Inheritance of mitochondrial DNA in the rotifer *Brachionus plicatilis*

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Abstract

By crossing *Brachionus plicatilis* s.s. NH1L strain and German strain, we obtained two types of hybrids, NH1L female × German male designated as NXG and German female × NH1L male designated as GXN. To confirm the crossing of the two hybrid strains at the genetic level, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis using 10 kinds of primers (10 and 12 mers) was carried out. Some amplified DNA fragments from RAPD of the hybrid strain showed mixed patterns of both parental strains, thus confirming that both hybrids were crossbreeds of the NH1L and German strains. Using these hybrids, we investigated the mode of mitochondrial inheritance in *B. plicatilis*. Full length mtDNA of the four strains was amplified by PCR, and digested with restriction enzymes to obtain restriction fragment length polymorphism (RFLP) patterns. Both hybrid strains had the same RFLP patterns as their female parents. This result shows that mitochondrial inheritance in rotifers is maternal.

Introduction

Mitochondrial DNA (mtDNA) has been extensively studied in various groups of animals. The inheritance of mtDNA is predominantly maternal, and mtDNA sequences reflect a lineage rather than a genomic relationship. In addition, since the mtDNA molecule occurs in 1,000-10,000 copies per cell, it is easy to isolate. For these reasons, mtDNA is widely used as molecular marker in intraspecies and interspecies phylogenetic analyses (Kocher et al., 1989).

In spite of the widely reported maternal inheritance of mtDNA, paternal inheritance does occur in a few animals. In *Drosophila*, paternal leakage was detected at a low level,

but frequently enough to explain the observed levels of heteroplasmy in natural populations (Kondo et al., 1990). Using PCR, Gyllensten et al. (1991) showed that paternally inherited mtDNA molecules in mice occurred at a frequency of 10^{-4}, relative to maternal inheritance. Moreover, in mussels of the genus *Mytilus*, female progeny always inherit mtDNA maternally (homoplasmic mtDNA), while male progeny inherit both maternal and paternal mtDNA (heteroplasmic mtDNA, Zouros et al., 1994). This type of inheritance was first discovered by Skibinski et al. (1994), who observed that sperm of a heteroplasmic male contains only the mtDNA of the father. These results suggested that only the male maintains the mtDNA from sperm after fertilization. This special inheritance type is called doubly uniparental inheritance (Zouros et al., 1994), and is common in *Mytilus*.

The mode of *Brachionus* mtDNA inheritance may have important implications for taxonomic studies and in population genetics. In rotifers, the mode of inheritance of mtDNA is still unknown, but it has been hypothesized that the inheritance type of *Brachionus* mtDNA is maternal. Based on this, some mtDNA genes such as 16S ribosomal RNA and cytochrome oxidase subunit-I have been used in phylogenetic analysis (Gomez et al., 2002). The size of the sperm of *B. plicatilis* is about 40 μm, which is about 1/3 of the male body size (Snell & Hoff, 1987). The number of mitochondria in its sperm is not yet determined, but based on the size of the sperm, the number of mitochondria in the sperm might be similar to that in the oocyte. In humans, it has been suggested that maternal inheritance of mtDNA depends on the number of mtDNA; the mtDNA copy number of the oocyte is at least 1,000 times higher than the sperm (Ankel-Simons & Cummins, 1996). Based from the above information, we hypothesized that paternal inheritance of mtDNA may also exist in *B. plicatilis*.

We investigated the mode of inheritance of mtDNAs in the rotifer *B. plicatilis* using hybrid strains.

Materials and Methods

Rotifers

The Parental NH1L strain used in this study originated from an outdoor eel culture pond in Mie Prefecture (Hagiwara et al., 1988), and was clonally cultured in Nagasaki University for more than 15 years. The Parental German strain was collected from Schlei-Fjord Island in 1988, and was cultured in Nagasaki University in this same manner (Fu et al., 1991). The two hybrid strains, NH1L female × German male and German female × NH1L male (designated NXG and GXN, respectively) were obtained in the crossbreeding study of Kotani et al. (2006). All four strains were cultured in 200 ml flasks containing diluted sterilized seawater (22 ppt) at 25^{o}C, and were fed *Nannochloropsis oculata* or *Chlorella vulgaris*.

Isolation of genomic DNA

Cultured rotifers were harvested separately by filtration using a plankton net (45 μm mesh) until 150 mg (wet weight) of rotifer were obtained. The rotifers were washed with sterilized artificial seawater and kept in 400 ml seawater for 24 h in order to let them excrete any remaining algae from their guts. After 24 h starvation, the rotifers were washed with sterilized MilliQ water using a plankton net, and collected by centrifugation (20,000 × g for 1 min at 4^{o}C). Harvested rotifers were homogenized using a sterile pestle in 450 μl lysis buffer (10 mM Tris-HCl pH 8.0, 25 mM EDTA, 10

mM NaCl) and 25 μl 10% SDS. After homogenization, 25 μl proteinase K (10 mg/ml) was added, and the solution was incubated at 50°C for 3 h. The digested solution was treated with an equal volume of organic solvents: phenol (saturated with TE buffer; 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), phenol/chloroform/isoamylalchohol (25:24:1, v/v/v) and chloroform/isoamylalchohol (24:1, v/v) for extraction of protein and cell debris. Genomic DNA was subsequently precipitated by ethanol and resuspended in TE buffer.

RAPD-PCR analysis

In the preliminary experiment, twelve 12-mer (A-01 to A-12; Wako Pure Chemical, Japan) and twenty 10-mer primers (O-01 to O-10 and W-01 to W-10; Operon Technologies, USA) were assayed. Primers that produced a well-resolved DNA band patterns on agarose gel were used for RAPD analysis. Three 12-mer primers (A-01, A-09, A-12) and seven 10-mer primers (O-03, O-05, O-07, W-02, W-03, W-05, W-10) were selected for further analysis. Their sequences and Tm values are shown in Table 1. PCR was performed in a 20 μl reaction solution containing 1 × reaction buffer, 48 ng of genomic DNA, 0.15 mM dNTPs, 0.25 unit KOD Dash DNA polymerase (Toyobo, Japan) and 5 μM of each primer using a Px2 Thermal Cycler (Thermo Electron Corporation, USA) at initial denaturation of 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 38°C for 30 sec and 74°C for 20 sec for 12-mer primers, and 35 cycles of 95°C for 30 sec, 30°C for 30 sec and 74°C for 20 sec for 10-mer primers. PCR products were electrophoresed in 2.5% MetaPhor agarose gel (Cambrex, USA) using TBE buffer (89 mM Tris-Boric acid, pH 8.3, 2 mM EDTA), followed by SYBR Green I (Cambrex) staining according to the manufacturer’s instructions.

RAPD data analysis

To assess the levels of genetic similarity among the four strains, pairwise comparisons of the RAPD patterns of each strain were compared according to Nei & Li (1979). Band sharing index (BSI) was calculated according to the formula, $BSI = 2N_{XY} / (N_X + N_Y)$, where N_{XY} refer to the number of DNA bands shared in common by individuals X and Y, and N_X and N_Y refer to the total number of bands for individuals X and Y, respectively. BSI values range from 0, when there are no bands shared between the RAPD patterns of two individuals, to 1, when no differences are observed in the RAPD patterns, i.e. when they are identical.

PCR amplification of full length of mtDNA

To amplify the full length mtDNA by inverse PCR, sense and antisense primers were designed from the 3'- and 5'-region of 16S rRNA genes from EST database of *B. plicatilis* NH1L (DDBJ/EMBL/GenBank accession number BJ979769) and other rotifer strains in the GenBank database, respectively. The sense and antisense primers were 5'-CGATAAATATATCTACTACCTCGATGTTGG-3' and 5'-GATTTCTAATTAAGAAAACAAGAATCACGCTACC-3', respectively. PCR was performed in a 25 μl reaction solution containing 1 × reaction buffer, 12 ng of genomic DNA, 0.2 mM dNTPs, 0.25 unit KOD Dash DNA polymerase and 0.2 μM of primer using a Px2 Thermal Cycler at initial denaturation of 95^{o}C for 2 min, followed by 35 cycles of 98^{o}C for 10 sec and 73^{o}C (this temperature was decreased at 0.2^{o}C per cycle during the 35 cycles) for 15 min. The amplified PCR products were fractionated on 0.5% SeaKem GTG agarose gel (Cambrex) and the DNA fragments were detected by

ethidium bromide staining.

Partial sequencing of amplified full length mtDNA

The amplified product from *B. plicatilis* NH1L was digested with *Bgl*II restriction enzyme (Fermentas, Canada). The digested products were fractionated on 1.5% agarose-LE classic type gel (Nacalai tesque, Japan) and then about 900 bp fragment was purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The purified fragment was ligated into *Bam*HI-digested pBluescript SK (-) plasmid vector, and the plasmid was used for the transformation of *Escherichia coli* JM109 strain. The recombinant plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen), sequenced with ABI Big Dye 1.1 chemistry using standard M13 forward and reverse primers, and analyzed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA).

PCR-RFLP analysis of full length of mtDNA

Amplified full length of mtDNAs from *B. plicatilis* NH1L, German and two hybrid strains were digested with *Eco*RI or combination of *Bgl*II and *Sal*I restriction enzymes (Fermentas). The digested products were fractionated by electrophoresis on 1.5% SeaKem GTG agarose gel (Cambrex) using TBE buffer and the DNA fragments were detected by SYBR Green I staining according to the manufacturer's instructions. The sizes of the amplification products were estimated using a 2-Log DNA ladder marker (New England Biolabs, UK).

Results

RAPD analysis

The number of bands generated by each primer was variable, ranging from 12 to 33, with size ranging from 80 to 1,400 bp (Fig. 1). Some amplified DNA fragments from RAPD of both hybrid strains showed mixed patterns of Parental NH1L and German strains (Fig. 1). From the RAPD data, the genetic distance between the two parental strains and the hybrid strains was calculated (Table 2) according to Nei & Li's method (see Materials and Methods). It is suggested that common RAPDs are inherited from common or assortative ancestral DNA sequences on RAPD patterns of the two evaluated strains (Liu et al, 1999), therefore, increase of common RAPDs, BSI values close to 1, shows high genetic similarity, and decrease of common RAPDs, while BSI values close to 0, shows low genetic similarity. The values are lowest in the parental pair (0.061-0.417), medium in the combination of parental and hybrid strains (NH1L-NXG, 0.286-0.621; NH1L-GXN, 0.235-0.667; German-NXG, 0.364-0.929; German-GXN, 0.378-0.741) and highest in the pair of hybrid strains (0.563-0.929).

Analysis of the DNA fragment from amplified full length mtDNA

The amplified fragment by inverse PCR showed about 13 kbp length which was estimated by comparison with DNA marker. BLAST analysis showed that the deduced amino acid sequence of the cloned 900 bp fragment (DDBJ/EMBL/GenBank accession number AB303309) from the amplified fragment digested with *Bgl*II has high similarity to Cytochrome*b* of *Pogonomyrmex californicus* (taxing score and E-value were 158 and 8e-38, respectively), add other organisms.

RFLP analysis

The amplified full length mtDNAs (about 13 kbp) showed no length variation between the parental and hybrid strains. However, when mtDNA digested was with *Eco*RI, we obtained two bands of 10.8 and 1.1 kbp DNA fragments in NH1L and NXG strains, and three bands of 6.3, 3.7 and 1.6 kbp DNA fragments in German and GXN strains (Fig. 2A). When mtDNA of all strains were double digested with *Bgl*II and *Sal*I, we obtained the same RFLP patterns from both German and GXN strains. In all RFLP patterns of mtDNAs, one DNA fragment was detected exclusively in NXG (Fig. 2B).

Discussion

In this study, the inheritance of mtDNA in *B. plicatilis* was investigated using hybrid strains. The two parental (NH1L and German) strains are genetically different from each other as detected by allozyme analysis (Fu et al., 1991). These strains were crossbreed by Kotani et al. (2006) using males and females from both strains, and they successfully produced the two hybrid strains, which are morphologically distinct from the parental strains. The hybrids are intermediate in size and have lower mictic female production than the parental strains (Kotani et al, 2006). However, this study did not confirm the crossing of both hybrids at the genetic level. In our study, using RAPD, we could confirm that these hybrids are a cross between both parental strains. RAPD has been widely used in genetic analysis because of its ease, universal applicability and high frequency of polymorphic bands (Williams et al., 1990; Liu et al., 1999; Werner et al., 2001; Yotsukura et al., 2001). The results of RAPD analysis in our study showed that some polymorphic DNA fragments from parental strains were also found in each hybrid (Fig. 1). The BSI values calculated from RAPD fingerprinting using 10 primers were

near to 1 in order of comparison with both hybrids, each hybrid and parent, and both parents (Table 2). A BSI value close to 1 means a high genetic similarity, and this similarity decreases as the value closes on 0. The BSI values of two hybrid strains were highest (0.563-0.929), indicating that the 2 hybrid strains are indeed a crossed of both parental strains.

Animal mtDNA is usually a small (~ 16 kbp), single, circular molecule containing 37 genes: two rRNA genes, 13 protein-coding genes and 22 tRNA genes (Boore, 1999). The size of amplified DNA fragment of *B. plicatilis* by inverse PCR was about 13 kbp, and the deduced amino acids sequence of a clone taken from a fragment of *Bgl*II-digested of this DNA was sequenced, we found that it had high similarity to Cytochrome*b*. Thus, we are certain that the amplified DNA product was mtDNA.

The RFLP analysis showed that the fingerprinting patterns between NH1L and NXG (NH1L female parent), and German and GXN (German female parent) were identical, when the mtDNA was digested with *Eco*RI, indicating that mtDNA is inherited maternally. However, when mtDNA was double-digested with *Bgl*II and *Sal*I, we detected a distinct DNA fragment in NH1L and NXG strains. We hypothesize that a point mutation occurred during amplification of mtDNAs by PCR, or this is a mutated mtDNAs and is inherited by the hybrid.

In many organisms, inheritance of mitochondria is predominantly maternal (Hutchison et al., 1974; Giles et al., 1980; Martin, 1989; Vaillancourt et al., 2004). Ankel-Simons and Cummins (1996) suggested that the copy number of mtDNA is different between oocyte and sperm; the mtDNA copy number of the oocyte is at least 1,000 times higher than that of the sperm, based on maternal inheritance of mtDNA in humans. Sutovsky et al. (1999 and 2000) showed that the recycle marker protein

ubiquitin-dependent proteolysis has been implicated in the recognition and selective elimination of paternal mitochondrial and mtDNA after fertilization in cow and monkey embryos. In this study, we demonstrated that the inheritance of mtDNA in *Brachionus* is maternal, as in many other organisms. Although the exact regulatory mechanism of mtDNA inheritance is not clear, we suggest that these mechanisms in *Brachionus* are similar to those in most other organisms.

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Labels of Figures:

Fig. 1 RAPD amplification profiles generated using primers A (A), primers O (B), and primers W (C). N, Paternal NHIL strain; G, Paternal German strain; NXG and GXN are hybrid strains; M, 100 bp DNA ladder marker (New England Biolabs). Arrows with the same letter indicate common band between parent and hybrid; a, matched with Parental NH1L strain; b, matched with Parental German strain.

Fig. 2 RFLP pattern of mtDNA of parental and hybrid rotifer strains investigated in this study. A, mtDNA digested by *EcoR*I; B, mtDNA double digested by *Bgl*II and *Sal*I. N, Parental NH1L strain; G, Parental German strain; NXG and GXN are hybrid strains; M, 2-Log DNA ladder marker. An arrow indicates DNA fragments detected exclusively in NXG strain.

Table 1 RAPD primers and their Tm values used in this study.

Primer name	Sequence (5’-3’)	Tm (^{o}C)
A-01	TGCACTACAACA	36.2
A-09	CCGCGATTAGAT	39.6
A-12	TTCGGACGAATA	36.2
O-03	ACTTCGCCAC	32.0
O-05	GGCTCATGTG	32.0
O-07	GACCCTAGTC	32.0
W-02	CAACAACTGC	30.0
W-03	GGAGGACTTC	32.0
W-05	CACCGGTATC	32.0
W-10	CCCCATGCTC	34.0

Table 2 Band sharing index (BSI) of RAPD-PCR analysis.

	Combination of strains					
Primer name	NH1L-German	NH1L-NXG	NH1L-GXN	German-NXG	German-GXN	NXG-GXN
A-01	0.263	0.488	0.439	0.606	0.485	0.722
A-09	0.150	0.439	0.378	0.537	0.378	0.684
A-12	0.217	0.613	0.586	0.480	0.565	0.871
O-03	0.229	0.286	0.235	0.929	0.593	0.593
O-05	0.417	0.621	0.667	0.552	0.444	0.563
O-07	0.238	0.426	0.400	0.372	0.390	0.652
W-02	0.333	0.516	0.483	0.828	0.741	0.929
W-03	0.061	0.308	0.316	0.529	0.545	0.769
W-05	0.359	0.372	0.512	0.700	0.450	0.636
W-10	0.200	0.359	0.439	0.364	0.514	0.765

Fig. 1 Koushirou Suga et al.

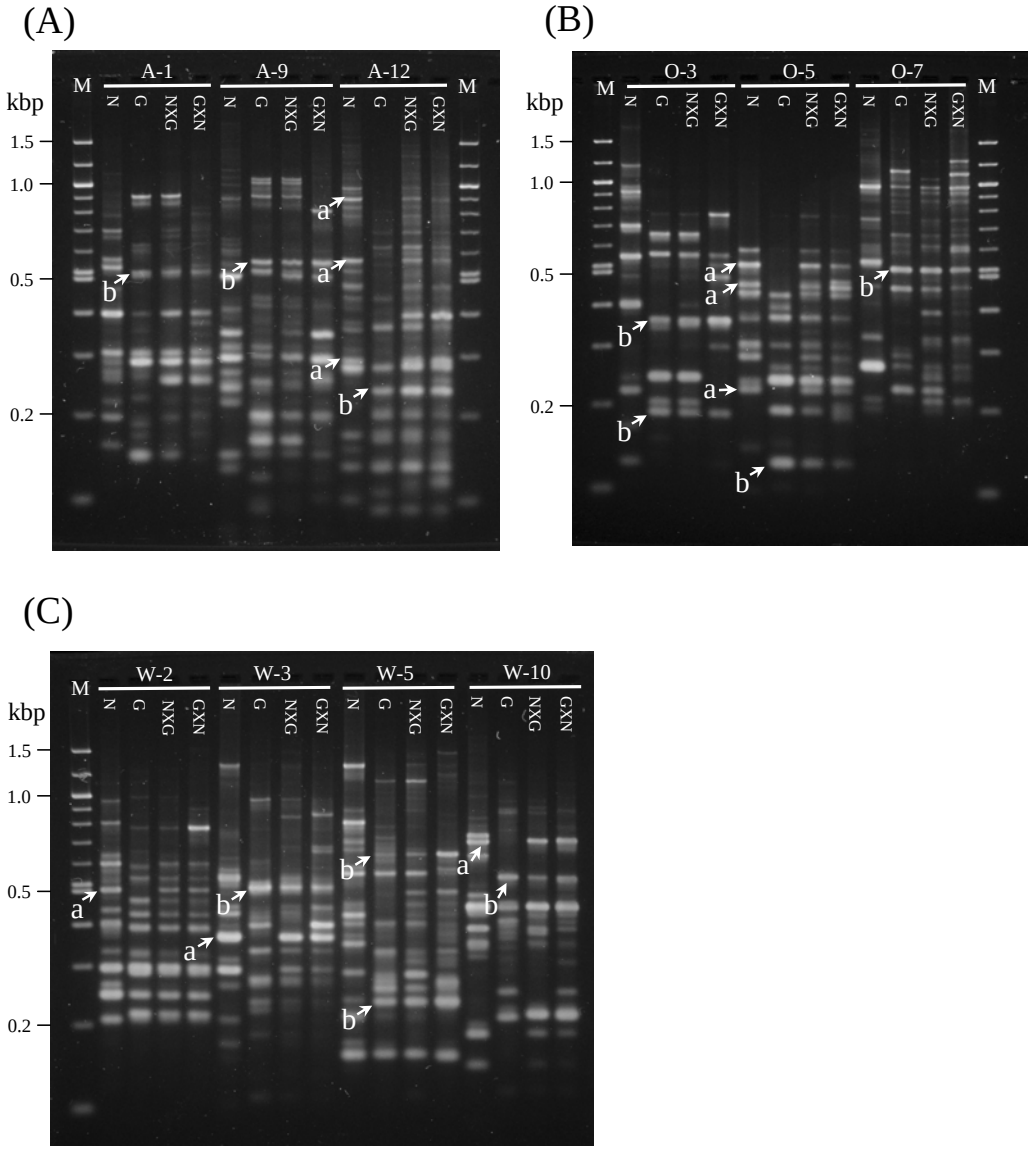

Fig. 2 Koushirou Suga et al.