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Abstract	The cosmopolitan diatom Chaetoceros is well-known as one of the most abundant and species-rich microalgal genera, and considered important from the viewpoints of biogeochemistry, marine ecology, biodiversity and so on. Thus, accurately distinguishing Chaetoceros species is essential in understanding the microbial ecology of aquatic environments. However, precise identification of Chaetoceros at the species level is extremely difficult, which makes the comprehension of the ecological dynamics of this diatom genus practically impossible. In this study, in order to overcome this problem, we applied polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to distinguish 18 isolates of Chaetoceros species targeting the rbcL region of chloroplast DNA encoding RubisCO large subunit. Suitable restriction enzymes to distinguish the PCR samples were selected based on rbcL DNA sequences. As a result, the tested Chaetoceros isolates were successfully distinguished by treatment with restriction enzymes, DdeI, Csp45I and RsaI. This result indicates that PCR-RFLP method is applicable for identification of hard-to-identify diatoms groups.
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PCR-RFLP Analysis for Species-Level Distinction of The Genus *Chaetoceros* Ehrenberg (Bacillariophyceae)

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Summary——The cosmopolitan diatom *Chaetoceros* is well-known as one of the most abundant and species-rich microalgal genera, and considered important from the viewpoints of biogeochemistry, marine ecology, biodiversity and so on. Thus, accurately distinguishing *Chaetoceros* species is essential in understanding the microbial ecology of aquatic environments. However, precise identification of *Chaetoceros* at the species level is extremely difficult, which makes the comprehension of the ecological dynamics of this diatom genus practically impossible. In this study, in order to overcome this problem, we applied polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to distinguish 18 isolates of *Chaetoceros* species targeting the *rbc*L region of chloroplast DNA encoding RubisCO large subunit. Suitable restriction enzymes to distinguish the PCR samples were selected based on *rbc*L DNA sequences. As a result, the tested *Chaetoceros* isolates were successfully distinguished by treatment with restriction enzymes, *Dde*I, *Csp*45I and *Rsa*I. This result indicates that PCR-RFLP method is applicable for identification of hard-to-identify diatoms groups.

Keyword: Diatom, *Chaetoceros*, PCR-RFLP, distinction, restriction enzyme, *rbc*L

1. Introduction

Diatoms (Bacillariophyceae) account for a large part of the marine primary production and have been key players in the oceanic carbon cycles for over forty million years (Smetacek 1999, Falkowski *et al*. 2000). Because they are precise indicators of water quality and habitat conditions, diatoms are considered significant also in the fields of environmental studies, geohistory and geochemistry (Pan *et al*. 2004, Harding *et al*. 2005). Since the explosive diversification across the Eocene/Oligocene and Oligocene/Miocene boundaries, the genus *Chaetoceros* has been one of the most successfully

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propagating diatom groups (Suto 2006), with more than 400 species so far described (Rines & Hargraves 1988). Species in *Chaetoceros* are distributed globally and often dominate in diverse environments, both coastal and open waters (Hasle and Syvertsen, 1996). Because of their high abundance and worldwide distribution, the genus is recognised as one of the most important algae for environmental studies.

Nevertheless, one of the most serious obstacles in examining the ecology of *Chaetoceros* is the difficulty in precisely identifying and distinguishing at the species level. Especially, identification of small-sized *Chaetoceros* species only by using optical microscopy (OM) are virtually impossible; transmission electron microscope is necessary to achieve correct identification. However, electron microscope is generally not suitable for routine observations for natural environmental samples. This has been the bottleneck in examining the ecology of *Chaetoceros.*

Here, a relatively quick and economical method for the distinction of multiple strains of *Chaetoceros* is proposed; it is based on a polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis, which requires no expensive equipment. As a PCR-target, the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) large subunit gene (*rbc*L) was selected considering its suitability for interspecific comparison (Cooper & Hausman 2009). In this paper, a new method for distinguishing local strains of *Chaetoceros* using PCR-RFLP patterns of *rbc*L is introduced.

2. Materials and Methods

Eighteen clonal culture strains of *Chaetoceros* were used in this study (Tab. 1); they were established from western Japanese coastal waters. 7 strains among them were identified based on their morphological features (Pl. 1, Tab. 1). Each culture was grown in modified SWM3 medium (Chen *et al.,* 1969, Itoh & Imai 1987) enriched with 2nM Na_2SeO_3 using a 12-h light/12-h dark cycle with ca. 110 μmol photons m^{-2} s^{-1} provided as cool white fluorescent illumination at 15℃.

DNA extraction from diatom cultures - Total DNA was extracted from each *Chaetoceros* culture as described below. 500 ul (around 5,000 cells) of the cell cultures in a microcentrifuge tube were centrifuged at 6,500 ×*g* for 5 min, and the resultant cell pellet was preserved at -80℃ until analysis; the pellet was treated in 100 μL of Tris–EDTA buffer (pH 8.0) at 100℃ for 10 min then stored at 4℃. After centrifugation at 17,000 ×*g* for 5 min, 100 μL of the supernatant was collected and used for further tests as an extracted DNA sample.

PCR and restriction endonuclease treatment of amplified products - In this study, DNA

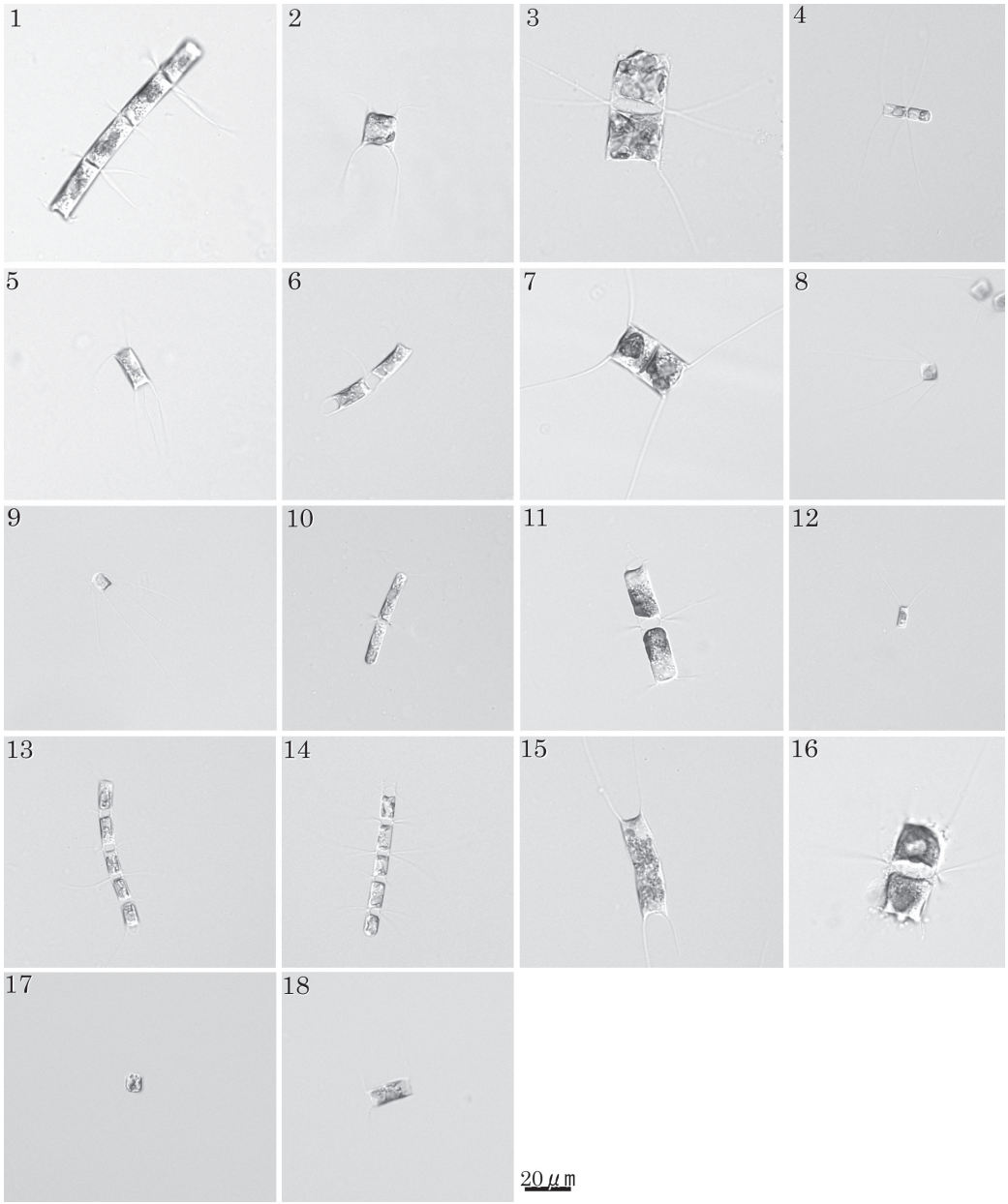

Plate 1. Optical micrographs of *Chaetoceros* spp. cells tested in this study.

polymorphism was detected in *rbc*L region of chloroplast DNA encoding RubisCO. This well-known enzyme functions in the Calvin cycle to catalyze the first major step of carbon fixation, and is a major sink for plant nitrogen (Jensen 2000). Because of the relatively low level of polymorphism within the *rbc*L target region, it is often used for interspecific comparisons (*e.g.* Xu & Tabita 1996). The primer pair used for the amplification of

Table 1. Original locality of *Chaetoceros* strains tested in this study; all samples are from Japan.

Strain no.	Sample code	Species name	Locality
1	IT07-C37	*Chaetoceros* aff. *pseudocurvisetus* L.Mangin	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
2	SS08-C09	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
3	IT07-C34	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
4	Chaet.sp.	*Chaetoceros* sp.	unknown
5	TG07-C28	*Chaetoceros* sp.	Ago Bay, Mie Pref.
6	Ch42	*Chaetoceros salsugineum* Takano	Ariake Sound, Northwest side of Kyushu
7	Chae5	*Chaetoceros* cf. *affinis* Lauder	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
8	SS08-8-21	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
9	SS08-0624-1	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
10	SS08-C03	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
11	TG07-C12	*Chaetoceros* sp.	Ago Bay, Mie Pref.
12	2-10	*Chaetoceros tenuissimus* Meunier	Harimanada, Seto Inland Sea, Hyogo Pref.
13	Ch48	*Chaetoceros debilis* Cleve	Ariake Sound, Northwest side of Kyushu
14	IT07-C11	*Chaetoceros setoensis* Ikari	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
15	D51	*Chaetoceros lorenzianus* Grunow	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
16	SS08-C01	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
17	SS08-8-20	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.
18	SS08-8-23	*Chaetoceros* sp.	Hiroshima Bay, Seto Inland Sea, Hiroshima Pref.

*Chaetoceros rbc*L fragment was “Diatom_rbcL_01F (5’- GGA GAA ATC AAT GTC TCA ATC TG -3’)” and “Diatom_rbcL_03R (5’- CAA RCA ACC TTG TGT AAG TCT C -3’)”. For the PCR reaction, 50 μL of reaction mixture contained as a final concentration, the two primers at 15 pmole each; 5–20 ng DNA template; 0.2 mM each dATP, dCTP, dGTP and dTTP; 5 μL of 10×PCR buffer for Blend Taq (Mg^{2+} concentration); and 2.5 units of Blend Taq polymerase (Toyobo, Osaka, Japan). The PCR condition was as follows: an initial step at 94°C for 2 min followed by 30 cycles with a denature temperature of 94°C for 30 s, an annealing temperature of 53°C for 30 s and an extension temperature of 72°C for 1.5 min. With this condition, PCR fragments with the expected size (～1.55 kbp) was successfully obtained from each DNA sample. PCR products purified by using MinElute PCR Purification Kit (Qiagen, Tokyo, Japan) were directly sequenced. The restriction site maps of the resultant 18 sequences were compared by using Genetyx version 5.1.0 software (Genetyx Corporation, Tokyo, Japan) and restriction enzymes suitable for distinguishing the 18 sequences were analyzed. As a result, the following three enzymes were selected (restriction site in parenthesis): *Dde*I (C | TNAG), *Csp*45I (TT | CGAA) and *Rsa*I (GT | AG).

For restriction enzyme reaction, aliquots of PCR reaction mixtures were incubated for 1 h at 37°C with addition of 20 units of each enzyme in each suitable buffer condition. Each enzymatically-digested DNA was subjected to electrophoresis on 1.2% agarose gel in Tris-

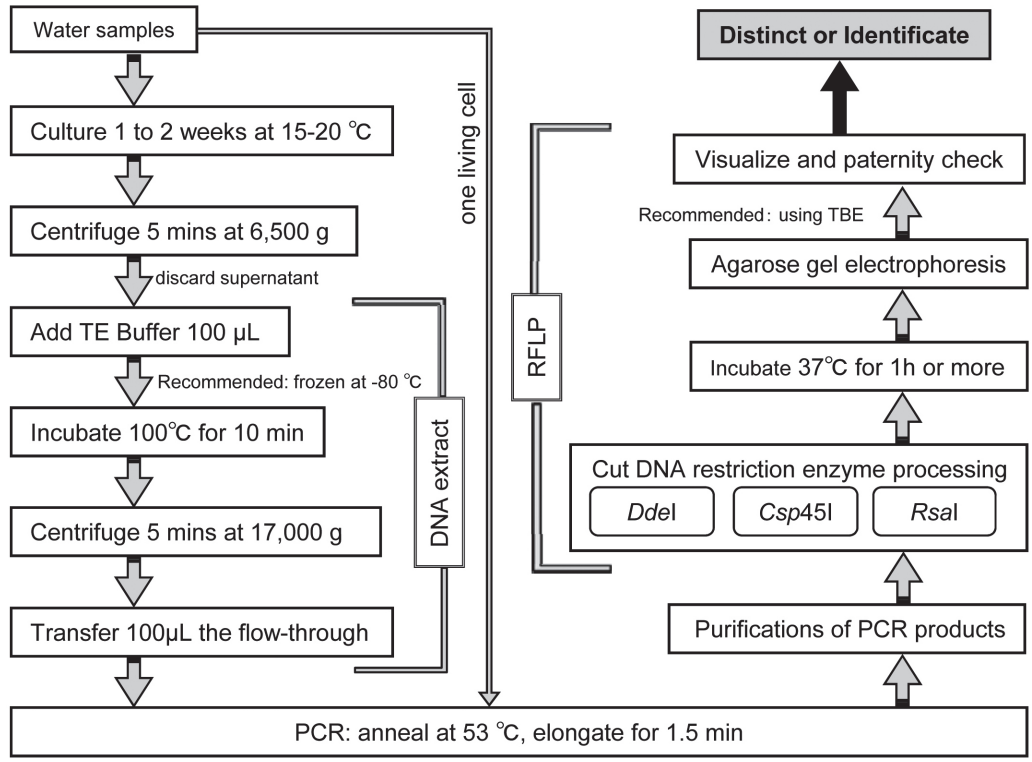

Plate 2. Flow chart illustrating step in the process of the PCR-RFLP method.

borate-EDTA at 50 V for 1 h; then, the DNA fragments were stained with ethidium bromide, visualized under UV light and photographed. The flow chart is given in Plate 2.

3. Results and Discussion

Plate 3 shows the PCR-RFLP patterns observed with *Dde*I, *Csp*45I and *Rsa*I; the estimated size of each restriction fragment is shown in Table 2.

Each of the amplicons of IT07-C37, SS08-C09, IT07-C34, Chaet.sp., TG07-C28, Ch42, Chae5, SS08-8-21 and SS08-0624-19 digested with *Dde*I showed its specific RFLP-pattern within the 18 *Chaetoceros* strains tested in the present study; the RFLP patterns of the other 9 strains were divided into three groups: A) SS08-C03, TG07-C12, 2-10 and Ch48; B) IT07-C11, D51 and SS08-C01; C) SS08-8-20 and SS08-8-23 (Tab. 2 and Pl. 3, Fig. a). Although the smallest restriction fragment of SS08-C03 (~0.14 kbp) was slightly larger than those of the other strains in group A (~0.12 kbp), further distinction was considered to be necessary (see below).

Each of the *Csp*45I-digested amplicons of Chaet.sp., SS08-0624-1 and 2-10 showed its specific RFLP patterns; the RFLP patterns of the other 15 strains were divided into three

groups D-F; D) IT07-C37, IT07-C34, Ch42, Chae5, Ch48, D51 and SS08-8-23; E) SS08-C09, TG07-C28, SS08-8-21, SS08-C03, TG07-C12 and IT07-C11; F) SS08-C01 and SS08-8-20 (Tab. 2 and Pl. 3, Fig. b).

When *Rsa*I was used for digestion, each of Ch42, SS08-C03, TG07-C12 and IT07-C11 amplicons showed its specific RFLP pattern; the RFLP patterns of the other 14 strains were divided into two groups: G) IT07-C37, SS08-C09, SS08-0624-1 and SS08-8-20; H) IT07-C34, Chaet.sp., TG07-C28, Chae5, SS08-8-21, 2-10, Ch48, D51, SS08-C01 and SS08-8-23 (Tab. 2 and Pl. 3, Fig. c). Although the pattern of Ch42 resembled that of group H, the largest and the smallest fragments (0.97 and 0.05 kbp, respectively) were different in size.

Considering the morphological distinctiveness of the 18 tested *Chaetoceros* strains and the low sequence homology (<96%) of their *rbc*L fragments, each of them was considered belonging to different species. Based on the above PCR-RFLP results, the 18 strains were successfully distinguished by using the three restriction enzymes. Overall, *Dde*I distinguished 9 strains with certainty; 7 among the remaining 9 were separately

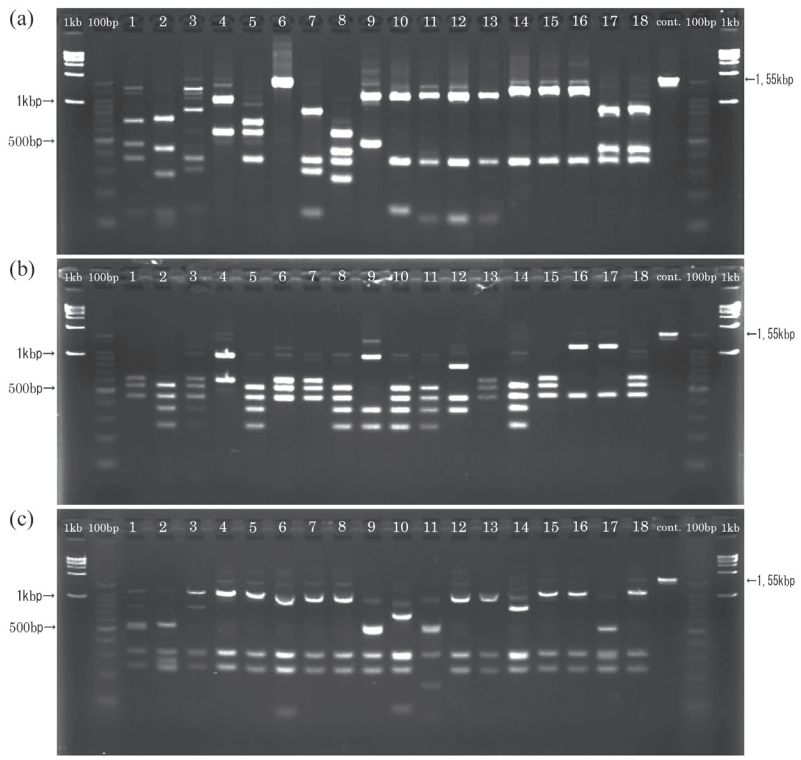

Plate 3. RFLP of *rbc*L fragments treated with *Dde*I (Fig. a), *Csp*45I (Fig. b) and *Rsa*I (Fig. c). Species numbers are shown in Table 1. “1kb” and “100bp” respectively indicate 1 kilobase and 100 base ladder markers.

Table 2. Major restriction fragments from *rbc*L.

Strain no.	Sample code	*Dde*I (kbp)	*Csp*45I (kbp)	*Rsa*I (kbp)	enzymes used for distinction
1	IT07-C37	0.75/0.45/0.35	0.60/0.53/0.42	0.51/0.51/0.31/0.22	*Dde*I
2	SS08-C09	0.87/0.41/0.27	0.53/0.42/0.35/0.25	0.51/0.51/0.31/0.22	*Dde*I
3	IT07-C34	0.90/0.35/0.30	0.60/0.53/0.42	1.02/0.31/0.22	*Dde*I
4	Chaet.sp.	1.01/0.54	0.95/0.60	1.02/0.31/0.22	*Dde*I or *Csp*45I
5	TG07-C28	0.66/0.54/0.35	0.53/0.42/0.35/0.25	1.02/0.31/0.22	*Dde*I
6	Ch42	not digested	0.60/0.53/0.42	0.97/0.31/0.22/0.05	*Dde*I
7	Chae5	0.77/0.35/0.30/0.13	0.60/0.53/0.42	1.02/0.31/0.22	*Dde*I
8	SS08-8-21	0.54/0.41/0.35/0.25	0.53/0.42/0.35/0.25	1.02/0.31/0.22	*Dde*I
9	SS08-0624-1	1.06/0.49	0.95/0.35/0.25	0.51/0.51/0.31/0.22	*Dde*I or *Csp*45I
10	SS08-C03	1.06/0.35/0.14	0.53/0.42/0.35/0.25	0.65/0.31/0.31/0.22 /0.05	*Rsa*I
11	TG07-C12	1.08/0.35/0.12	0.53/0.42/0.35/0.25	0.51/0.51/0.31/0.11 /0.11	*Rsa*I
12	2-10	1.08/0.35/0.12	0.78/0.42/0.35	1.02/0.31/0.22	*Csp*45I
13	Ch48	1.08/0.35/0.12	0.60/0.53/0.42	1.02/0.31/0.22	*Dde*I and *Csp*45I
14	IT07-C11	1.20/0.35	0.53/0.42/0.35/0.25	0.70/0.31/0.31/0.22	*Rsa*I or *Dde*I and *Csp*45I
15	D51	1.20/0.35	0.60/0.53/0.42	1.02/0.31/0.22	*Dde*I and *Csp*45I
16	SS08-C01	1.20/0.35	1.13/0.42	1.02/0.31/0.22	*Dde*I and *Csp*45I
17	SS08-8-20	0.79/0.41/0.35	1.13/0.42	0.51/0.51/0.31/0.22	*Dde*I with *Csp*45I
18	SS08-8-23	0.79/0.41/0.35	0.60/0.53/0.42	1.02/0.31/0.22	*Dde*I with *Csp*45I

distinguished by using *Csp*45I, and the remaining 2 were distinguished by *Rsa*I; hereby, we developed a new PCR-RFLP method for species-level distinction for the genus *Chaetoceros*. Even when artificial morphological transformation was induced in the algal culture due to long-term culturing, this PCR-RFLP method is applicable. In addition, the method is inexpensive and quick (required only 5 hours, the actual work are only less than 1 hour), and requires no special equipments other than an ordinary electrophoresis system. It is not necessary to use axenic algal culture for DNA extraction because the target *rbc*L PCR fragments are easily amplifiable with the highly specific primers mentioned above. Furthermore, even single-cell PCR is also applicable (data not shown); hence, the method is available for distinguishing *Chaetoceros* cells from natural environments. Of course morphological investigation will remain being most important in diatom classification and distinction; still, the PCR-RFLP method developed in this study may help to identify quickly or distinguish *Chaetoceros* cells based on differences in DNA sequence.

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