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Sequence-based diagnostics and phylogenetic approach of uncultured freshwater dinoflagellate *Peridinium* (Dinophyceae) species, based on single-cell sequencing of rDNA

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Abstract

The armoured dinoflagellate *Peridinium* is widely distributed in freshwater environments worldwide and contains a large number of species. Their identity, however, has remained elusive, since the small cells tend to be morphologically similar. To help resolve this, a sequence-based diagnostics for uncultured *Peridinium* cells from field samples was applied, using single-cell PCR and direct DNA sequencing of the PCR products. Single cells were isolated randomly from field samples, and PCR successfully amplified the target rDNA regions from the crude lysates. Phylogenetic trees showed that all the cells were strongly grouped into the same clade (>99% bootstrap value), including the previously identified *P. bipes* f. *occultatum*, and apparently separated from relatives such as *P. cinctum*, *P. volzii* and *P. willei*. All 17 isolates were genotypically identified as *P. bipes* f. *occultatum*, based on over than 99% of sequence similarities, and the organism was responsible for water blooms at different seasons in Korean waters. The sequence-based typing could clearly resolve *P. bipes* f. *occultatum* from the various *Peridinium* cells, and that the method is accurate and more labor-saving than the conventional method to monitor *Peridinium* species. This protocol may be useful for the application of molecular tools to uncultured *Peridinium* cells.

Introduction

The armoured dinoflagellate *Peridinium* Ehrenberg is responsible for freshwater red tides that cause nuisance odors to drinking water and economically serious problems for agricultural industries (Kawabata & Hirano, 1995). In Korean waters, dense blooms have occurred regularly every year (Ki et al., 2005). Organisms at three different sites have been identified as *P. bipes* Stein f. *occultatum* (Lindem.) Lef., based on their morphology (Ki et al., 2005). However, many different types of cell, particularly with respect to the size range (29–63 μm), were observed among the *Peridinium* samples collected monthly.

The diagnosis of *Peridinium* is based mainly on morphological features, particularly the plate arrangement and apical pore complex (Elster & Ohle, 1968). However, the thecal formula in this species is extremely difficult to construct, and it is necessary to observe many times by light microscopy, since these plates are small. In addition, many morphological features are known to vary in response to the environment as well as during different growth stages. For these reasons, their morphological identification of *Peridinium* cells collected from natural samples remains confused. Therefore, in order to clearly discriminate the species, it is necessary to have considerably taxonomic experiences and skills on the pattern and number of plate.

The above problems were in part resolved by employing molecular techniques (e.g. PCR, hybridization assay, restriction method, or antibody probes) for the identification of harmful algal blooms species (Adachi et al., 1994; Scholin et al., 1994; Penna & Magnani, 2000; Godhe et al., 2001). Of these methods, PCR has been commonly adopted in many protocols due to its simplicity and high specificity (Godhe et al., 2001).

Recently, an advanced technique, sequence-based species diagnosis, has been developed for more accurate identification of harmful algae (Ki et al., 2004), based on the ribosomal DNA (rDNA) sequence comparisons.

The DNA marker regions are usually targeted to the rDNAs, because their nucleotide sequences are more conserved than the other coding regions within the various organisms. The rDNAs have already been sequenced from many dinoflagellate (Chesnick et al., 1997; Daugbjerg et al., 2000), and increased gradually in public resources. However, only few studies concerning the rDNA sequences for the taxonomic aspects of the genus *Peridinium* have so far been conducted (Hansen et al., 2000), owing to relatively small number of *Peridinium* rDNA sequences. Furthermore, most studies with regard to the DNA sequence of *Peridinium* have been performed on evolutionary relationships of their plastid (Chesnick et al., 1997; Daugbjerg et al., 2000; Saldarriaga et al., 2001).

With the rapid progress of molecular technologies, several methods have been described to obtain sequences from single dinoflagellate cells (Bolch, 2001; Marín et al., 2001; Ruiz Sebastián & O'ryan, 2001; Edvardsen et al., 2003; Ki et al., 2004). These techniques have been a major step in resolving the scarcity of DNA sequences from the dinoflagellates collected from various locations worldwide. However, they have so far been limited in their application for the marine organisms, and have never been employed to study DNA sequences or molecular diagnostics of freshwater dinoflagellate isolates.

The objectives of this study were to determine the rDNA nucleotide sequences of individual *Peridinium* cells isolated directly from freshwater samples, and to genotypically identify the morphological variants at the species level using the sequences as genetic markers. Furthermore, the two discrete sequences of LSU and SSU rDNA regions were used independently for construction of phylogenetic trees in order to deepen understanding of their taxonomic relationships within the Peridiniales.

Materials and methods

Sampling and morphological observations

Water samples were collected using a Van Dorn water sampler and plankton nets in two geographically adjacent regions (Sang-sa and Juam Reservoirs), Korea, as described previously (Ki et al., 2005), when seasonal *Peridinium* blooms occurred. The cells were immediately fixed with Lugol's solution and formalin at 1% final concentration. The preserved samples were used for morphological diagnosis of *Peridinium* species together with sequence-based species identification.

Single cell isolation and enzymatic DNA extraction

Single cells of *Peridinium*, approximately 40–60 μm in length and brownish color, were randomly isolated from the preserved or un-preserved field samples under a dissecting microscope (Carl-Zeiss Ltd, Stuttgart, Germany) at 40 to 200$\times$ magnification by capillary isolation (Ki et al., 2004). Code numbers of the individual cells were as follows: PJA-1 (Juam Reservoir at Nov. 11, 2003); PJ0311-1, -2, -3, -4, -5, -6 (Juam Reservoir at Mar. 11, 2004); PJ0401-1, -2, -3, -4, -5, -6 (Juam Reservoir at Apr. 1, 2004); PS0312-1, -2, -3, -4 (Sang-sa Reservoir at Mar. 12, 2004). Individual cells were transferred to sterile 1 $\times$ TE buffer droplets three to six times to facilitate the removal of contaminants, suspended in $\sim$1 μL of TE buffer, and then placed in 200-μL thin-walled PCR tubes containing a drop of mineral oil. Samples were then frozen at –20 °C until analysis.

DNA extraction followed the method described in our previous work (Ki et al., 2004b) with slight modifications. Proteinase K (1 μL, 200 μg mL^{-1}) was then added to the tubes previously prepared, and they were maintained at 55 °C for 30 min with a UNO-II Thermoblock (Biometra, Göttingen, Germany). Samples were then incubated at 95 °C for further 5 min to inactivate the proteinase K and facilitate DNA denaturation. The tubes were then cooled to 4 °C in preparation for PCR amplification.

PCR amplification and direct DNA sequencing

PCR primers, based on conserved sequences among related dinoflagellate species, were designed and synthesized. Two sets of PCR primers were used for PCR amplification of SSU [a forward AT18F01; 5′-YACCTGGTTGATCCTGCCAGTAG-3′ and a reverse AT18R02; 5′-GTTTCAGCCTTGCGACCATAC TCC-3′ (International Union of Pure and Applied Chemistry degeneracy symbols were used)] and LSU rDNA (forward AT28F01, 5′-CYGCTGARTTT AAGCATATAAGTAAG-3′; reverse PM28R1318, 5′-TCGGCAGGTGAGTTGTTACACAC-3′) from single *Peridinium* cells, respectively.

Table 1. rDNA sequences analyzed from a single cells, their GenBank accession numbers, and sequence-based diagnosis. Each nucleotide sequence was searched by BLAST, and a sequence having the highest sequence similarity score was selected as the reference sequence. Molecular diagnosis was performed using both genetic distance and similarity score between the aligned sequences. A sequence similarity of 99% or greater was considered as an acceptable identification.

Code	Morpho-species	Accession no.	Target rDNA	Size (bp) used	Genetic distance	Similarity score (%)	Sequence-based diagnosis
Between AF231805[a]							
PJ0311-6	*P.* sp.	AY682797	SSU	388	0.0052	99	*P. bipes* f. *occultatum*
PJ0401-5	*P.* sp.	AY682798	SSU	978	0.0020	99	*P. bipes* f. *occultatum*
PJ0401-6	*P.* sp.	AY682799	SSU	889	0.0022	99	*P. bipes* f. *occultatum*
PJA-1	*P.* sp.	AY682800	SSU	978	0.0093	99	*P. bipes* f. *occultatum*
PS0312-2	*P.* sp.	AY682801	SSU	836	0.0012	99	*P. bipes* f. *occultatum*
PS0312-4	*P.* sp.	AY682802	SSU	978	0.0020	99	*P. bipes* f. *occultatum*
Between AY359682[b]							
PJ0311-1	*P.* sp.	AY682703	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0311-2	*P.* sp.	AY682704	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0311-3	*P.* sp.	AY682705	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0311-4	*P.* sp.	AY682706	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0311-5	*P.* sp.	AY682707	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0401-1	*P.* sp.	AY682708	LSU	836	0.0012	99	*P. bipes* f. *occultatum*
PJ0401-2	*P.* sp.	AY682709	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0401-3	*P.* sp.	AY682710	LSU	836	0	100	*P. bipes* f. *occultatum*
PJ0401-4	*P.* sp.	AY682711	LSU	836	0	100	*P. bipes* f. *occultatum*
PS0312-1	*P.* sp.	AY682712	LSU	836	0.0012	99	*P. bipes* f. *occultatum*
PS0312-2	*P.* sp	AY682713	LSU	835	0	100	*P. bipes* f. *occultatum*

[a]Partial SSU rDNA of *P. bipes* sensu lato (Inagaki et al., 2000).
[b]Partial LSU rDNA of *P. bipes* f. *occultatum* (unpublished data).

PCR reactions were performed using all the crude lysates (each 2 μL) extracted from single cells (Table 1). Eight microliters of PCR master mix [0.5 U *Taq* polymerase (Promega, Madison, WI), 0.25 mM each of all four dNTP's, 1 $\times$ reaction buffer, and 1.5 mM $MgCl_2$], including 10 pmoles of each primer, were added to the PCR tube containing approximately 2 μL lysate at 4 °C. Thermocycling was as follows: initially 95 °C for 5 min, followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 60 s. After the completion of the cycles, extension was facilitated at 72 °C for 5 min. Two microliters of each PCR product were loaded onto a 1.5% agarose gel in 1 $\times$ TBE buffer along with 2 μL of loading buffer. Agarose gels were stained with ethidium bromide and photographed according to standard methods (Sambrook et al., 1989).

The unpurified PCR products (3–6 μL) were subjected to DNA cycle sequencing using a Thermo Sequenase™ 2.0 Sequencing Kit (USB, Cleveland, OH) in the presence of 1.5 pmole sequencing primers nested within the PCR primers. Four nested sequencing primers were used for sequencing reactions of the PCR products of SSU rDNA (forward SAT18F01, 5′-CCAGTAGTCATATGCTTGTC-3′; reverse SAT18R02, 5′-AGCCTTGCGACCATACTCC-3′) and LSU rDNA (forward SPM28F01, 5′- AAGCATAT AAGTAAGCGGAG-3′; reverse SPM28R01, 5′-GATGGTTCGATTAGTCTTTC-3′). All of the primers were labeled with near infrared dye (IRD) at the 5′ end. The four base-specific reactions were subjected to 50 cycles, consisting of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 60 s in the UNO-II Thermoblock. When completed, the reactions were stopped by adding 4 μL of IR2 stop/loading buffer (Li-cor, Lincoln, NE), and the products were heat-denatured and analyzed on a Model 4200 Dual Dye Automated Sequencer (Li-cor).

DNA sequence-based diagnosis and phylogenetic analysis

For sequence analysis, a database of rDNA sequences belonging to the order Peridiniales, including our previously reported SSU and LSU rDNA sequences of Korean *P. bipes* f. *occultatum* (AY359682,

AY733008-AY733012), was constructed privately. All the rDNA sequences were retrieved from GenBank. For sequence-based species diagnosis, nucleotide sequence analyzed from the single cells was added to the dataset, and their sequences were aligned with the default settings for gap inclusion and extension, using the Clustal W (Thompson et al., 1994). Molecular diagnosis of the unidentified *Peridinium* was performed with both genetic distance estimated by a Kimura 2-parameter distance model and similarity scores by GeneDoc 2.6 (http://www.psc.edu/biomed /genedoc/) between pairs of algal species from the aligned sequence data.

Phylogenetic analysis was performed using the aligned rDNA sequences contained in the genus *Peridinium*. Various regions were further aligned manually, using the BioEdit 5.0.6 (North Carolina State University, NC), and regions that could not be unambiguously aligned were excluded from the analysis. Phylogenetic tree was inferred by the use of the neighbor-joining (NJ) algorithm, using MEGA 2.0 (Charrel et al., 1999). The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis from 1000 bootstrap replications.

Results

Single-cell PCRs and DNA sequence

PCRs followed the standard protocols and successfully amplified the target rDNA regions from the genomic DNA, extracted from single *Peridinium* cells (Figure 1). The PCR fragments in length were approximately 1100 bp for SSU rDNA and 1200 bp for LSU rDNA with each PCR primer set. DNA sequencing was performed single or bi-directionally by using the nested sequencing primers and revealed the PCR products to be 388–978 and 836 nucleotides sequences for SSU rDNA and LSU rDNA, respectively (Table 1). The nucleotide sequences determined have been deposited in the GenBank database under accession numbers AY682797 to AY682813 (Table 1).

Sequence-based species diagnostics

The nucleotide sequence was searched by BLAST for sequence-based species diagnosis, and a sequence with the highest sequence similarity score was selected as a reference. The partial SSU rDNA sequences from six isolates were 0.0012–0.0093 of genetic distance and >99% sequence similarity with a sequence of accession no. AF231805 (NIES 364, *Peridinium bipes* f. *occultatum*) obtained from GenBank (Table 1). Similarly, the partial LSU rDNA sequence from 11 isolates were recorded at 0–0.0012 of genetic distance and nearly 100% sequence similarity with a reference sequence (HY971028T; *P. bipes* f. *occultatum*; accession no. AY359682). Based on the sequence similarities, all the isolates were genotypically identified as *P. bipes* f. *occultatum* (Table 1).

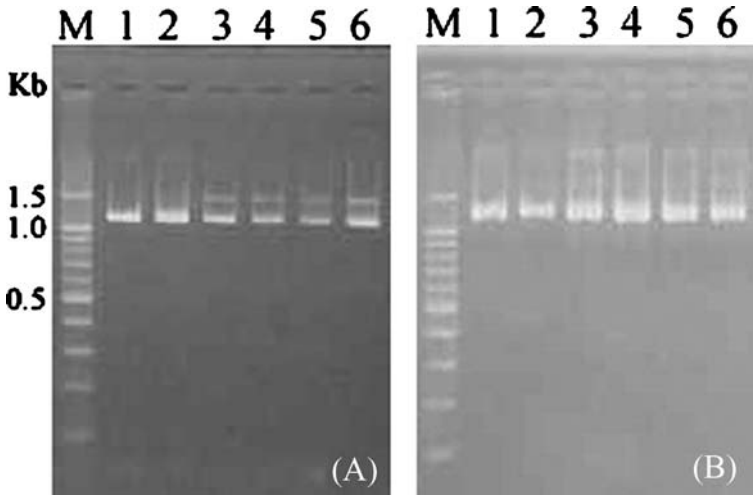

Figure 1. PCR products amplified from the DNA extracts of single-cells isolated from the field samples using AT18F01 and AT18R02 primers for SSU rDNA (A) and using AT28F01 and PM28R1318 primers for LSU rDNA (B). Lane 1–6: unidentified *Peridinium* species. M: 100-bp ladder size marker. Note: panel (B) shows the PCR fragments of re-amplification.

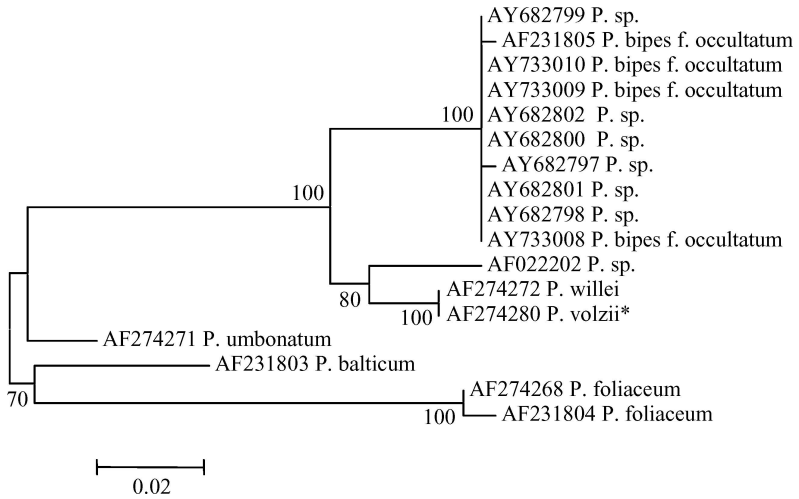

Figure 2. Phylogenetic tree constructed by NJ method using a Kimura 2-distance model and partial SSU rDNA sequences revealed in the present study (six isolates) and from the database (11 strains). The complete deletion option was selected for the gabs/missing data. Numbers above branches represent percentages of 1000 bootstrap repetitions, and bootstrap values are shown above the internodes, when higher than 50%. Branch lengths represent genetic distance among the taxa. Asterisk (*) represents the species, based on the name of original strain (NIES 365). Note: AF274268, AF274271, AF274272, AF274280 (Saldarriaga et al., 2001); AF231803, AF231804, AF231805 (Inagaki et al., 2000); AF022202 (Saunders et al., 1997); AY733008, AY733009, AY733010 (unpublished data).

Phylogenetic analyses

A phylogenetic tree was constructed using the NJ method and the SSU rDNA (Figure 2). Individual *Peridinium* species (e.g. *P. balticum*, *P. foliaceum*, *P. umbonatum* and *P. willei*) was strongly clustered into the same species-group, and apparently separated from the other different species, as judged by the genetic distances among the sequences. All the sequences revealed here were grouped with the sequences of *P. bipes* f. *occultatum* (100% bootstrap value), and their sequences were also significantly separated from two sequences of *P. willei* and *P. volzii*, supported strongly by the bootstrap value of 100%.

In LSU rDNA-inferred tree (Figure 3), all the sequences obtained here were strongly grouped with the

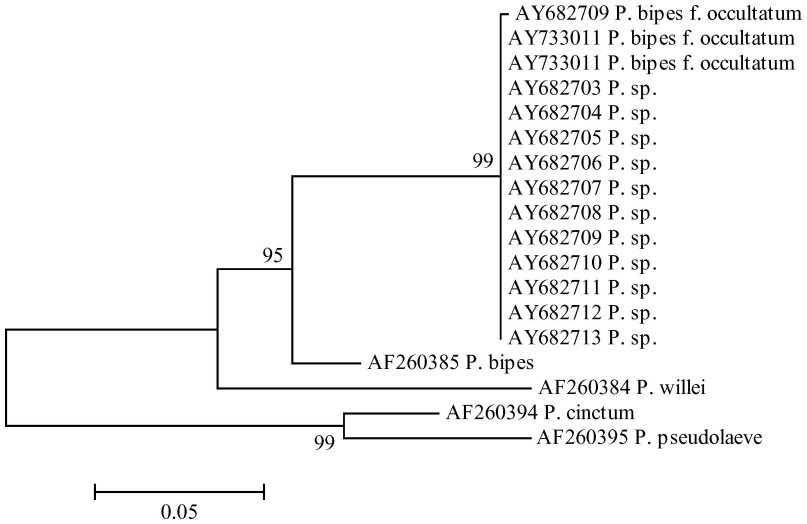

Figure 3. A NJ tree of the partial LSU rDNA sequences revealed in the present study (11 isolates) and from the database (7 strains). The complete deletion option was selected for the gabs/missing data. Distances were calculated by the Kimura 2-distance method with 1000 bootstrapped replicates in MEGA 2.0. Bootstrap values less then 50% are not shown. Branch lengths represent genetic distance among the taxa. Note: AF260384, AF260385, AF260394, AF260395 (Daugbjerg et al., 2000); AY359682, AY733011, AY733012 (unpublished data).

pre-analyzed sequences of *P. bipes* f. *occultatum* (99% bootstrap value). *P. willei* in mid-point rooted phylogeny was grouped into a cluster with *P. bipes* sensu lato rather than *P. cinctum*.

Discussion

The single cell PCR technique, including single cell isolation, cell storage, DNA extraction and PCR amplification, was performed by adding directly the subsequent reagent into the same reaction tube. These successive additions were highly crucial for DNA extraction and the following PCR amplification from single *Peridinium* cells with little DNA loss. In this method, a treatment of proteinase K effectively extracted the genomic DNA from the rigid cell walls of *Peridinium*. Rarely, the standard PCRs insufficiently amplified the target rDNA regions from the crude lysates, and the DNA band could not be visualized with ethidium bromide or its signal was weak. In such cases the PCR products were diluted with 1:100 ratio of 1 × TE buffer and re-amplified with the same PCR primers under identical cycle conditions. The further reaction was efficient in improving the yield of copy DNA (Figure 1B).

For sequencing reactions, when a DNA band of the expected size was seen on a gel, the products were diluted with 1 × TE buffer to generally be adjusted the same concentration (approximately 50 ng μL^{-1}). Sequencing reactions were performed with the diluents as the DNA template, and commonly yielded the high-quality sequence ladders of the PCR fragments. In cases where the quality of the PCR products was poor, their sequencing reactions yielded inadequate base-signals. Thus, the DNA sequences in length varied from 388 to 978 nucleotides (17 strands).

The various sequences in length, which were 388 to 978 for SSU rDNA and about 836 for LSU rDNA, were used for sequence comparisons. The different-sized sequence did not sensitively affect the result of BLAST search and genotypic identification, and genetic distance was also recorded sufficiently high among the other species within the genus *Peridinium*. In fact, the SSU rDNA sequences revealed here were nearly identical with the corresponding sequences of *P. bipes* f. *occultatum* (Accession no. AY733008-10), whereas they significantly differed from several sequences of the closely related species [*P. umbonatum* (Accession no. AF274271, 0.103 of genetic distance), *P. willei* (AF274272, 0.061), *P. volzii* (AF274280, 0.061)].

Phylogenetic trees graphically showed that all the Korean cells tested were grouped into the same clade, including the previously identified *Peridinium bipes* f. *occultatum*, and clearly separated from its relatives such as *P. willei* and *P. cinctum*. Particularly, analysis of the LSU rDNA, covering the D1/D2 regions, showed that *P. bipes* f. *occultatum* (Korean strains) was significantly separated from *P. bipes* (European strain, accession no. AF260385), based on considerably low sequence similarity (91%) and high genetic variation (0.069), despite the *P. bipes* f. *occultatum* can be regarded as part of *P. bipes*. With present results, the reasons are unclear, and further studies are needed to determine the nucleotide sequences of the rDNA or other genes of increased number of samples, collected worldwide from different geographical regions.

The cells at the genus level were genotypically identified to the species level, as judged by the sequence similarity. It was considered that an acceptable identification is a sequence similarity of over than 99% ($<$0.0093 genetic distance), since the genetic variations, which might be caused by its geographical separation of the same species, were slightly low within the intra-species level rather than within the inter-species level. In a previous study, Ki et al. (2004) reported that sequence homology was very high ($>$99% sequence similarity) among the same species of a marine dinoflagellate *Alexandrium catenella* collected from the geographically segregated regions, whereas it was significantly low ($<$94% sequence similarity) among other species belonging to the same genus *Alexandrium*.

The sequence-based species diagnostics is mainly based on the sequence comparison with the pre-revealed sequence, and the method could therefore be applied to identify several already-known-to-be-different species. For sequence-based typing, the database used here could not include numerous representative sequences of *Peridinium* cells occurring in natural environments. Fortunately, six rDNA sequences of the Korean *P. bipes* f. *occultatum*, which were isolated from water samples collected from Juam, Sang-sa and Togyo Reservoirs, have been deposited in GenBank database (unpublished data). In addition, a SSU rDNA sequence from Japanese *P. bipes* f. *occultatum* (Accession no. AF231805) is available on-line in the GenBank. Based on the sequence similarity or genetic distance, the 17 *Peridinium* variants in size had the same genotype and the sequence-based typing could unambiguously identify the various types of cells as *P. bipes* f. *occultatum*. The results showed that the causative

organisms of water blooms in different seasons (spring and autumn) in Korea were *P. bipes* f. *occultatum*, and a single bloom was genetically homogeneous, although several other *Peridinium* species also occur in Korean freshwaters (Ki, 1998).

As noted previously, the sequences from *Peridinium* species are markedly insufficient. Thus, sequence-based diagnostics can at present be applied to several species. Wider application of this method requires nucleotide sequences from many *Peridinium* species. The single cell PCR described here can partly resolve the scarcity of *Peridinium* nucleotide sequences, which is especially important where the cells cannot yet be grown in culture or are available only in small numbers from field samples.

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