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A sequencing protocol of some DNA regions in nuclear, chloroplastic and mitochondrial genomes with an individual colony of *Thalassiosira nordenskioeldii* Cleve (Bacillariophyceae)

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Abstract: From cultured cells of micro algal species of *Thalassiosira nordenskioeldii* Cleve, total DNA was extracted with a buffer containing a chelating agent, Chelex 100. Each DNA fragment for 18s rDNA, 16s rDNA and *coxI* in nuclear, chloroplastic and mitochondrial genomes, respectively, were amplified from the DNA extracts by polymerase chain reactions, and their sequences were determined. This protocol for determining DNA sequences, was scaled down for the DNA extracts from small amounts of cells. Finally, DNA sequences in genomes were determined with 10 cells collected by a micromanipulation technique. The quantity of cells corresponded to an individual colony of this species. With this protocol, DNA sequences in various regions in genomes of each individual micro algal species living in natural environments can be determined directly, without isolation of strains and cultures.

key words: Bacillariophyceae, chelating agent, DNA sequence, micromanipulation, *Thallassiosira nordenskioeldii*

Introduction

Genetic information on DNA sequences in genomes is different not only between species but also between individuals in the same species. This information is often used both for interspecific analyses such as estimation of phylogenic relationships and for intraspecific analyses such as estimation of gene flow and identification of individuals (Karp *et al*., 1998). Among micro algal species of diatoms, the interspecific and intraspecific differences were also found. Their interspecific differences have been shown by DNA sequences directly (Zechman *et al*., 1994; Kooistra and Medlin, 1996; Medlin *et al*., 1996; Ehara *et al*., 2000a). To show intraspecific differences, more precise analyses of DNA sequences are needed. Intraspecific differences of diatoms have often been observed indirectly with various methods for genomewide analyses. Different genetic information between populations of the same species blooming in different seasons has been suggested by allozyme analysis (Gallagher, 1980). Diversity among populations of the same diatom species has been determined by restriction fragment length polymorphisms (RFLP) and allozyme analysis (Stabie *et al*., 1992). Genetic diversities among individuals in an algal population has been estimated by random amplified polymorphism DNA (RAPD) and by microsatellite (Lewis *et al*.,

1997; Rynearson and Armbrust, 2000).

These methods are usually applied only to cultured cells, as they require large amount of DNA. However genetic information on small amount of DNA in each individual could be essential for analyses of the natural populations and individuals, especially for microorganisms, as their individuals are hard to identify by means of morphological characteristics. Adequate amounts of DNA can be prepared by isolations of strains and cultures. However, some species are hard to isolate and culture, and there is no way to obtain genetic information on these species. Most of microorganisms living in polar regions are in this category. For these organisms, sequencing protocols of some DNA regions without isolations of strains and cultures is potentially important.

With a polymerase chain reaction (PCR), large amounts of DNA fragments can be amplified from a small amount of DNA. Although the fragments are restricted to a particular region in genomes and the methods mentioned above are not generally applied to fragments, genetic information can be obtained directly from their sequences. For the analyses we have to amplify DNA fragments from small amounts of DNA in each individual microorganism. In the present study we aimed to demonstrate a possible protocol for determining the DNA sequences in objective regions in nuclear, chloroplasitic and mitochondrial genomes of each individual microorganism and indicate the possibility of intraspecific analyses of microorganisms in natural habitats.

Materials and methods

Materials

Thalassiosira nordenskioeldii Cleve (Bacillariophyceae) was used as a model microorganism in this study. Species of this genus are characteristically arctic (Cupp, 1977). Many results of DNA sequences in nuclear, chloroplastic and mitochondrial genomes of these species are in the DNA data base now (*cf*. DNA Data Bank of Japan, DDBJ hereafter). This species is distributed over various sea areas (*cf*. Cupp, 1977). The strain in this study was isolated by the author from a sea ice biota of Saroma Ko lagoon, Hokkaido, Japan in February 1996. This species grows at temperatures between -2 and 15°C (Suzuki and Takahashi, 1995). After culturing at 0°C for more than 5 years, the strain did not show morphological changes and sustained the same growth rate as those determined soon after isolation of the strain in 1996. We were able to prepare adequate amounts of the cells in this study. Cells of many species of this genus were united in flexible chains by organic threads (Cupp, 1977). This strain also forms chain colonies of 10 and more cells in a natural habitat. As the cells were united by a single organic thread, the colonies were easily broken by shaking the culture, making it possible to control the colony size approximately. Colonies made of *ca*. 10 intact cells were prepared and used. In each cell of this strain, 12.2 ± 1.2 (average $\pm$S.D., n=50) of chloroplasts were found by confocal laser microscopic observation. A single colony made of 10 cells contains 122 copies of chloroplastic genome sets on average.

Culture conditions and cell collection

The strain was cultured in F/4 medium (Guillard and Ryther, 1962). All the cells for the following experiments were cultured at 5°C under 50 μmol photons m^{-2} s^{-1} on a 14:10 h L:D (light:dark) cycle (fluorescent light, FL40S:D, National) and were growing in exponential

phase at a density of *ca*. 8000 cells ml^{-1}. Cells in the 1000 m*l* cultures were collected by centrifugation at 2000G×10 min and were obtained as *ca*. 200 μl of pellets. Cells in the 1.0 m*l* cultures were also collected by centrifugation in 1.5 m*l* volume tubes. After removing the supernatant, pellets of 8000 cells in the tubes were directly used for the following experiments. Small amounts of the cells were collected in the same way from 1 m*l* of the cultures at densities of 8000～100 cells ml^{-1} prepared by dilution of the cultures by the F/4 medium. After centrifugation, pellets of 4000, 1600, 800, 400, 160 and 80 cells was used in the following experiments. To prepare pellets of 100 cells or less, cells were counted and picked up by micromanipulation under a stereoscopic microscope. The cells picked up were transferred into 20 μl of the culture medium in 200 μl tubes and were collected in the same way as those in the 1.0 m*l* cultures. Pellets of precisely 100, 50, 20 and 10 cells were prepared.

DNA extraction

An extraction protocol with proteinase K and phenol-chloroform was adopted to prepare DNA from 200 μl of cell pellets. Pellets ground in liquid nitrogen were transferred to 5.0 m*l* of the extraction buffer containing 50 mM Tris-HCl at pH 8.0, 20 mM EDTA and 1.0% of Sodium dodecylsulfate, and were incubated at 50˚C for more than 8 hours with *ca*. 5 units ml^{-1} of Proteinase K (Wako Pure Chemical Industries, Ltd.). Proteins in the samples were denatured with phenol and phenol-chloroform, and nucleic acids were purified by precipitation with ethanol and were dissolved in TE buffer containing 10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA (Dellaporta *et al*., 1983; Sambrook *et al*., 1989). This DNA extraction protocol was also applied to small amounts of the cells. To the pellets of 80 to 4000 cells in 1.5 m*l* volume tubes, 200 μl of the extraction buffer were added and incubated with Proteinase K. Proteins in extracts were denatured with phenol and phenol-chloroform, and the precipitated DNA was dissolved in TE buffer.

A DNA extraction protocol with Chelex 100 (Bio-Rad) as the chelating agent (Walsh *et al*., 1991) was also adopted. This protocol was designed for small amounts of tissues such as blood stain and plucked hair, and was rapid and simple without multiple tube transfers. To distilled water or TE buffer, 5% (wt/vol) of Chelex 100 was added and autoclaved at 120˚C for 10 min. Soon after suspending the Chelex 100 particles, 200 μl of 5% Chelex 100 suspension was added to each pellet of 80 to 4000 cells in a 1.5 m*l* volume tube. The solution was incubated at −20˚C for 20 min. It was vortexed at high speed for 5 s and incubated again at 95˚C for 15 min followed by an additional incubation at 4˚C for more than 10 min. After centrifugation the supernatant was transferred to a new 1.5 m*l* tube. Extracted total DNA in the supernatant was used as a template for PCR. For smaller amounts of cells collected in 200 μl volume tubes, the protocols were scaled down precisely and DNA was extracted with 20 μl of 5% Chelex 100.

DNA amplification

Primers for PCR were designed in highly conservative regions on already-known 18s rDNA, 16s rDNA and cytochrome oxidase subunit I (*coxI*) in nuclear, chloroplastic, and mitochondrial genomes of diatom species, respectively (Kowallik *et al*., 1995; Kooistra and Medlin, 1996; Medlin *et al*., 1996; Nubel *et al*., 1997; Inagaki *et al*., 1998; Ehara *et al*., 2000a, b; Yager *et al*., 2001). In this study we chose the rDNA regions in nuclear and chloroplastic genomes, both of which are multicopy (Rowe *et al*., 1996; White and McLaren,

Table 1. Sequences of each primer.

Primer name	Sequence (5´ - 3´)
18sF	GATAACCGTAGTAATTCTAGACTAA
18sR	TTTAATATACGCTATTGGAGCTG
16sF	CCATATGCTTTCGAGTGAAAT
16sR	CTACTATACTCTAGTCTAATAGTTTC
CoxF	ATGATTTTTTTTATGGTGATGCC
CoxR	CCAAACCCAGGTAAAATTAAAAT

2000). There are many copies of 18s rDNA and 16s rDNA in a cell of this strain. Especially, the copy numbers of 16s rDNA could be high, as a cell contains more than 10 chloroplasts. Copy numbers of *coxI* should be lower than those of rDNA, although there should be more than one copy as the cell usually contains more than one mitochondrion. The forward and reverse primers for 18s rDNA, 16s rDNA and *coxI* have been named 18sF and 18sR, 16sF and 16sR, and CoxF and CoxR, respectively (Table 1). These primers were designed to have similar melting points, between 50 and 54˚C. One μl each of forward and reverse primer (20 pmol μl^{-1}), and 1.0 μl of template DNA (*ca*. 20 ng μl^{-1}), were added to 47 μl of the reaction mixture (Premix Ex *Taq*, TaKaRa). The cycling reaction was done in a programmable heat block (PCR Thermal Cycler PERSONAL, TaKaRa) controlled by a program to incubate at 95˚C for 30 s (denature), incubate at 51˚C for 30 s (anneal) and incubate at 72˚C for 60 s (extend). After 30 incubation cycles, the samples were incubated an additional 10 min at 72˚C. Molecular weights of DNA fragments amplified by the PCR were estimated by electrophoresis in 2.0% agarose gel with 100bp DNA Ladder (TaKaRa). Both the gel buffer and running buffer were TAE (Tris-acetate and EDTA buffer) containing 40 mM Tris, 40 mM acetate and 1 mM EDTA (pH 8.0).

DNA sequencing

PCR products were purified by an ultrafiltration column (SUPREC-02, TaKaRa) before sequencing reactions, and directly used as templates for Sanger's method (Sanger *et al*., 1977). The same primers used for the above-mentioned PCR were used for each reaction. The sequence reactions were conducted in 20 μl of Terminator Ready Reaction Mix (PE Applied Biosystems) with *ca*. 3.2 pmol of the primers and 50 ng of template DNA. For the reactions 30 cycles of incubations at 96˚C for 10 s, 51˚C for 5 s and 60˚C for 4 min were conducted with the programmable heat block. DNA sequences were determined with ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Sequences determined in this study were compared with corresponding DNA sequences of other diatom species previously determined and submitted to the DNA database (DDBJ).

Results

The most common extraction protocol with proteinase K and phenol-chloroform (Dellaporta *et al*., 1983) was applied for *ca*. 8.0×10^6 cells in 1000 m*l* of culture. The extracted DNA was used as PCR templates with primers for the region of 16s rDNA in chloroplastic genome, at first, and fragments were amplified. The molecular weight of the fragments

determined by electrophoresis corresponded well to that expected from the previously determined sequences of other diatom species. The DNA extraction protocol with proteinase K and phenol-chloroform was also applied for the small amount of 8000 cells in 1.0 m*l* of culture collected in 1.5 m*l* tubes. The protocol was precisely scaled down, but no DNA fragments were amplified by PCR. This extraction protocol was not efficient when the volume of solutions was small. To precipitate and eliminate the denatured protein layer between phenol and water solution, most of the solution remained in tubes. After 3 treatments, more than 100 in 200 μl solution was lost.

Instead of the protocol with proteinase K and phenol-chloroform, a simple extraction protocol with Chelex 100 (Walsh *et al*., 1991) was applied for 8000 cells in the 1.0 m*l* cultures. With the extract, a region in 16s rDNA was amplified by the same PCR protocol that was used for extracts from 8.0×10^6 cells. Template DNA in 1.0 μl solution for one application of PCR contained DNA from 1/200 of 8000 cells, which was only 40 cells, and similar numbers for large size single colonies of this strain.

With the same protocol as described above we tried to extract total DNA from smaller amounts of cells. Extracted total DNA was applied for PCR with a pair of primers for a region in 16s rDNA, at first. DNA fragments were amplified with the extracts from 800 cells;

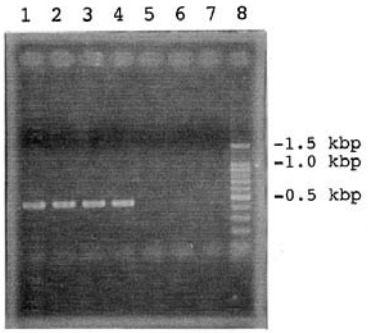

Fig. 1. Electrophoretic image of DNA fragments amplified by PCR with a pair of primers for 16s rDNA in 2% agarose gel. Lanes 1, 2, 3, 4, 5, 6 and 7 show fragments amplified with the extracts in Chelex 100 buffers from 8000, 4000, 1600, 800, 400, 160 and 80 cells, respectively. Lane 8 shows molecular markers of the 100 bp DNA Ladder (TaKaRa).

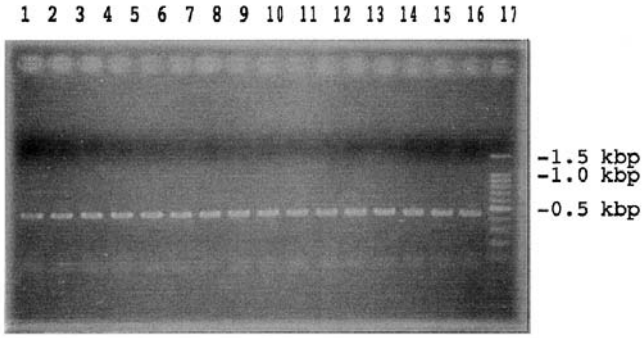

Fig. 2. Electrophoretic image of DNA fragments amplified by PCR with a pair of primers for 16s rDNA in 2% agarose gel. Lanes 1 to 4, 5 to 8, 9 to 12 and 13 to 16 show fragments amplified with the extracts in Chelex 100 buffers from 100, 50, 20 and 10 cells, respectively. Lane 17 shows molecular markers of the 100 bp DNA Ladder (TaKaRa).

no fragments were amplified from 400 cells or less (Fig. 1). To decrease cell numbers for DNA extractions, we reduced the volumes of extraction buffers and collected the cells by micromanipulation. Precise numbers of cells were collected under a stereoscopic microscope and samples containing correct numbers of the cells were prepared. Then fragments for 16s rDNA could be consistently amplified by PCRs even from extractions of 10 cells (Fig. 2). With primers for *coxI* and 18s rDNA, fragments were also amplified with DNA extracts both from 8.0×10^6 cells and from 10 cells.

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T. eccentrica          101 TATATCAGTTATAGTTTATTTGATAGTCCCTTACTACTTGGATACCCGTAGTAATTCTAG  160
T. rotula              101 ....................................................  160
S. costatum            103 --.....................A.....C............A.........  158
T. nordenskioeldii         ------------------------------------------------------
      (this study)           ..................... ..... ........... ..........

T. eccentrica          161 AGCTAATACATGCATCAATACCCAACTGTTCGCGGAAGGGTAGTATTTATTAGGTATAGA  220
T. rotula              161 ....................................................  220
S. costatum            159 ........T.........AG.GG....-C...-....CC.CC..G........TAT..A.  216
T. nordenskioeldii         -------------------------.................................
      (this study)         ........ .........  .  ..**..***.****..*..**.********...**.*

T. eccentrica          221 CCAACCCTCTTCGGAGGTGCTTTGGTGATTCATAATAACTTTTCGAATCGCATGGCTCCA  280
T. rotula              221 ....................................................  280
S. costatum            217 ..TT.A.........T..A........G..........C........T.....CACATG  276
T. nordenskioeldii         ......G........C.....................................
      (this study)         **..*..********..**.********.**********.********.*****.....

T. eccentrica          281 TGCCGGCGATGGATCATTCAAGTTTCTGCCCTATCAGCTTTGGATGGTAGTGTATTGGAC  340
T. rotula              281 ....................................................  340
S. costatum            277 .TG....A..............................G...............  336
T. nordenskioeldii         ....................................................
      (this study)         *..****.*********************************.**********

T. eccentrica          341 TACCATGGCTTTAACGGGTAACGAATTGTTAGGGCAAGATTTCGGAGAGGGAGCCTGAGA  400
T. rotula              341 ....................................................  400
S. costatum            337 ....................................................  396
T. nordenskioeldii         ....................................................
      (this study)         ************************************************

T. eccentrica          401 GACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGG  460
T. rotula              401 ....................................................  460
S. costatum            397 ....................................................  456
T. nordenskioeldii         ....................................................
      (this study)         ************************************************

T. eccentrica          461 AGGTAGTGACAATAAATAACAATGCCGGGCCTTTACAGGTCTGGCAATTGGAATGAGAAC  520
T. rotula              461 ....................................................  520
S. costatum            457 ....................................................  516
T. nordenskioeldii         ..............................A.....................
      (this study)         ******************************.**********************

T. eccentrica          521 AATTTAAATCCCTTATCGAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTA  580
T. rotula              521 ....................................................  580
S. costatum            517 ....................................................  576
T. nordenskioeldii         ....................................------------------
      (this study)         ****************************************..... ......

T. eccentrica          581 ATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTT                619
T. rotula              581 ....................................                 619
S. costatum            577 ....................................                 615
T. nordenskioeldii         ------------------------------------
      (this study)         ....................................
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Fig. 3a. Part of the 18s rDNA sequence of *T. nordenskioeldii* in this study was compared with the sequences of *T. eccentrica*, *T. rotula* and *S. costatum* determined by Kooistra and Medlin (1996) and Medlin *et al.* (1996), respectively. Numbers indicate the base numbers from the initiation codon. The underline indicates primers for PCR.

The sequences of each fragment amplified from the extracts of 10 cells were determined (Figs. 3a, 3b, 3c). The sequences were completely matched with those determined with fragments amplified from extracts of 8.0×10^6 cells. These sequences also showed higher homologies to the corresponding sequences of other species previously determined (Figs. 3a, 3b, 3c), although some variations were observed among the DNA regions. Homologies between the sequence of the fragment for 18s rDNA and those of some diatom species of *Thalassiosira eccentrica* (Kooistra and Medlin, 1996), *T. rotula* (Kooistra and Medlin, 1996)

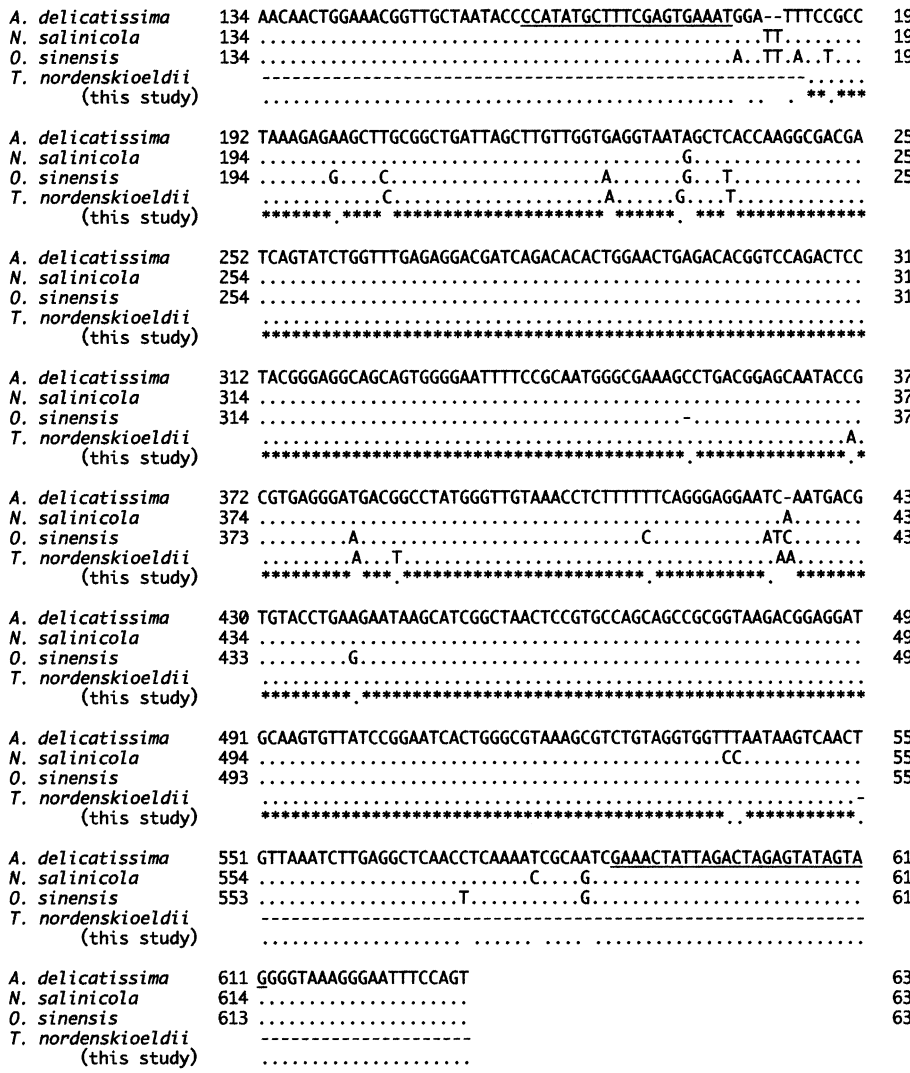

Fig. 3b. Part of the 16s rRNA sequence of *T. nordenskioeldii* in this study was compared with the sequences of *A. delicatissima*, *N. salinicola* and *O. sinensis* determined by Kowallik *et al*. (1995), Nubel *et al*. (1997) and Yager *et al*. (2001), respectively. Numbers indicate the base numbers from the initiation codon. The underline indicates primers for PCR.

and *Skeletonema costatum* (Medlin *et al*., 1996) were 99.2, 99.2 and 92.0%, respectively. The fragment corresponded well to the part of 18s rDNA from 186 to 562 bases (Fig. 3a). The fragments for 16s rDNA and those for *coxI* corresponded well to the part of 16s rDNA from 188 to 551 bases and that of *coxI* from 204 to 658 bases, respectively (Figs. 3b, 3c).

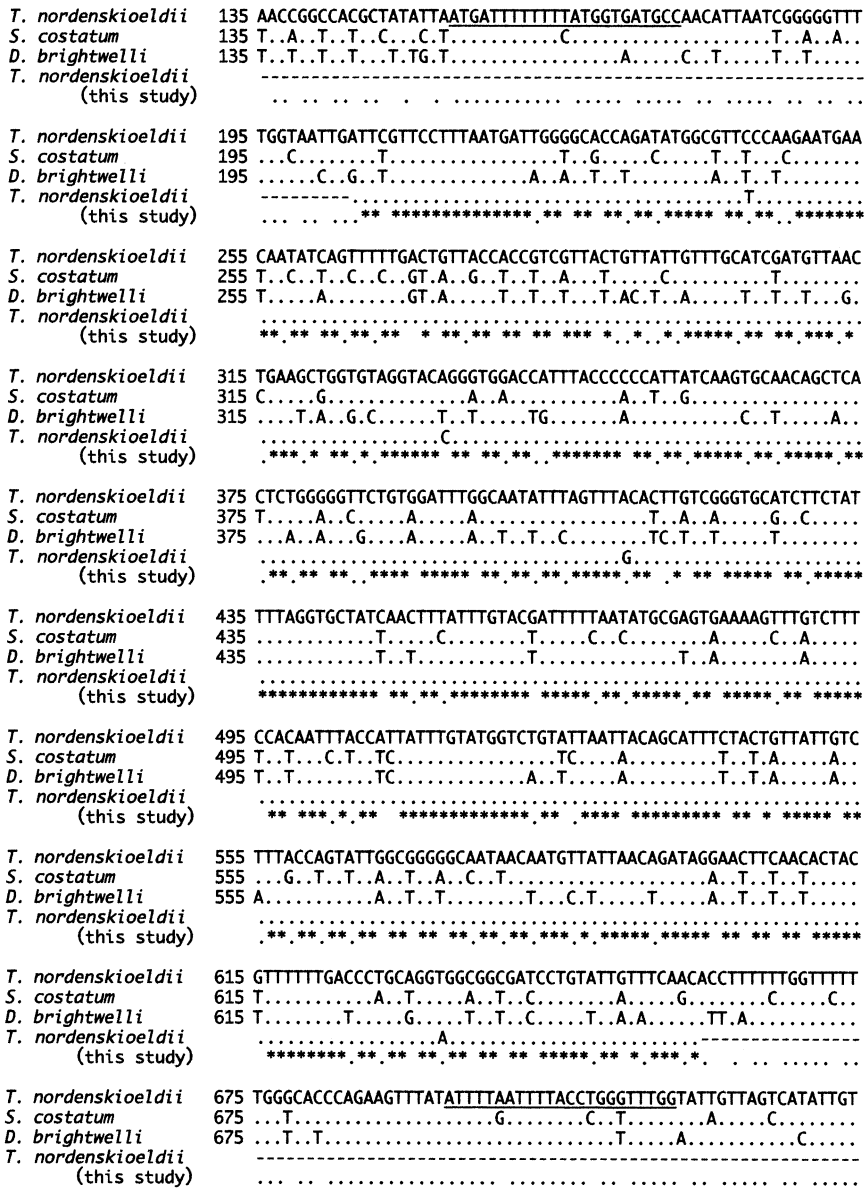

Fig. 3c. Part of the *coxI* sequence of *T. nordenskioeldii* in this study was compared with the sequences of *T. nordenskioeldii*, *S. costatum* and *D. brightwelli* determined by Inagaki *et al*. (1998), Ehara *et al*. (2000a) and Ehara *et al*. (2000b). Numbers indicate the base numbers from the initiation codon. The underline indicates primers for PCR.

Homologies between the sequence of the fragment for 16s rDNA and those of the diatom species *Amphora delicatissima* (Kowallik *et al*., 1995), *Navicula salinicola* (Nubel *et al*., 1997) and *Odontella sinensis* (Yager *et al*., 2001) were 97.5, 97.0 and 96.7%, respectively. Homologies of 99.1, 82.0 and 80.4% were determined between the sequence of the fragment for *coxI* and those of the diatom species *T. nordenskioeldii* (Inagaki *et al*., 1998), *S. costatum* (Ehara *et al*., 2000a) and *Ditylum brightwelli* (Ehara *et al*., 2000b), respectively.

Discussion

Strains of most microorganisms living in the polar region can not be isolated and cultured, and it is hard to collect adequate amounts of their DNA for common protocols to analyze genetic information such as allozyme analysis, RELP, RAPD and determination of DNA sequences. In this study we tried to determine the sequences of some regions in nuclear, chloroplastic and mitochondirial genomes from each individual of a strain of *T. nordenskioeldii* isolated from sea ice biota. This species often found in Arctic regions. As the common DNA sequencing protocol, Sanger's method (Sanger *et al*., 1977), usually requires a much larger amount of DNA such as ca. 500 ng, than amonts in individual colonies, DNA fragments for objective regions in genomes were amplified before sequencing. Instead of extraction protocols with proteinase K and phenol-chloroform, a simple extraction protocol with the chelating agent Chelex 100 was adopted. The protocols are simple, rapid, involve no organic solvents and do not require multiple tube transfers of samples (Walsh *et al*., 1991). Total DNA was extracted from 8000 cells and was applied for PCR with primers for some objective regions, and DNA fragments for all regions were amplified. DNA fragments were also amplified with the DNA extracts from 10 cells by a scaled down protocol with Chelex 100 and all of the sequences of these fragments were determined. Their sequences matched perfectly with those determined from a large amount of cultured cells. Even from 10 cells, DNA sequences of 3 regions were determined without error.

These sequences showed higher homologies with the sequences of regions of diatoms previously determined (Fig. 3a, 3b, 3c), although some differences were observed among the regions. Most higher homologies were observed with the 16s rDNA sequence. Homologies between this strain and other diatom species, *Amphora delicatissima*, *Navicula salinicola* and *Odontella sinensis*, were 97.5, 97.0 and 96.7%, respectively. This region was quite conservative and showed higher homologies even between species of different genera. Homologies of the fragment for 18s rDNA were also high. Those between this strain and other species of *Thalassiosira eccentrica*, *T. rotula* and *Skeletonema costatum* were 99.2, 99.2 and 92.0%, respectively. Species in the same genus, *Thalassiosira*, showed higher homologies than the species in a different genus of *Skeletonema*. The least conservative region was *coxI* in this study. Homologies of 99.1, 82.0 and 80.4% were found between the strain and other species, *T. nordenskioeldii*, *S. costatum*, and *Ditylum brightwelli*, respectively. Even among different populations of the same species, *T. nordenskioeldii*, differences of 3 bases were found in 517 bases of the fragment in *coxI* (Fig. 3c). Characteristic different homologies were determined even with 3 regions of genomes. These results strongly suggest that we could select DNA regions having appropriate homologies for objectives of various studies.

Total DNA was extracted from 10 cells with 20 μl of the extraction buffer. Then 1.0 μl

of extract for 1 time PCR contained total DNA from 0.5 diploid vegetative cells of *T. nordenskioeldii* and corresponded only to one molecule of the nuclear DNA. The fragment amplified from one molecule DNA with PCR might vary its sequence because of the error of *Taq* DNA polymerase, which misreads the bases of DNA at a rate of 8.0×10^{-6} mutation frequency bp^{-1} duplication^{-1} on average (Cline *et al*., 1996). However, sequences of 18s rDNA in the nuclear genome were determined without error. Many copies of 18s rDNA, which we consciously chose, exits in a genome (Rowe *et al*., 1996; White and McLaren, 2000) and 1.0 μl of DNA extract could contain more than 1 copy of the region of 18s rDNA. The sequences of 16s rDNA and *coxI* in chloroplastic and mitochondrial genomes, respectively, were also determined without error. The extracts for 1 time PCR also could contain more than 1 copy of the regions, as 1 cell could have more than 1 chloroplast and 1 mitochondrion. When we analyze genetic information with small amount of microorganisms, both the multicopy regions in the genome and regions in organellar genomes would be proper candidates for analyses without error.

It is difficult to identify individual microorganisms such as diatoms using morphological characteristics. Some populations of microalgae often had different genetic information, although they showed the same morphological characteristics (Gallagher, 1980; Brand *et al*., 1981; Gallagher, 1982; Stabie *et al*., 1992; Rynearson and Armbrust, 2000). In contrast, some strains having the same origin and genetic information showed different morphological characteristics (Medlin, 1991). Genetic information on microorganisms could be one of the most important characteristics to identify the population and individuals. However, information on microorganisms living in polar regions is hard to use, as adequate amounts of DNA could not be collected by isolation of strains and cultures. In this study we made it possible to use genetic information in individual microorganisms of diatom species without isolation of strains and cultures. DNA sequences in various regions in genomes of each individual diatom species living in natural environments could be determined directly by using precise numbers of cells and efficiently extracting DNA and PCR.

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