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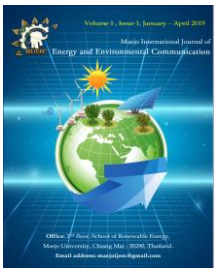

ARTICLE

Sequencing of 18S rRNA gene of Bdelloid rotifers and design of the primers for real-time PCR

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ABSTRACT

Three individuals of Bdelloid rotifer (J1, J2 and J3) were isolated from a MBR system in Nagasaki University and one individual of rotifer (J4) in the original seed sludge collected from a wastewater treatment plant for the MBR was isolated. The four rotifer species were able to proliferate in toxic Microcystis cell suspension. The partial sequence of 18S rRNA gene of each isolated rotifer was determined using In-fusion cloning and searched by BLAST. The gene of the four rotifers J1, J2, J3 and J4 showed the same sequence, then the consensus sequence was in the branch of Bdelloid rotifers in the phylogenetic tree. Furthermore, a specific Bdelloid forward primer 55F and reverse primer 395R for real-time PCR was designed based on the consensus sequence for quantitative researches on the Bdelloid rotifers population. We succeeded to quantify the population of a Bdelloid rotifer cultured in toxic Microcystis cell suspension using the new designed primer pairs.

1. Introduction

The order Bdelloidea consists of three families Habrotrochidae, Adinetidae and Philodinidae. Bdelloid rotifers were inhabiting on the bottom of lotic and lentic waters (Fontaneto and Ricci, 2004). The rotifers take the food particle such as bacteria, unicellular algae, yeasts or particulate organic matter by mainly filtering (Geng et al., 2006; Garcia et al., 2009). It is known that some kinds of Bdelloid rotifers are known as major micro-fauna in biological wastewater treatment system such as activated sludge and biofilm system (Sugiura et al., 1990; Inamori et al., 1998; Itayama et al., 2008). Moreover, some studies found that Bdelloid rotifer *Philodina erythrophthalma* could graze toxic Microcystis cells (Inamori et al., 1998). Of course, rotifers are common and important members of freshwater zooplankton communities in lake and reservoirs (Jame and Alan, 2010). The higher abundance of rotifers was often observed as higher abundance of cyanobacteria in eutrophicated aquatic ecosystem (Satori et al., 2009; Maria and Miquel, 2010). Therefore, it is important to find effective micro-fauna for biological water treatment to aim the degradation of toxic cyanobacteria. Actually, toxic Microcystis cells were effectively removed in some bioreactor

experiments, where Bdelloid rotifer *Philodina erythrophthalma* was grown in sponge carriers of the bioreactor (Iwami et al., 1999; Iwami et al., 2000; Itayama et al., 2008). Thus, if Bdelloid rotifers in activated sludges in wastewater treatment plants can prey on toxic cyanobacteria and degrade the toxin, the sludge can play an important role as a micro-fauna seed for the biological treatment of toxic cyanobacteria.

On the other hand, in the studies on micro-fauna in such bioreactors, rotifers were morphologically identified and enumerated under the microscope. However, the dynamics of rotifer couldn't be exactly quantified if rotifers were inhabiting in the carries. Molecular ecological methods can provide the solution. For example, real-time polymerase chain reaction (PCR) was examined to quantify the nitrification bacteria in carriers for wastewater treatment (Geets et al., 2007). Therefore, molecular ecological methods are very useful for the study of micro-fauna in bioreactors.

In this study, we isolated Bdelloid rotifers from a MBR as a wastewater treatment facility, because Bdelloid rotifers were focused on as specific species which may degrade toxic cyanobacteria. The sequence of 18S rRNA (Ribosomal ribonucleic acid) gene of the isolated rotifers were determined using a molecular cloning method. Based on the determined sequence, a new specific primer for real-time PCR was designed to apply for the molecular ecological studies of Bdelloid rotifers.

2. Experimental setup and methods

2.1. Isolation and Identification of rotifers

The rotifers were collected from, a membrane bioreactor (MBR) for a cafeteria wastewater treatment in Bunkyo campus of Nagasaki University (sample name J1, J2 and J3), Japan and a sludge of a wastewater treatment plant in Nagasaki, Japan (sample name J4). This sludge was used as the seed sludge for the MBR. The samples were transported to our laboratory at the room temperature within 10 minutes from the MBR for the isolation in the next step.

Collected water samples of 500 µl containing rotifers were transferred to each well of 24 well microplate (Iwaki, Asahi Glass co., Ltd.). The each well was filled with new sterile BG11 medium (Kratz and Myers, 1955; Stanier et al, 1971) of 1.0 ml and toxic cyanobacteria *Microcytis aeruginosa* (NIES 843) cell suspension with $3x10^4$ cells·ml^{-1} of 500 µl cultured by the BG11 medium, in order to perform an enrichment culture at room temperature and with dark condition. After 7 days, one droplet of 100 µl was transferred from each well to a plastic petri dish. Then we applied the single individual isolation method for moving rotifer individual using a glass capillary tube of the tip diameter of 100 um under the inverted microscope (ECLIPSE TS100, Nikon Co., Japan) with 100 ~ 400 magnification (Pringsheim, 1946; Thakong et al., 2019). Then captured rotifer individual was again cultured in the same *Microcystis aeruginosa* (NIES 843) cell suspension. The 24 well culture plates were covered and placed in darkness under room temperature. The culture was run for 4 weeks, then the number of rotifers in each well was counted under the inverted microscope with x100. The wells containing enough number of rotifers were chosen for the next DNA (Deoxyribonucleic acid) extraction.

2.2. DNA extraction and PCR

The proliferated rotifers were harvested by a nylon net (50 µm mesh size). Rotifers on the net were rinsed by 1 ml autoclave water with several times and carefully transferred to 2 ml centrifuge tubes. Then the tube was centrifuged with 11,000xg for 5 minutes (Model 3520, Kubota Corporation Co., Ltd., Japan). The supernatant was carefully discarded. Then DNA of collected rotifers was extracted using DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the instruction manual of the kit.

The concentration of the extracted DNA was measured using Qubit™ dsDNA HS Assay Kits (Molecular Probes®, Inc. Invitrogen™, Ltd., USA) with the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Schwerte, Germany). The condition of PCR were as follows: 0.5 µl of PrimeSTAR® HS DNA Polymerase (Takara Bio Inc., Japan) was added in reaction mixture of 50 µl which contained forward and revere primers of 1 µl of 10 pmol, 2.5 mM dNTP Mixture of 4 µl, 5x PrimeSTAR® Buffer (Mg^{2+} plus) of 4 µl and template DNA of 1 µl. The forward and reverse primers were designed from a *Rotaria Rotatoria* 18S rRNA gene (accession No. JX494744) in GeneBank of NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine, USA) in this study. The designed rotifer specific forward and reverse primers were rotifer_1F (5'-ATGCATGTCTAAGTATGA ACTGG-3') and rotifer_1541R (5'-GGGCGGTGTGTAC AAAGGACAG -3'), respectively.

An additional sequence (5'-TCGAGCTCGGTACCC-3') was bonded at 5' end of the forward primer and an additional sequence (5'-GCAGGTCGACTCTAG-3') was bonded at 5' end of the reverse primer for In-fusion cloning according to the manufacture protocol as described later. Veriti™ (Thermo Fisher Scientific, Schwerte, Germany) thermal cycler was used for the PCR. The thermal cycle condition was as follows: 96°C for 10 seconds, 30 cycles; 96°C, 10 seconds, annealing Tm 55°C for 5 seconds, and 72°C for 30 seconds, and a final extension step of 72°C for 7 minutes, 1 cycle for incubation; 15°C, hold for products refrigeration.

PCR products were resolved by electrophoresis on a 1.5% agarose gel stained, which was comprised of 0.75 g agarose dissolved by 50ml 1× TAE buffer (40 mmol/l Tris, 20mmol/l Acetic acid, and 1mmol/l EDTA, pH=8.0), 2µl of SYBR® Safe DNA Gel Stain was mixed in during the process of gel making. Agarose gel was submerged in 1× TAE buffer and the electrophoresis was completed by Mupid®-2plus (Takara Bio Inc., Japan). The PCR products were visualized on a Gel Doc system (ProDoc-LED505-TR60W with Transilluminator scope WD, Japan) was checked using UV light and photographed. DNA content was quantified by comparison to a precision molecular mass standard. PCR

amplicons of the expected size were purified using NucleoSpin® Gel and PCR clean-up (MACHEREY-NAGEL GmbH & Co. KG, Germany).

2.3. Cloning of the PCR product and the sequencing

In-Fusion® HD Cloning Kit (Takara Bio Inc., Japan) was used for the cloning of the purified PCR product of 2.5 µl from each sample. The series of transformation process was performed according to the manufactures protocol manual, where *E-coli* HST08 (Takara Bio Inc., Japan) was used as competent cell. In the cloning, pUC19 plasmid (Takara Bio Inc., Japan) was used as a vector containing with an ampicillin resistance gene (Yanisch-Perron et al 1985).

Transformed cells were selected by SOC medium (Thermo Fisher Scientific, Schwerte, Germany) agar plates containing carbenicillin disodium salt (Invitrogen™, Thermo Fisher Scientific cooperation, Schwerte, Germany) with the final concentration of 50 ug/ul. After the incubation of the agar plates (37°C, 12 hours), twenty colonies were randomly picked up from the plate. The each selected colony was suspended with 50 ul sterile water, then the direct PCR was performed using each colony suspension of 1 ul to check the insert of the expected size, where the forward primer M13 M4 forward primer and M13 RV reverse primer for pUC19 vector plasmid was used (Takara Bio Inc., Japan). The PCR reagent mixture and thermal cycler were the same as in the former PCR.

The thermal cycler condition was as follows: 95°C for 2 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minutes, then a final extension step of 72°C for 5 minutes. The amplicons were checked by the same electrophoresis described in the former section. The short incubation with LB medium (37°C, 8 hours) was performed for the colonies, which showed the specific band at the expected size in the electrophoresis gel, to obtain the plasmid DNA which have a 18S rRNA gene of a rotifer. The plasmid was extracted and purified using Gene Elute™ HP Plasmid Miniprep Kit (SIGMA-ALDRICH Co. LLC., USA) from the colony suspensions, then the PCR was carried out under the same condition of the colony direct PCR in order to determine the sequences of PCR products by a company service (Hokkaido System Science, Co., Ltd., Japan). The determined sequences were identified by BLAST () search by NCBI (National Center for Biotechnology Information, U.S. National Library of Medicine, USA).

Multiple alignments of sequences, which contained the determined rotifer 18S rRNA gene sequences and other rotifer sequences collected from the database, were carried out by the Tamura-Nei method (Tamura and Nei 1993; Nei and Kumar., 2000). The phylogenic analysis was conducted by the Neighbor- Joining method (Saitou and Nei 1987). The analysis was conducted using the software Geneious (ver 8.1.9, Biomatters Ltd., New Zealand).

2.4. Real-time PCR of Bdelloid rotifer

The design of oligonucleotide for real time PCR primers based on the consensus sequence of Bdelloid rotifers which were determined in this study. The design was carried out using a free software Primer3 plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). An in-silico test for several primer sets was performed to check the specificity for 18S rRNA genes of several species from NCBI database.

The artificial gene of rotifer 18S rRNA gene including the sequence region between the selected forward and reverse was synthesized and inserted in a plasmid vector pEX-A2J2 of 2659 bp by a company service (FASMAC, Co., Japan). The artificial gene was diluted with sterile TE buffer to make a concentration series of $2.98x10^8$, $2.98x10^7$, $2.98x10^6$, $2.98x10^5$, $2.98x10^4$, $2.98x10^3$ copy/ml as the standard for real-time PCR.

One individual of *Philodina* sp. as Bdelloid rotifer was identified and isolated from a collected sludge of the same MBR in Nagasaki University (Thakong et al., 2019). The isolated *Philodina* sp. was cultured with the same condition using *Microcytis aeruginosa* (NIES 843) cell suspension as already described. After the proliferation of *Philodina* sp., the population density was enumerated in a counting chamber of 1ml (RIGOSYA, Ltd., Japan) under the inverted microscope (ECLIPSE TS100, Nikon Co., Japan) with 100 magnification. Then 40 ml, 20 ml, 10 ml and 5 ml of the *Philodina* sp. culture suspension were centrifuged to correct different number of the individuals for the DNA extraction by the same kit as described the former paragraph. Duplicate tubes for 40 ml and 20 ml were prepared. Regarding to variance of rotifer population for few number samples, triplicate tubes for 10 ml and 5 ml were prepared.

In the DNA extraction, three replicates were prepared. A thermal cycler Dice II (Takara Bio Inc., Japan) was used for the real-time PCR of SYBR method. Following the conditions specified in the manual of THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co., Ltd. Japan), 0.6 ul of forward and reverse primers of 10 pmol and 1 ul of DNA template of *Philodina* sp. samples were added in PCR mixture of the kit. For each standard and each DNA sample, three replicate wells were used. Two step PCR was applied regarding Tm value adjusted for the obtained primer pairs. According to the manufactures protocol of the kit, two step PCR thermal cycle condition was set as follows: initial denaturation at 95°C for 30 seconds, 40 cycles of 95°C for 10 seconds and the determined 60.3°C for 30 seconds.

In the quantification by real-time PCR, the prepared artificial gene dilution solutions were used as standard copy number of 18S rRNA gene of Bdelloidea rotifer. Ct value from each amplification curve was calculated by the attached software of Dice II (Takara Bio Inc., Japan).rtificial gene of rotifer 18S rRNA gene including the sequence region between the selected forward and reverse was synthesized and inserted in a plasmid vector pEX-A2J2 of 2659 bp by a company service (FASMAC, Co., Japan). The artificial gene was diluted

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3. Results and Discussion

3.1. Isolation and Morphological identification of Bdelloid rotifers

We isolated Bdelloid rotifers from the MBR in Nagasaki University and the seed sludge for the MBR from a wastewater treatment plant. In this study, we specially isolated Bdelloid rotifers because it was known that Bdelloidea *Philodina erythrophthalma* grazed and degraded toxic cyanobacteria cells (Iwami et al., 1999; 2000). The enrichment culture using 24 well microplates containing *Microcystis aeruginosa* (strain NIES 843) cell suspension was carried out. Microcystis strain NIES843 produces cyanotoxin microcystins. Therefore, we regarded that the proliferated micro-fauna in the cell suspension must have an ability to graze the toxic cell and the strong resistance character for the toxin. As a result, Bdelloid rotifers were found in the enrichment culture of each samples.

Each isolated Bdelloid rotifers were again cultured with the *M. aeruginosa* cell suspension. We succeeded to mass culture for the isolated Bdelloid rotifers which were identified *Philodina* sp. morphologically. Three individuals of *Philodina* sp. (J1, J2 and J3) were isolated from the MBR and one individual (J4) was isolated from the seed sludge from a wastewater treatment plant.

3.2. In-fusion cloning and 18Sr DNA sequencing of the isolated rotifers

In order to obtain enough concentration of DNA template for the PCR, we extracted DNA from around 100 individuals of the cultured rotifers. Then we carried out PCR of 18Sr RNA gene in the purified DNA from J1, J2, J3 and J4 samples using the rotifer specific primer pairs. As the primers was designed from a 18S rRNA gene of *Rotaria* sp. which belongs in order Bdelloidea, the primer pairs could amplify 18S rRNA gene of the isolated Bdelloid rotifer *Philodina* sp.. An optimum annealing temperature of the PCR was examined, because the forward and reverse primers were bonded to specific tails for In-fusion cloning to insert the plasmid pUC-19. Using the optimum annealing temperature 55 °C of rotifer_1F and rotifer_1541R, we succeeded to obtain a single band at expected size position in the electrophoresis gel.

Then the bands were cut to purify the PCR products. Then *E.coli* competent cells incorporating the plasmid pUC-19 could only form colonies on the SOC agar plate containing antibiotics carbenicillin due to an ampicillin resistance gene in pUC-19, where carbenicillin was used instead of ampicillin in this study. Figure 1(a) showed the results of electrophoresis of the PCR amplicons of colony direct PCR with the primer pairs of M13 M4 and M13 RV for the 20 colonies from J1 and J2 samples. 16 colonies in J1 showed the bands around 1650 bp position which was close to the expected size of the PCR amplicon in the plasmid. However, there were bands for the colony number 6, 16, 18 at 850 bp position, then the number 7 colony didn't show any brand. The colony number 8, 9, 17 in J2 didn't showed any band in the gel.

The bands of remaining colonies of J2 were found at the expected position. The all colonies in J3 sample showed the success of the cloning as shown in Figure 1(b). On the other hand, the bands of the colony number 2, 6-10 and 12 in J4 sample were found at 850 bp position. The remaining 13 bands were at around 1650 bp. 10 colonies were selected from the colonies of J1, J2, J3 and J4 which showed the band at the expected position. Then, the plasmid DNA was extracted and purified from each *E.coli* cell suspension by the short incubation for each selected colony.

PCR by the same primer pairs (M13 M4 and M13 RV) was carried out for each extracted plasmid to determine the sequences. The obtained sequences were compared after the multiple alignment, then a consensus sequence of J1 (J2, J3 and J4) of around 1k bp was determined from the correct sequences for J1 (J2, J3 and J4) colonies by the Geneious software. The consensus sequences for J1, J2, J3 and J4 were again compared each other. As a result, the four DNA sequences showed the same sequence in the obtained region of 18S rRNA gene.

One consensus sequence of J1, J2, J3 and J4 was compared to 18S rRNA gene sequences of several rotifers in the GenBank nucleotide collection using BLAST search. A molecular phylogenetic tree of 18S rRNA gene was constructed from the consensus sequence of J1, J2, J3 and J4 and the collected sequences from GenBank. The sequence of the isolated rotifers was found in the branch of Bdelloid rotifers in which *Philodina acuticornis* was found as similar species as shown in Figure 2.

The result was reasonable because we isolated *Philodina* sp. based on the morphological identification. *Adineta vaga* also was close to the isolated rotifer. However, we can morphologically distinguish *Adineta vaga* from *Philodina* sp. (Ricci and Melone 2000). Bdelloidea *Adineta vaga* was studied as an unique organisms which can continue asexual reproduction for more than million years (Flot J-F et al., 2013).

We can understand that Bdelloidea is at an unique position in the evolution of eukaryote organisms, because , actually, the branch of Bdelloid rotifers was apart from the branch of the other rotifers such as *Brachionus* sp. (Order Ploimida), *Lepadella* sp. (Order Ploimida) and *Lecane* sp. (Order Ploimida) in the phylogenic tree based on the 18S rRNA genes. In addition, Yeast is at mid position between Bdelloidea and Ploimida. Therefore, a specific primer can be designed based on 18S rRNA gene to identify Bdelloid rotifer. However, it may be difficult to identify each Bdelloid rotifer species considering the resolution of 18S rRNA gene base identification.

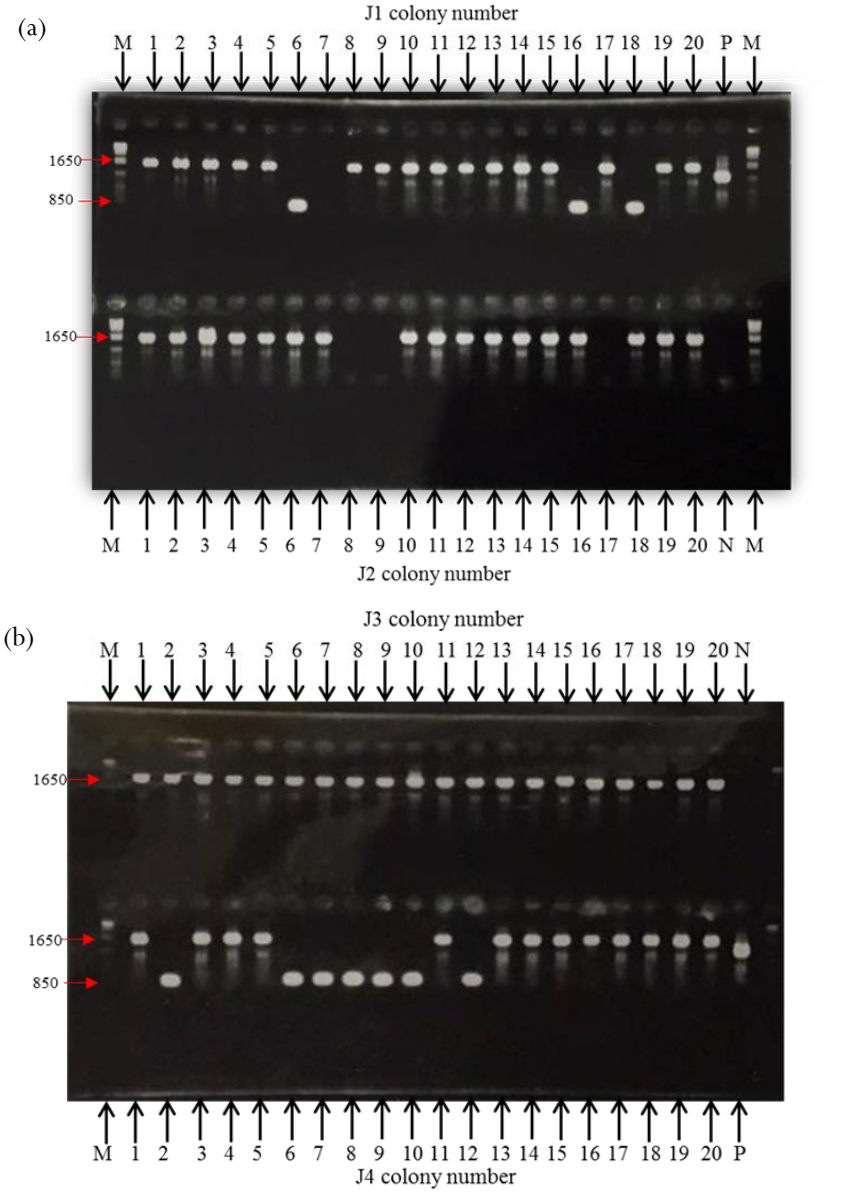

Figure 1. The results of colony direct PCR to confirm the inserted 18S r RNA gene in pUC-19 plasmid from J1, J2, J3 and J4 samples. The upper part in the gel photograph (a) shows the results of J1 sample. The lower part shows the results of J2 sample. The upper part in the gel photograph (b) shows the results of J3 sample. The lower part shows the results of J4 sample. The number beside the molecular maker (M) at left side of gel photograph shows the base pair DNA size (bp). Then the band of P shows a xylE gene (924bp) as a positive control. The N mean the negative control.

3.2. Real-time PCR of Bdelloid rotifer by real time PCR

In order to investigate the population of Bdelloid rotifers using real time PCR for the quantification in the molecular ecological studies, a specific forward primer and a reverse primer were designed in the obtained consensus sequence of 18S rRNA gene using the free software Primer 3 plus. In the several candidate positions, 55F forward primer (5'-GCCTGGGCTAATACATGCGA-3') and 395R reverse primer (5'-CTGCTGCCTTCCTTGGAAGT -3') were selected. Then the primers were confirmed the specificity for Bdelloid rotifers by in-silico test using BLAST search.

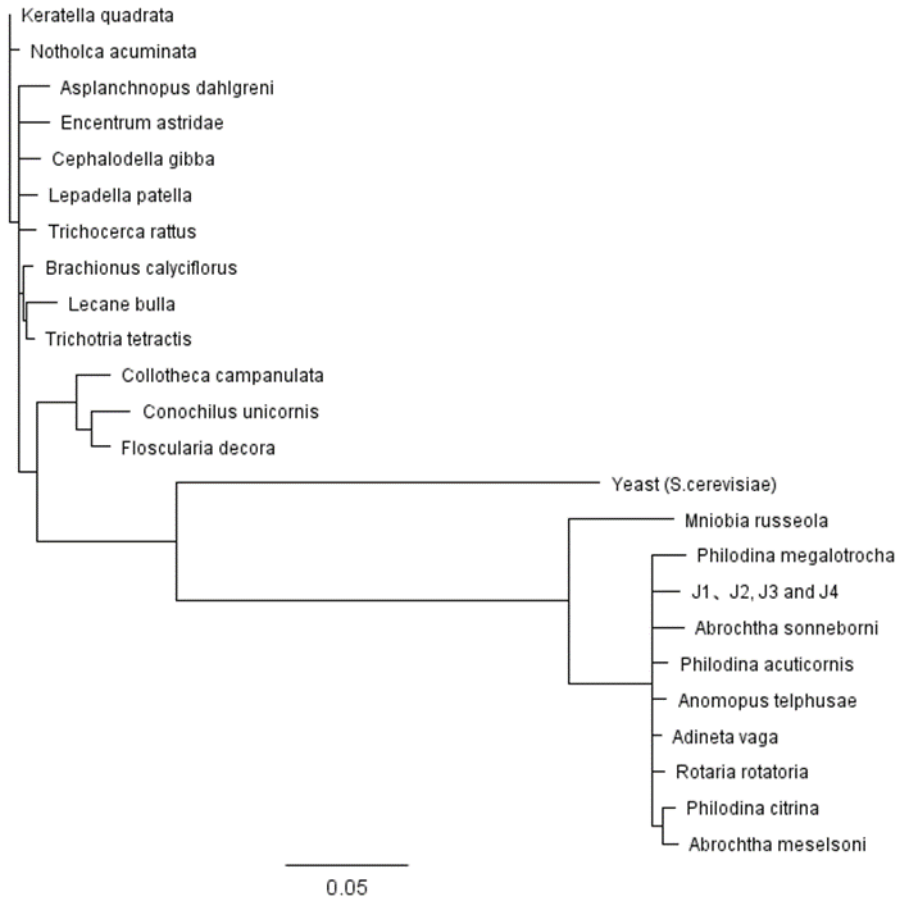

Figure 2. Phylogenic tree of 18S rRNA gene sequences of the isolated Bdelloidea rotifers from MBR in Nagasaki, Japan for for with the sequence of closest species obtained from NCBI

We examined the primer pairs for the real-time PCR of SYBR green method. One individual Bdelloid rotifer, which was morphologically *Philodina* sp., was again isolated from the MBR in Nagasaki University. The mass culture of the isolated rotifer in toxic Microcystis (NIES102) cell suspension was carried out to obtain the enough number of rotifer, then we collected the culture suspension of the rotifer population density around 2.6 individual per ml. 52 rotifers (in 20 ml suspension), 26 rotifers (in 10 ml), 13 rotifers (in 5 ml) and 6.5 rotifers (in 2.5 ml) were used for the DNA extraction. Total eluted DNA after extraction is 60 ul.

Each extracted DNA of 1µl was used as DNA template for a well in a real-time PCR plate. The amplification plots of the standards of the real-time PCR using the new designed primers (55F and 395R) were shown in Figure 3(a). The vertical axis is a logarithmic scale of fluorescence intensity of SYBR green. The standard curve for 2.98 $x10^8$ copy/ml started the increment at 12 cycle, then the almost linear increment part was appeared from 13 cycle to 15 cycle though a small bend was found. Then the standard curve for 2.98 $x10^6$ copy/ml started the increment at 17 cycle, then the almost linear increment part was appeared from 18 cycle to 22 cycle. Of course, the linear part in the semi-logarithmic plot means the exponential increment of DNA product. The PCR efficiency of 85.6% was evaluated from the standard curves using the attached software of Dice II (Takara Bio Inc., Japan). The obtained efficiency showed the lower value than that we expected. However, the plot of Ct values vs. copy number of standard DNA of the artificial gene showed very good linear regression result with $R^2 = 0.998$ as shown Figure 4(a). Therefore, we judged that the new designed primer pairs can be applied to quantify the population of Bdelloid rotifers by real-time PCR.

Figure 3(b) presents the amplification curves for the extracted DNA from the cultured rotifer (*Philodina* sp.) samples with different population. Figure 4(b) presents the regression result between the population enumerated by the optical microscope and 18S rRNA gene copy number quantified by the real-time PCR.

The R^2 value of 0.987 is enough good regression quality in case no rotifer is zero gene. From the regression coefficient, one individual of the isolated *Philodina* correspond 1.35$x10^6$ gene copy of 18S rRNA gene in average. Here, we have to consider the total cell number consisting one individual and the number of repeats of ribosomal RNA gene in a chromosome in a cell, when the gene copy number is converted to the number of individual. Of course, it can be depending on a body size of a rotifer.

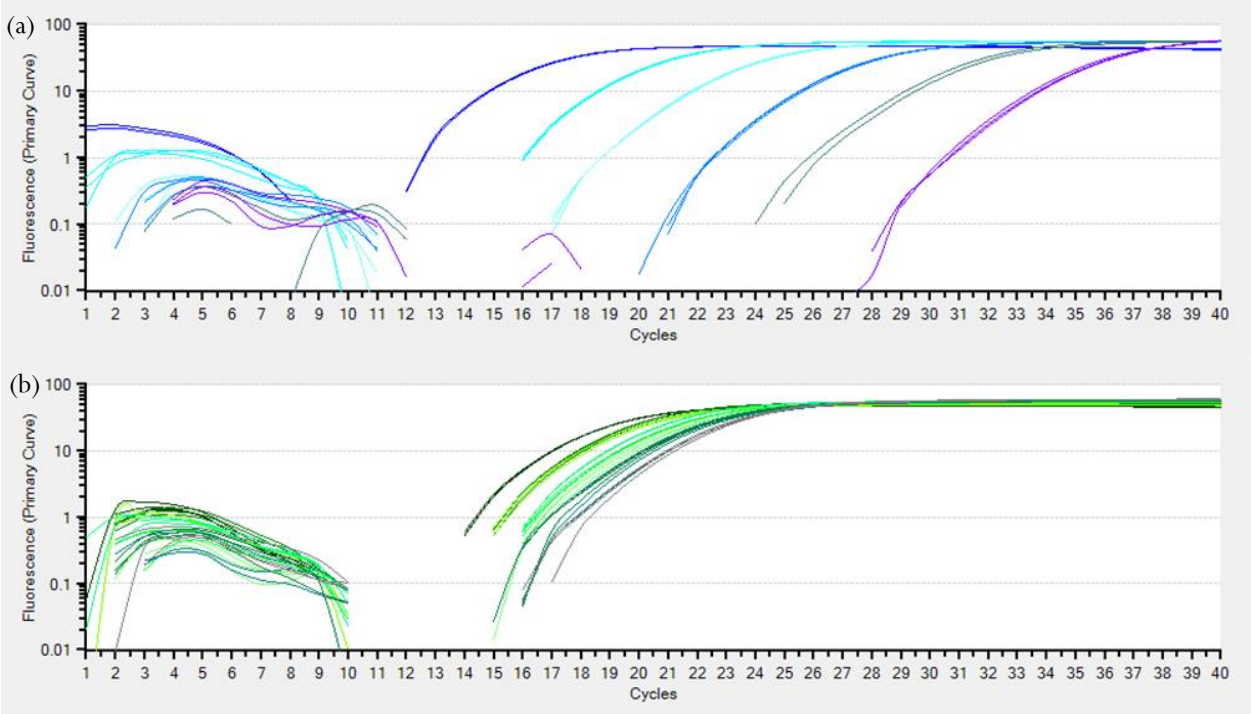

Figure 3. Amplification curves of the real-time PCR by new designed primers for 18S rDNA of Bdelloid rotifer (a) artificial gene for standards (from left side, 2.98 x10^8, 2.98 x10^7, 2.98 x10^6, 2.98 x10^5, 2.98 x10^4, 2.98 x10^3 copy/ml), (b) extracted DNA of *Philodina* sp. culture samples

According to the observation of the isolated *Philodina* sp., the body can be approximated the cylinder shape of the length 300 µm and the diameter 30 µm. If it is assumed that a half of the body is a cavity and the cell can be approximated by a cube on 3 µm side, 2500 cell a rotifer is estimated. Therefore, the number of repeats of ribosomal RNA gene per cell is estimated as 540 copy /cell (= 1.35x10^6 copy/ 2500 cells) under the assumption of 100% DNA recovery in DNA extraction and purification. The value may be reasonable because it is known that the number of the repeats of ribosomal RNA gene of eukaryote microorganisms in a chromosome largely varies depending of the species (Torres-Machorro., 2010). Furthermore, the copy number can vary in a species. For example, the repeats of 18S rRNA gene of Daphnia pulex varied from 94 to 489.5 copy (Eagle and Crease., 2012). Therefore, we need to consider on this point when the rotifer population is estimated by real-time PCR based on 18S rRNA gene.

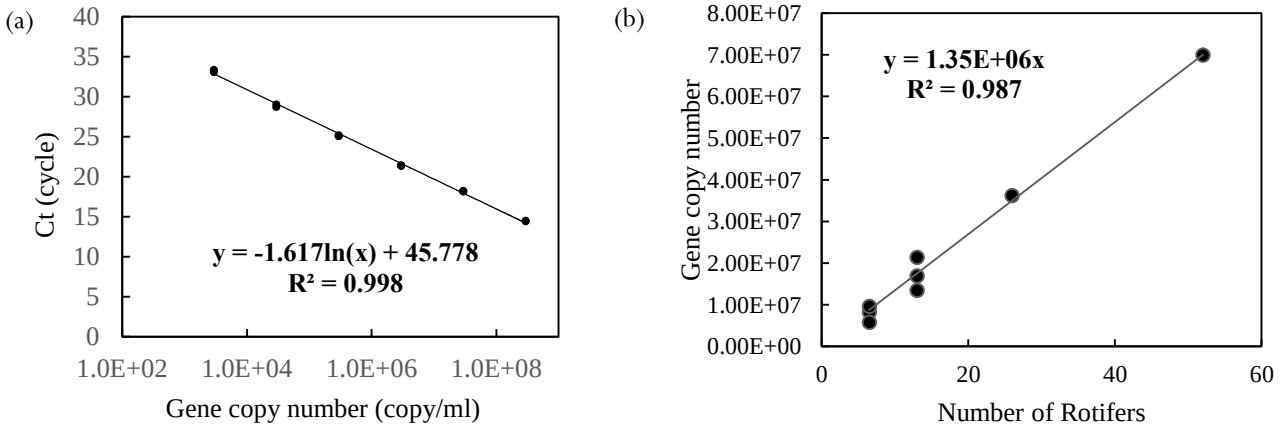

Figure 4. The regression between Ct and the copy number of standards for artificial gene (a), and (b) between the copy number of 18Sr RNA gene and the number of rotifers

4. Conclusion

We succeeded to isolate three individuals of Bdelloid rotifer from MBR in Nagasaki University and the seed sludge for the MBR. Enrichment culture of each isolated rotifer was separately carried out using toxic Microcystis cell suspension as food source. The partial sequence of 18S rRNA gene of the each isolated rotifer was determined by a cloning method. Because the obtained four sequences showed the same sequence, one consensus sequence of the isolated Bdelloid rotifer was compared to the rotifer sequences in GenBank of NCBI by BLAST search. As a result, *Philodina acuticornis* was found as a similar species to the isolated rotifer. Then we designed a new forward primer and reverse primer for real-time PCR of Bdelloid rotifer. We succeeded to quantify the gene copy number of Bdelloid rotifer samples isolated from the MBR with different population by the real-time PCR using the new designed primer pairs.

Nomenclature and Abbreviation

PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
MBR	Membrane bioreactor
NCBI	National Center for Biotechnology Information

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References

Eagle S. H., Crease T. J., 2012. Copy number variation of ribosomal DNA and Pokey transposons in natural populations of Daphnia. Mobile DNA 3(4): 1-12.

Flot J-F., Hespeels B., Li X., et al., 2013. Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. Nature 500: 453-457.

Fontaneto D., Ricci C., 2004. Rotifera: Bdelloidea. In: Yule C.M., Yong H.S., (eds.), Freshwater invertebrates of the Malaysian Region. Academy of Sciences Malaysia Kuala Lumpur Malaysia 121-126.

Garcia C.E., Nandini S., Sarma S.S., 2009. Seasonal dynamics of zooplankton in Lake Huetzalin, Xochimilco (Maxico city, Mexico). Limnologica 39: 283-291.

Geets J., de Cooman M., Wittebolle L., Heylen K., Vanparys B., De Vos P., Verstraete W., Boon N., 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Applied Microbiology and Biotechnology 75(1): 211-221.

Geng H., Xie P., Xu J., 2006. Effect of Microcystis aeruginosa PCC7820 in combination with a green alga on the experimental population of *Brachionus calyciflorus* and *B. rubens*. Bulletin of Environmental Contamination and Toxicology 76: 963-969.

Inamori Y., Sugiura N., Iwami N., Matsumura M., Hiroki M., Watanabe M., 1998. Degradation of the toxic cyanobacterium, *Microcystis viridis* using predaceous function of micro-animals combined with bacteria, Phycological Research 46 (Suppl.): 37-44.

Itayama T., Iwami N., Koike M., Kuwabara T., Whangchai N., Inamori Y., 2008. Measuring the effectiveness of a pilot scale bioreactor for removing Microcystis in an outdoor pond system. Environmental Science and Technology 42(22): 8498-8503.

Iwami N., Itayama T., Sugiura N., Inamori Y., Matsumura M., 1999. Predation and degradation characteristics of Microcystis forming water bloom by combination of functional microanimals. Japanese Journal of Water Treatment Biology 35(4): 225-233.

Iwami N., Sugiura N., Itayama T., Inamori Y., Matsumura M., 2000. Control of cyanobacteria Microcystis using predatory microanimals inhabiting in bioreactor. Environmental Technology 121(5): 591-596.

James H.T. Alan C., 2010. Ecology and Classification of North American Freshwater Invertebrates (Third Edition), Academic Press, 173-235. ISBN 9780123748553.

Kratz W.A., and Myers J., 1955. Nutrition and growth of several blue green algae. American Journal of Botany 42(3): 282-287.

Maria C.S.S., Miquel L., Vera L.M.H., 2010. Responses of the rotifer *Brachionus calyciflorus* to two tropical toxic cyanobacteria (*Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*) in pure and mixed diets with green algae. Journal of Plankton Research 32(7): 999-1008.

Nei M., Kumar S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Pringsheim E.G., 1946. The Biphasic or Soil-Water Culture Method for Growing Algae and Flagellata. Journal of Ecology 33(2):193-204.

Ricci C., Melone G., 2000. Key to the identification of the genera of bdelloid rotifers. Hydrobiogia 418: 73-80.

Saitou N., Nei M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Satori L.P., Nogueira M.G., Henry R., Moletto E.M., 2009. Zooplankton fluctuations in Jurumirim Reservoir (Sao Paulo, Brazil): a three-year study. Brazilian Journal of Biology 69(1): 1-18.

Stanier R.Y., Kunisawa R., Mandel M., Cohen-Bazire G., 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological Reviews 35(2):171-205.

Sugiura N., Inamori Y., Sudo R., Oouchiyama T., Miyoshi Y., 1990. Degradation of blue green alga, *Microcytis aeruginosa* by flagella, *Monas guttula*. Environmental Technology 11: 739-46.

Tamura K., Nei M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10: 512-526.

Thakong W., Yuenyongkirimard D., Shimizu K., Iwami N., Whangchai N., Ramaraj R., Itayama T., 2019. Development of one cell or one individual direct PCR of

protozoan or metazoan 18S rRNA gene for molecular ecology. Indian Journal of Ecology 46(3): 486-492.

Torres-Machorro A.L., Hernández R., Cevallos A.M., López-Villaseñor I., 2010. Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny?. FEMS Microbiology Reviews 34(1): 59-86.

Yanisch-Perron C., Vieira J., Messing J., 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33(1): 103-119.

Appendix-1

Consensus identity of J1, J2, J3 and J4 18S rRNA gene sequences isolated from MBR system.