Two Circular Chromosomes of Unequal Copy Number Make Up the Mitochondrial Genome of the Rotifer *Brachionus plicatilis*

Koushirou Suga,† David B. Mark Welch,‡ Yukari Tanaka,*‖ Yoshitaka Sakakura,§ and Atsushi Hagiwara‖

*Nagasaki Industrial Promotion Foundation, Omura, Japan; †Institute for East China Sea Research, Nagasaki University, Nagasaki, Japan; ‡Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, MA; §Faculty of Fisheries, Nagasaki University, Nagasaki, Japan; and ‖Graduate School of Science and Technology, Nagasaki University, Nagasaki, Japan

The monogonont rotifer *Brachionus plicatilis* is an emerging model system for a diverse array of questions in limnological ecosystem dynamics, the evolution of sexual recombination, cryptic speciation, and the phylogeny of basal metazoans. We sequenced the complete mitochondrial genome of *B. plicatilis sensu strictu* NH1L and found that it is composed of 2 circular chromosomes, designated mtDNA-I (11,153 bp) and mtDNA-II (12,672 bp). Hybridization to DNA isolated from mitochondria demonstrated that mtDNA-I is present at 4 times the copy number of mtDNA-II. The only nucleotide similarity between the 2 chromosomes is a 4.9-kbp region of 99.5% identity including a transfer RNA (tRNA) gene and an extensive noncoding region that contains putative D-loop and control sequence. The mtDNA-I chromosome encodes 4 proteins (ATP6, COB, NAD1, and NAD2), 13 tRNAs, and the large and small subunit ribosomal RNAs; mtDNA-II encodes 8 proteins (COX1–3, NAD3–6, and NAD4L) and 9 tRNAs. Gene order is not conserved between *B. plicatilis* and its closest relative with a sequenced mitochondrial genome, the acanthocephalan *Leptorhynchoides thecatus*, or other sequenced mitochondrial genomes. Polymerase chain reaction assays and Southern hybridization to DNA from 18 strains of *Brachionus* suggest that the 2-chromosome structure has been stable for millions of years. The novel organization of the *B. plicatilis* mitochondrial genome into 2 nearly equal chromosomes of 4-fold different copy number may provide insight into the evolution of metazoan mitochondria and the phylogenetics of rotifers and other basal animal phyla.

Introduction

Members of the phylum Rotifera are major components of nonmarine aquatic ecosystems throughout the world. Rotifers are also the most experimentally tractable members of the assemblage of basal metazoan phyla known as the Gnathifera, which also includes Micrognathozoa, Gnathostomula, and possibly Cycliophora and Gastrotricha. The position of Gnathifera, as a group of lophotrochozoans, a sister taxon to Lophotrochozoa, or a more basal superphylum, is not known (Zrzavy et al. 1998; Giribet et al. 2000, 2004; Mark Welch 2001; Funch et al. 2005). Rotifera itself is composed of 4 major groups: Acanthocephala, Bdelloidea, Monogononta, and Seisonidea, although the relationship of these taxa has not been resolved (Garey et al. 1996; Mark Welch 2000, 2001, 2005; Herlyn et al. 2003; Funch et al. 2005). Monogononta is the best studied rotifer group, and monogononts have become a model system for studies of aquatic food webs, predator prey dynamics, population dynamics, speciation, the evolution of sex, and ecotoxicology (Snell et al. 1999; Kotani et al. 2001; Preston and Snell 2001; Gomez et al. 2002; Yoshida et al. 2003, 2007; De Meester et al. 2004; Serra et al. 2004; Gomez 2005; Marcial et al. 2005; Suatoni et al. 2006). Monogononts also play a major role in the international aquaculture industry as an essential live food for the initial stage of larval rearing of marine fishes (Hagiwara 1994; Hagiwara et al. 2001).

The most studied monogonont rotifer is the euryhaline *Brachionus plicatilis*, which is now recognized to be a species complex consisting of as many as a dozen species (Gomez et al. 2002; Gomez 2005; Suatoni et al. 2006). As morphological differences between some of these species are extremely subtle, species discrimination is largely based on molecular phylogenetics. The principal gene used for phylogenetic discrimination of the *B. plicatilis* species complex is the mitochondrially encoded *cytochrome* c *oxidase subunit 1* (*cox1*), which can be used to efficiently define cryptic phylotypes. In fact, assigning isolates of *B. plicatilis* to established phylotypes was one of the early applications of *cox1*-based barcoding (reviewed in Gomez 2005). However, the relatively small region generally amplified from *cox1* does not adequately resolve relationships between phylotypes. Therefore, additional regions of the mitochondrial genome are required to elucidate the evolution of the *B. plicatilis* complex.

In part because of ease of isolation, small size, and simple structure, a metazoan mitochondrial genome has become relatively straightforward to sequence; there were more than 1,200 complete mitochondrial genomes available from GenBank in 2007, and the number is rapidly increasing. Analysis of the sequence and structure of these genomes has led to a greater understanding of mitochondrial function and origin and has proven useful in elucidating the phylogeny of their host organisms (Wolstenholme 1992; Boore and Brown 1998; Helfenbein and Boore 2004; Valles and Boore 2006). The complete mitochondrial genome of the acanthocephalan rotifer *Leptorhynchoides thecatus* was published in 2005 and found to contain several atypical features, including unusually short ribosomal RNA (rRNA) genes, transfer RNA (tRNA) genes missing the TΨC arm, and no obvious control region (Steinauer et al. 2005). With the hope that the first description of the mitochondrial genome of a monogonont rotifer would pave the way for the sequencing of other gnathiferan mitochondrial genomes and lead to a better understanding of the evolution of Gnathifera, Rotifera, and the *B. plicatilis* species

Key words: aneuploidly, mitochondrial genome structure, metazoan evolution, Gnathifera, Rotifera, *Brachionus plicatilis*.

E-mail: dmarkwelch@mbl.edu

Mol. Biol. Evol. 25(6):1129–1137. 2008
doi:10.1093/molbev/msn058
Advance Access publication March 7, 2008

complex, we sequenced the complete mitochondrial genome of *B. plicatilis sensu strictu*. Surprisingly, we found that the genome is composed of 2 circles of similar size but unequal copy number, a structure that has not been reported previously for any metazoan. We found evidence for a similar genome structure in an additional 17 strains from the *B. plicatilis* species complex, indicating that it is relatively stable. Our results suggest that sequencing additional gnathiferan mitochondrial genomes may provide important information about an unexpected alternative to the traditional organization of the metazoan mitochondrial genome.

Materials and Methods

Rotifers and Rotifer Culturing

Brachionus plicatilis (Müller 1786) is a cryptic species complex of at least a dozen species, 3 of which have been formally described (Ciros-Perez et al. 2001; Suatoni et al. 2006; Fontaneto et al. 2007). The complex has been divided into 3 major divisions, L, S, and SS, based on allozyme variation (Fu et al. 1991). In this study, we examined 6 strains from each division: *B. plicatilis sensu strictu* strains NH1L (Japan), j-MS (Japan), sp-VG (Spain), c-GC2 (Cayman Islands), a-AU (Australia), and *Brachionus manjavacas* (Fontaneto 2007) strain RUS (Russia) from the L division; strains c-GC10 (Cayman Islands), y-YS (Yugoslavia), p-OT (Philippines), s-SI (Singapore), us-WSP (United States), and *Brachionus ibericus* (Ciros-Pérez 2001) strain sp-KC (Spain) from the S division; and *Brachionus rotundiformis* (Tschugunoff 1921) strain m-LK (Malaysia) and related strains j-JS, j-OK and j-KJ (Japan), i-IN (Indonesia), and a-ML (Australia) from the SS division. We sequenced the complete mitochondrial genome from *B. plicatilis sensu strictu* NH1L, which originated from an outdoor eel culture pond in Mie Prefecture (Hagiwara et al. 1988) and has been clonally cultured at Nagasaki University for more than 15 years.

Strains were cultured as previously described (Suga, Mark Welch, et al. 2007) in a Jarfermentor (MBS, Yamaguchi, Japan) containing diluted sterile artificial seawater (SAS; 17 ppt) at 25 °C, and fed 3–7 $\times$ 10^6 cells/ml *Chlorella vulgaris*, which had been cultured in bacteria-free medium, harvested by centrifugation, and suspended in SAS to a density of 8.6 $\times$ 10^9 cells/ml supplemented with 800 ng/ml vitamin B12 (which is essential for rotifer growth) prior to introduction to the rotifer culture.

Rotifer Harvesting and Isolation of Total DNA

Rotifer cultures were harvested by filtration using a plankton net (45 micron mesh) until 150 mg (wet weight) of rotifer was obtained. The rotifers were washed with SAS and kept in 400 ml SAS for 24 h in order to allow them to excrete any remaining algae from their guts. Starved rotifers were washed with sterilized MilliQ water and collected by centrifugation (20,000 $\times$ g for 1 min at 4 °C). Total DNA was prepared as described previously (Suga, Tanaka, et al. 2007), essentially using Proteinase K digestion and phenol–chloroform extraction from harvested rotifers.

Amplification and Sequencing of Mitochondrial DNA

Mitochondrial DNA was obtained from a series of polymerase chain reactions (PCRs) using primers listed in supplementary table S1 (Supplementary Material online). First, approximately 20 ng of total DNA was used as template in a PCR with primers rrnL-for and rrnL-rev, which were designed to the large subunit rRNA gene (*rrnL*) sequence previously obtained from a cDNA library (Suga, Mark Welch, et al. 2007). Amplification was performed in 25-μl volumes containing 0.2 mM of primers; 0.2 mM deoxynucleoside triphosphates; 120 mM Tris–HCl pH 8.0; 10 mM KCl; 6 mM $(NH_4)_2SO_4$; 0.1% Triton X-100; 0.001% bovine serum albumin; and 0.25 unit KOD Dash DNA polymerase (Toyobo, Osaka, Japan), using a Px2 Thermal Cycler (Hybaid, Ashford, UK) at an initial denaturation of 95 °C for 2 min followed by 35 cycles of 98 °C for 10 s and a 15 min annealing/extension step, beginning at 73 °C and decreasing at 0.2 °C per cycle. The approximately 11-kb amplification product was digested with *Bgl*II and digested DNA fragments were fractionated on 1.2% agarose gel. An approximately 1.5-kbp fragment was purified and ligated into *Bam*HI-digested pBluescript SK(−) plasmid vector. The recombinant plasmid was sequenced with M13 forward and reverse primers using ABI Big Dye 1.1 chemistry and separated on a ABI Prism 310 genetic analyzer and found to contain the small subunit rRNA gene (*rrnS*). The primer pairs (rrnS-for + rrnS-rev) and (rrnL-for + rrnL-rev) were then used to produce approximately 4-kbp and 8-kbp amplification products, respectively. These were ligated into pCR-XL TOPO (Invitrogen, Carlsbad, CA) and cloned, and 2 plasmid inserts from each size amplicon were sequenced by primer walking. As the resulting sequences lacked many mitochondrial genes, including those encoding the cytochrome *c* oxidase subunits, we designed primers to *cox1* and *cox3* and used them in amplification reactions as described above. The primer pairs (cox1-for + cox3-rev) and (cox3-for + cox1-rev) produced amplification products of approximately 5.5 and 6.5 kbp, respectively, which were then sequenced by primer walking.

Total DNA isolated from each of the 18 strains was amplified using the primer pairs (rrnL-for + rrnL-rev) and (cox1-for + cox1-rev) using the procedure described above.

Sequence Analysis and Annotation of Mitochondrial DNA

The locations of protein-coding and rRNA genes were determined by BLAST searches to National Center for Biotechnology Information (NCBI) databases; a set of 1,281 complete, curated metazoan mitochondrial genomes; and a databases of *B. plicatilis* cDNA (Suga, Mark Welch, et al. 2007). Most tRNA genes were identified using tRNAscan-SE (Lowe and Eddy 1997); remaining tRNA genes were identified by manual inspection for anticodon sequences and secondary structure (Kumazawa and Nishida 1993). The EMBOSS suit of programs was used to translate genes, determine codon usage, identify repeat regions and similarity between contigs, and perform other standard analysis tasks. The noncoding region (NCR) was examined for

secondary structure using MFOLD with default parameter values (Zuker 2003).

Purification of Mitochondria and Isolation of Mitochondrial DNA

All steps of the purification of mitochondria were carried out at 4 °C. Harvested *B. plicatilis* NH1L rotifers (700 mg wet weight) were homogenized using a DIAX 100 homogenizer (Heidolph, Schwabach, Germany), and the homogenate was centrifuged (1,000 × g for 10 min at 4 °C) to separate the cell mixture from extracellular debris such as the mastax. Mitochondria were isolated from the cell mixture using a Qproteome mitochondria isolation kit for eukaryotic cells (Qiagen, Hilden, Germany), following the manufacturer's protocol, except that after resuspension in disruption buffer the lysate was passed through a 45 micron mesh to remove large, low-density cellular debris such as the intracytoplasmic lamina. The total cytochrome c oxidase activity of each fraction was assayed using cytochrome c and n-dodecyl β-D-maltoside (final concentration 1 mM) according to the instructions provided by Sigma-Aldrich, St Louis, MO. Estimated protein concentrations were measured by DC protein assay (Bio-Rad, Hercules, CA) using bovine gamma globulin as a standard. DNA was isolated from purified mitochondria using a mtDNA extractor CT kit (Wako Pure Chemical, Osaka, Japan).

Southern Blots of Purified and Amplified Mitochondrial DNA

Mitochondria were isolated from harvested *B. plicatilis* NH1L (200 mg wet weight) as described above and purified mitochondrial DNA treated with DNase free ribonuclease A (Wako Pure Chemical). For each digest, ~10 ng of mitochondrial DNA was digested in a 50-μl reaction volume containing 1× reaction buffer and 2 units of *Sal*I, *Hin*dIII, *Eco*RI, *Not*I, or *Xho*I (Fermentas, Berlington, ON) at 37 °C for 3 h. Each digestion reaction was then split into 2 equal fractions and separated on two 0.5% Seakem GTG agarose gels (Cambrex, East Rutherford, NJ), followed by SYBR Green I (Cambrex) staining according to the supplier's instructions. The gels were then blotted onto Amersham Hybond N^+ nylon membranes following the supplier's protocol (GE healthcare, Chalfont St Giles, UK). Amplification products from PCR using (rrnL-for + rrnL-rev) and (cox1-for + cox1-rev) for each of the 18 strains were similarly gel fractionated and transferred to membranes. Regions of *cob* and *nad4* were amplified (supplementary table S1, Supplementary Material online) from NH1L and labeled with DIG-11-dUTP and used as probes according to the DIG Easy Hyb protocol (Roche, Indianapolis, IN). Hybridization was detected using the DIG Nucleic Acid Detection Kit (Roche).

Dot Blots of Purified Mitochondrial DNA

The concentration of 3 independent mitochondrial DNA preparations and of plasmids consisting of pCR-XL ligated to either a (rrnL-for + rrnS-rev) amplicon (which included *rrnL*, *cob*, NCR, and *rrnS* of mtDNA-I) or a (cox1-for + cox3-rev) amplicon (which included *cox1*, *nad6*, *nad4*, *nad4L*, *nad5*, *cox2*, and *cox3* of mtDNA-II) was determined by optical density. Five dilutions of each plasmid and 3 dilutions of each mtDNA preparation were made, and 7.5 ul of each mtDNA dilution and 8 ul of each plasmid dilution were immobilized on 2 Hybond N^+ membranes following the supplier's protocols (GE Healthcare). The *cob* and *nad4* PCR products (supplementary table S1, Supplementary Material online) were labeled using DIG-11-dUTP and used as probes to one or the other membrane, as described above. Hybridization was detected by CDP-*Star* (GE Healthcare), and the blot was scanned with the FluorChemR Imaging System (Alpha Innotech, San Laredo, CA) and analyzed with Quantity One software (Bio-Rad). The relationship of hybridization intensity to plasmid copy number was used to create standard curves to determine the number of molecules of each mtDNA in the mitochondrial DNA based on intensity of hybridization to each probe as previously described (Mark Welch and Meselson 1998).

Results

Structure, Organization, and Nucleotide Composition of the *B. plicatilis* Mitochondrial Genome

When we assembled sequences derived from PCR of *B. plicatilis* NH1L mitochondrial DNA, we obtained 2 circular contigs, designated mtDNA-I (11,153 bp, DNA Data Bank of Japan [DDBJ]/EMBL/GenBank accession number AP009407) and mtDNA-II (12,672 bp, DDBJ/EMBL/GenBank accession number AP009408). We identified genes for 4 proteins: ATP6, COB, NAD1, and NAD2; 13 tRNAs: tRNA(D), tRNA(E), tRNA(G), tRNA(H), tRNA(L-cun), tRNA(M), tRNA(P), tRNA(Q), tRNA(S-agn), tRNA(T), tRNA(V), tRNA(W), and tRNA(Y); and 2 rRNAs: rRNA(L) and rRNA(S), on mtDNA-I, and the genes for 8 proteins: COX1–3, NAD3–6, and NAD4L; and 9 tRNAs: tRNA(A), tRNA(C), tRNA(F), tRNA(I), tRNA(K), tRNA(L-uur), tRNA(N), tRNA(R), and tRNA(S-ucn), on mtDNA-II (fig. 1). There is a 4.9-kb region of 99.5% identity between the 2 contigs, which contains the tRNA(L) genes and a large non-coding region. For each contig, all genes would be transcribed from the same DNA strand, and the shared region is in the same orientation relative to the direction of transcription in both contigs. There is little or no intergenic space between genes and no overlap of genes. As shown in table 1, the contigs are A + T rich, and the transcribed strands are slightly lighter (55.2% and 56.7% pyrimidine) than the untranscribed strands. All regions of the transcribed strands are very G poor and T rich, a phenomenon that is most apparent at 4-fold degenerate codon positions.

We assessed the possibility that the 2 contigs represent a misassembly of a single circular contig or that there are additional mitochondrial DNAs by Southern hybridization to DNA extracted from mitochondria. Isolated mitochondrial DNA from strain NH1L was digested with *Sal*I, *Hin*dIII, *Eco*RI, *Not*I, and *Xho*I and probed with a PCR fragment of a gene found on mtDNA-I (*cob*) and with a PCR fragment of a gene found on mtDNA-II (*nad4*).

Table 1
Nucleotide Composition in *Brachionus plicatilis* Mitochondrial DNA

	Region	A%	C%	G%	T%	AT%	CT%
mtDNA-I	protein coding	23.3	21.8	14.1	40.8	64.2	62.6
	4-fold	24.6	23.0	9.8	42.6	67.2	65.6
	rRNA	33.4	14.4	16.4	35.8	69.2	50.2
	tRNA	33.4	13.5	15.8	37.3	70.7	50.8
	non-NCR	27.6	18.7	15.0	38.7	66.3	57.4
	Full length	28.6	19.9	16.2	35.2	63.9	55.1
mtDNA-II	protein coding	24.0	20.8	15.7	39.5	63.5	60.3
	4-fold	24.7	21.0	13.0	41.3	66.0	62.3
	tRNA	36.0	13.2	15.1	35.7	71.7	48.9
	Full length	26.9	20.7	16.4	36.0	62.9	56.7
	Non-NCR	25.1	20.1	15.6	39.2	64.3	59.3
Common	NCR	30.0	21.5	17.8	30.7	60.7	52.2

As illustrated in figure 1, mtDNA-I should not be digested with *Not*I or *Xho*I, should be linearlized with *Sal*I and *Hin*dIII, and should be digested into fragments of 97, 1,486, and 9,570 bp with *Eco*RI; mtDNA-II should not be digested with *Eco*RI, should be linearized with *Sal*I, *Not*I, and *Xho*I, and should be digested to fragments of 2,299 and 10,373 bp with *Hin*dIII. As seen in figure 2, hybridization of mtDNA-I probe to *Not*I and *Xho*I digests produced the same 2-band pattern as hybridization to undigested DNA, and hybridization of the mtDNA-II probe produced a similar but slightly higher 2-band pattern in the *Eco*RI digest and undigested DNA. Thus, the 4 bands visible in the undigested lane of the stained gel (fig. 2*A*) correspond to relaxed and supercoiled forms of the 2 circles. The probe to mtDNA-I hybridized to a ~11-kb band in the *Sal*I and *Hin*dIII digests, as expected for a linearized 11,153-bp circle. The probe to mtDNA-II hybridized to a ~12.5-kb band in the *Sal*I, *Not*I, and *Xho*I digests, as expected for a linearized 12,672-bp circle, and to a ~10.3-kb band in the *Hin*dIII digest, as expected for a 10,373-bp fragment containing the *nad4* probe region. Multiple digest reactions under a variety of DNA and enzyme concentrations using 6 different purifications of mtDNA consistently produced the same pattern of complete digestion using *Hin*dIII, *Not*I, and *Xho*I and incomplete digestion using *Sal*I and *Eco*RI. This could indicate sequence polymorphism in the mtDNA pool, although there was no evidence of this from examination of chromatograms of the sequencing reactions. It is more likely indicative of cytosine methylation of the *B. plicatilis* mitochondrial genome as *Eco*RI and *Sal*I are both impaired by overlapping CpG methylation. The evidence suggests that not all CpG sites are methylated, as *Not*I is blocked by CpG methylation but produced a complete digest, and that not all methylation sites are always methylated, as *Eco*RI and *Sal*I produced partial digests. In any case, there were no bands visible in the gel or on the Southerns

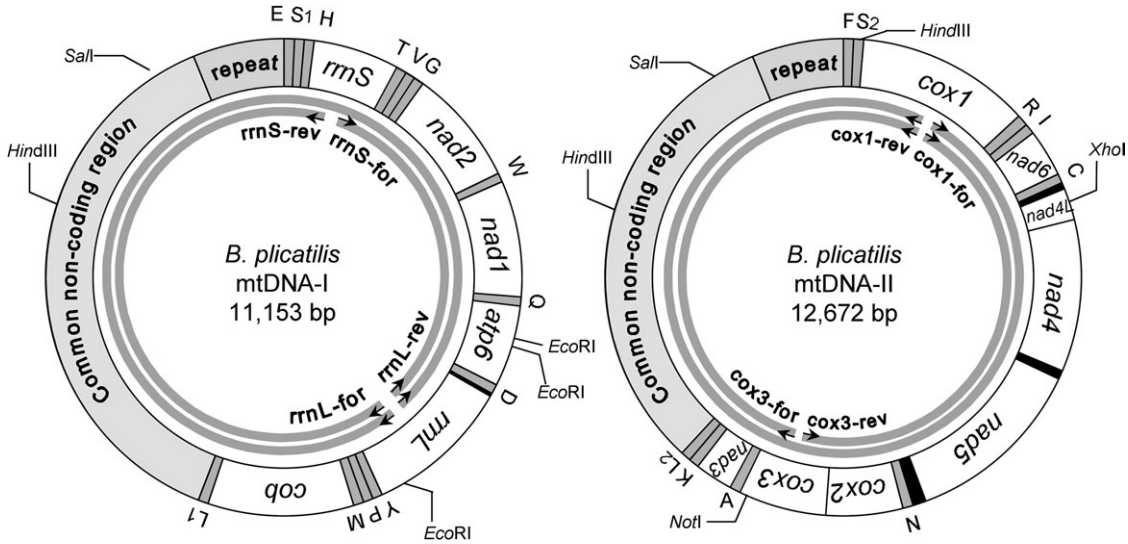

FIG. 1.—Maps of the 2 mitochondrial chromosomes in the rotifer *Brachionus plicatilis*. All genes are transcribed from the same DNA strand, clockwise as these maps are oriented. Protein-coding genes and rRNA genes are indicated in white and are designated by standard nomenclature; tRNAs are indicated in gray and are identified by 1-letter code for the corresponding amino acid except the 2 serine and 2 leucine tRNAs: S1, S2, L1, and L2 are *trnS(agn)*, *trnS(ucn)*, *trnL(cun)*, and *trnL(uur)*, respectively. The common NCR is indicated in light gray, with the repeat region indicated by lateral stripes; other NCRs are indicated in black. Sites for restriction enzymes used for Southern blot analysis in figure 2, *Eco*RI, *Hin*dIII, *Not*I, *Sal*I, and *Xho*I are shown. Arrows and gray curves within each circle indicate primers and amplicons, respectively, used for sequencing. Scaling is approximate.

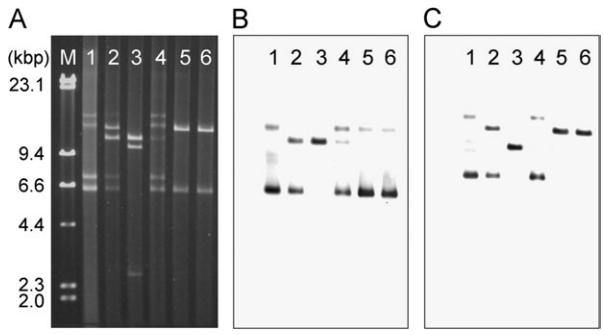

FIG. 2.—Restriction analysis of mitochondrial DNA of *Brachionus plicatilis*. (*A*) Ethidium bromide–stained agarose gel, showing Lambda DNA/*Hin*dIII marker (M), mitochondrial DNA from *B. plicatilis* that was undigested (1) or digested with *Sal*I (2), *Hin*dIII (3), *Eco*RI (4), *Not*I (5), or *Xho*I (6). (*B*) Southern blot of the gel probed with NH1L-*cob* PCR product. (*C*) Southern blot of a second gel containing the second half of the same digests, probed with NH1L-*nad4* PCR product.

that were not accounted for by the digest patterns predicted for mtDNA-I and mtDNA-II.

Codon Usage

All identified genes can be translated without interruption using the standard invertebrate mitochondrial code. Nine of the 12 protein-coding genes begin with the orthodox initiation codon ATG (*cox1*, *cox2*, *cob*, *nad1*, *nad2*, *nad5*, *nad6*, *nad4L*, and *atp6*), and 2 genes are initiated by ATA (*nad4* and *cox3*). One gene, *nad3*, was determined to begin GTG because 1) there is no in-frame upstream ATR codon before a stop codon, 2) this codon position matches the first codon position of most other metazoan *nad3* sequences, and 3) it is the first 3 bases after the preceding gene. Eight protein-coding genes terminate with the complete stop codons TAA (*cox1*, *cox2*, *cob*, *nad4*, *nad5*, *nad6*, and *nad4L*) or TAG (*cox3*). The other 4 genes have incomplete termination codons: TA- (*nad2*) and T– (*nad1*, *nad3*, and *atp6*), which could be completed to TAA by post-transcriptional polyadenylation (Ojala et al. 1981).

We investigated codon usage on each mtDNA to determine if this could account for the higher copy number of mtDNA-I. Codon usage in mtDNA-I and mtDNA-II is similar and overall codon usage is not biased (the effective number of codons, N_c [Wright 1990; Fuglsang 2006], is 50 for each mtDNA). The most common amino acids encoded by the *B. plicatilis* mitochondrial genes are leucine (17%) and serine (11%), which are also the only amino acids with 2 tRNAs. Both have a tRNA gene on each contig: the *tRNA(L)* and *tRNA(S)* on mtDNA-I recognize 58% and 11.5% of the total leucine and serine codons, respectively, in the *B. plicatilis* mitochondrial genome.

In total, the tRNA genes on mtDNA-I recognize 55% of the codons in the mitochondrial genome and 52% of the codons on mtDNA-I. In both mtDNAs, T is strongly favored over G at third codon positions, with the exception of those codons ending −AG (supplementary table S2, Supplementary Material online). The bias against G extends to the 6 possible codons for serine, where TC[ACT] is strongly favored over AGY and TCG, and to the use of TAG as a stop codon by only 1 protein-coding gene.

The tRNAs and rRNAs

Nineteen of 22 tRNA genes were detected by tRNAscan-SE, with cove scores of 9.86–32.47. The remaining 3, *trnS(agn)*, *trnS(ucn)*, and *trnR*, were located manually (Kumazawa and Nishida 1993). Unlike many metazoan genomes which have clusters of 5–7 tRNA genes, the tRNA genes of the *B. plicatilis* mitochondrion are dispersed, with no clusters larger than 3. The tRNA genes range in size from 61 to 69 nt, with and average A + T content of 71.2%, which is higher than the overall A + T composition of the mtDNAs (table 1). In some metazoans, including the acanthocephalan rotifer *L. thecatus*, some mitochondrial tRNA genes have lost either T or D arms or have other unusual features (Steinauer et al. 2005). However, the tRNAs of *B. plicatilis* have almost complete cloverleaf structures.

The genes for the large and small ribosomal subunits, 1,107 bp and 716 bp, respectively, are found on mtDNA-I. The full length of each gene was estimated by comparison to curated metazoan mitochondrial genomes in the NCBI RefSeq database. In addition, the 5′ end of the large subunit rRNA gene was known from rRNA contamination of a cDNA library (Suga, Mark Welch, et al. 2007). No intergenic space is predicted in the *trnH–rrnS–trnT* region, and no intergenic space is predicted between *rrnL* and *trnY*.

The Common Non-Coding Region

Both contigs have a 4.9-kb region that is 99.5% identical between them. Following the orientation of transcription, this region begins with 3 bases followed by a gene encoding tRNA(L), *trnL(cun)* in mtDNA-I and *trnL(uur)* in mtDNA-II. The 2 genes differ at only 3 positions, one of which is in the anticodon and confers their respective specificities. The remainder of the common region contains no open reading frames of significant length, or with significant similarity to any sequence in GenBank non-redundant databases, *B. plicatilis* cDNA databases, or a database of 1,281 complete mitochondrial genomes, and is therefore considered noncoding. This is the only NCR of significant length in either contig. The common NCR ends with perfect repeats of a 168-base motif, beginning with positions 71–168 of the motif followed by 3 cytosines then 3 complete motifs followed by positions 1–52 of the motif; in mtDNA-I, this partial repeat extends for an additional 44 bases beyond the region shared by the 2 mtDNAs. There are about 10 bases between the final motif and a tRNA gene in each mtDNA, with no discernable homology. Scanning the NCR with MFOLD predicted extensive secondary structure in the form of 3 large fold-back domains of 240–560 nt. Major features of this region are summarized in supplementary figure 1 (Supplementary Material online).

The base composition of the NCR is significantly different than the base composition of either mtDNAs outside the NCR (χ^2 $P \ll 0.01$), due to a decrease in the amount of T in favor of all 3 other bases. There are regions at the beginning and end of the NCR where the proportion of cytosine exceeds 60%, generally at the expense of guanine. In particular, there are multiple stretches in the last approximately 700 bases of the region that are 70–80% cytosine and

Table 2
Purification of Mitochondria from *Brachionus plicatilis*

Extract or Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Activity Yield (%)	Purification (fold)
Homogenized rotifer	1.055	0.076	0.072		
Cell mixture	0.525	0.086	0.164	113	2.3
Organelle mixture	0.319	0.094	0.294	124	4.1
Cytosolic proteins	0.246	0.042	0.169	55	
Mitochondria + microsomal fraction	0.181	0.074	0.408	97	5.7
Microsomal fraction	0.083	0.026	0.313	34	
Mitochondrial fraction	0.043	0.050	1.168	66	16

0–10% guanine. Several hundred bases before this terminal C-rich region, there is an AT repeat of 9 units in mtDNA-I and 7 units in mtDNA-II, similar to the AT repeats that may play a role in transcriptional regulation in other mitochondrial genomes (Jacobs et al. 1988; Cao et al. 2004; Scouras and Smith 2006).

Relative Copy Number of mtDNA-I and mtDNA-II

The cytochrome oxidase activity of each fraction obtained during mitochondrial purification is shown in table 2. The final mitochondrial fraction was a pale yellow color similar to the mitochondria purified from cells of other metazoans and represented a 16-fold purification of cytochrome oxidase activity. The cytosolic protein and microsomal fractions had some cytochrome c oxidase activity, indicating that some of the mitochondria were fragmented during purification. Hybridization of dot blots of purified mitochondrial DNA with probes to mtDNA-I or mtDNA-II showed a molar ratio of mtDNA-I to mtDNA-II of 3.96 with a standard deviation (SD) of 0.88 (table 3).

Conservation of Mitochondrial Genome Structure in the *B. plicatilis* Species Complex

To determine if the structure of the mitochondrial genome was conserved across species of the *B. plicatilis* complex, we amplified total DNA from 18 strains of *Brachionus*, representing each of the major 3 divisions of the complex, each of the 4 named species, and at least 10 phylotypes. We used inverted primers designed to hybridize to strain NH1L *rrnL* (found on mtDNA-I in NH1L) and to hybridize to strain NH1L *cox1* (found on mtDNA-II in NH1L). Amplification with any combination of *rrnL* + *cox1* primers was never successful. However, all amplifications with *rrnL* inverted primer pairs or with *cox1* inverted primer pairs were successful and produced products of similar size in all 18 strains (although there may be some variation in the *cox1* amplicon lengths; fig. 3*A*). This indicates that all strains have 2 circular mitochondrial chromosomes, 1 with *rrnL* and 1 with *cox1*.

We then probed a Southern blot of the amplicons with NH1L-*cob*, found on mtDNA-I in NH1L, and with NH1L-*nad4*, found on mtDNA-II in NH1L. Hybridization of NH1L-*cob* to all *rrnL* amplicons was easily detected, indicating that *cob* and *rrnL* are on the same circle in all strains. No hybridization of NH1L-*cob* to *cox1* amplicons was visible (fig. 3*B*). When hybridization with NH1L-*nad4* was performed at the same temperature as the NH1L-*cob* hybridization, 42 °C, only the *cox1* amplicons from the strains belonging to the same major division as NH1L ("L type") were detected (data not shown). At 30 °C, hybridization of NH1L-*nad4* to the *cox1* amplicons of all L-type and SS-type strains could be detected, and there was no hybridization to the *rrnL* amplicons visible for any strain (fig. 3*B*).

Discussion

We sequenced cloned PCR amplicons from mitochondrial DNA of the monogonont *B. plicatilis sensu strictu* NH1L and found that all sequences could be assembled into 2 circular contigs. These can encode the large and small subunit rRNAs, the 22 tRNAs sufficient to translate the mitochondrial protein code, and the core 12 metazoan mitochondrial proteins, a complement similar to the mitochondrial genome of many other metazoans. Restriction digests of DNA isolated from *B. plicatilis* mitochondria, combined with hybridization to Southern blots of these digests, suggest that there is no other major mitochondrial DNA molecule in the *B. plicatilis* mitochondrion and that both mtDNA-I and mtDNA-II are partially methylated. Hybridization to Southern blots of PCR amplification products from a total of 18 strains of the *B. plicatilis* species complex indicates that the sizes and gene content of these 2 contigs are relatively constant in the complex. Based on nucleotide divergence at *cox1*, the *B. plicatilis* group is millions of years old and the 18 strains from each of the major divisions represent tens of millions of years of independent evolution (Gomez et al. 2002; Suatoni et al. 2006). We conclude that the mitochondrial genome of *Brachionus* is composed of 2 circular chromosomes, mtDNA-I and mtDNA-II, that these chromosomes are relatively stable and that there is no other major DNA present, such as a master circle. Furthermore, dot blot analysis shows that, in *B. plicatilis sensu strictu* NH1L, mtDNA-I is present at 4 times the copy number of mtDNA-II.

The organization of the mitochondrial genome into 2 circular chromosomes of unequal copy number has not been reported for any other organism. The metazoan mitochondrial genome is generally a single circular molecule, ranging in size from 14 to 17 kbp, encoding the large and small subunit rRNAs, 22 tRNAs, 12 or 13 proteins (COX1–3, NAD1–6 plus 4L, COB, and ATP6, with or

Table 3
Dot Blot Analysis of mtDNA-I and mtDNA-II

mtDNA Isolation	Molecular Number ($\times 10^6$)		
	mtDNA-I[a]	mtDNA-II[b]	mtDNA-I/mtDNA-II
1	4.69 ± 1.41	0.99 ± 0.31	4.74
2	6.85 ± 1.40	1.66 ± 0.57	4.13
3	4.44 ± 1.27	1.47 ± 0.29	3.02

[a] Given pCR-XL-*rrnL–rrnS* of 10,760 bp and mtDNA-I of 11,153 bp.
[b] Given pCR-XL-*cox1–cox3* of 9,379 bp and mtDNA-II of 12,672 bp.

without ATP8), and a NCR containing controlling elements for replication and transcription (Wolstenholme 1992). The only known cases of multiple mitochondrial chromosomes in bilaterian metazoans are in the nematodes *Globodera pallida* and *Globodera rostochiensis*, each with 6 small circular mtDNAs sharing varying amounts of sequence identity and which probably arose via recombination (Armstrong et al. 2000; Gibson, Blok, and Dowton 2007; Gibson, Blok, Phillips, et al. 2007), and in the mesozoan *Dicyema japonicum*, which has single gene minicircles that are believed to be derived from master circles found in germ cells (Watanabe et al. 1999; Awata et al. 2005). There is no evidence from Southern hybridization of mitochondrial gene probes to mitochondrial DNA of a master circle in *Brachionus*.

Whether the 4:1 ratio of the 2 mitochondrial chromosomes in *B. plicatilis* NH1L is constant in all mitochondria over all stages of development or represents an average value is not known. The advantage, if any, of this unequal ratio is not immediately obvious. The 2 rRNA genes are located on mtDNA-I, enabling equal and high levels of expression for these genes, and the 3 cytochrome *c* oxidase subunit genes are on mtDNA-II. However, the subunits of NADH dehydrogenase, which are each present once per holoenzyme (Sazanov and Hinchliffe 2006), will be produced in unequal number unless some sort of regulatory mechanism is present. Examining codon usage and amino acid content of translated genes reveals no need to have the tRNAs of mtDNA-I overexpressed compared with those of mt-DNA-II (supplementary table S2, Supplementary Material online).

The evolution and maintenance of the 2-chromosome genome structure may be related to the 4.9-kb region of near identity shared by the 2 chromosomes. An obvious hypothesis is that the shared region is kept nearly identical by gene conversion. However, one terminus of the region contains a gene for tRNA(L), *trnL(cun)* on mtDNA-I and *trnL(uur)* on mtDNA-II. These 2 genes differ by only 3 dispersed nucleotides, unlikely to be sufficient to stop a gene conversion tract. Thus, gene conversion between the 2 chromosomes in this region could result in the loss of 1 or the other *trnL*, yet both appear to be needed to translate the *B. plicatilis* mitochondrial transcriptome (supplementary table S2, Supplementary Material online). Other than *trnL*, this region is noncoding and shows no similarity to any of the 1,281 completely sequenced metazoan mitochondrial genomes available from NCBI. However, this NCR contains some structural features common to the D-loop or control region, where replication originates in other metazoan mitochondria. Many arthropod D-loop regions have terminal repeats (Zhang and Hewitt 1997), and 20 nt strings of very high GC are found in both sea urchins (GenBank accession numbers NC_001453, NC_001572, and NC_001770) and mussels (GenBank accession numbers NC_006161, NC_006886, and NC_007687), which may play a role in formation of the D-loop structure (Cao et al. 2004). The putative control region of the mussels *Mytilus* spp. has an internal stem-loop structure similar to one found in the central part of the human control region (Cao et al. 2004), although both are less extensive than the stem-loop structures predicted in the *B. plicatilis* NCR. Many control regions contain a motif of TA or AT repeats, which may play a role in transcriptional regulation (Jacobs et al. 1988; Cao et al. 2004; Scouras and Smith 2006). If the AT repeats in *B. plicatilis* have a similar role, the difference in the number of repeats in the 2 chromosomes could provide a means of regulation compensating for the dosage difference that would otherwise ensue from the 4:1 copy number ratio. Finally, given that both chromosomes would require a control region and that

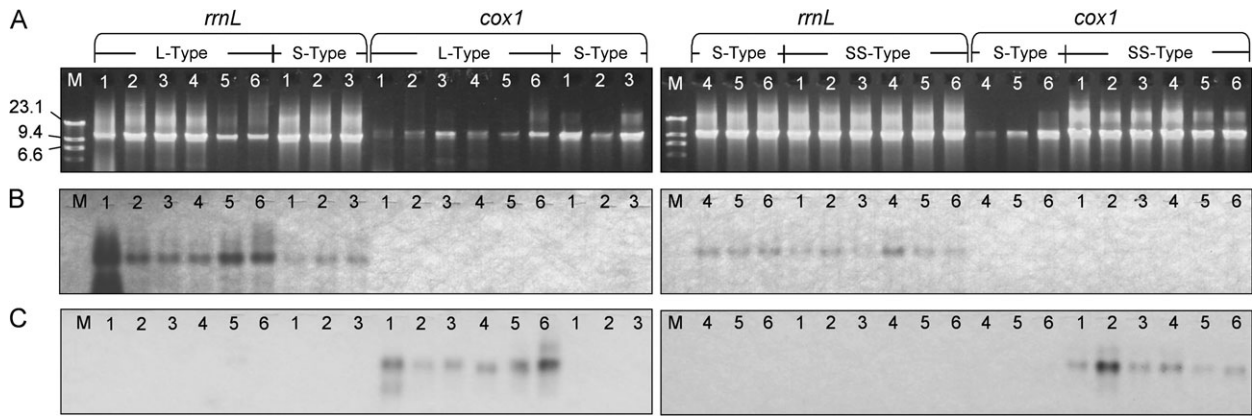

FIG. 3.—Conservation of mitochondrial genome structure. (*A*) Agarose gel electrophoresis of total DNA from 18 different *Brachionus* strains amplified with inverted primers to *rrnL* or *cox1*, as indicated above the gel. M, lambda DNA/*Hin*dIII marker, the size of bands in kilobase are indicated; L1, NH1L; L2, j-MS; L3, RUS; L4, a-AU; L5, sp-VG; L6, c-GC2; S1, y-YS; S2, p-OT; S3, s-SI; S4, sp-KC; S5, us-WSP; S6, c-GC10; SS1, j-JS; SS2, j-OK; SS3, j-KJ; SS4, i-IN; SS5, m-LK; and SS6, a-ML. (*B*) A Southern blot of the gel probed with a *cob* PCR product. (*C*) A Southern blot of an identical gel probed with an *nad4* PCR product.

the NCR is the only region they have in common other than a tRNA gene, it is likely that the NCR is, or contains, the D-loop and control regions. The NCR, at 4,854 bp in mtDNA-I and 4,850 in mtDNA-II, is much larger than annotated D-loop regions for most metazoans. Among 565 annotated mitochondrial D-loops in GenBank, the average length was 1,110 (SD 579); only amphibians had D-loops of similar length to the NCR in *B. plicatilis*.

Several diverse species are known to have had a duplication of mitochondrial DNA resulting in 2 copies of the D-loop region on a single circular chromosome (Arndt and Smith 1998; Kumazawa et al. 1998; Jeyaprakash and Hoy 2007). This leads to a model for the creation of the *Brachionus* mitochondrial genome structure: a duplication of the NCR/putative D-loop, perhaps mediated by homology between the 2 *trnL* genes, resulted in 2 D-loop regions on a single chromosome and intramolecular crossing-over with proper resolution of the Holliday junction gave rise to 2 chromosomes, each with a complete D-loop.

The hybridization intensities of NH1L probes to mitochondrial chromosomes of other *Brachionus* strains were broadly consistent with current ideas about the phylogeny of the group. The NH1L-*cob* probe bound about equally well to all strains from the L division and showed less affinity for the strains from the S and SS divisions. The NH1L-*nad4* probe showed variable hybridization even within the L division, weak hybridization to the SS division, and no hybridization to the S division, suggesting that *nad4* is more rapidly evolving than *cob* and that the SS division may be more closely related to the L division than previously thought. This demonstrates the potential utility of using additional regions of the mitochondrial genome, including entire chromosomes, for phylogenetic analysis of this cryptic species group.

The only other rotiferan mitochondrial genome in GenBank is the acanthocephalan *L. thecatus*, which has a single circular chromosome with noncanonical tRNA genes, no obvious D-loop region, and 2 possible but unorthodox copies of *atp8* (Steinauer et al. 2005). There is no conservation of gene order between *L. thecatus* and *B. plicatilis* or between either species and other sequenced mitochondrial genomes. The lack of similarity to Lophotrochozoan mitochondria and the unusual features of both acanthocephalan and monogonont genomes suggest that Gnathifera may be a sister group to Lophotrochozoa or even more basal. However, the mitochondria of additional taxa from this poorly studied group will need to be further sampled in order to understand these unusual mitochondrial features as well as the early events in the evolution of Metazoa.

Supplementary Material

Supplementary figure 1 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We thank Jessica Mark Welch, Richard Thomas, and 2 anonymous reviewers for helpful comments that improved the manuscript. This research was financially supported in part by the Ministry of Education, Science, Sports, and Culture, Grant-in-Aid for Scientific Research (C), 2004–2005, No. 16580153, Grant-in-Aid for Scientific Research (B), 2006–2008, No. 18380118, the Nagasaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, Japan Science and Technology, and United States National Science Foundation grant EF-0412674.

Literature Cited

Armstrong MR, Blok VC, Phillips MS. 2000. A multipartite mitochondrial genome in the potato cyst nematode *Globodera pallida*. Genetics. 154:181–192.

Arndt A, Smith MJ. 1998. Mitochondrial gene rearrangement in the sea cucumber genus *Cucumaria*. Mol Biol Evol. 15:1009–1016.

Awata H, Noto T, Endoh H. 2005. Differentiation of somatic mitochondria and the structural changes in mtDNA during development of the dicyemid *Dicyema japonicum* (Mesozoa). Mol Genet Genomics. 273:441–449.

Boore JL, Brown WM. 1998. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev. 8:668–674.

Cao L, Kenchington E, Zouros E, Rodakis GC. 2004. Evidence that the large noncoding sequence is the main control region of maternally and paternally transmitted mitochondrial genomes of the marine mussel (*Mytilus* spp.). Genetics. 167:835–850.

Ciros-Perez J, Gomez A, Serra M. 2001. On the taxonomy of three sympatric sibling species of the *Brachionus plicatilis* (Rotifera) complex from Spain, with the description of *B. ibiricus* n. sp. J Plankton Res. 23:1311–1328.

De Meester L, Gomez A, Simon JC. 2004. Evolutionary and ecological genetics of cyclical parthenogens. In: Moya A, Font E, editors. Evolution: from molecules to ecosystems. Oxford, UK: Oxford University Press. p. 122–134.

Fontaneto D, Giordani I, Melone G, Serra M. 2007. Disentangling the morphological stasis in two rotifer species of the *Brachionus plicatilis* species complex. Hydrobiologia. 583:297–307.

Fu Y, Hirayama K, Natsukari Y. 1991. Morphological differences between two types of the rotifer *Brachionus plicatilis* O.F. Müller. J Exp Mar Biol Ecol. 151:29–41.

Fuglsang A. 2006. Estimating the "effective number of codons": the Wright way of determining codon homozygosity leads to superior estimates. Genetics. 172:1301–1307.

Funch P, Sørensen MV, Obst M. 2005. On the phylogenetic position of Rotifera—have we come any further? Hydrobiologia. 546:11–28.

Garey JR, Near TJ, Nonnemacher MR, Nadler SA. 1996. Molecular evidence for Acanthocephala as a subtaxon of Rotifera. J Mol Evol. 43:287–292.

Gibson T, Blok VC, Dowton M. 2007. Sequence and characterization of six mitochondrial subgenomes from *Globodera rostochiensis*: multipartite structure is conserved among close nematode relatives. J Mol Evol. 65:308–315.

Gibson T, Blok VC, Phillips MM, Hong G, Kumarasinghe D, Riley IT, Dowton M. 2007. The mitochondrial subgenomes of the nematode *Globodera pallida* are mosaics: evidence of recombination in an animal mitochondrial genome. J Mol Evol. 64:463–471.

Giribet G, Distel DL, Polz M, Sterrer W, Wheeler WC. 2000. Triploblastic relationships with emphasis on the acoelomates and the position of Gnathostomulida, Cycliophora, Platyhelminthes, and Chaetognatha: a combined approach of 18S rDNA sequences and morphology. Syst Biol. 49:539–562.

Giribet G, Sørensen MV, Funch P, Kristensen RM, Sterrer W. 2004. Investigations into the phylogenetic position of Micrognathozoa using four molecular loci. Cladistics. 20:1–13.
Gomez A. 2005. Molecular ecology of rotifers: from population differentiation to speciation. Hydrobiologia. 546:83–99.
Gomez A, Serra M, Carvalho GR, Lunt DH. 2002. Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). Evolution. 56:1431–1444.
Hagiwara A, Hino A, Hirano R. 1988. Effects of temperature and chlorinity on resting egg formation in the rotifer *Brachionus plicatilis*. Nippon Suisan Gakkaishi. 54:569–575.
Hagiwara A. 1994. Practical use of marine rotifer cysts. Isr J Aquacult Bamidgeh. 46:13–21.
Hagiwara A, Gallardo WG, Assavaaree M, Kotani T, Araujo AB. 2001. Live food production in Japan: recent progress and future aspects. Aquaculture. 200:111–127.
Helfenbein KG, Boore JL. 2004. The mitochondrial genome of *Phoronis architecta*—comparisons demonstrate that phoronids are lophotrochozoan protostomes. Mol Biol Evol. 21: 153–157.
Herlyn H, Piskurek O, Schmitz J, Ehlers U, Zischler H. 2003. The syndermatan phylogeny and the evolution of acanthocephalan endoparasitism as inferred from 18S rDNA sequences. Mol Phylogenet Evol. 26:155–164.
Jacobs HT, Elliott DJ, Math VB, Farquharson A. 1988. Nucleotide sequence and gene organization of sea urchin mitochondrial DNA. J Mol Biol. 202:185–217.
Jeyaprakash A, Hoy MA. 2007. The mitochondrial genome of the predatory mite *Metaseiulus occidentalis* (Arthropoda: Chelicerata: Acari: Phytoseiidae) is unexpectedly large and contains several novel features. Gene. 391:264–274.
Kotani T, Ozaki M, Matsuoka K, Snell TW, Hagiwara A. 2001. Reproductive isolation among geographically and temporally isolated marine *Brachionus* strains. Hydrobiologia. 446/447:283–290.
Kumazawa Y, Nishida M. 1993. Sequence evolution of mitochondrial tRNA genes and deep-branch animal phylogenetics. J Mol Evol. 37:380–398.
Kumazawa Y, Ota H, Nishida M, Ozawa T. 1998. The complete nucleotide sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two identical control regions. Genetics. 150:313–329.
Lowe TM, Eddy SR. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25:955–964.
Marcial HS, Hagiwara A, Snell TW. 2005. Effect of some pesticides on reproduction of rotifer *Brachionus plicatilis* Müller. Hydrobiologia. 546:569–575.
Mark Welch DB. 2000. Evidence from a protein-coding gene that acanthocephalans are rotifers. Invertebr Biol. 119:17–26.
Mark Welch DB. 2001. Early contributions of molecular phylogenetics to understanding the evolution of Rotifera. Hydrobiologia. 446/447:315–322.
Mark Welch DB. 2005. Bayesian and maximum likelihood analyses of rotifer-acanthocephalan relationships. Hydrobiologia. 546:47–54.
Mark Welch DB, Meselson M. 1998. Measurements of the genome size of the monogonont rotifer *Brachionus plicatilis* and of the bdelloid rotifers *Philodina roseola* and *Habrotrocha constricta*. Hydrobiologia. 387/388:395–402.
Ojala D, Montoya J, Attardi G. 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature. 290: 470–474.
Preston BL, Snell TW. 2001. Full life-cycle toxicity assessment using rotifer resting egg production: implications for ecological risk assessment. Environ Pollut. 114:399–406.
Sazanov LA, Hinchliffe P. 2006. Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. Science. 311:1430–1436.
Scouras A, Smith MJ. 2006. The complete mitochondrial genomes of the sea lily *Gymnocrinus richeri* and the feather star *Phanogenia gracilis*: signature nucleotide bias and unique *nad4L* gene rearrangement within crinoids. Mol Phylogenet Evol. 39:323–334.
Serra M, Snell TW, King CE. 2004. The timing and proportion of sex in monogonont rotifers. In: Moya A, Font E, editors. Evolution: from molecules to ecosystems. Oxford, UK: Oxford University Press. p. 135–146.
Snell TW, Serra M, Carmona MJ. 1999. Population genetics and ecotoxicology of rotifers. In: Forbes VE, editor. Genetics and ecotoxicology. Washington, DC: Taylor and Francis. p. 169–185.
Steinauer ML, Nickol BB, Broughton R, Ortí G. 2005. First sequenced mitochondrial genome from the phylum Acanthocephala (*Leptorhynchoides thecatus*) and its phylogenetic position within Metazoa. J Mol Evol. 60:706–715.
Suatoni E, Vicario S, Rice S, Snell TW, Caccone A. 2006. An analysis of species boundaries and biogeographic patterns in a cryptic species complex: the rotifer *Brachionus plicatilis*. Mol Phylogenet Evol. 41:86–98.
Suga K, Mark Welch DB, Tanaka Y, Sakakura Y, Hagiwara A. 2007. Analysis of expressed sequence tags of the cyclically parthenogenetic rotifer *Brachionus plicatilis*. PLoS ONE. 2:e671.
Suga K, Tanaka Y, Sakakura Y, Hagiwara A. 2007. Inheritance of mitochondrial DNA in the rotifer *Brachionus plicatilis*. Hydrobiologia. 593:167–173.
Valles Y, Boore JL. 2006. Lophotrochozoan mitochondrial genomes. Integr Comp Biol. 46:544–557.
Watanabe KI, Bessho Y, Kawasaki M, Hori H. 1999. Mitochondrial genes are found on minicircle DNA molecules in the mesozoan animal *Dicyema*. J Mol Biol. 286:645–650.
Wolstenholme DR. 1992. Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. 141:173–216.
Wright F. 1990. The 'effective number of codons' used in a gene. Gene. 87:23–29.
Yoshida T, Ellner SP, Jones LE, Bohannan BJM, Lenski RE, Hairston NGJ. 2007. Cryptic population dynamics: rapid evolution masks trophic interactions. PLoS Biol. 5:e235.
Yoshida T, Jones LE, Ellner SP, Fussmann GF, Hairston NGJ. 2003. Rapid evolution drives ecological dynamics in a predator-prey system. Nature. 424:303–306.
Zhang DX, Hewitt GM. 1997. Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. Biochem Syst Ecol. 25:99–120.
Zrzavy J, Mihulka S, Kepka P, Bezdek A, Tietz D. 1998. Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. Cladistics. 14:249–285.
Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31:3406–3415.

Richard Thomas, Associate Editor

Accepted February 27, 2008