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Comparative Molecular Analysis of Freshwater Centric Diatoms with Particular Emphasis on the Nuclear Ribosomal DNA of *Stephanodiscus* (Bacillariophyceae)

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DNA-based discrimination of species is a powerful way for morphologically otherwise similar species, like centric diatoms. Here, the author sequenced long-range nuclear ribosomal DNAs, spanning from the 18S to the D5 region of the 28S rDNA, of *Stephanodiscus*, particularly including a Korean isolate. By comparisons, high DNA similarities were detected from the rDNAs of nine *Stephanodiscus* (>99.4% in 18S rDNA, >98.0% in 28S rDNA). Their genetic distances, however, were significantly different (Kruskal-Wallis test, $p < 0.01$) compared to two related genera, namely *Cyclotella* and Discostella. In addition, genetic distances of 18S rDNAs were significantly different (Student's *t*-test, $p = 0.000$) against those of the 28S rDNAs according to individual genera (*Cyclotella*, *Discostella*, and *Stephanodiscus*). Phylogenetic analyses showed that *Stephanodiscus* and *Discostella* showed a sister taxon relationship, and their clade was separated from a cluster of *Cyclotella* (1.00 PP, 100% BP). This suggests that *Stephanodiscus* has highly conserved sequences of both 18S and 28S rDNA; however, *Stephanodiscus* is well-separated from other freshwater centric diatoms, such as *Cyclotella* and *Discostella*, at the generic level.

Key Words: freshwater diatom, nucleotide divergence, phylogeny, ribosomal DNA, *Stephanodiscus*

INTRODUCTION

The centric diatom *Stephanodiscus* Ehrenberg 1846 is commonly present in freshwater environments, and several species are important bio-indicators of water quality, particularly for eutrophicated waters (Ha *et al*. 2002). Conventionally, their taxonomic identities are determined by microscopic observations of certain morphological characters, such as the pattern of the central area of the exoskeleton, and density and branching of the striae (Oliva *et al*. 2008). However, morphological discrimination of these species is very difficult, because of small size (less than 15 μm) and a number of recorded different *Stephanodiscus* species (approximately 124 taxa) according to Guiry and Guiry (2009). In addition, morphologies of *Stephanodiscus* are similar to those of other freshwater centric diatoms, e.g. *Cyclotella* and *Discostella* (formerly, these represented the stelligeroid group of *Cyclotella* [Houk and Klee 2004]). Moreover, several centric diatoms of different species sometimes are co-occurring. Many uncertainties about the proper identities of the centric diatoms are, therefore, remaining.

Recently, DNA-based taxonomy is widely used for the discrimination of small-size organisms, including diatoms and dinoflagellates (Karsten *et al*. 2005; Ki *et al*. 2009). Indeed, molecular analyses (e.g. immunoassays, PCR assay, DNAchip), including phylogenetic inferences, are very effective to discriminate morphologically similar, microscopic-size organisms. In most cases, these molecular approaches are based on the DNA sequences of the nuclear ribosomal DNA (rDNA), because it occurs in all living organisms, and many rDNA sequences are available, compared to other genes (e.g. *actin*, α-, β,-*tubulin*, and *Hsp90*). The rDNA sequences have been used for the discrimination of centric diatoms and for the phylogenetic analyses (Alverson *et al*. 2007; Kaczmarska *et al*. 2007). Most studies on the freshwater centric diatoms have been biased to *Cyclotella*, particularly for the systematics and phylogenetic relationships of cyclotelloid diatoms (Beszteri *et al*. 2005, 2007; Alverson *et al*. 2007). More recently, genetic divergence between *Cyclotella* and *Discostella* has been studied, by comparisons of a wide range of rDNA sequences (Jung *et al*. 2009). With regard to molecular analyses of *Stephanodiscus*, Kaczmarska *et al*. (2005) showed for the first time that *Cyclotella* and

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Stephanodiscus were not belonging to the same phylogenetic clade, making the family Stephanodiscaceae paraphyletic. Recently, Alverson *et al*. (2007) reported phylogenetic relationships of thalassiosiroid diatoms, representing the separations of the freshwater centric diatoms, e.g. *Cyclotella*, *Discostella* and *Stephanodiscus*. Recently, the author reported high molecular genetic divergences between *Cyclotella* and *Discostella*, suggesting that rDNA may be a suitable molecular marker for the discrimination of the two genera and species (Jung *et al*. 2009). Excluding these works, little attention has been paid on the molecular analyses of *Stephanodiscus*.

In the present study, the author sequenced nuclear rDNA, spanning the 18S to the 28S rDNA, of *S. hantzschii* and *Stephanodiscus* sp., including a Korean isolate, and characterized molecular features of various rDNA regions according to each rDNA. Comparative analyses of individual 18S, 28S rDNAs were performed with some rDNAs of selected *Stephanodiscus* to reveal the rDNA relationships of the freshwater centric diatoms. In addition, molecular divergences between *Stephanodiscus* with other centric diatoms *Cyclotella* and *Discostella* were compared to evaluate their usefulness for the discrimination of freshwater centric diatoms. The studied *S. hantzschii* is one of the planktonic, cosmopolitan species and the *Stephanodiscus* blooms were recorded annually in Korean waters, particularly in the Paldang Reservoir and Nakdong River (Kim 1998; Ha *et al*. 2002; Han *et al*. 2002; Kim *et al*. 2008).

MATERIALS & METHODS

Cultures of *Stephanodiscus*

Water samples were collected from Paldang Reservoir (a reservoir in Han River) of Korea, when *Stephanodiscus* blooms occurred. The author isolated single cells of *Stephanodiscus* from field samples using the capillary method (Ki and Han 2005), and established a clonal culture (KHR001) of *Stephanodiscus*. An additional strain (UTCC 267) of *S. hantzschii* was commercially obtained from the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC). All the cultures were routinely maintained in Diatom Medium, DM, (Beakes *et al*. 1988), and were grown at 15°C, 12:12 h light:dark cycle, with a photon flux density of about 65 μmol photons m^{-2} s^{-1}.

DNA extraction and PCR amplification

A total of 50 ml clonal cultures were harvested by centrifugation at 8,000 rpm for 15 min. The concentrated cells were transferred to 1.5 ml micro tubes, 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) was added and the tubes were stored at –20°C until DNA extraction. Genomic DNA was isolated from the stored cells using the DNeasy Plant mini kit (Qiagen, Valencia, CA).

Polymerase chain reaction (PCR) was subject to amplify the 18S-28S rDNA of *Stephanodiscus* genomic DNA. In this case, the author used a set of PCR primers that targeted to bind nuclear 18S rDNA (a forward AT18F01, 5′-ACC TGG TTG ATC CTG CCA GTA G-3′) and 28S rDNA (a reverse PM28-R1318, 5′-TCG GCA GGT GAG TTG TTA CAC AC-3′), which are specific for diatoms (Jung *et al*. 2009). PCR was performed with 50 μl reaction mixtures containing 30.5 μl sterile distilled water, 5 μl 10 x LA PCR buffer II (TaKaRa, Kyoto, Japan), 8 μl dNTP mix (4 mM), 5 μl of each primer (5 M), 0.5 μl LA Taq polymerase (2.5 U), and 1 μl of template. PCR cycling was performed in a Bio-Rad iCycler (Bio-Rad, Hercules, CA) with 94°C for 2 min, following 35 cycles of 94°C for 20 sec, 55°C for 30 sec, and 72°C for 2 min, and a final extension at 72°C for 10 min. Resulting PCR products were electrophoresed in a 1.0% agarose gel (Promega, Madison, WI), stained with ethidium bromide, and visualized by ultraviolet transillumination.

DNA sequencing

For DNA sequencing, desired PCR products were purified with a QIAquick PCR purification Kit (Qiagen GmbH, Germany). DNA sequencing reactions were performed in a ABI PRISM® BigDye^{TM}, Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) using the PCR products (2 μl) as the template and 10 picomoles of the above PCR and internal walking primers. Labeled DNA fragments were analyzed on an automated DNA sequencer (Model 3700, Applied Biosystems, Foster City, CA).

Editing and contig assembly of DNA sequences were performed using Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI). The coding rDNAs were identified by comparison with those of other diatoms, including *Cyclotella meneghiniana* (GenBank No. GQ148712) and *Discostella* sp. (GQ148713). DNA sequences determined here have been deposited to GenBank as accession numbers GQ844873 and GQ844874.

Comparisons of *Stephanodiscus* rDNA

BLAST (The Basic Local Alignment Search Tool)

Table 1. Origins of the centric diatoms, *Stephanodiscus*, *Cyclotella* and *Discostella*, and their DNA sequence GenBank accession numbers

Species	Strain	18S	28S	
Stephanodiscus sp.	KHR001	GQ844873#		This study
S. hantzschii	UTCC 267	GQ844874#		This study
S. agassizensis	CHTC1	DQ514895	DQ512451	Alverson *et al*. 2007
S. binderanus	ESB2	DQ514896	DQ512452	Alverson *et al*. 2007
S. hantzschii	WTC21	DQ514914	DQ512470	Alverson *et al*. 2007
S. minutulus	Y98-1	DQ514916	DQ512472	Alverson *et al*. 2007
S. neoastraea	Sneo4	DQ514906	DQ512462	Alverson *et al*. 2007
S. niagarae	J95-16	DQ514907	DQ512463	Alverson *et al*. 2007
S. reimerii	WLO-11	DQ514909	DQ512465	Alverson *et al*. 2007
S. yellowstonensis	Y7	DQ514910	DQ512466	Alverson *et al*. 2007
C. atomus	ROR01-04	DQ514858	DQ512407	Alverson *et al*. 2007
C. choctawhatcheeana	L1840	AM712618	AM778964	Bruder and Medlin 2007
C. distinguenda	Tiplady	DQ514859	DQ512408	Alverson *et al*. 2007
C. gamma	cygamma	DQ514852	DQ512400	Alverson *et al*. 2007
C. meneghiniana	HYK0210-A1	GQ148712#		Jung *et al*. 2009
C. striata	CCMP1586	DQ514851	DQ512399	Alverson *et al*. 2007
D. pseudostelligera	ROR01-1	DQ514905	DQ512461	Alverson *et al*. 2007
D. stelligera	L1360	DQ514903	DQ512459	Alverson *et al*. 2007
Discostella sp. HYK0210-A2	HYK0210-A2	GQ148713#		Jung *et al*. 2009
Discostella sp. L435	L435	DQ514902	DQ512458	Alverson *et al*. 2007

represents DNA sequences including 18S to 28S rDNA.

searches were performed with the present rDNA sequence data and the available DNA sequences in the National Center for Biotechnology Information (NCBI) database. In addition, DNA sequences of *S. hantzschii* and a Korean *Stephanodiscus* were compared with those of other *Stephanodiscus* (see Table 1). DNA similarity scores of individual rDNA molecules were calculated by using pairwise sequences among nine selected species of *Stephanodiscus* in BioEdit 5.0.6 (Hall 1999). In addition, dot-plot analysis was carried out using the MegAlign 5.01 (DNAstar Inc., Madison, WI). Molecular genetic divergences of the nine species were measured with the Kimura two-parameter model in MEGA 4.0 (Tamura *et al*. 2007). Statistical analyses on the nucleotide comparisons were performed using SPSS 10.0.7 (SPSS Inc., Chicago, IL).

Phylogenetic relationships of *Stephanodiscus* species

Phylogenetic analyses of the freshwater centric diatoms were carried out, following our previous work (Jung *et al*. 2009). In the present case, the author constructed two new data matrixes of individual 18S and 28S rDNAs, including ten *Stephanodiscus*, four selected *Discostella*, and six selected *Cyclotella* rDNAs (see Table 1). A total of 21 sequences, including the outgroup (*Thalassiosira gessneri* #AN02-08), were aligned with the Clustal W 1.8 (Thompson *et al*. 1997). The aligned sequences were trimmed each end to the same length. In addition, various regions and uncertain sequences were further corrected manually. Finally, only unambiguous positions of the aligned sequences were used in the subsequent analyses: 1,689 out of 1,813 alignment positions for 18S, 506 out of 1,264 for 28S). MrModeltest2 (Nylander 2004) was used to find the optimal model of DNA substitution for the Bayesian tree construction. As best-fit models for the present 18S rDNA dataset, the author selected the General Time Reversible plus Invariant sites plus Gamma distributed model (GTR+I+G) for 18S (–lnL = 3859.3) and for 28S (–lnL = 2260.4) from the Akaike Information Criterion (AIC), respectively. A Bayesian tree of the 18S was implemented with the selected GTR+I+G substitution model in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The Markov chain Monte Carlo process was set to two chains (MCMC), and 1,000,000 generations were conducted. The sampling frequency was assigned as every 100 generations. After analysis, the first 2,000 trees were deleted as burn-in and a consensus tree was constructed. The phylogenetic tree was visualized with TreeView ver.1.6.6 (Page 1996). Bayesian posterior probabilities (PP) of more than 0.50 were indicated at each branch node. An additional Neighbor-Joining (NJ) tree was constructed with

Table 2. Sequence length and G+C content (%) measured from the *Stephanodiscus* rDNA determined in the present study

		Length (bp)	Nucleotide (%)			
			A	T	G	C
	18S	1,740 (1,805*)	26.5	28.4	25.7	19.4
Stephanodiscus sp.	ITS1	235	23.4	19.6	24.7	32.3
#KHR001	5.8S	165	29.7	26.1	22.4	21.8
	ITS2	290	18.3	24.8	33.4	23.4
	28S	1,252	26.3	24.2	28.8	20.7
	18S	1,798 (1,805*)	26.6	28.3	25.7	19.5
S. hantzschii	ITS1	233	22.7	21.0	23.2	33.0
#UTCC 267	5.8S	165	29.7	26.1	22.4	21.8
	ITS2	291	19.9	24.7	31.3	24.1
	28S	1,281	26.8	24.2	28.6	20.5

*Putative length of complete 18S rDNA, based on the conserved region of the 5′ end.

the same data matrix of 18S rDNA using the Maximum Composite Likelihood model in MEGA 4.0 (Tamura *et al*. 2007). For the 28S rDNA tree, Bayesian and NJ analyses were performed the same way as in the 18S analysis.

RESULTS AND DISCUSSION

Nuclear rDNA of *S. hantzschii* and Korean *Stephanodiscus*

In the present study, DNA sequences of nuclear rDNAs, spanning the 18S to the D5 domain of the 28S rDNA, were determined from *S. hantzschii* #UTCC 267 (3,768 bp; 47.9% GC) and a Korean *Stephanodiscus* sp. #KHR001 (3,682 bp; 48.3% GC), as shown in Table 2. Their gene structures were organized in the typical eukaryotic fashion of rDNA (i.e. 18S-ITS1-5.8S-ITS2-28S). In general, the 28S rDNA, the largest rDNA coding region, contains twelve hyper-variable domains (Hassouna *et al*. 1984; Lenaers *et al*. 1989), often designated as divergent (D) domains. Of them, the present sequences included D1 to D5 of the 28S rDNA. Upon comparisons, most sequences available in public databases (e.g. DDBJ, EMBL, NCBI) were revealed from D1/D2 domains of the 28S rDNA, while the others contain much genetic information (Ki and Han 2007). The present data included wider range of the 28S rDNA from the genus *Stephanodiscus*. With these data, the author evaluated their molecular characteristics and compared 2 representatives of *Stephanodiscus* with freshwater centric diatom data available in the NCBI. Particularly, complete lengths of the 18S rDNA sequences of *S. hantzschii* #UTCC 267 and *Stepahnodiscus* sp. #KHR001 were estimated to be 1,805 bp, after incorporating undetermined nucleotides of the 18S rDNA 5′end into the present 18S sequences, taking into account of available data (e.g. AM712618, DQ093370) recorded in GenBank (e.g. AM712618, DQ093370). These were nearly identical to those of other relatives, including *Cyclotella* and *Discostella* (Jung *et al*. 2009).

By database searches, the author found many partial 18S, 28S rDNA sequences revealed from *Stephanodiscus*, particularly by the work of Alverson *et al*. (2007). The rDNA ITS regions were only revealed from a few species, including *S. hantzschii* (U03078), *S. niagarae* (U03074-6, AF455267-9), and *S. yellowstonensis* (U03077). By using the ITS data, Wolf *et al*. (2002) demonstrated the same species of *S. neoastraea* and *S. heterostylus*. All the DNA sequence data (e.g. 18S, ITS, 28S) available in databases have been partially sequenced at a given locus. Here the author compared the present data with available partial sequences reported from *Stephanodiscus* (Tables 3, 4). Firstly, these sequences were compared with those of the NCBI database using the BLAST search algorithm. BLAST searches of individual rDNA sequences showed that a Korean *Stephanodiscus* sp. #KHR001 was highest matched with *S. hantzschii* #CCAP 1079/4 (GenBank DQ093370) with 99.5% similarity by 18S rDNA comparison, and was also matched to *S. hantzschii* #AT-N2 (AJ878502) with 98.5% similarity by 28S comparison. On the other hand, the ITS rDNA comparison showed that the top hit was recorded with *S. niagarae* (U03076) of 89.9% DNA similarity. BLAST searches of individual rDNA of *S. hantzschii* #UTCC 267 were similar to those of Korean *Stephanodiscus* spp. In the latter, the rDNA ITS was highly matched at 94.1% similarity with *S. yellowstonensis* (U03077) and at 96.9% similarity with *S. hantzschii* (U03078), respectively. Overall similari-

Table 3. Similarity scores between 9 pairs of the aligned sequence data of the nearly complete 18S rDNA (above diagonal) and partial 28S rDNA (below diagonal) from nine selected species of *Stephanodiscus*

Species	Strains	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]
		[18S rDNA: 1,689 sites]								
[1] *Stephanodiscus* sp.	KHR001		99.5	99.5	99.5	99.5	99.5	99.4	99.4	99.4
[2] *S. hantzschii*	UTCC 267	99.0		100	100	100	100	99.8	99.8	99.8
[3] *S. agassizensis*	CHTC1	98.8	99.4		100	100	100	99.8	99.8	99.8
[4] *S. binderanus*	ESB2	98.8	99.8	99.2		100	100	99.8	99.8	99.8
[5] *S. minutulus*	Y98-1	99.0	100	99.4	99.8		100	99.8	99.8	99.8
[6] *S. neoastraea*	Sneo4	98.8	99.4	100	99.2	99.4		99.8	99.8	99.8
[7] *S. niagarae*	J95-16	98.0	98.4	99.0	98.2	98.4	99.0		99.9	99.9
[8] *S. reimerii*	WLO-11	98.0	98.4	99.0	98.2	98.4	99.0	100		100
[9] *S. yellowstonensis*	Y7	98.0	98.4	99.0	98.2	98.4	99.0	100	100	
		[28S rDNA: 507 sites]								

ties of the coding rDNAs, e.g. 18S and 28S, were high within the genus *Stephanodiscus*.

Molecular similarities and genetic divergence of rDNA

Molecular comparisons showed that a Korean isolate of *Stephanodiscus* had a different genotype compared with other *Stephanodiscus* (Table 3), including *S. hantzschii*. The present Korean isolate was presumably identified as *S. hantzschii* based on routine morphological observations and previous studies (Han *et al*. 2002). As noted previously, morphological characteristics of *Stephanodiscus* are similar to each other, and a number of species (at least 124 species) have been described so far. In the present study, the author tentatively discriminated *Stephanodiscus* sp. #KHR001 (or named as *Stephanodiscus* sp. cf. *S. hantzschii*). Considering these molecular and morphological characteristics of *Stephanodiscus*, the author selected nine species of *Stephanodiscus*, including Korean one, and measured DNA similarities of both 18S and partial 28S rDNA sequences (Table 3). High DNA similarities were recorded among rDNA pairs of nine *Stephanodiscus* (>99.4% in 18S rDNA, >98.0% in 28S rDNA). Strikingly, DNA similarities of 18S rDNAs were considerably similar to one another. The present Korean isolate (KHR001) showed more than 99.5% similarity with *S. agassizensis*, *S. binderanus*, *S. hantzschii* and *S. minutulus*, respectively. By 28S comparisons, DNA similarities were highly recorded among the *Stephanodiscus*, while these data included the most variable domain D1/D2 within the 28S rDNA (Ki and Han 2007). These suggest that molecular genetic divergences within the *Stephanodiscus* are considerably low with approximately 1% in 18S rDNA and 2% in 28S rDNA, respectively. However, we detected high genetic divergences of other freshwater centric diatoms, e.g. *Cyclotella* and *Discostella* (Jung *et al*. 2009). Genetic divergences of freshwater centric diatoms may, therefore, be taxon-dependent rather than general molecular characteristics in the three diatom groups.

Phylogenetic relationships of freshwater centric diatoms

Molecular relationships of three major freshwater centric diatoms, namely *Cyclotella*, *Discostella* and *Stephanodiscus*, were inferred from Bayesian, Neighbor-Joining analyses, using their available 18S and partial 28S rDNA sequences, respectively (Figs 2, 3). Recently, we reported the phylogenetic relationships of *Cyclotella* and *Discostella*, in which phylogenetic trees were inferred with Bayesian method, using 18S and 28S rDNA data (Jung *et al*. 2009). In the present study, the author focused on *Stephanodiscus* relationships against the two other genera, as well as inter-species relationships within the genus *Stephanodiscus*. Considering our previous work (Jung *et al*. 2009), the author constructed new data matrices, including certain members of *Cyclotella* and *Discostella*. In the present analyses, a total of 20 species, including six *Cyclotella*, four *Discostella* and ten *Stephanodiscus*, with the outgroup of *Thalassiosira*, were subjected to phylogenetic analyses with Bayesian and NJ methods (Fig. 1). Phylogenetic analyses showed that the three genera included here were well separated (1.00 PP, 100% BP). Overall topologies of the Bayesian tree were compatible with those of the NJ tree. All the species of *Stephanodiscus* formed a cluster (1.00 PP, 100% BP), of which clade was separated from a *Discostella* cluster.

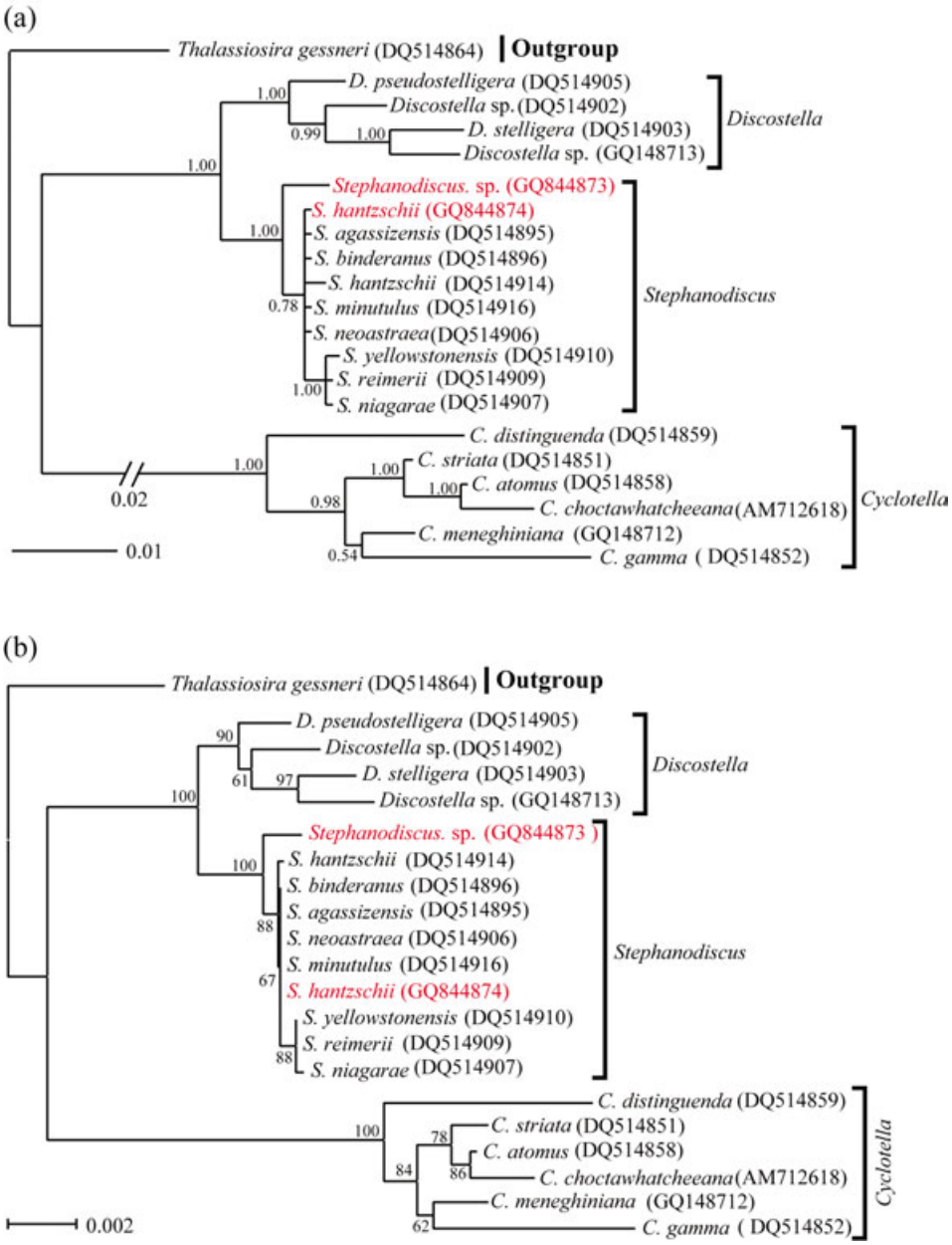

Fig. 1. Phylogenetic relationships of three centric diatom genera, *Cyclotella*, *Discostella*, and *Stephanodiscus*, inferred by nearly complete 18S rDNA sequences with (a) Bayesian and (b) NJ algorithms, respectively. Both analyses were used as the same data matrix, with different nucleotide substitution models (e.g. GTR + I + G in Bayesian, and Maximum Composite Likelihood in NJ algorithm). Likelihood scores as the Bayesian tree were calculated at –lnL = 3,898.6. The centric diatom, *Thalassiosira gessneri* #AN02-08 (GenBank no. DQ514864), was used as the outgroup. Bayesian posterior probabilities less than 0.50 and bootstrap proportion less then 50% were not shown.

Stephanodiscus and *Discostella* are a sister relationship, separating a clade of *Cyclotella*, showing that these patterns were in agreement with Alverson *et al*. (2007). In the *Stephanodiscus* linage, most species, excluding a cluster of *S. niagarae*, *S. reimeri*i and *S. yellowstonensis*, formed a polytomy including six species. These were caused by low genetic divergences and high DNA similarities, detected in Table 3. Within this linage, a Korean *Stephanodiscus* isolate was positioned at an early divergent place, clearly being separated from other *Stephanodiscus* (1.00 PP, 100% BP).

In addition to this, phylogenetic analyses of partial 28S rDNA of the three centric diatom groups showed similar branch patterns, when compared with those of 18S rDNA phylogenies. *Stephanodiscus* was a sister relationship with *Discostella*, of which clade was clustered with

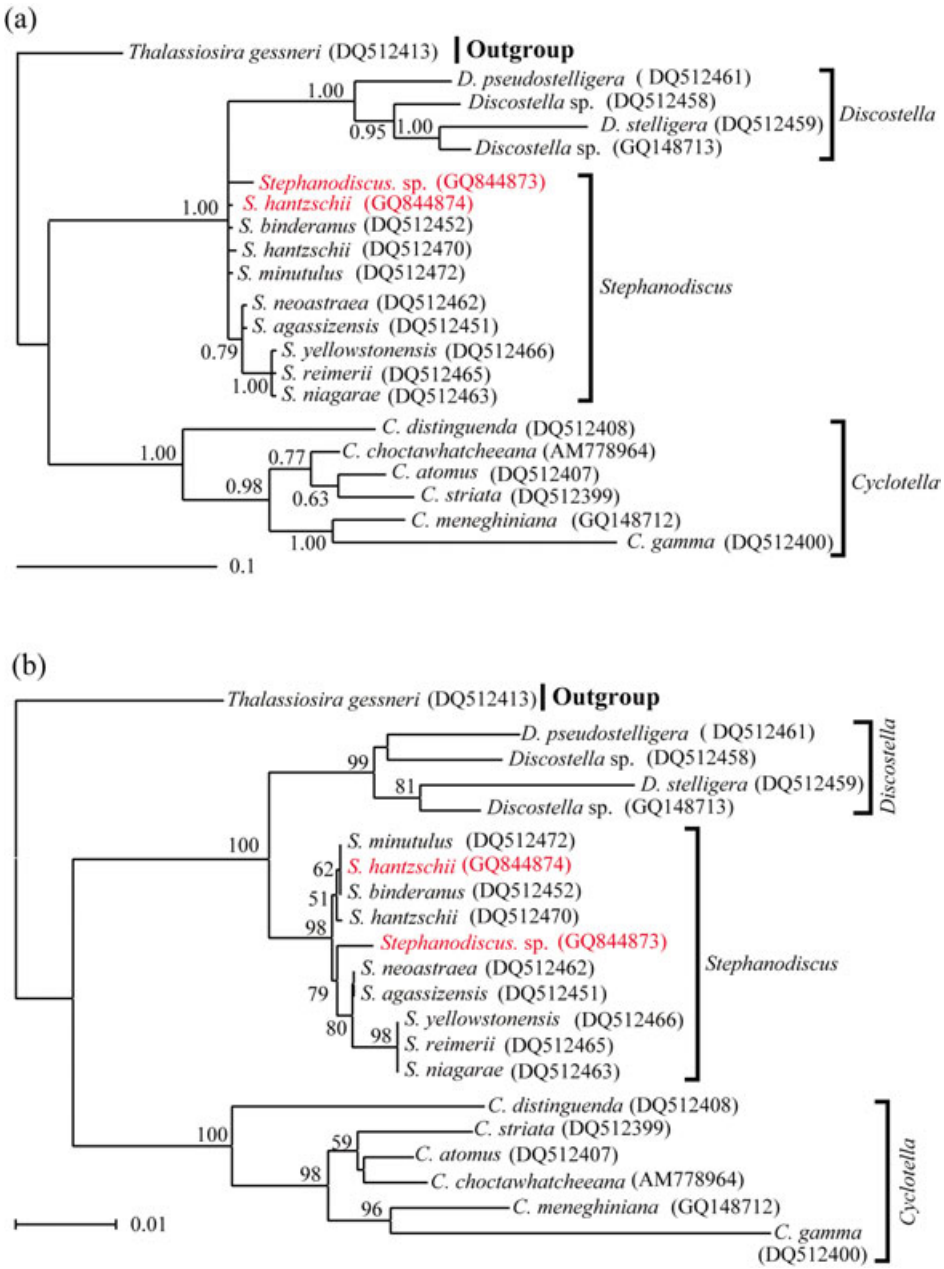

Fig. 2. Phylogenetic relationships of three centric diatom genera, *Cyclotella*, *Discostella*, and *Stephanodiscus*, inferred by partial 28S rDNA sequences with (a) Bayesian and (b) NJ algorithms, respectively. Both analyses were used as the same data matrix, with different nucleotide substitution models (e.g. GTR + I + G in Bayesian, and Maximum Composite Likelihood in NJ algorithm). Likelihood scores of Bayesian tree was calculated at –lnL = 2,296.2. The centric diatom, *Thalassiosira gessneri* #AN02-08 (GenBank no. DQ512413), was used as the outgroup. Bayesian posterior probabilities less than 0.50 and bootstrap proportion less then 50% are not shown.

Cyclotella (1.00 PP, 100% BP). Within theses analyses, *Stephanodiscus* formed a polytomy, excluding a cluster of *S. niagarae*, *S. reimerii* and *S. yellowstonensis* (Fig. 2). The 28S rDNA phylogeny showed that the Korean isolate was not separated from other *Stephanodiscus*. Overall 28S phylogeny was in good accordance with the 18S phylogeny described above.

Molecular divergences of 18S, ITS, 28S rDNAs

The present rDNA sequences of *Stephanodiscus* were graphically compared with those of the *Cyclotella* sensu lato, by using dot-matrix and entropy-plot analyses (Fig. 3). Here the dot-plot was obtained using sliding windows of 60 nucleotides along the compared rDNAs. The

Table 4. Comparisons of 18S and 28S rDNA nucleotide divergences based on corrected *p*-distances of *Stephanodiscus* (St), *Stephanodiscus* versus *Cyclotella* (St vs. Cy) and *Stephanodiscus* versus *Discostella* (St vs. Di). Genetic distances between each paired sequence from 20 species listed in Table 1 were calculated with Kimura two-parameter model

	18S			28S			Student's *t*-test
	Mean	SD	N	Mean	SD	N	
St	0.2	0.16	45	1.0	0.65	45	$p = 0.000$*
St vs. Cy	5.4	0.45	60	15.6	2.90	60	$p = 0.000$*
St vs. Di	1.7	0.28	40	7.7	1.27	40	$p = 0.000$*
Kruskal-Wallis Test	$p < 0.01$*			$p < 0.01$*			

* Significantly difference in 95% confidence interval of the difference. Abbreviation: SD, Standard Deviation; N, Number.

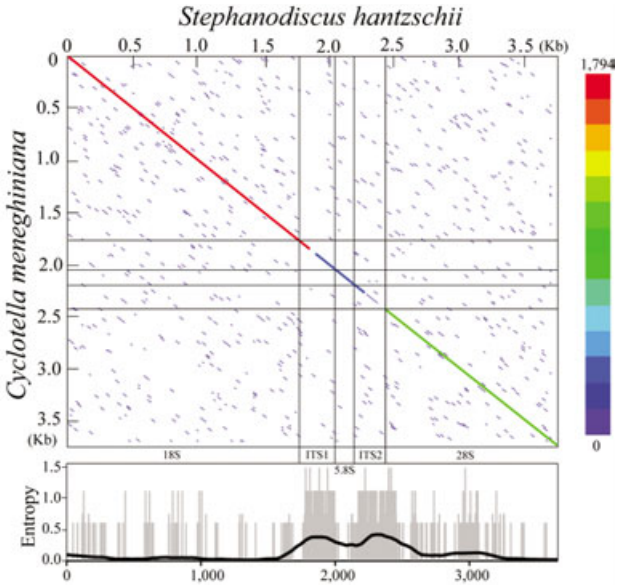

Fig. 3. A dot-matrix plot and an entropy-plot of the nuclear rDNA of *Stephanodiscus* and other close relatives. The dot-matrix plot was drawn with the rDNA sequence comparison of *S. hantzschii* (UTCC 267) and *C. meneghiniana* (HYK0210-A1). In addition, the entropy plot was drawn by calculating the amount of nucleotide variability among four rDNAs, including *C. meneghiniana*, *Discostella* sp., *S. hantzschii*, and *Stephanodiscus* sp. (see Table 1). Color scale bar represents consecutive sequence length of some regions detected similarly between the two sequence pair. A line on the entropy plot displays the normalized curve on each histogram.

plot showed a clear distribution of both variable and conserved positions along the rDNA sequences: the coding regions were conserved, the other non-coding regions were highly variable (Fig. 3). This was in good accordance with our previous study (Jung *et al*. 2009).

Nucleotide divergences of the 18S and 28S rDNA sequences were compared, using pairwise genetic distances calculated with the Kimura two-parameter model (Table 4). In most cases, DNA divergences within nine *Stephanodiscus* (listed in Table 3) were considerably low both in 18S (less than 0.2%) and in 28S rDNA (less than 1.0%). By comparisons, divergences of the 28S rDNA were significantly different compared to the 18S rDNA (Student's *t*-test, $p = 0.000$). In addition, divergences of individual 18S, 28S rDNA among the three groups, *Cyclotella*, *Discostella*, and *Stephanodiscus*, were significantly different according to the Kruskal-Wallis Test ($p < 0.01$). By comparisons of *Stephanodiscus* with *Cyclotella* and *Discostella*, high genetic divergences were calculated from 18S (*Stephanodiscus* versus *Cyclotella*, 5.4%, SD = 0.45) and 28S rDNA (*Stephanodiscus* versus *Cyclotella*, 15.6%, SD = 2.9). These support that *Stephanodiscus* has high similarities of both 18S and 28S rDNA (Table 3), but *Cyclotella* and *Discostella* shows low similarities in both genes (Jung *et al*. 2009).

DNA identity of *Stephanodiscus* from Paldang Reservoir

The centric diatoms, including *Cyclotella*, *Discostella*, and *Stephanodiscus*, commonly occur in freshwaters, including the Han River (Han *et al*. 2002; Jung *et al*. 2009). According to the previous studies (Kim 1998; Han *et al*. 2002), high abundance of the centric diatoms were frequently observed in water samples collected from Paldang Reservoir and Han River during early spring. Some blooms were caused by *Cyclotella* and *Discostella* (e.g. Jung *et al*. 2009), and sometimes *Stephanodiscus* blooms occurred in Paldang Reservoir (Kim 1998; Han *et al*. 2002). The blooming *Stephanodiscus* in Paldang Reservoir were morphologically considered as *S. hantzschii* (Han *et al*. 2002). In addition, the author isolated a Korean *Stephanodiscus* cell (KHR001) from a water sample of Paldang Reservoir when *Stephanodiscus* cells were present predominantly, and identified them as *S. hantzschii*, judging by routine morphological observa-

tions and previous reports (Kim 1998; Han *et al*. 2002). However, comparative molecular data done with BLAST searches and similarity scores (Table 3) were not in accordance with morphological identity. Upon rDNA comparisons between *Stephanodiscus* sp. #KHR001 with other *S. hantzschii* (the present UTCC 267, WTC21, AT-N2), the present Korean isolate should be a different species, than *S. hantzschii*, judging from the present phylogenetic analyses and molecular similarities (Table 3; Figs. 1, 2). Previously, Kim (1998) discriminated three species of *Stephanodiscus*, e.g. *S. hantzschi* f. *tenuis*, *S. parvus*, *S. invistatus*, from spring water samples of Paldang Reservoir. These suggest that the blooming species may be some of these recorded species (e.g. *S. hantzschii*, *S. hantzschi* f. *tenuis*, *S. parvus*, *S. invistatus*) possibly including unrecorded species, while *S. hantzschii* have been considered only to be the blooming species in Paldang Reservoir for a long time. Thus, existing ecological and morphological discrimination of the blooming *Stephanodiscus* may be reinvestigated, considering the present molecular data available.

In conclusion, the present study determined long-range sequences of rDNA from *S. hantzschii* #UTCC 267 and a Korean *Stephanodiscus* sp. #KHR001. Molecular comparisons showed high genetic similarities (or low genetic divergence) within the genus *Stephanodiscus* compared with those of *Cyclotella* and *Discostella*. From these facts, the author concludes that nuclear rDNA sequences of *Stephanodiscus* are considerably similar to each other, but they are significantly different ($p < 0.01$) from other freshwater centric diatoms (e.g. *Cyclotella* and *Discostella*).

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REFERENCES

Alverson A.J., Jansen R.K. and Theriot E.C. 2007. Bridging the Rubicon: phylogenetic analysis reveals repeated colonization of marine and fresh waters by thalassiosiroid diatoms. *Mol. Phylogenet. Evol*. **45:** 193-210.

Beakes G., Canter H.M. and Jaworski G.H.M. 1988. Zoospore ultrastructure of *Zygorhizidium affluens* Canter and *Z. planktonicum* Canter, two chytrids parasitizing the diatom *Asterionella formosa* Hassall. *Can. J. Bot*. **66:** 1054-1067.

Beszteri B., Ács É. and Medlin L.K. 2005. Conventional and geometric morphometric studies of valve ultrastructural variation in two closely related *Cyclotella* species (Bacillariophyceae). *Eur. J. Phycol*. **40:** 89-103.

Beszteri B., John U. and Medlin L.K. 2007. An assessment of cryptic genetic diversity within the *Cyclotella meneghiniana* species complex (Bacillariophyta) based on nuclear and plastid genes, and amplified fragment length polymorphisms. *Eur. J. Phycol*. **42:** 47-60.

Bruder K. and Medlin L.K. 2007. Molecular assessment of phylogenetic relationships in selected species/genera in the Naviculoid diatoms (Bacillariophyta). I. The genus *Placoneis*. *Nova Hedwigia* **85:** 331-352.

Guiry M.D. and Guiry G.M. (2009). "*Stephanodiscus*". AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org/. Retrieved on 2009-08-15.

Ha K., Jang M.H. and Joo G.J. 2002. Spatial and temporal dynamics of phytoplankton communities along a regulated river system, the Nakdong River, Korea. *Hydrobiologia* **470:** 235-245.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser*. **41:** 95-98.

Han M.-S., Lee H.R., Hong S.S., Kim Y.O., Lee K., Choi Y.K., Kim S. and Yoo K.I. 2002. Ecological studies on Togyo reservoir system in Chulwon, Korea. V. Seasonal changes of size-fractionated standing crops and chlorophyll a of phytoplankton in Kyungan Stream of Pal'tang Reservoir, Korea. *Korean J. Environ. Biol*. **20:** 91-99.

Hassouna N., Michot B. and Bachellerie J.-P. 1984. The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Res*. **12:** 3563-3583.

Houk V. and Klee R. 2004. The stelligeroid taxa of the genus *Cyclotella* (Kützing) Brebisson (Bacillariophyceae) and their transfer into the new genus *Discostella* gen. nov. *Diatom Res*. **19:** 203-28.

Huelsenbeck J.P. and Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17:** 754-755.

Jung S.W., Han M.-S. and Ki J.-S. 2009. Molecular genetic divergence of the centric diatom *Cyclotella* and *Discostella* (Bacillariophyceae) revealed by nuclear ribosomal DNA comparisons. *J. Appl. Phycol*. DOI 10.1007/s10811-009-9462-5.

Kaczmarska I., Beaton M., Benoit A.C. and Medlin L.K. 2005. Molecular phylogeny of selected members of the order Thalassiosirales (Bacillariophyta) and evolution of the Fultoportula. *J. Phycol*. **42:** 121-138.

Karsten U., Schumann R., Rothe S., Jung I. and Medlin L. 2006. Temperature and light requirements for growth of two diatom species (Bacillariophyceae) isolated from an Arctic macroalga, *Polar Biol*. **29:** 476-486.

Ki J.-S., Cho S.-Y., Katano T., Jung S.W., Lee J., Park B.S., Kang S.-H. and Han M.-S. 2009. Comprehensive comparisons of

three pennate diatoms, *Diatoma tenuae*, *Fragilaria vaucheriae*, and *Navicula pelliculosa*, isolated from summer Arctic reservoirs (Svalbard 79°N), by fine-scale morphology and nuclear 18S ribosomal DNA. *Polar Biol*. **32:** 147-159.

Ki J.-S. and Han M.-S. 2005. Sequence-based diagnostics and phylogenetic approach of uncultured freshwater dinoflagellate Peridinium species based on single-cell sequencing of rDNA. *J. Appl. Phycol*. **17:** 147-153.

Ki J.-S. and Han, M.-S. 2007. Informative characteristics of 12 divergent domains in complete large subunit rDNA sequences from the harmful dinoflagellate genus, *Alexandrium* (Dinophyceae). *J. Eukaryotic Microbiol*. **54:** 210-219.

Kim M.-C., La G.-H., Kim. H.-W., Jeong K.-S., Kim D.-K. and Joo G.-J. 2008. The effect of water temperature on proliferation of *Stephanodiscus* sp. in vitro from the Nakdong River, South Korea. *Korean J. Limnol*. **41:** 26-33.

Kim Y.J. 1998. Ecological characteristics of phytoplankton community in lake Paltang Dam. *Korean J. Limnol*. **31:** 225-234.

Lenaers G., Maroteaux L., Michot B. and Herzog M. 1989. Dinoflagellates in evolution. A molecular phylogenetic analysis of large subunit ribosomal RNA. *J. Mol. Evol*. **29:** 40-51.

Nylander J.A.A. 2004. MrModeltest v2 (Evolutionary Biology Centre, Uppsala Univ, Uppsala, Sweden.

Oliva M.G., Lugo A., Alcocer J. and Cantoral-Uriza E.A. 2008. Morphological study of *Cyclotella choctawhatcheeana* Prasad (Stephanodiscaceae) from a saline Mexican lake. *Saline Syst*. **4:** 17.

Page R.D.M. 1996. TREEVIEW: an application to display of phylogenetic trees on personal computers. *Comput. Appl. Biosci*. **12:** 357-358.

Tamura K., Dudley J., Nei M. and Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. **24:** 1596-1599.

Thompson J.D., Higgins D.G. and Gibbson T.J. 1997. Clustal X: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res*. **22:** 4673.

Wolf M., Scheffler W. and Nicklisch A. 2002. *Stephanodiscus neoastraea* and *Stephanodiscus heterostylus* (Bacillariophyta) are one and the same species. *Diatom Res*. **17:** 445-451.

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