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Single-cell PCR on protargol-impregnated euplotid ciliates: a combined approach of morphological and molecular taxonomy

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Ciliates are considered one of the most diverse protozoa and play significant roles in ecology. For successful taxonomic study of these microscopic eukaryotes, a staining procedure is necessary, due mainly to intrinsic difficulties in recognizing characteristics from living cells. Although molecular taxonomy has been used to resolve the ambiguities associated with traditional morphology-based taxonomy, extraction of genomic DNA from stained ciliate cells is not available yet. In the present study, we describe a method to extract genomic DNA from a single protargol-impregnated euplotid cell. By using $HgCl_2$ as a fixative and modulating the exposure time of bleach solution in the protargol impregnation, high-quality genomic DNA can successfully be extracted from a stained single cell with minimal loss of morphological integrity. This technique will contribute to the effectiveness of combined approaches of molecular and morphological taxonomy from single ciliate cells.

Keywords: euplotids; molecular data; morphology; protargol impregnation; single-cell PCR

Introduction

Ciliates (Protozoa, Ciliophora) are one of the most highly differentiated single-celled eukaryotes and are abundant in almost every aquatic environment (Fenchel 1987; Foissner 1991). However, because of the high morphological variability at environmental conditions, biogeographical variants, and sibling species (Caprette and Gates 1994; Schlegel and Meisterfeld 2003; Coleman 2005), the identification of ciliates based on morphology alone is extremely difficult. Many phylogenetists and taxonomists have begun to accept that molecular markers based on a comparison of DNA sequences present a promising and helpful tool to resolve some taxonomic ambiguities in microscopic eukaryotes such as ciliates (Barth et al. 2006; Chantangsi et al. 2007). Until recently, the taxonomic study of ciliates has largely been limited to species it is possible to monoculture or temporarily culture stocks from environmental samples. As a result, most uncultivated ciliates, which represent at least 80% of extant ciliate species, have not been studied relative to their morphological and molecular taxonomy (Moon-van der Staay et al. 2001; Stoeck et al. 2003; Countway et al. 2005).

In general, the recognition of characteristics in cortical and internal structures, i.e. basal bodies, cirri, various fibrillar systems, and the nuclear apparatus, is essential for successful taxonomic studies of most ciliates, and these characteristics can be precisely revealed through various staining methods (Foissner 1991). Although there is no method that is uniformly appropriate for all kinds of ciliate species and characteristics, protargol impregnation (Wilbert 1975; Foissner 1991) among the various staining techniques is the most widely applied method and has proven indispensable for descriptive research of ciliates. Bouin's solution is the most frequently used fixation solution for preserving samples prior to the protargol impregnation. This solution, however, includes formaldehyde, which is known to adversely affect the quality of DNA (Grafstrom et al. 1983; Auinger et al. 2008). As such, it is still not clear whether any of the intermediate steps in the protargol impregnation cause DNA damage.

Euplotids are known as one of the most highly differentiated groups in ciliates and can be found in nearly every habitat, including marine, estuarine, freshwater, and edaphic environments (Fenchel 1987;

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Brusca and Brusca 2002). Furthermore, euplotids have been more comprehensively investigated than other ciliates. Nevertheless, euplotid species cannot be easily distinguished on the basis of morphological differentiation due to dimorphism or sibling species, and consequently taxonomical debates continue to arise (Nobili 1966; Machelon et al. 1984; Génermont et al. 1985; Valbonesi et al. 1988). Notably, because of the sensitive response of cell activities to xenobiotic compounds, euplotids can be used as pollution indicators in aquatic ecosystems. They can similarly be used in the laboratory to determine the cellular effects of drugs in pharmacological assays, pheromone evolution in mating processes, and telomere research (Nilsson 1989; Wang et al. 2002; Trielli et al. 2007; Vallesi et al. 2008).

The efficiency of the single-cell PCR method may be highly dependent upon the amount of template DNA, because the amount of starting material is inevitably very small. Ribosomal DNA has been used most frequently as a target substrate for single-cell PCR methods, as several hundred or thousand copies of this DNA exist in the cells of most eukaryotes. This can thus compensate for the inevitable shortage of genomic DNA existing in single-cell protists. Also, in molecular taxonomic studies of ciliates, small subunit ribosomal DNA (SSU rDNA) has been widely used as genetic diagnostic characteristics and thousands of SSU rDNA sequences have been determined and deposited in GenBank (Strüder-Kypke et al. 2000; Petroni et al. 2002; Puitika et al. 2006; Bourne et al. 2008).

Several PCR amplification techniques have already been introduced in conjunction with a single cell of diverse protist groups (Dyal et al. 1995; Marín et al. 2001; Takano and Horiguchi 2005; Auinger et al. 2008; Duff et al. 2008; Kim and Min 2009). Previous techniques, however, were suitable only for living or preserved specimens. Here, we report a novel method that obtains a sufficient amount of DNA suitable for PCR amplification from a protargol-impregnated cell of euplotids. This method can be used in combination with molecular and morphological techniques based on correctly identified specimens.

Materials and methods

Sampling and ciliate culture

Ciliates were collected from the coastal waters of the Yellow Sea (37°27′N, 126°36′E), Incheon, Republic of Korea, and culture was performed using seawater collected from the sampling site, filtered through a 0.45 μm nitrocellulose filter (Millipore), and autoclaved. The monocultured clone of *Euplotes crassus* was maintained at room temperature for up to 10 days with rice grains as a food source to enrich bacterial growth.

Protargol impregnation

Protargol impregnation was performed as described in Foissner (1991) with minor modifications. Specific modifications that have been applied are described in the following subsections. The concentration and volume of the four reagents used in protargol impregnation were as follows and distilled water was added to a volume of 500 μL: 20 μL bleach solution (0.1% NaClO); 250 μL protargol solution (1% protargol); 20 μL developer (1% Na_2SO_3 and 0.2% hydroquinone); and 20 μL fixation solution (5% $Na_2S_2O_3$). After treatment with the above-mentioned reagents, the cells were washed three times with distilled water and then transferred into 1.5 mL microcentrifuge tubes for genomic DNA extraction.

Effect of fixatives on the PCR efficiency

To test the possible effects of fixatives on PCR efficiency, cultured *E. crassus* cells were fixed with $HgCl_2$ and Bouin's solution. The fixed samples were isolated after 5 min, 10 min, 1 h, and 1 d, and then washed three times with distilled water. Each sample set consisted of three tubes, and each tube contained a single *E. crassus* cell. After washing, genomic DNAs were extracted and PCR amplification was then performed.

Effect of each protargol impregnation reagent in DNA extraction

To evaluate reagents which are deleterious to DNA extraction, the four reagents used in protargol impregnation described by Foissner (1991) were tested via two methods. Before treatment with the reagents, ciliate cells were fixed with $HgCl_2$ and washed three times with distilled water in advance. First, to verify the independent effect of each reagent on the quality of extracted DNA, each sample set was treated by one of four different reagents. Next, to determine the effect of reagents in the staining procedures, one out of the four steps in protargol impregnation was omitted in series. Thus, the bleaching step in the first sample set, the protargol step in the second set, the development step in the third set, and the fixation step in the fourth set were individually eliminated. After treating the cells with the appropriate reagents, the cells were washed three times with distilled water. For this experiment, the entire sample set consisted of five tubes, and each tube contained a single *E. crassus* cell. After extracting

genomic DNAs from cleaned cells, the SSU rDNA region was amplified.

Effect of bleach exposure time on the DNA extraction and staining quality

To verify whether bleaching time had any effect on the genomic DNA or staining intensity, four sample sets were allocated. After $HgCl_2$ fixation, they were exposed to the bleach solution for 1, 3, 5, and 10 min, respectively. The conditions of the other steps in the protargol impregnation were not changed. Each sample set consisted of five tubes that contained a single *E. crassus* cell. Genomic DNA was extracted from each treatment and a PCR analysis was conducted.

Microscopic observation

For microscopic observation, stained ciliates were transferred into a droplet of distilled water on a coverslip of a HS-slide (Higgins-Shirayama slide: Shirayama et al. 1993). After adjusting the sample position with a fine needle, the slide was loaded upside down on the stage of a microscope without a coverslip on the other side of the slide. As one side of the HS-slide is open, the examined samples can be picked up easily using a micropipette. Observation of the slides was performed at 650-fold magnification under a microscope (Leica DM2500). The visibility of the diagnostic characteristics including nuclei, cirri position, cirri number, body size, adoral zone length, and the numbers of adoral membranelles and kineties were compared among *E. crassus* samples.

DNA extraction

Reagent-treated cells or microscopically observed cells on the coverslip of HS-slides were picked up using a micropipette under the dissecting microscope (Leica MZ 12.5) and then transferred into a distilled water droplet on a slide. The mounted water was changed three times to remove various contaminants in the samples. Cleaned single cells were transferred into 1.5 mL microcentrifuge tubes by a micropipette. Genomic DNA extraction was carried out using a RED Extract-N-Amp^{TM} Tissue PCR kit (Sigma-Aldrich) according to a procedure reported by Kim and Min (2009).

PCR amplification

Two different-sized PCR fragments of the SSU rDNA region were amplified, i.e. a partial (416 bp) and a nearly complete SSU rDNA region. For amplification of the partial SSU rDNA region, two primers of 18S + 1370 (5′-TGGTGCATGGCCGTTCTT-3′) and 18S-1770 (5′-CGACGGGCGGTGTGTACA-3′) were used. These are designed based on the conserved sequences of the eukaryotic SSU rDNA. Primers of EukA (Medlin et al. 1988) and Cili-2000 (5′-CGACGGGCGGTGTGTACAAAGGGCAGGG-3′) were used for amplification of the nearly complete SSU rDNA region. Thirty microliters of each PCR mixture was prepared from 1 μL of the genomic DNA, 200 μM of dNTP, 0.5 μL of each primer (20 pmol), 1 × reaction buffer with 1.5 mM $MgCl_2$ (Promega, Madison, WI), and 2.5 U *Taq* DNA polymerase. PCR amplification was performed with initial denaturation for 2 min at 94°C followed by 35 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 55°C, extension for 30 s for partial and 2.5 min for nearly complete SSU rDNA regions at 72°C, and a final 10 min extension at 72°C. Five microliters of each PCR product was run on 1.5% agarose gel and visualized under UV light.

Application to the environmental sample

Two euplotid species were isolated individually from an environmental sample from Sokcho (38°13′N, 128°36′E), Gangwon province in the East Sea of the Republic of Korea. After live observation, ciliates were fixed in $HgCl_2$ for 10 min. Protargol impregnation for detailed identification was performed under a dissecting microscope. Stained ciliates were transferred into a droplet of distilled water on a coverslip of a HS-slide. After adjusting the sample position by a fine needle, the opened side of the HS-slide was loaded upside down on the stage of a microscope. Specimens were identified by visible diagnostic characteristics and photomicrographs were taken at 650-fold magnification (Leica DM2500). After morphological identification, each cleaned single cell was transferred into a 1.5-mL microcentrifuge tube using a micropipette. Using the single-cell PCR method described in this study, nearly complete SSU PCR fragments were obtained and sequenced using BigDye Terminator version 3.1 and an ABI 3730xl sequencer (Applied Biosystems). Along with EukA and Cili-2000, additional internal primers, i.e. Pro + 930 and Cili-1300 (Gong et al. 2007), were used for sequencing. These sequences along with others retrieved from GenBank were aligned with ClustalX employing the default option. A phylogenetic tree was constructed by employing the neighbor-joining (NJ) tree method and nucleotide diversity among the sequences was calculated with the Kimura two-parameter model using MEGA 4.0 (Tamura et al. 2007).

Results

Genomic DNA extraction from a single protargol-impregnated cell

To test the effect of fixative on the quality of extracted genomic DNA, Bouin's solution and $HgCl_2$ were compared. When DNA was extracted from specimens that were preserved in $HgCl_2$ for 5 min, 10 min, 1 h and 1 d, and used as templates for PCR, obvious differences were not found in the intensity of the PCR bands. When the samples were preserved in Bouin's solution, PCR products were recovered only from the samples exposed for 5 min (Figure 1).

Bleach was also found to be a deleterious reagent to PCR analysis. When samples were exposed to a bleach solution for 10 min, no PCR bands were observed from the extracted DNAs, while the other three reagents, i.e. protargol solution, developer, and fixation solution, had no such effects on the efficacy of PCR (Supplementary Figure S1[1]). The damaging effect of bleach solution on the extracted DNA quality could be eliminated by omission of a single step in the protargol-impregnation process. When the bleaching step was removed, PCR amplification was successful without any reduction of band intensity. However, no PCR bands were obtained when any of the other three staining steps were eliminated from the staining procedures (Supplementary Figure S2[1]).

The success of the PCR reaction was variable according to the time intervals of bleach treatments. In this experiment, except for the bleaching time, all the other steps of staining were identical and carried out in accordance with the protocols described in this study. We observed that the intensity of stained macronuclei descended stepwise in four cases (Figure 2). Optimal observation of the diagnostic characteristics was obtained with between 3 and 5 minutes bleach treatment (Figure 2D and 2E), whereas there were difficulties in observing such diagnostic characteristics as the kineties and kinetosome in samples treated between 1 and 10 minutes by weak and strong bleach (Figure 2C and 2F). When cells were exposed to bleach for less than 3 minutes, the DNA remained stable and good PCR products were obtained (Figure 3). However, when the cells were exposed to the bleach solution for more than 5 minutes, the PCR bands were very weak or did not appear at all (Figure 3).

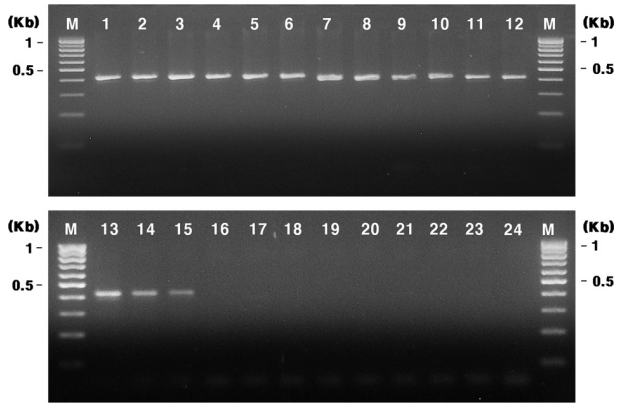

Figure 1. The effect of $HgCl_2$ and Bouin's solution on PCR amplification. *Euplotes crassus* cells were treated only by fixatives. The genomic DNA was extracted and then PCR amplification was performed by 18S + 1370 and 18S-1770. M, 100 bp ladder; lanes 1–3, cells were treated with $HgCl_2$ for 5 min; lanes 4–6, treated with $HgCl_2$ for 10 min; lanes 7–9, treated with $HgCl_2$ for 1 h; lanes 10–12, treated with $HgCl_2$ for 1 d; lanes 13–15, treated with Bouin's for 5 min; lanes 16–18, treated Bouin's for 10 min; lanes 19–21, treated Bouin's for 1 h; lanes 22–24, treated Bouin's for 1 d.

Application to two euplotid species in the environmental sample

Two euplotid species isolated from an environmental sample of Sokcho in the East Sea were identified as *Diophrys scutum* (*D. scutum* specimen A and B) and *E. crassus* (*E. crassus* specimen A and B) based on morphological criteria (Table 1 and Supplementary Figure S3[1]), and their newly determined SSU rDNA sequences were deposited in the GenBank under the accession numbers HQ413691 to HQ413694. Sequences of the specimen A and B of *D. scutum* were identical: the length was 1651 bp and the percentage of GC content 44.5%, and it showed 99.8% similarity with *D. scutum* (DQ353851) retrieved from GenBank. Sequences of the specimen A and B of *E. crassus* and known *E. crassus* (AJ305255) were also identical: the length was 1781 bp and the percentage of GC content 43.9%.

Discussion

There is a strong need to develop a novel taxonomic approach for uncultivable ciliate species that can be combined with morphological and molecular study using a single individual. To this end, it is necessary to extract genomic DNA from a single specimen of correctly identified ciliates based on a staining technique. Toward meeting this goal, the effects of the individual steps of protargol impregnation upon DNA damage were monitored. We found that the modified protargol impregnation does not interfere with morphological examination and secures high-quality genomic DNA. First, we replaced Bouin's solution with $HgCl_2$, a commonly used alternative. We did not observe significant differences between the samples preserved by Bouin's solution and $HgCl_2$ in terms of staining quality (data not shown). However,

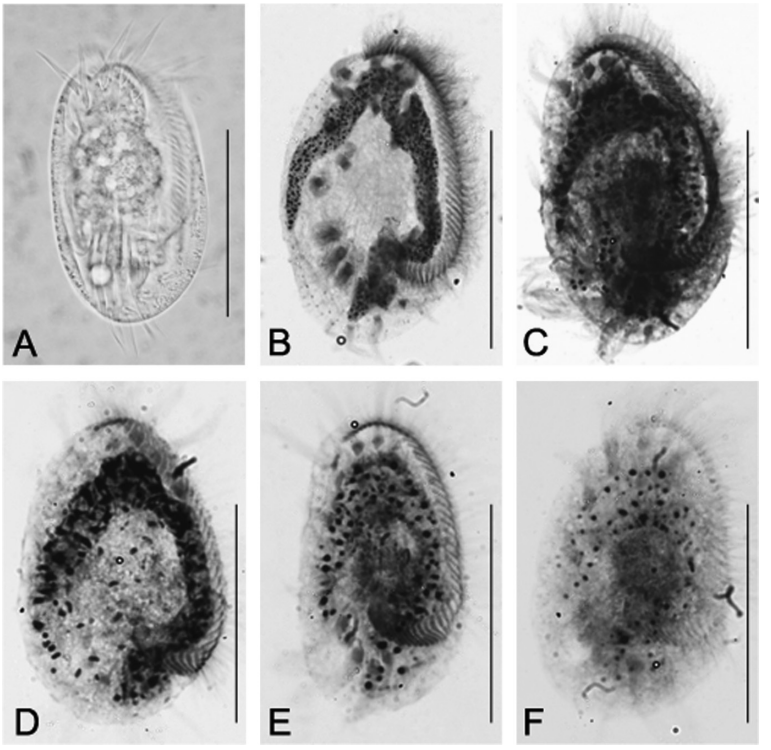

Figure 2. Photomicrographs of living (A) and protargol-impregnation *Euplotes crassus* (B–F). (A) Living specimen. (B) Normally stained individual (fixed by Bouin's solution and treated with bleach for 3 min). (C–F) The effect of bleaching time on the intensity of protargol impregnation; C, treated with bleach for 1 min; D, treated for 3 min; E, treated for 5 min; F, treated for 10 min.

only DNA obtained from cells exposed to Bouin's solution for less than 5 minutes was able to amplify PCR products while genomic DNA from cells fixed with $HgCl_2$ could successfully amplify fragments even when samples were preserved for one day (Figure 1).

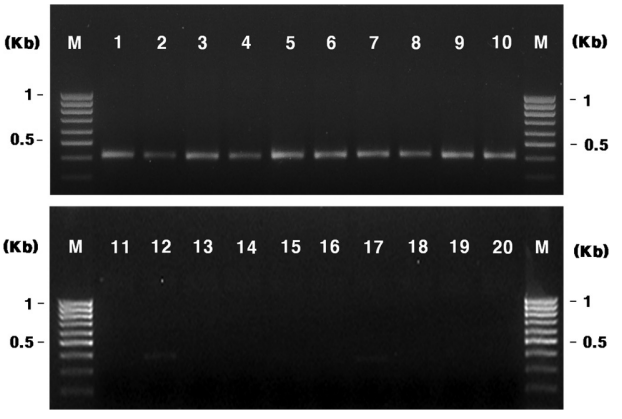

Figure 3. The effect of bleaching time on PCR amplification. PCR amplification was performed via primers 18S + 1370 and 18S-1770 from the genomic DNA of single-cell stained *Euplotes crassus*. M, 100 bp ladder; lanes 1–5, treated with bleach for 1 min; lanes 6–10, treated for 3 min; lanes 11–15, treated for 5 min; lanes 16–20, treated for 10 min.

Second, we found that the PCR amplification process is also affected by the bleach procedure using sodium hypochlorite (NaOCl), a well-known DNA and protein destroyer (Hayatsu et al. 1971; Abdul-Baki 1974). Bleach addition, however, is an essential step for improving the examination quality in portargol impregnation, because it removes excess dye in tissues that mask diagnostic characteristics (Figure 2C and 2F). In the present study, we confirmed that high-quality genomic DNA can be isolated by adjusting the bleach treatment time and successfully achieved PCR products. When two stained euplotid species, *D. scutum* and *E. crassus*, were bleached for less than 3 minutes, DNA degradation was dramatically reduced or completely eliminated. It was found that increased time of exposure to bleach solution increased the likelihood of damaging the genomic DNA, resulting in failed PCR amplifications (Figures 2 and 3).

In our experiments, we also found that optimal examination of diagnostic characteristics could be achieved with bleach treatment lasting between 3 and 5 minutes on euplotids. This time interval is almost equal to or slightly longer than the maximal bleach time allowed for successful PCR amplification in euplotid species. On the other hand, the bleach step may be affected by the particular species under

Table 1. Characteristics of four protargol-impregnation specimens directly isolated from the environmental sample. They were identified *Diophrys scutum* and *Euplotes crassus* based on morphological criteria and SSU rDNA sequences.

	Species					
	Diophrys scutum			*Euplotes crassus*		
Characteristics	Specimen A	Specimen B	Mean	Specimen A	Specimen B	Mean
Length of body*	157.5	160	158.75	117.5	114.5	116
Width of body*	87.5	83.0	85.3	77.5	80	78.75
No. of Ma (Shape)	2 (sausage-shaped)	2 (sausage-shaped)	2	1 (‘3’-shaped)	1 (‘3’-shaped)	1
No. of kinety	6	6	6	10	10	10
No. of AM**	67	64	65.5	57	59	58
Accession no.	HQ413691	HQ413692	99.8***	HQ413693	HQ413694	100***

*Measurements in μm.
**Adoral membranelles.
***Similarity to compare with *D. scutum* (DQ353851) and *E. crassus* (AJ305255) retrieved from GenBank in %.

consideration such as the condition of the specimens as well as by the commercial components of the bleach solution (Foissner 1991; Lee and Soldo 1992). Therefore, discovering the optimal bleach conditions, i.e. the treatment time and concentration of solution, is impossible for all ciliates. In order for the new technique to be applicable to most ciliate species, moderate conditions of bleach treatment should be investigated for each group.

For identification purposes, samples of living ciliates are usually picked up one by one under a dissecting microscope by pipettes. The magnification ratio of the dissection microscope, however, is insufficient to diagnose the important taxonomical characters of ciliates clearly. Therefore, if purely cultured and stained ciliates are not used, it is difficult to completely eliminate the chance of misidentification of species. Furthermore, when the whole individual is completely used for a PCR analysis, re-examination of morphological characteristics is impossible. The most important strengths of the new technique are that the same specimen of single-celled organisms is used for both morphological examination and molecular analysis and re-identification is possible based on images with higher-magnitude resolution from stained samples (Supplementary Figure S4[1]). Therefore, the conflict between molecular and morphological data can easily be resolved, and this method will be very helpful in obtaining accurate genetic diversity and phylogenetic information among diverse ciliate groups (Supplementary Figure S4[1]).

In summary, we have devised DNA extraction techniques from stained single ciliate cells that may be effectively applied to combined taxonomical approaches based on molecular and morphological properties of ciliates. Although we have only employed euplotid ciliates as the model organism, this technique has potential application for taxonomic studies of rare ciliates, including tiny and uncultivable ciliates.

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Note

1. Supplementary material can be found by clicking on the Supplementary Content tab at http://dx.doi.org/10.1080/19768354.2011.604943.

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