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Optimization of DNA Extraction from a Single Living Ciliate for Stable and Repetitive PCR Amplification

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Abstract: Ciliates are undoubtedly one of the most diverse protozoans that play a significant role in ecology. However, molecular examination, based on comparing the DNA sequences, has been done on a limited number of the species. Because most ciliates are uncultivable and their population sizes are often too small, it is usually difficult to obtain sufficient genomic DNA required for PCR based experiments. In the present study, we evaluated the effectiveness of four commercial DNA extraction procedures that extract high quality genomic DNA from a single ciliate cell. It was discovered that RED Extract-N-Amp^{TM} PCR kit is the best method for removing PCR-inhibiting substances and minimizing DNA loss during purification. This method can also amplify more than 25 reactions of PCR. In addition, this technique was applied to single cells of 19 species belonged to 7 orders under 5 classes that isolated from mixed natural populations. Their small subunit ribosomal DNA (SSU rDNA) was successfully amplified. In summary, we developed a simple technique for the high-yield extraction of purified DNA from a single ciliate cell that may be more useful for rare ciliates, such as tiny and uncultivable marine microbes.

Key words: Ciliophora, SSU rDNA, single cell PCR

INTRODUCTION

Ciliates are known as one of the most highly differentiated single-celled organisms among eukaryotes. They are found in a range of aquatic and wet environments, including oceans, marine sediments, lakes, ponds, rivers, as intestinal parasites in humans or animals, and even in soils (Fenchel, 1987; Foissner, 1991; Brusca and Brusca, 2002). Because of species-specific variation toward pollution tolerance, ciliates can be used as pollution indicators in aquatic ecosystems, or to determine the effects of drugs on cellular function and/or structure in pharmacological assays (Nilsson, 1989).

Many phylogenetists and taxonomists have recently come to accept the notion that molecular techniques based on comparing DNA sequences obtained by polymerase chain reaction (PCR) may represent a promising and helpful tool in resolving taxonomic ambiguities in microscopic eukaryotes such as ciliates (Prescott, 1994; Tautz et al., 2003; DeSalle et al., 2005; Barth et al., 2006; Chantangsi et al., 2007; Kim et al., 2007).

In order for molecular biological applications to be used on single cell ciliates, stable DNA extraction methods should be developed to reduce inhibitors and to minimize the loss of the genomic DNA for the purposes of molecular taxonomy on uncultivable species that were individually isolated from field sample. Several single cell DNA extraction techniques have already been introduced and these have been found to be effective on treating diverse single protists (Marín et al., 2001; Ruiz Sebastián and O'rian, 2001; Ki et al., 2005; Takano and Horiguchi, 2005; Auinger et al., 2008; Duff et al., 2008). They has been used most frequently ribosomal DNA as a target substrate for single cell PCR methods because several hundreds and even more than ten thousand copies of this DNA exist in the cell of most eukaryotes including ciliates, therefore this can compensate the inevitable shortage of genomic DNA that exists in single cell protists. Previous approaches, however, were performed by directly incorporating the subsequent reagents into the same reaction tube, and therefore could only be used once. Therefore, previous techniques were not ideal in determining the multi-gene variability from multiple sequences. Since there has been no congruent opinion on which genes (or gene-regions) are suitable to serve as species discrimination markers in the

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ciliate groups (DeSalle et al., 2005; Kim et al., 2007), identifying appropriate gene(s) for species discrimination had to be the first task to be carried out. For this task, several candidate genes, such as internal transcribed spacer (ITS), SSU, and large subunit (LSU) rDNA, as well as several mitochondrial genes, whose use was well justified from other taxa as suitable genes for species discrimination, can be amplified several times using genomic DNA determined a single cell of diverse ciliates including the uncultivable species.

In this study, we compared the efficiency of four commonly used DNA extraction methods in extracting DNA from single ciliates cells. From this study, we also propose an optimal protocol that can provide suitable DNA from each one of various ciliate species isolated from mixed natural populations.

MATERIALS AND METHODS

Monoculture

Ciliates were collected from the coastal water of the Yellow Sea (37°27'N and 126°36'E), Incheon, the Republic of Korea. Individual sample identified as *Euplotes crassus* was cultured using seawater collected from the sampling site, filtered through a 0.45 μm Nitrocellulose filter (Millipore Co., Bedford, MA) and autoclaved. The culture was maintained at room temperature for up to ten days with rice grains as a food source to enrich bacterial growth.

Sampling and ciliate culture

Ciliates were collected from several coastal waters of the Republic of Korea: Eoyeong-pogu (JeJu-do: 33°30'N and 126°28'E); Ggotji Beach (Taean, Chungcheongnam-do: 36°19'N and 126°30'E); Haenam (Haenam, Jeollanam-do: 34°42'N and 126°21'E); Hajeo-ri (Yeongdeok, Gyeongsang-buk-do: 36°23'N and 129°24'E); Munam 2-ri (Goseong, Gangwon-do: 38°17'N and 128°33'E); Songjeong Beach (Busan: 35°10'N and 129°12'E); The Incheon public waterfront (Incheon: 37°26'N and 126°35'E); and Yeong-geumjeong (Sokcho, Gangwon-do: 38°12'N and 128°36'E). Sampling was performed using 20 μm-mesh plankton net, a polyurethane foam enveloped slide (PFE-S) system (Xu et. al., 2009), and the natural slides. After sampling, specimens were directly isolated while the raw cultures were maintained at 17°C in a chamber adjusted with a photoperiod of 12:12 h light and dark.

Single cell isolation and washing

Each individual ciliate was isolated using a micropipette under the dissecting microscope (Leica MZ 12.5), and then transferred to a filtered seawater droplet on a slide glass. The mounted seawater was changed 4 to 6 times in order to remove various contaminants from the samples. The cleaned single cells were transferred to 1.5-mL micro-centrifuge tubes, and the seawater was removed as far as possible.

DNA extraction

Each single cell of *E. crassus* ciliates isolated from the raw culture media was transferred to 1.5-mL microcentrifuge tubes. After three freeze-thaw cycles (–20 to 20°C), the following four DNA extraction methods were employed:

(i) DNA extraction by RED Extract-N-Amp™ PCR kit (Sigma-Aldrich Co., Brooklyn, NY) was performed according to the manufacturer's instruction. The reaction volume was scaled down to one tenth of the originally prescribed volume for each tube (Gong et al., 2007).

(ii) DNA was extracted using proteinase K digestion. The sample was transferred to a 0.2-mL PCR tube and the volume was adjusted to 24 μL using TE buffer. This was followed by addition of 1 μL proteinase K (200 μg/mL). The tube was then incubated at 55°C for 30 min using a GeneAmp PCR system 2700 thermal cycler (PE Applied Biosystems, Mississauga, ON). Proteinase K was inactivated by incubating the sample tube at 95°C for 3 min. Enzyme based this method is hereafter referred to as the 'Proteinase K method' in this paper.

(iii) DNA was extracted by column based DNeasy® Tissue kit (Qiagen Science, INC., Germantown, MD) according to the guidelines of the kit, and the extracted DNA was re-suspended in 25 μL of distilled water.

(iv) DNA was extracted by DNAzol® Genomic DNA Isolation Reagent (MRC Molecular Research Center, INC., Cincinnati, OH), using one fifth of the volume of reagents mentioned in the manufacturer-recommended extraction procedures and eluted in 25 μL of distilled water. This method use guanidine as a component of lysis solution

PCR amplification and sequencing

Three different sized PCR fragments of ribosomal DNA (rDNA) region were amplified, e.g., partial (416 bp) small subunit (SSU), nearly complete (1,887 bp) SSU, and from 5' end of SSU to 3' end of internal transcribed spacer 2 (ITS2) (2,391 bp) rDNA regions. Thirty micro liters of each PCR mixture was prepared, containing 1 μL of the genomic DNA, 200 μM of each dATP, dCTP, dGTP, dTTP, 0.5 μL of each primer (20 pmol), 1x reaction buffer with $MgCl_2$ (Promega, Madison, WI), and 2.5 U Takara *Ex Taq*® DNA polymerase (Takara Bio Inc., Otsu, Shiga). The optimized PCR conditions for the partial SSU region using primers 18S+1370 (5'-TGGTGCATGGCCGTTCTT-3') and 18S-1770 (5'-CGACGGGCGGTGTGTACA-3') involved an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. A final extension step also took place at 72°C for 2 min. Secondly, PCR conditions for

the nearly complete SSU region using primers EukA and EukB (Medlin et al., 1988) involved an initial denaturation at 94°C for 2 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 1 min and extension at 72°C for 2.5 min. This was followed by a final extension step at 72°C for 5 min. Finally, a third PCR condition for SSU~ITS2 region was conducted using primers EukA (Medlin et al., 1988) and Pro28S-30 (5'-CGCTTAVTRATA TGCTTAARTYCAGCG-3'), with an initial denaturation at 94°C for 2 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 1 min and extension at 72°C for 3 min with a final extension step at 72°C for 5 min. Five μL of each PCR product was run on 1.5% (the partial SSU) and 0.8% (the SSU and SSU~ITS2) agarose gel and visualized under UV light.

PCR fragments of the nearly complete SSU rDNA were sequenced using the BigDye Terminator version 3.1 and an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA), along with 18S+1370.

Sequence data analysis

Sequences determined from this study and sequences retrieved from GenBank were aligned by ClustalX (Thompson et al., 1997) with the default option and adjusted by eye. Gaps were treated as a fifth characteristic. Nucleotide diversity among the species was calculated by the MEGA 4.0 program (Tamura et al., 2007) with a Kimura 2-parameter.

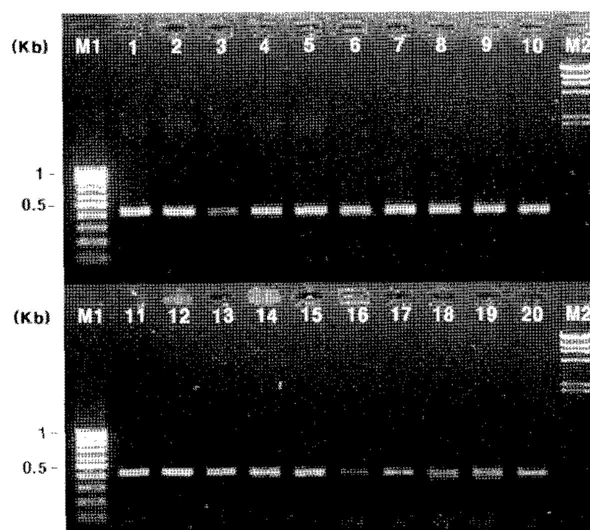

Fig. 1. Comparison of four different DNA extraction methods. Genomic DNA for PCR amplification was extracted from living single cells of *E. crassus*. M1, 100 bp ladder; M2, λ/Hind III ladder. Lanes 1-5, extracted by RED Extract-N-Amp™ PCR kit; Lanes 6-10, by Proteinase method; Lanes 11-15, by DNeasy® Tissue kit; Lanes 16-20, by DNAzol® Genomic DNA Isolation reagent kit.

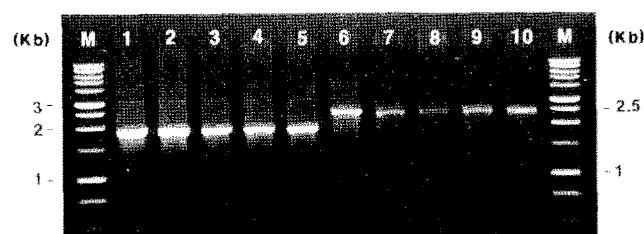

Fig. 2. Long and repetitive PCR amplification of DNA extracts from a living single cell of *E. crassus* by RED Extract-N-Amp™ PCR kit. M, 1 kb ladder; Lanes 1-5, complete SSU rDNA (1,887 bp); Lanes 6-10, SSU~ITS2 rDNA (2,391 bp).

RESULTS AND DISCUSSION

Comparison of four DNA extraction methods

DNA extractions were performed by four different common DNA extraction methods with five replicates allocated from a monoculture stock of *E. crassus*. The efficiencies of the different DNA extraction methods were determined by amplifying the partial SSU rDNA. The DNA yield from the extraction methods was indicated the PCR bands intensity. PCR amplification was performed successfully in all four methods and the concentration of PCR products (416 bp in length) obtained by these methods was over 10 ng/μL (Fig. 1). The amplifications yield of DNA obtained from DNAzol® Genomic DNA isolation reagent kit, however, was slightly lower than the other three methods. This confirmed that high quality genomic DNA from the single ciliate cells for PCR amplification could be extracted, even by the regular DNA extraction methods used here.

The above extracted genomic DNA samples were suitable for longer PCR fragment amplifications, so that the complete SSU rDNA (1,887 bp in length) and the region of 5' end of SSU to ITS2 rDNA (2,391 bp in length) were successfully amplified using the genomic DNA extracted by the RED Extract-N-Amp™ PCR kit (Fig. 2). Although long fragments were amplified from the other DNA extraction methods, the intensity of the bands was fainter than those of the former one (data not shown). For this reason, the RED Extract-N-Amp™ PCR kit was the most preferred method due to the entire DNA extraction procedure was conducted within a single tube without transfer of material to other tubes, thereby, reducing any loss of the genomic DNA material.

The most important merit of our new technique is its fast application and it's comprised of only three simple steps. Only one out of the 25 μL of each total genomic DNA extracted by the four DNA extraction methods was used and repetitive PCR amplifications are possible over 25 times. The procedures also displayed efficiency in cost and time, which may be very important if these procedures were to be employed on a larger scale. As the reaction volume for single cell DNA extraction can be reduced to one tenth or one twentieth of the original, the cost of DNA extraction per sample can also be reduced dramatically.

Application to various ciliate species

The existing molecular taxonomies of ciliates are mostly culture-based studies that are known to take a lot of time and to be labor-consuming (Strüder-Kypke et al., 2000a, b; Strüder-Kypke et al., 2001; Petroni et al., 2002; Puitika et al., 2006; Bourne et al., 2008). On the other hand, molecular approaches of ciliates from environmental samples are known to be rich in PCR inhibitors, such as humic acids, which could be extracted together with DNA during the DNA isolation and purification process, and which could interfere with PCR amplification (Tebbe and Vahjen, 1993; Jiang et al., 2005).

For solving the above problems, the efficiency of RED Extract-N-Amp™ PCR kit was confirmed in the subsection

Table 1. The single cell PCR method that was described in this paper was applied to the diverse ciliate species

Class / Order / Family / Species	Source	Body length (μm)	No. of Ma[a]	Closest species in congener (NCBI Acc. No.)	Similarity
Spirotrichea					
Euplotida					
Aspidiscidae					
Aspidisca sp.	Field sample[b]	30-40	1	*Aspidisca steini* (AF305625)	93.1
Euplotidae					
Euplotes crassus	Field sample	90-110	1	*Moneuplotes crassus* (AF508761)	99.4
Euplotes minuta	Field sample	50-60	1	*Euplotes minuta* (AJ310490)	99.9
Euplotes woodruffi	Raw culture[c]	100-140	1	*Euplotes woodruffi* (AF452710)	99.5
Uronychiidae					
Diophrys appendiculata	Raw culture	60-90	2	*Diophrys appendiculata* (AY004773)	99.9
Diophrys scutum	Field sample	150-200	2	*Diophrys scutum* (EU189069)	99.8
Uronychia binucleata	Raw culture	70-110	2	*Uronychia binucleata* (EF198667)	99.8
Uronychia multicirrus	Raw culture	150-200	several	*Uronychia multicirrus* (EU267929)	99.9
Uronychia setigera	Raw culture	50-80	2	*Uronychia setigera* (EF198669)	99.5
Heterotrichida					
Condylostomatidae					
Condylostoma magnum	Raw culture	200-300	several	*Condylostoma* sp. (AM295496)	96.2
Spirostomidae					
Spirostmum sp.	Field sample	250-300	several	*Spirostomum ambiguum* (AM398201)	94.5
Tintinnida					
Codonellidae					
Tintinnopsis sp.	Field sample	Unrecorded	-	*Tintinnopsis tubulosoides* (AF399111)	98.2
Pseudokeronopsidae					
Pseudokerenopsis sp.	Raw culture	180-230	several	*Pseudokeronopsis carnae* (AY881633)	96.8
Urostylidae					
Holosticha sp.	Raw culture	70-100	2	*Holosticha* sp. *parawarreni* (EF123707)	99.9
Litostomatea					
Pleurostomatida					
Litonotidea					
Litonotus paracygnus	Raw culture	150-300	2	*Litonotus paracygnus* (EU242509)	99.8
Loxophyllum rostratum	Raw culture	100-130	2	*Loxophyllum rostratum* (DQ190465)	97.2
Phyllopharyngea					
Dysteriida					
Dysteriidae					
Dysteria brasiliensis	Raw culture	100-120	1	*Dysteria* sp. (AY331801)	99.5
Karyorelictea					
Protostomatida					
Trachelocercidae					
Trachelocerca sp.	Field sample	200-900	several	NA[d]	-
Prosromatea					
Prorodontida					
Colepidae					
Coleps sp.	Field sample	95	1	NA	-

[a]Number of Macronuclear nodules (Ma) were followed by Carey (1992) and Corliss (1979).
[b]Specimens were isolated directly from field collected samples.
[c]specimens were isolated from the temporally cultured media less than seven days.
[d]No congeneric sequence is available from NCBI

of the 'Comparison of four DNA extraction methods'. In addition, genomic DNAs were extracted from a single ciliate isolated directly from the field collected samples and the temporally cultured media less than seven days. The SSU rDNA sequences were amplified from the 19 species that belonged to 7 orders under the 5 classes here (Table 1). The newly determined sequences were compared to the GenBank retrieved sequences of the next closest species. The sequences similarities between the closest species were ranged from 94.7 to 100% (Table 1). While *Trachelocerca* sp. and *Coleps* sp. were not exited congeneric sequences from NCBI and were not available to compare with the other sequences.

Most uncultivated ciliates, which represent at least 80% of the extant ciliate species, have not been studied relative to their molecular studies (Foissner, 1999; Moon-van der Staay et al., 2001; Stoeck et al., 2003; Strüder-Kypke and Lynn, 2003; Countway et al., 2005). The procedure described here is simple to perform and yields an excellent recovery of high-purity DNA of a single ciliate cell from natural mixed populations. When these methods will be applied, they will be very helpful in obtaining genetic and phylogenic information among diverse ciliate groups without any culture steps. Our results also provide useful guidelines that may be applied to developing protocols for other protists as well.

In the single cell DNA extraction method, the efficiency of PCR may have been highly dependent on the recovery rate of the extracted genomic DNA because the amount of starting material was inevitably very small. The success of this new technique described in this study may also be dependent on the multi-copy property of the rDNA gene.

The recovery technique was able to obtain genomic DNA of high quality from a single ciliate. The development of a method that obtains pure and optimum amounts of DNA from a few or single ciliate samples may prove to be useful, as this would effectively eliminate the constraints and difficulties imposed by the need for cultivation (Dyal et al., 1995). This technique may be much more useful for rare ciliates, such as tiny and uncultivable ones, because only a few specimens are needed for amplification.

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