Journal of Nematology 37(2):236–241. 2005.

Rolling Circle Amplification of Complete Nematode Mitochondrial Genomes

Sha Tang[1] and Bradley C. Hyman[1,2]

Abstract: To enable investigation of nematode mitochondrial DNA evolution, methodology has been developed to amplify intact nematode mitochondrial genomes in preparative yields using a rolling circle replication strategy. Successful reactions were generated from whole cell template DNA prepared by alkaline lysis of the rhabditid nematode *Caenorhabditis elegans* and a mermithid nematode, *Thaumamermis cosgrovei*. These taxa, representing the two major nematode classes Chromodorea and Enoplea, maintain mitochondrial genomes of 13.8 kb and 20.0 kb, respectively. Efficient amplifications were conducted on template DNA isolated from individual or pooled nematodes that were alive or stored at −80°C. Unexpectedly, these experiments revealed that multiple *T. cosgrovei* mitochondrial DNA haplotypes are maintained in our local population. Rolling circle amplification products can be used as templates for standard PCR reactions with specific primers that target mitochondrial genes or for direct DNA sequencing.

Key words: Caenorhabditis elegans, mermithid, mitochondrial DNA, molecular nematology, nematode, rolling circle amplification, systematics, *Thaumamermis cosgrovei.*

Nematode mitochondrial DNA (mtDNA) is usually a circular molecule that encodes a defined set of genes typical of metazoan mitochondrial genomes. One known exception to this organization is *Globodera pallida* mtDNA, which is organized as a set of sub-genomic circles (Armstrong et al., 2000). Irrespective of architecture, gene content includes 12 or 13 protein coding genes (Lavrov and Brown, 2001) that specify subunits of the mitochondrial electron transport chain proteins and the F1-ATPase and two rRNA and 22 tRNA genes that contribute to an organelle-specific translation system. Sequence analysis of this orthologous gene set has shown considerable utility for examining phylogenetic affinities and population structure of diverse nematode species (Blouin et al., 1998).

Direct estimates of nematode mtDNA mutation rates and patterning of nucleotide substitution are essential for effective evolutionary and population studies (Denver et al., 2000). Beyond that of nucleotide sequence divergence, comparative mitochondrial gene order is another character that may show promise for predicting the evolutionary histories of some taxa, with emphasis on deep divergence within a phylogeny (Boore and Brown, 1998). Moreover, deducing the transcriptional organization of mitochondrial genes has the potential to provide insight into the molecular events sponsoring the evolution of mitochondrial genome architecture. Each of these mtDNA-based areas of inquiry requires the isolation and analysis of complete mitochondrial genomes. When sufficient numbers of nematodes can be collected in their native habitats or propagated in laboratory cultures and colonies, mtDNA can be purified in sufficient yields by equilibrium centrifugation for subsequent analysis. The advent of conventional and long-distance PCR methodologies has enabled targeted regions of the mitochondrial genome to be amplified from one or a few individuals. On occasion, lengthy PCR products covering one third to one half of the entire molecule can be amplified (Hu et al., 2002). "Whole" mitochondrial genomes are then reconstructed by conducting several long-distance reactions that result in a set of several overlapping PCR fragments, collectively capturing the entire nematode mtDNA molecule.

Analysis of representative Enoplean nematodes has revealed that lengthy repeated sequences result in mtDNA molecules that are considerably larger than typical metazoan mitochondrial genomes (Hyman et al., 2004). As such, even more efficient amplification protocols would be beneficial for analysis of lengthy, contiguous nematode mtDNA sequence. One useful tool would be a single-step replication methodology that results in the amplification of complete nematode mitochondrial genomes.

To develop such a method, we reasoned that mtDNA may be the smallest, most abundant, and very likely the only circular molecule in the nematode cell. As such, it would serve as the primary DNA template for rolling circle amplification (RCA) in total cell DNA preparations. Rolling circle amplification is an isothermal reaction that employs DNA polymerase from the bacteriophage ϕ29. One important property of this enzyme is that its processive transit along the DNA template strand is accompanied by strand displacement from the double helix of previously replicated DNA. This feature creates new single-stranded template; in the presence of random primers, the displaced parental strands become template for new synthesis. This methodology had been developed for the rapid amplification of small, circular plasmid DNA from bacteria to facilitate high-throughput DNA sequencing efforts (Dean et al., 2001; Reagin et al., 2003). The RCA reaction can be readily biased to replicate small circular molecules in the presence of large chromosomal DNA by reducing

Received for publication 21 December 2004.

[1] Interdepartmental Graduate Program in Genetics, Genomics and Bioinformatics and [2]Department of Biology, University of California, Riverside, CA 92521.

The authors thank M. Maduro, E. Platzer, and Z. Wu for critical reading of the manuscript. This work was supported in part by awards from the Agricultural Experiment Station, the Committee on Research, and the Institute for Integrative Genome Biology of the University of California Riverside.

E-mail: bhyman@ucr.edu

This paper was edited by D. McK. Bird.

the concentration of primers, deoxyribonucleotide triphosphate building blocks (dNTPs), and the ϕ29 DNA polymerase (Amersham Biosciences Technical Support, pers. comm.). Because RCA relies on random primers and target specificity is achieved by the size of the template and concentration of the reactants, prior knowledge of the nucleotide sequence to develop PCR primers is unnecessary. This feature is advantageous because mitochondrial gene order varies among nematode taxa (Hyman et al., 2004), nucleotide divergence between nematode mtDNAs is considerable, and the opportunity to develop "universal primers" for standard PCR reactions is limited. In this report, we describe the successful amplification of complete nematode mtDNA molecules of several contour lengths. These include the mitochondrial genomes of *Caenorhabditis elegans* (13.8 kb) and the mermithid nematode *Thaumamermis cosgrovei* (20.0 kb).

Materials and Methods

Nematodes: Caenorhabditis elegans strain N2 was propagated on NGM agar seeded with *Escherichia coli* OP50 (Brenner, 1974). *Thaumamermis cosgrovei* is an obligate parasitic nematode of the common terrestrial isopod *Armadillidium vulgare* (pillbug) (Poinar, 1981). Isopod hosts were collected from the Botanic Garden on the University of California-Riverside campus. The isopods were inverted by hand, splitting the exoskeleton, and submerged into 0.9% NaCl. Slim white post parasitic-stage nematodes emerged from the thoracic cavity of the host and were transferred with a dental pick into fresh saline.

DNA isolation: A rapid alkaline lysis procedure (Raimond et al., 1999) was adapted to prepare an enriched population of circular molecules from nematodes that was suitable template for RCA reactions. Nematodes were crushed with a glass rod in 300 µl of extraction buffer (0.1 M NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA). A total of 600 µl 1% SDS, 0.2 N NaOH was added next and the nematode lysate was incubated on ice for 5 minutes, followed by the addition of 450 µl 3M potassium acetate. The lysate was incubated on ice for an additional 10 minutes. The mixture was spun at high speed in a micro-centrifuge and DNA was further purified from the supernatant by phenol/chloroform extraction. This procedure was used to prepare total cellular DNA from both individual nematodes and pooled samples containing 5 to 1,000 nematodes. For individual nematodes, reaction volumes were scaled down 10-fold.

Rolling circle amplification: Rolling circle amplification reactions were conducted using the TempliPhi kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. In brief, 5 µl of sample buffer and 0.4 µl of diluted, enriched nematode mtDNA template were mixed, denatured at 95°C for 3 minutes, and chilled on ice; subsequently, 5 µl reaction buffer and 0.2 µl enzyme mix were added and incubated at 30°C for approximately 18 hours.

Restriction enzyme digestion and gel electrophoresis: Restriction enzyme digestions and gel electrophoresis were carried out using standard procedures. Restriction fragment products were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Results and Discussion

Template input: Several methods were employed to prepare total cellular template suitable for RCA reactions. These included modifications of a standard sodium dodecyl sulfate/proteinase K nematode mini-lysate procedure (Powers et al., 1986) as well as a method based on alkaline lysis (Raimond et al., 1999). Successful RCA reactions, defined as the generation of a high-molecular weight product that can subsequently be cleaved into a collection of discrete restriction fragments, were achieved only with alkaline lysis, using either live or frozen (–80°C) nematodes. Because ribosubstitution occurs during normal replication of animal mitochondrial genomes, one initial concern with the alkaline lysis procedure was the possibility of compromising template integrity by nicking covalently closed circular mtDNA molecules. However, enough mtDNA remains intact to provide sufficient template for RCA reactions. Alkaline lysis is self-selecting for convalently closed, small, circular template molecules; large linear chromosomal segments produced by random sheer during DNA extraction would be effectively eliminated in this procedure. The ratio of small circular:large linear template concentrations appears to be an important requirement for successful reactions.

For small nematodes such as *C. elegans,* template DNA was prepared by alkaline lysis from 0.1 cm^3 packed volume. The final volume of the template preparation was 40 µl; 1.0% (0.4 µl) of this extract was then employed in a typical RCA reaction (Fig. 1). Serial dilutions of this same template were tested for the ability of RCA to amplify mtDNA. A 1:5 dilution of the template (equivalent to 0.005 cm^3 packed nematode volume) generated an amount of mtDNA product equivalent to that of the undiluted preparation. Mitochondrial DNA product could still be observed after a 50-fold dilution of the template, although the yield was significantly diminished to a level that would not support further analysis. For larger mermithid nematodes, individual nematodes can be employed. Small segments (10–20%) of the body length are routinely used for template preparation, preserving the head and tail region for morphological analysis and preservation as conventional or digitized voucher specimens.

Reaction output: RCA reaction products uncleaved by restriction enzymes generate a high-molecular weight

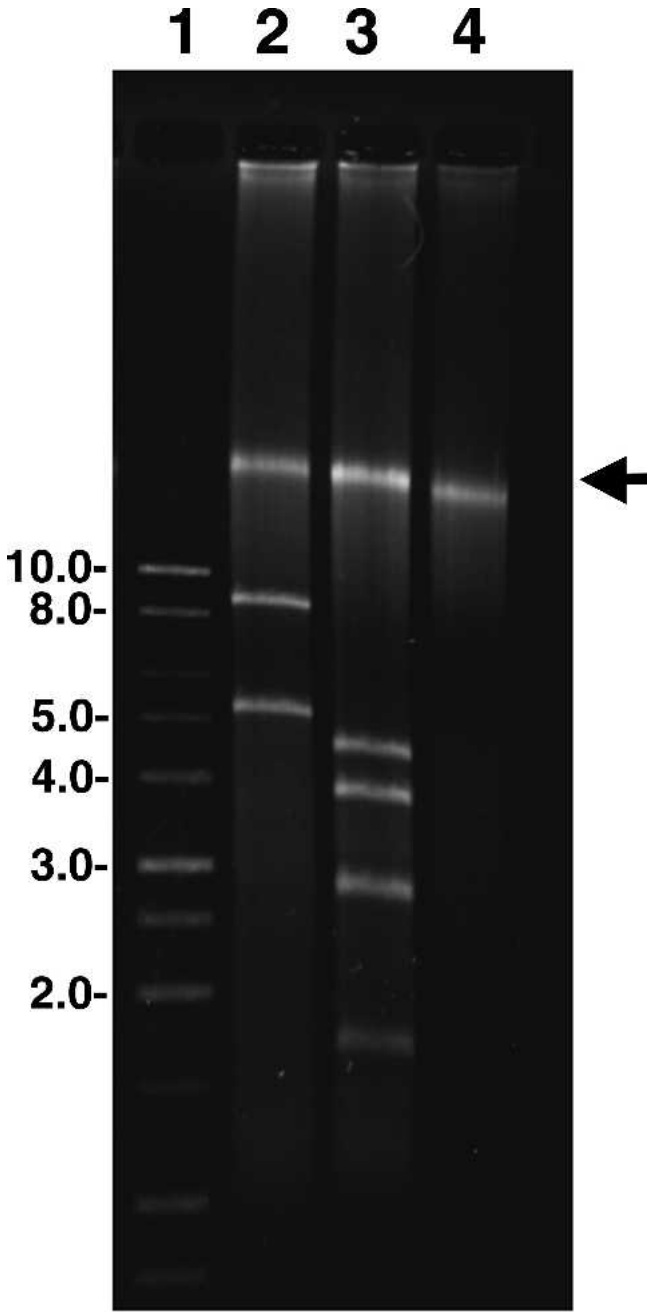

FIG. 1. Rolling circle amplification of *C. elegans* mtDNA. Total cellular DNA template was isolated by alkaline lysis from approximately 0.1 cm^3 packed nematodes. Rolling circle amplification reaction products were fractionated on a 1.0% agarose gel. Lane 1) 1-kb DNA ladder (Promega, Madison, WI) molecular weight marker. Lane 2) Eco RI-cleaved RCA product. Lane 3) Bgl II-cleaved RCA product. Lane 4) Uncleaved RCA product (arrow). A predicted 90-bp Eco RI fragment was not detected (lane 2), as it has migrated off the bottom of the gel. Sizes of molecular markers are shown in kilobase pairs (kbp).

band when fractionated by agarose gel electrophoresis (Fig. 1). This is anticipated, as the reaction products, formed after random priming events, are circularly permuted linear copies of the circular mtDNA molecule and remain associated as a large network of molecules. Resolution of this complex structure requires size reduction into specific sub-genomic fragments by restriction enzyme cleavage. The resultant restriction fragments collectively comprise the entire mitochondrial genome. A DNAase-sensitive high-molecular weight band often persists after restriction enzyme cleavage (Fig. 1, arrow). The reason for the insensitivity to restriction enzyme cleavage is unknown.

Rolling circle amplification faithfully amplifies the C. elegans mitochondrial genome: To determine whether RCA can be used to amplify complete nematode mtDNA molecules, the mitochondrial genome from the model genetic nematode *C. elegans* was chosen as a test template. At 13.8 kb, the molecule is among the smaller nematode mtDNA molecules characterized and may pose less of a challenge than larger mitochondrial genomes for efficient amplification. Moreover, the complete nucleotide sequence of *C. elegans* mtDNA is available (Okimoto et al., 1992), and restriction fragment product sizes can be predicted to verify that mtDNA has been amplified.

Figure 1 displays the results of successful amplification of the *C. elegans* mitochondrial genome, using total cellular DNA isolated from approximately 0.1 cm^3 packed nematodes. The nucleotide sequence predicts restriction enzyme products of 8,463; 5,250; and 90 bp when *C. elegans* mtDNA is cleaved by Eco RI. Digestion of the RCA product with Eco RI revealed DNA fragments whose electrophoretic migration precisely matches these predicted lengths (Fig. 1). Similarly, eight digestion products ranging in size from 0.1 to 4.6 kb are predicted when *C. elegans* mtDNA is cleaved with BgI II. The four largest expected products of 4,648; 3,909; 2,658; and 1,683 bp were easily resolved in the gel system employed (Fig. 1).

One feature of the mtDNA digestion patterns generated by restriction enzyme cleavage of RCA reaction products deserves additional comment. Ethidium bromide binds DNA on a stoichiometric basis (LePecq and Paoletti, 1967), and it is anticipated that the largest bands would exhibit the highest fluorescent intensity after illumination by ultraviolet light. However, when mtDNA was amplified by RCA, the 8.5–kb Eco RI fragment (Fig. 1) and the 4.6-kb Bgl II fragment (Fig. 1) revealed a band intensity substoichiometric to smaller restriction products. These observations can be explained when the nature of the rolling circle amplification reaction is considered. Random priming from a circular template results in a collection of double-stranded products. A high percentage of these molecules do not represent full-length circles. The larger the expected restriction fragment, the lower the probability that an individual RCA product will contain both target sites necessary to generate an authentic restriction fragment. Hence, the longest predicted restriction fragments were present in substoichiometric yields.

This same reasoning likely explains a light background smear sometimes visible after electrophoretic fraction when RCA-generated products are digested with restriction enzymes (Figs. 1–3), when a population

of different-sized fragments with one restriction enzyme-generated and one random end can be generated. Chromosomal DNA amplification by RCA (Dean et al., 2002) may also contribute to the background smear. However, this background DNA hybridizes with mtDNA probes in DNA transfer-hybridization experiments. In addition, the enrichment for small, circular mtDNA templates by alkaline lysis, together with the template specificity imposed by reduced reactant concentrations characteristic of TempliPhi RCA reactions, suggests nuclear DNA is likely a minor component of the background smear observed after restriction enzyme cleavage of RCA products.

RCA of mermithid nematode mtDNA indicates large mitochondrial genomes can be amplified from individual nematodes and indicates extensive size polymorphism: The mermithid nematode *T. cosgrovei* is an obligate parasitic nematode of terrestrial isopods. In North America, its range appears to be restricted to Southwestern California populations of *Armadillidium vulgare* (the common pillbug) and *Porcellio scaber* (the common sowbug) (Poinar, 1981). Although these hosts were introduced to the Americas from Europe (Jass and Klausmeier, 2000), this nematode has not been found there. These observations suggest a recent host switch. For the purpose of studying this potentially interesting life history, mtDNA-based molecular markers are under development.

Total cellular DNA was prepared from five pooled *T. cosgrovei* nematodes and used as template for an RCA reaction. A complex array of restriction products was obtained when the RCA products were digested with the restriction enzyme Bgl II (Fig. 2). The sizes of these restriction fragments sum to a value that far exceeds any known metazoan mitochondrial genome length. Importantly, when RCA was performed on template derived from three individual nematodes in our *T. cosgrovei* population, the Bgl II restriction fragment patterns were reduced to a subset of the fragments observed when pooled template was employed (Fig. 2). Within each individual analyzed, the molecular weights of these restriction fragments sum to a genome size of approximately 20 kb with all enzymes tested, a contour length that is more typical of mermithid mtDNA molecules (Hyman et al., 2004). The complete nucleotide sequences of two *T. cosgrovei* mtDNA haplotypes have been assembled (Tang and Hyman, unpubl.) and predict restriction fragments whose lengths precisely match those sized in Figure 2, lanes 2 and 5. These results indicate that mtDNA molecules of several different sizes are maintained within the local *T. cosgrovei* population and that a single nematode can carry one of these haplotypes. Restriction enzyme and Southern blot analyses were performed on template DNA derived from 30 additional individuals, further confirming the presence of multiple haplotypes maintained in *T. cosgrovei* (Tang and Hyman, unpubl.).

A 1.5-kb DNA band was present at an elevated stoichiometry relative to other restriction products in Eco

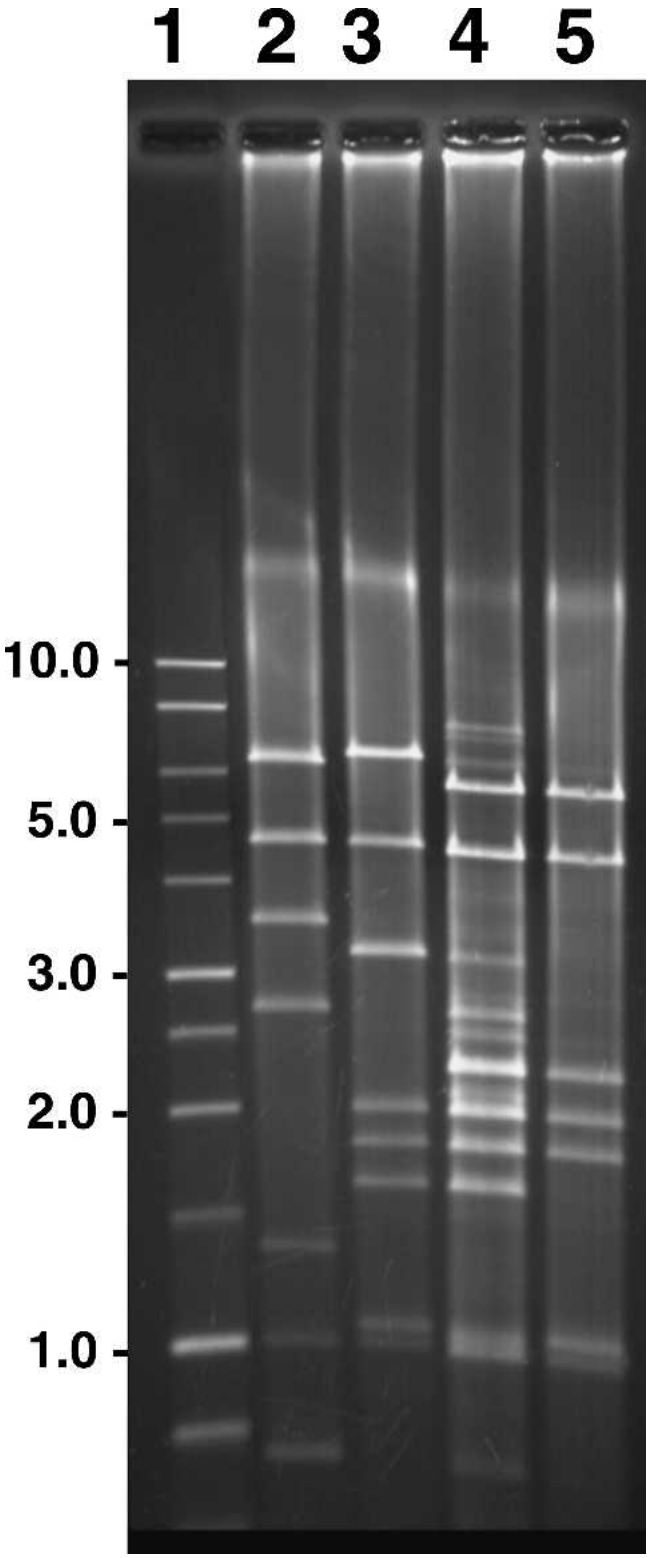

FIG. 2. Rolling circle amplification of *T. cosgrovei* mtDNA from pooled and individual nematodes. Total cellular DNA template was prepared by alkaline lysis from three individual nematodes or from five pooled individuals. Rolling circle amplification reaction products were digested with Bgl II and fractionated on a 1.0% agarose gel. Lane 1) 1-kb DNA molecular weight marker (Promega, Madison, WI). Lanes 2, 3, 5) Bgl II-cleaved RCA product using template from a single nematode. Lane 4) Bgl II-cleaved RCA product using template from five pooled nematodes. Sizes of molecular weight markers are shown in kilobase pairs (kb).

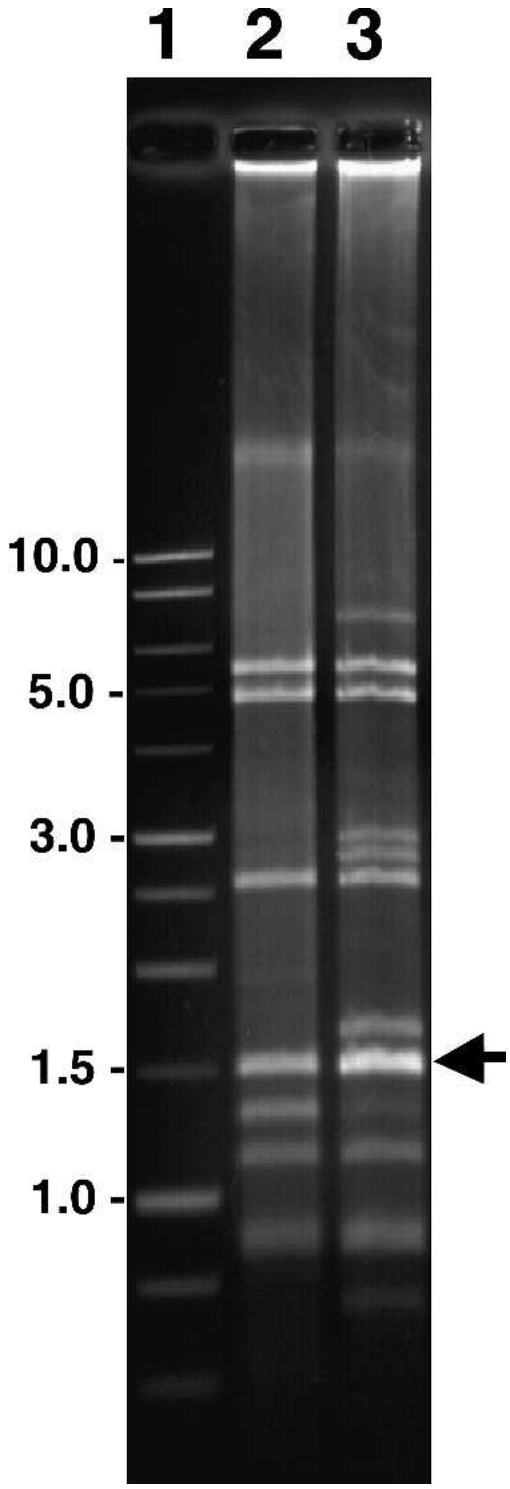

FIG. 3. Rolling circle amplification faithfully duplicates lengthy repeated mtDNA segments. Total cellular DNA templates were prepared by alkaline lysis from five pooled individuals (same template as in Fig. 2) and from the same individual nematode as characterized in Fig. 2, lane 5. Rolling circle amplification products were digested with Eco RV and fractionated on a 1% agarose gel. Lane 1) 1-kb DNA molecular weight marker (Promega, Madison, WI). Lane 2) Eco RV-cleaved RCA product using template amplified from an individual nematode. Lane 3) Eco RV-cleaved RCA product using template from five pooled nematodes. Arrow indicates 1.5-kb Eco RV cleavage product derived from repeated DNA within the *T. cosgrovei* mitochondrial genome. Sizes of molecular weight markers are shown in kilobase pairs (kb).

RV digestions of *T. cosgrovei* mtDNA amplified by RCA using template DNA prepared from individual or pooled nematodes (Fig. 3). The complete nucleotide sequence of the *T. cosgrovei* mitochondrial genome (Tang and Hyman, unpubl.) revealed lengthy duplicated segments containing the NADH subunit 4 (ND4), F_1-ATPase subunit 6 (ATP6), and 12S ribosomal RNA (rrnS) mitochondrial genes. Internal to these repeating units are two Eco RV restriction sites separated by 1.5 kb. Cleavage of mtDNA with Eco RV produces two identical 1.5-kb fragments, visualized as a more intensely stained 1.5-kb DNA band after fractionation by gel electrophoresis. This result indicates that RCA faithfully amplifies large nematode mitochondrial genomes, including mtDNA molecules with unusual architectural features such as lengthy repeating units. The 20-kb *T. cosgrovei* mitochondrial genome is not the upper limit of template size that can be successfully amplified using RCA, as a complete 26.2-kb mitochondrial genome from the mosquito mermithid *Romanomermis culicivorax* (Hyman and Beck-Azevedo, 1996) has been efficiently amplified by RCA (data not presented).

Concluding remarks: The RCA products described here have been subsequently employed in a variety of analyses, including restriction site mapping and molecular cloning, as template in conventional PCR with primers that target specific mitochondrial genes, and as template in direct sequencing reactions. Rolling circle amplification of complete nematode mtDNA molecules, as with other methods designed to duplicate entire genomes in preparative yields from small amounts of material (Lasken and Egholm, 2003), facilitates genotyping, archiving voucher specimens, and sharing of samples among laboratories. Given these advantages, RCA provides a highly practical and time-efficient strategy to support investigation of mitochondrial genome evolution across the phylum Nematoda.

LITERATURE CITED

Armstrong, M. R., V. C. Blok, and M. S. Phillips. 2000. A multipartite mitochondrial genome in the potato cyst nematode *Globodera pallida*. Genetics 154:181–192.

Blouin, M. S., C. A. Yowell, C. H. Courtney, and J. B. Dame. 1998. Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Molecular Biology and Evolution 15:1719–1727.

Boore, J. L., and W. M. Brown. 1998. Big trees from little genomes: Mitochondrial gene order as a phylogenetic tool. Current Opinion in Genetics and Development 8:668–674.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

Dean, F. B., S. Hosono, L. Fang, X. Wu, A. F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, M. Driscoll, W. Song, S. F. Kingsmore, M. Egholm, and R. S. Lasken. 2002. Comprehensive human genome amplification using multiple displacement amplification. Proceedings of the National Academy of Sciences USA 99:5261–5266.

Dean, F. B., J. R. Nelson, T. L. Giesler, and R. S. Lasken. 2001. Rapid amplification of plasmid and phage DNA using phi29 DNA

polymerase and multiply-primed rolling circle amplification. Genome Research 11:1095–1099.

Denver, D. R., K. Morris, M. Lynch, L. L. Vassilieva, and W. K. Thomas. 2000. High direct estimate of the mutation rate in the mitochondrial genome of *Caenorhabditis elegans*. Science 289:2342–2344.

Hu, M., R. B. Gasser, N. B. Chilton, and R. B. Gasser. 2002. Long PCR-based amplification of the entire mitochondrial genome from single parasitic nematodes. Molecular and Cellular Probes 16:261–267

Hyman, B. C., and J. L. Beck-Azevedo. 1996. Similar evolutionary patterning among repeated and single-copy nematode mitochondrial genes. Molecular Biology and Evolution 13:221–232.

Hyman, B. C., E. G. Platzer, S. Tang, and Z. Wu. 2004. Comparative mermithid mitochondrial genomics. Journal of Nematology 36:324–325 (Abstr.).

Jass, J., and B. Klausmeier. 2000. Endemics and immigrants: North American terrestrial isopods (Isopoda, Oniscidea) north of Mexico. Crustaceana 73:771–799.

Lasken, R. S., and M. Egholm. 2003. Whole genome amplification: Abundant supplies of DNA from precious samples or clinical specimens. Trends in Biotechnology 21:531–535.

Lavrov, D. V., and W. M. Brown 2001. *Trichinella spiralis* mtDNA: A nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAs and has a gene arrangement relatable to those of coelomate metazoans. Genetics 157:621–637.

LePecq, J.-B., and C. Paoletti. 1967. A fluorescent complex between ethidium bromide and nucleic acid: Physical-chemical characterization. Journal of Molecular Biology 27:87–106.

Okimoto, R., J. L. Macfarlane, D. O. Clary, and D. R. Wolstenholme. 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics 130:471–498.

Poinar, G. Jr. 1981. *Thaumamermis cosgrovei* n. gen., n. sp. (Mermithidae: Nematoda) parasitizing terrestrial isopods (Isopoda: Oniscoidea). Systematic Parasitology 2:261–266.

Powers, T. O., E. G. Platzer, and B. C. Hyman. 1986. Large mitochondrial genome and mitochondrial DNA size polymorphism in the mosquito parasite, *Romanomermis culicivorax*. Current Genetics 11:71–77.

Raimond, R., I. Marcadé, D. Bouchon, T. Rigaud, J.-P. Bossy, and C. Souty-Grosset. 1999. Organization of the large mitochondrial genome in the isopod *Armadillidium vulgare*. Genetics 151:203–210.

Reagin, M. J., T. L. Giesler, A. L. Meria, J. M. Resetar-Gerke, K. M. Kapolka, and J. A. Mamone. 2003. A sequencing template preparation procedure that eliminates overnight cultures and DNA purification. Journal of Biomolecular Techniques 14:143–148.