strains, KMMCC P-6 was the largest with 14.25 μm, and P-12 was the smallest with 7.42 μm. This result means that relationships between 18S rDNA sequences and the size of the strains do not always coincide.

To analyze the relationship of 18S rDNA sequences among the strains of *Tetraselmis*, a phylogenetic tree was constructed using the NJ and MP methods with 2000 bootstrap values (Fig. 3). KMMCC P-36 and P-2 were selected as representative strains from identical sequences of groups A and C, respectively. Most strains were contained within two clades although separation was supported with less than 50%; 38% and 43% bootstrap values from NJ and MP, respectively. Groups A and B were clustered with *Tetraselmis striata* and *T. convolutae* excepting the branch order for KMMCC P-31 and P-57 strains from the clade with a 72% bootstrap value in NJ tree. They had two and five different sequence positions respectively. Other clades were groups C and D, which were comprised of *Tetraselmis chuii* and *Tetraselmis* sp. Tsbre. Group E, comprised of KMMCC P-6 and P-12 was separated from clades of groups C and D with 54% and 50% bootstrap support in NJ and MP tree, respectively. This corresponded with the results on different sequence positions.

Tetraselmis currently contains about 30 species identified by morphological inspection from public microalgal culture collections (e.g., UTEX, CCMP, CCAP). However,

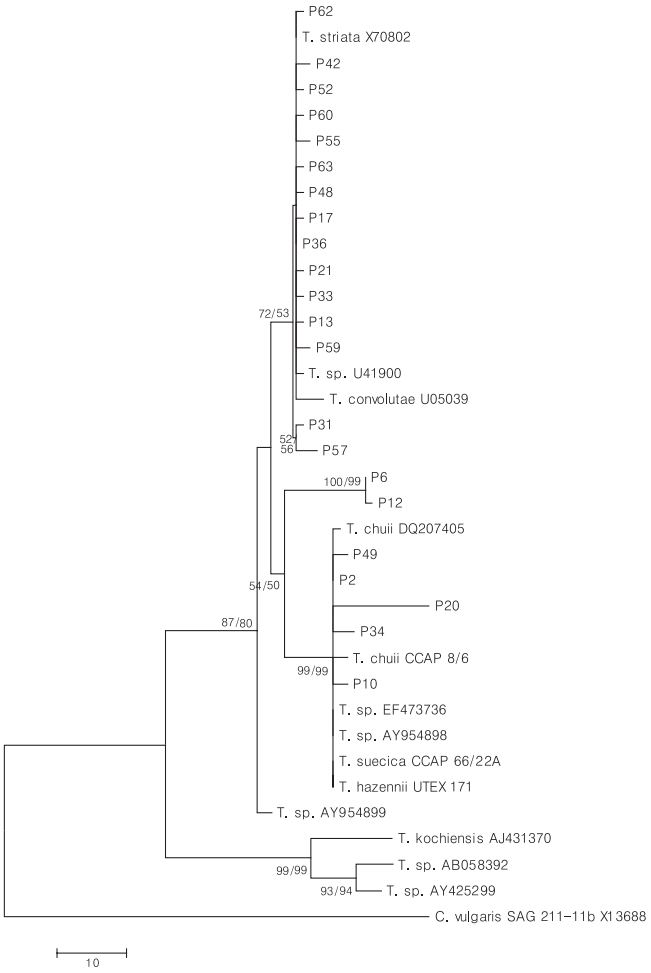

Fig. 3. Phylogenetic tree using the maximum parsimony method inferred from 18S ribosomal DNA sequences of the genus *Tetraselmis*. P-36 and P-2 are indicated as a representative strain for group A and group C, which have identical sequences, respectively. Tree reliability is tested by 2000 replications of bootstraps, which indicate numbers at the nodes from neighbor-joining (NJ) (left) and maximum-parsimony (MP) (right) analysis. *Chlorella vulgaris* is indicated for out group.

only four species: *Tetraselmis striata* (Steinkötter *et al.* 1994), *T. convolutae* (de Jesus *et al.* 1995), *T. kochiensis* and *T. chuii* reported an 18S rDNA sequence. Other strains have not yet been identified as to the level of species.

One of the objectives of this research on their rDNA sequences was to identify strains of *Tetraselmis* among culture collections. Variation of life-history characteristics such as the flagellate phase, non-motile phase and cyst stage of *Tetraselmis* depends on environmental factors (Norris *et al.* 1980). In our experience, morphological discernment of *Tetraselmis* under a light microscope was often confused with the genera *Colacium* and *Chloromonas*. Therefore, analysis of the 18S rDNA sequence is evaluated as a simple tool for distinguishing the genus *Tetraselmis*. In this study, 13 strains were identical with *Tetraselmis striata* PLY 443 and 5 strains with *Tetraselmis* sp. Tsbre. This was more than 43% of the total strains examined. Grouping of *Tetraselmis* strains based on the result of the 18S rDNA sequence showed a partially corresponding tendency with the mean size variation of the strains. To understand the characterization and discrimination among various strains of *Tetraselmis*, further detailed molecular studies on the ITS regions or rbc L gene reflecting higher evolutionary rate, which was used in the genus *Nannochloropsis* (Suda *et al.* 2002) and DNA hybridization analysis or amplified fragment length polymorphism used in *Chlorella vulgaris* (Després *et al.* 2003; Müller *et al.* 2005), are needed.

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