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Short communication

Rolling circle amplification of metazoan mitochondrial genomes

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1. Introduction

Ever since Darwin (1859) envisioned his grander view of life, evolutionary biologists have been resolute in their determination to reconstruct the Tree of Life. This is a daunting, yet essential task, as many medical, economic, environmental, conservation, and other social issues depend on the accurate placement of an organism or lineage on the tree (Cracraft and Donoghue, 2004). Comparisons of fragments of nuclear and organellar genomes have been prominent in reconstructing evolutionary relationships (Wilson et al., 1977), but larger scale genome sequencing provides additional phylogenetic information above the nucleotide level, including gene order, gene content and organization, RNA and protein folding patterns, etc. While recent successes in sequencing complete eukaryotic genomes (*Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Plasmodium falciparum*, and others) (CBS Genome Atlas Database; Hallin and Ussery, 2004) have rightly garnered substantial attention, complete nuclear genome sequences for even an infinitesimal fraction of the Tree of Life remains as remote now as it was in Darwin's day. However, the smaller size of the mitochondrial genome has resulted in the publication of more than 600 complete mt genomes and that number is growing rapidly (CBS Genome Atlas Database; Hallin and Ussery, 2004).

The use of organelle DNA has been a mainstay in the reconstruction of phylogenetic relations (Simon et al., 1994) and has substantial advantages over nuclear genes (e.g., small compact genome, haploid with no recombination, typically maternally inherited, conserved amino acid sequences, etc). Moreover, complete organellar genomes provide new sources of phylogenetic information, such as gene order and secondary and tertiary structures of proteins or RNAs (Boore, 1999; Lydeard et al., 2000).

With the exception of the Vertebrata and Arthropoda, existing techniques to sequence complete metazoan mt genomes (such as long PCR) require unique solutions for each taxon under investigation—often in spite of close relationships (e.g., Rawlings et al., 2003). Transpositions, nucleotide substitutions, insertions, or deletions, and duplications of portions of the mtDNA, all contribute to the need for extensive troubleshooting and optimization for the production of complete mt genomes using long PCR approaches.

Here, we report the successful use of rolling circle amplification (RCA) for the amplification of complete metazoan mt genomes to make a product that is amenable to high-throughput genome sequencing techniques. The benefits of RCA over PCR are many and with further development and refinement of RCA, the sequencing of organellar genomics will require far less time and effort than current long PCR approaches.

2. Strand displacement rolling circle amplification

RCA has been used in the amplification of microbial genomes, for the amplification of signal from probes, and in plasmid amplification for sequencing (Dean et al., 2001; Hawkins et al., 2002; Lizardi et al., 1998; Nelson et al., 2002). The benefits of RCA lie in its universal priming conditions; RCA employs numerous random short primers, which anneal at many sites to a DNA template. Once the primers have annealed to the DNA, the phi29 DNA polymerase extends the annealed primers and, because of its strong strand displacement activity and high processivity, generates long concatenamers of the circular template DNA in a single isothermal reaction. The phi29 polymerase

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also has an inherent 3′–5′ proofreading exonculease activity, accounting for its high fidelity (Esteban et al., 1993; Paez et al., 2004). Despite the name, this reaction does not work only on circular DNA; linear DNA also will undergo repeated rounds of simple strand displacement amplification (Lizardi et al., 1998), so it is critical that the DNA preparation be highly enriched for mtDNA, although the amount can be minuscule.

Here, we describe the extraction protocols used for both RCA and long PCR on the same two gastropod mollusks, followed by their respective amplification protocols, a description of the shotgun sequencing protocol used, and the sequence annotation procedure to generate complete mtDNA sequences.

We tried four different DNA extraction protocols for somatic tissue: CTAB/phenol/chloroform, Qiagen DNeasy Tissue kit (Valencia, CA), mtDNA Extractor WB Kit, Wako Pure Chemical Industries (Japan), and our mt isolation protocol described below. All resulted in successful Long PCR products, yet all failed repeatedly for the RCA protocol. The only successful combination was our mt purification approach when we used fresh, unfertilized eggs dissected from aquarium-raised specimens.

Approximately 0.1 g eggs was mixed with 10 ml of homogenization buffer in a 50 ml Oakridge tube. The homogenization buffer was a 1:5 mixture of the following pH 7.5 buffers: (1) 1.5 M sucrose/10 mM Tris/100 mM EDTA and (2) 10 mM Tris/100 mM EDTA/10 mM NaCl. This mixture was homogenized with a Tekmar T25 Tissuemizer at a speed setting of one using 5–10 strokes of 10–15 s each. The homogenized sample was centrifuged for 5 min at 2300*g* and 4 °C to pellet and remove nuclei. The supernatant was transferred to a fresh Oakridge tube and centrifuged for 25 min at 23,000*g* and 4 °C. The mitochondrial pellet was resuspended in 3 ml room temperature (RT) TE, then 750 µl of 10% SDS was gently mixed into the suspension and incubated at RT for 10 min to lyse mitochondrial membranes. The sample was centrifuged for 10 min at 17,000*g* and 4 °C to pellet and remove membranous debris. The resulting supernatant was extracted first with phenol and then with chloroform, then a half-volume of 7.5 M ammonium acetate was added and the solution gently mixed. Two volumes of 100% ethanol were added and mixed by inversion. The mixture was incubated at −20 °C for 1 h and precipitated by centrifugation at 20,000*g* for 45 min at 4 °C. The pellet was air-dried and resuspended in 100 µl of ddH_2O.

Long PCR of the *Lottia digitalis* and *Ilyanassa obsoleta* mt genomes was completed using the Takara LA Taq PCR kit (Japan) and five and four sets of primers respectively (Table 1). Primers were designed from *rrnL* and *cox1* sequence fragments, which were generated using the 16Sa-L2510 and 16Sb-H3080 primers described by Palumbi (1996) and the HCO-2193 and LCO-1490 primers described by Folmer et al. (1994), respectively. From these short fragments, primers were designed using Oligo 4.0 (Molecular Biology Insights, Cascade, CO) to create long PCR products that would span the regions between *rrnL* and *cox1*. Some primer combinations were redundant, but required to established authenticity of unusual motifs such as tandem repeat regions. Long PCR cycling parameters started with a 94 °C step for 2 min followed by 10 cycles of 94 °C for 10 s, 55 ± 10 °C for 30 s and 72 °C for 10 min. This was followed by 20 cycles of 94 °C for 10 s, 55 ± 10 °C for 30 s and 72 °C for 10 min plus 10 s per cycle.

Rolling circle amplification was performed with the Amersham Biosciences TempliPhi DNA amplification kit (Piscataway, NJ). The reaction required two steps. The first step was a denaturation step, in which 2 µl of the product from the mitochondrial isolation protocol above was mixed with 2 µl of the TempliPhi denaturation buffer and 6 µl of

Table 1
Long PCR primers used to successfully amplify large portions of the *Lottia digitalis* and *Ilyanassa obsoleta* mitochondrial genomes

Taxon	No.	Primer	Sequence (5′–3′)
Lottia digitalis	1	LdigCOI456U20	ACCTTTATCGGCTCTACCTT
	2	Ldig16S169L25	AAATTAATGCAACTGAGACAGCGTC
	3	WBS307Urc	TAGCCAACTCCTATCTATGC
	4	WBS3240Lrc	TCAGATGAGTTAACCCGAAG
	5	L5444digit	ATTTTCGTTAGGTTGGCTTTTCTTTGTA
	6	U2326digit	AATTTCTGCTGTGGCGGTGGGTCTCGTC
	7	YLAYKZ14206L25	AGAAAAGAGATAAACCAAGTCAGGA
	8	YLAYKZ785U29	ACACGGGTACAGGATTTTTAGTCTCAGGT
	9	YLAYKZ17697L28	TATTACTTAGCCCCTGTTCTTGTCATTG
	10	YLAYKZ5210U25	GGTTCCCCCAAAAAGGCACATTAGT
Ilyanassa obsoleta	11	16SCOII366U28i	AAAGGAATTAGTTACCGTAGGGATAACA
	12	16SCOII815L24i	ATAAACAGTTCACCCAGTCCCAAC
	13	COI16SI902L28i	TTAAAGCTCGATAGGGTCTTCTTGTCTT
	14	COI16SI307U27i	TTGGGACTGGGTGAACTGTTTATCCAC
	15	COI16SI809L28i	TTCATGTCAAACCATTCATACTAGCCTT

Long PCR combinations for *L. digitalis* were 1 + 2, 3 + 4, 5 + 6, 7 + 8, and 9 + 10. *I. obsoleta* 11 + 12, 11 + 13, 13 + 14, and 14 + 15. See Fig. 1 for approximate primer positions.

H_20. The sample was then denatured at 95 °C for 5 min then brought to RT. 10 µl of TempliPhi amplification buffer (includes enzyme) was added to the sample and incubated at 34 °C for 18 h. The reaction enzyme was then inactivated at 65 °C for 10 min. The reaction product was purified using an Amicon Ultrafree-MC minicolumn kit (Fisher Scientific, Hampton, NH). The purified product was run on a 1% electrophoresis agarose gel to visualize the RCA reaction result. The product was also cut with Invotrogen *Taq*I restriction enzyme (Carlsbad, CA) and run on a 1% electrophoresis agarose gel, with the expectation that only mtDNA would yield a banding pattern, with amplified nuclear DNA, in contrast, expected to generate a smear, since there would be a very large number of randomly located *Taq*I sites.

The DNA from successful amplification reactions was sheared into small fragments by repeatedly passing it through a narrow aperture using a Gene Machines HydroShear. The DNA stretches as it passes through under high pressure, and breaks if it is longer than a size specified by the pressure, typically between 1.0 and 1.5 bp for mtDNA. Repairs to make the ends blunt were made using T4 DNA polymerase and Klenow fragment enzymes, then the products was electrophoresed in 1% agarose gels for size selection. The portion between 1.0 and 1.5 bp was excised from the gel and extracted using Qiagen Gel Extraction kits (Valencia, CA). These were ligated into pUC18 plasmid vectors using Fast-Link ligation kits (Epicentre Technologies, Madison, WI) and the resulting plasmids were used to transform *Escherichia coli* DH10b via electroporation. Colonies were grown overnight on LB/Amp/X-gal plates and then an automated Genetix colony picker was used to select colonies into 384-well plates of LB with 10% glycerol. These were incubated overnight in a static incubator, without shaking or enhanced aeration, and then a small aliquot was processed robotically through plasmid amplification using RCA. Sequencing was carried out using an ABI 3730xl automated capillary DNA sequencer with ABI BigDye chemistry (Foster City, CA), and SPRI reaction clean up (Elkin et al., 2002). Detailed protocols are available at http://www.jgi.doe.gov/Internal/prots_index.html. We determined at least 10 times the amount of sequence in the target template, and then assembled the sequencing reads using the assembler program PHRAP (Green, 1996). Quality of sequence and assembly were manually verified using the program CONSED (Green, 1996). Genes were annotated using DOGMA (Wyman et al., 2004), then imported into MacVector (Accelrys, San Diego, CA) for subsequent analyses.

3. The mitochondrial genomes of *I. obsoleta* and *L. digitalis* (Fig. 1)

The complete mitochondrial (mt) genomes of the gastropod mollusks *I. obsoleta* and *L. digitalis* were obtained through RCA. The sequences obtained through RCA were independently confirmed by comparing them to mt genome sequences obtained using long PCR techniques. Both genomes contain the typical 13 protein genes, two ribosomal RNA genes, and 22 tRNA genes (Fig. 1).

The 26,400 bp mt genome of *L. digitalis* contains several repeating units distributed across two regions of the genome (Fig. 1A). The first region spans ≈7000 bp between *trnY* and *cob* and contains a series of overlapping tandem repeats. The second region spans 1500 bp and lies between *trnC* and *nad6*. We suspect length polymorphism for the former repeat region because long PCR amplification across this region generated from eggs pooled from multiple individuals produced several bands of similar sizes, while the same PCRs of single individuals produced single

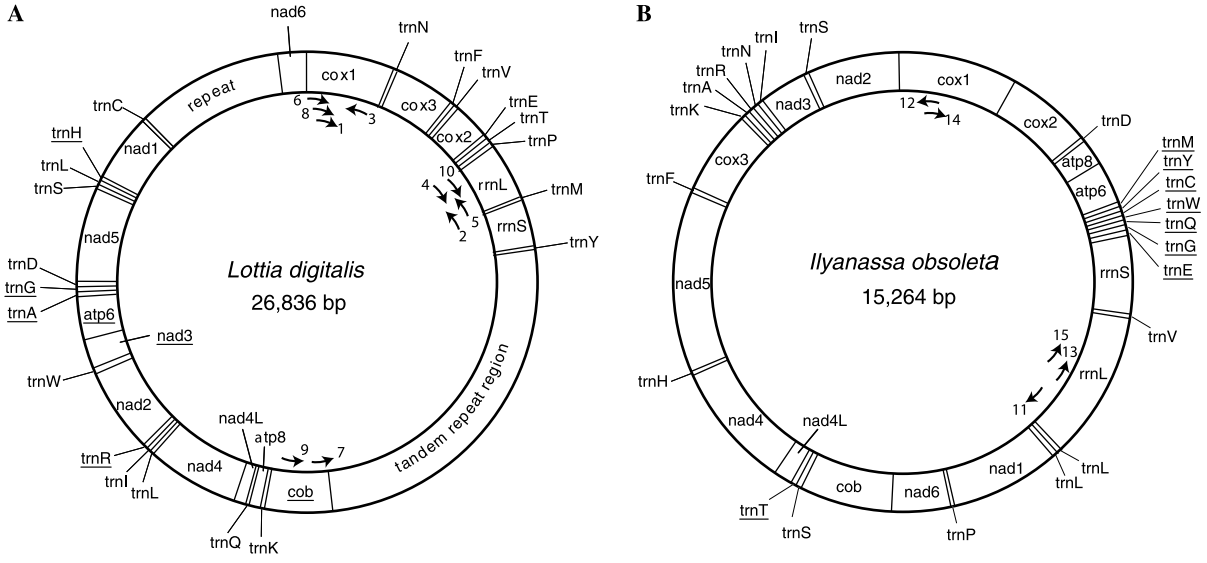

Fig. 1. Complete mt genomes obtained through rolling circle amplification for the gastropod mollusks. Underlined text indicates genes on the reverse strand. Arrows indicate the approximate position of primers listed in Table 1. (A) *Lottia digitalis* (GenBank Accession No. DQ238599), and (B) *Ilyanassa obsoleta* (GenBank Accession No. DQ238598).

bands of different sizes. Significant variation (0–6.2 bp) has been reported in tandem repeat regions in the mt genome of other organisms, including lizards (Mortiz and Brown, 1986), rabbits (Pfeuty et al., 2001), sheep (Hiendleder et al., 1998), and the blood fluke (Bieberich and Minchella, 2001), often involving the control region (D-loop), but sometimes including genes as well. Another oddity in the mitochondrial genome of *L. digitalis* is the lack of a traditional start codon for the *cox1* translation.

We found numerous single nucleotide polymorphisms (SNPs) in the *I. obsoleta* library created from the RCA product. Of the SNPs found in coding regions, 91.3% (105/115) were synonymous. This suggests to us that they are not due to any polymerase or sequencing errors, but rather, represent actual variation in mtDNA sequences. The *L. digitalis* assembly, on the other hand, revealed very few ambiguous positions. We also discovered that *I. obsoleta* has a frame shift in the middle of the *nad2* gene at position 14,613. At this position, there are two untranslated thymines between normal translating regions. Frames shifts in the *nad* genes have been reported in other taxa (Mindell et al., 1998; Parham et al., in press; Zardoya and Meyer, 1998). However, in all of the cases reported, a single extra nucleotide caused the frame shift; in this case, we have either two extra thymines or a missing nucleotide.

4. Extra-chromosomal replicas of the nuclear ribosomal RNAs?

In addition to the complete mt genome, we found that libraries generated from RCA of *L. digitalis* consistently recovered the sequences of the nuclear 18S, 5.8S, and 28S rRNA complex as a continuous unit including ETS 1 and 2 and ITS 1 and 2 (Fig. 2). The ends of this assembly match one another and therefore suggest that this complex exists as a tandem repeat or as a circle. The amount of this rRNA sequence product is much greater than one would expect from random sequences or background nuclear DNA amplification. Ribosome production is particularly critical during early embryonic development and Brown and Dawid (1968) have demonstrated that *Xenopus* oocyte nuclei may contain up to a million extra-chromosomal replicas of these genes for the production of rRNA during early development. They also reported extra copies of the genes for 18S and 28S rRNA in eggs of the bivalve mollusk *Spisula solidissima* and in the echiuran *Urechis caupo*. Therefore, it might not be too surprising to find that eggs of a gastropod would contain such extra-chromosomal DNA, or that it would amplify during RCA (especially if it is circular) and be found in the sequenced product. Unfortunately, sequence assemblies cannot differentiate between tandem repeats and circular entities. The results of Brown and Dawid (1968) suggest that the nuclear ribosomal complex exists in high copy number of tandem repeats while Hourcade et al. (1973) presented electron microscopy evidence suggesting that during the development of *Xenopus* oocytes, ribosomal DNA exists at elevated levels and replicates via rolling circle intermediates.

In contrast to the presence of all three ribosomes in *L. digitalis*, a BLAST search of the *I. obsoleta* RCA sequence library revealed only a small number of matches with the *I. obsoleta* 28S rRNA gene. This difference between copy numbers of different components of the nuclear ribosome complex in *L. digitalis* and *I. obsoleta* may have resulted from their respective eggs being at different stages of oogenesis. However, these two species also have substantially different modes of larval development—*L. digitalis* has lecitotrophic development, while *I. obsoleta* has planktotrophic development, and this marked difference in resource provisioning for the developing larvae may also affect production of ribosomes as well. Clearly, the possible role, implications, and mechanics associated with the presence of high copy numbers of nuclear and/or extra-nuclear ribosome genes in the Mollusca require further study.

5. Discussion

The benefits of RCA over long PCR are numerous and include: (1) the elimination of time-consuming primer design and testing, (2) the elimination of multiple PCR products to complete a genome, (3) a substantial reduction of the cost of PCR primer walking, (4) the elimination of thermal cycling, (5) the reduction of the number of shotgun libraries from between 4 and 12 to one, (6) the dramatic reduction of overall time investment in obtaining a complete mt genome, and (7) reduced equipment needs because RCA reactions require only standard bench top heat blocks.

Currently, there are only about 600 complete metazoan mt genomes published in GenBank and the vast majority of these are for vertebrates (>70%) (Helfenbein et al., 2004). Because of this limited sampling, what is now considered to be "typical" mt genomes may someday be considered unique to their respective lineages. This is especially apparent when we consider the complexity of gene rearrangements so far documented by the limited number of complete mt genomes in mollusks and other spiralians (e.g., Boore and Brown, 1995; Hoffmann et al., 1992; Kurabayashi and Ueshima, 2000). Additional data will undoubtedly

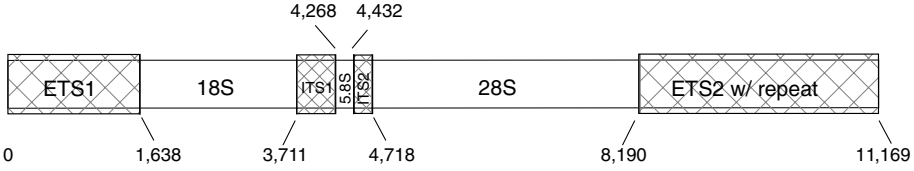

Fig. 2. Nuclear ribosomal complex for the gastropod mollusk: *Lottia digitalis* (GenBank Accession No. DQ248942).

provide a better understanding of the variation present in metazoan mt genomes, especially as continued development of RCA accelerates our progress in sequencing complete organellar genomes. Moreover, as the patterns of variation become better understood, it will also expand our knowledge of the mechanics of organellar evolution and reveal unexpected structures like the possibility of circular phases of nuclear ribosomal genes noted above.

Our demonstration of a successful application of RCA to metazoan mtDNAs may make possible the widespread use of organellar genomics in phylogenetic reconstruction as well as additional insights into the evolution of the mt genome and other circular DNA entities. Additional challenges remain, especially advancements in mitochondrial purification and continued development and refinement of RCA techniques. However, we predict that with a modest ramping up of investigator interest, RCA will make the retrieval and use of metazoan mitochondrial genomes commonplace in evolutionary and molecular studies, and Darwin's grand view may no longer be quite as distant.

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