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Association of clonal diversity and population growth in the small-type rotifer *Brachionus koreanus* during hatchery mass production

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ABSTRACT

In this study, we identify clones of S-type rotifers *Brachionus koreanus* based on haplotypes of mitochondrial gene cytochrome *c* oxidase subunit I (mtCOI) and microsatellite DNA genotypes. We then study how clonal diversity affects the population growth of rotifers in a mass culture at a hatchery using a lot of *B. koreanus* rotifers for analysis. Microsatellite DNA markers were also developed to identify clones of *B. koreanus*. Clones of *B. koreanus* were identified based on haplotypes of mtCOI, microsatellite genotypes, as well as a combination of these haplotypes and genotypes. Three haplotypes of mtCOI, three clones based on microsatellites, and six clones based on a combination of mtCOI haplotypes and microsatellite genotypes were identified. The population growth rate of mass-cultured rotifers was monitored for a month, and the correlation between population growth rate and these clonal diversities was analyzed. Observed was a significant positive correlation between the population growth rate and haplotype diversity ($r = 0.695$, $P = 0.004$), however no correlations were found between the population growth rate and clonal diversity based on either microsatellite genotypes ($r = 0.320$, $P = 0.245$) or a combination of mtCOI haplotype and microsatellite genotypes ($r = 0.435$, $P = 0.105$). Some clones shared mtCOI haplotypes and microsatellite genotypes suggesting sexual reproduction occurred in the hatchery stock of *B. koreanus*.

Statement of relevance: This study showed the correlation between population growth rate and clonal diversities based on mtCOI haplotypes and microsatellite DNA genotypes in *Brachionus koreanus*. There was a significant correlation between the population growth rate and haplotype diversity and suggested genetic factor is one of the possible causes affecting population growth rate. Our results will be useful in mass-production and maintenance of *Brachionus koreanus* at hatcheries.

1. Introduction

Small-morphotype (S-type) rotifers belonging to the *Brachionus* species have been used as larval food in the larviculture of many marine fish species (Hagiwara et al., 2001). Rotifers are used as an initial food in larval rearing for 10–30 days after mouth opening (Lubzens et al., 1989), before feeding with *Artemia* and formula diets. S-type rotifers are commonly used in hatcheries worldwide because this morphotype of rotifers is easily adopted and grown in hatchery environments (Papakostas et al., 2006). Several studies based on DNA barcoding analysis revealed that hatchery-used S-type rotifers in Europe and Japan belong to *B. koreanus*, formally called as *B.* sp. "Cayman" (Papakostas et al., 2006; Baer et al., 2008; Hwang et al., 2013; Moka et al., 2016; Mills et al., 2016).

Rotifers can suddenly die in very large numbers with a subsequent decrease in culture density, usually called a "Crash". Such Crashes have been one major obstacle to rotifer production since rotifer culture started. Once rotifer production has Crashed, sufficient numbers of rotifers for larval rearing are not produced in a hatchery, therefore, seed production is not able to continue. Crashes are thought to be caused by environmental factors such as: food quality and the absence of vitamin B12 (Scott, 1981; Hirayama, 1987), or competition with other organisms such as viruses, bacteria, and ciliates (Hagiwara et al., 1995; De Araujo et al., 2000). However, based on data, a few genetic factors has been considered a cause of such Crashes (Papakostas et al., 2007). We previously identified three haplotypes of mitochondrial gene cytochrome *c* oxidase subunit I (mtCOI) in the S-type rotifer *B. koreanus* of hatchery strains in Japan, and usually several haplotypes were mixed in a mass culture (Moka et al., 2016). This finding suggests that the genetic diversity of S-type rotifers changes during mass culturing and it may be one cause of the Crashes. Billions of rotifers are cultured in mass-production tanks every day. Although sexual recombination may occur in continuous and batch culture systems (Declerck et al., 2015), standing genetic variation may be a key factor affecting population growth in mass culture conditions. Therefore, genetic diversity using

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Nomenclature

S-type	small-morphotype
mtCOI	mitochondrial gene cytochrome *c* oxidase subunit I
MLG	multi-locus genotype
MLL	multi-locus lineage
*mt*MLL	mtCOI haplotype
*ms*MLL	microsatellite DNA based-MLL
ms + *mt*MLL	MLL combining information on both *mt*MLL and *ms*MLL

maternal DNA such as mtCOI may be underestimated. If rotifers in a mass culture possess large genetic diversity, genetic factors could affect population growth and be considered a cause of Crashes.

Microsatellite DNA markers are commonly used as a tool to identify clones of many living organisms (Guo and Gui, 2008; Halkett et al., 2005) including *Brachionus* rotifers (Gómez and Carvalho, 2000; Campillo et al., 2009; Papakostas et al., 2009; Declerck et al., 2015) because of high rates of polymorphisms. Papakostas et al. (2009) successfully identified the clonal composition of L-type rotifers used in aquaculture based on microsatellites. Therefore, microsatellite DNA markers are also expected to be a useful tool in identifying the clonal structure of the S-type rotifer *B. koreanus*. In this study, we developed microsatellite DNA markers for the S-type rotifer *B. koreanus*, and clonal lineages were estimated based on the polymorphisms of microsatellites. In addition, the possibility of sexual reproduction was estimated by comparing mtCOI haplotype with clonal lineages composed of microsatellite DNA polymorphisms. Correlations between population growth and clonal diversity in mass culturing were analyzed based on mtCOI and microsatellite genotype data.

2. Materials and methods

2.1. Rotifer culture and sampling

Rotifers were intensively cultured, at Marua Suisan Co., Ltd., in 3 kL tanks with a 3-day interval of batch culturing. Water temperature and salinity were set at 27 °C and 25 ppt, respectively. Rotifers were fed concentrated freshwater *Chlorella* (V12; Chlorella Industry Co., Ltd., Tokyo, Japan) twice a day.

To estimate population growth rate, the number of rotifer individuals per mL was manually calculated under a profile projector, V12 (Nikon, Tokyo, Japan). 1 mL of culturing media was sampled and the number of rotifer individuals counted. Rotifer individuals were counted three times, and the average number of rotifer individuals/mL was used as a measure of rotifer density (individuals/mL). Rotifer density was set at approximately 500 individuals/mL (1.5×10^9 individuals in 3 kL) on the first day.

Population growth rate of 3 days per batch was also calculated based on the following ratio:

$$\text{Population growth rate} = Nt/No$$

where: *No* is the initial rotifer density (individuals/mL)

Nt is the rotifer density on day *t*.

Sampling of rotifer individuals for further population genetic analysis was conducted at the end of each batch culturing of 31 days (batches I to XV). Culture media (1 mL) containing rotifers was taken from the culture tank, and 24 individuals per batch (total 356 individuals) were individually collected into a PCR tube, DNA was then extracted according to the method by Moka et al. (2015) and stored at −20 °C until genotyping.

2.2. Neutral marker analysis

We used two types of DNA markers to identify rotifer clones: mtCOI and microsatellite. PCR of mtCOI was performed according to the method by Moka et al. (2015). A partial mtCOI sequence (534 bp) was used for the genetic analysis. Partial COI sequences were aligned with ClustalW (Thompson et al., 1994), and the number of haplotypes was identified using MEGA 5.1 software (Tamura et al., 2011). Haplotype diversity (*h*) was also calculated using GenAlEx.

To conduct microsatellite analysis for S-type rotifer, we developed species-specific microsatellite DNA markers for *B. koreanus*. A hatchery strain of *B. koreanus* was collected from a private hatchery (Marua Suisan Co., Ltd., Ehime, Japan). This hatchery strain contained three haplotypes of *B. koreanus* (accession numbers LC004288 to LC004290) (Moka et al., 2016). Rotifers were stored in a 10 L container, and starved for one day before DNA extraction. Total DNA was extracted from 50 μg of well-washed rotifers using an Isotissue DNA extraction kit (Nippon Gene, Toyama, Japan). Di-nucleotide repeats (GT)n were isolated using dual-suppression polymerase chain reaction (PCR) (Lian et al., 2001). In brief, the DNA was separately digested with *Alu*I, *Eco*RV, *Hae*III, *Ssp*I, *Hin*CII, and *Afa*I blunt-end restriction enzymes. The DNA fragments were then ligated with a blunt adaptor using a DNA Ligation Kit (Takara Bio, Shiga, Japan). As the first step, fragments flanked by a microsatellite at one end were amplified by the (GT)10 primers and the adaptor primer designed from the longer strand of the adaptor. The amplified fragments were then cloned and sequenced. Next, two primers were prepared: primer IP1, designed from the sequenced region flanking the microsatellite, and, for nested PCR, primer IP2, based on the sequence between IP1 and the microsatellite. The primary PCR reaction was conducted with each constructed DNA library using IP1 and AP1 primers. The secondary PCR reaction was conducted using a 100-fold dilution of the primary PCR products using IP2 and AP2 primers, on the basis that nested PCR dramatically improves the success rate of amplifying the microsatellite flanking regions. The single-banded fragments were purified and then directly sequenced. All primer pairs for amplifying the microsatellites were designed by Primer3 (Rozen and Skaletsky, 2000).

In addition to *B. koreanus*, we also used another S-type rotifer strain belonging to the SM3 clade (*B.* sp. SM3) (Mills et al., 2016). This rotifer strain was previously genotyped (accession numbers LC004291 and LC004292) (Moka et al., 2016). The procedures to develop microsatellite DNA markers for *B.* sp. SM3 were the same as the method described above. All microsatellite sequences developed were deposited in GenBank (LC125210 – LC125217).

We performed PCR in 5 μL reaction mixture containing 1 μL of extracted template DNA, 2 × KOD Fx buffer, 0.2 mM dNTPs, 0.1 U of KOD Fx polymerase (Toyobo, Osaka, Japan) and 0.1 μM each of the designed primers. The PCR reaction was performed using the following cycling conditions: denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 68 °C for 30 s. The PCR products of non-labeled primers were separated using Fragment Analyzer (Advanced Analytical, Iowa, USA) at 8 kV for 70 min using a DNF-900 dsDNA Reagent Kit, and the band sizes calculated using *PRO Size*™ software (Advanced Analytical). Five out of ten primer pairs isolated from the *B. koreanus* genome DNA were successfully amplified. Three out of eight primer pairs isolated from *B.* sp. SM3 were successfully cross-amplified with *B. koreanus*. These forward and reverse primers of microsatellite DNA markers were then labeled with fluorescent dyes and tailed (Life Technologies). These eight primers were amplified using 46 individuals of the S-type rotifer population mass cultured at the hatchery, and the minimum combination of markers for identifying multi-locus genotypes (MLGs) was determined using GenClone 2.0 software (Arnaud-Haond and Belkhir, 2007). The PCR protocol using fluorescent-labeled primers was the same as that used for non-labeled primers, as described as above. The PCR products with fluorescent dyes were electrophoresed and quantified using an