

## Amplification of the complete mitochondrial genome of two protostome worms: a useful technique for comparative studies of metazoan mitochondrial DNA

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### Abstract

We report the first polymerase chain reaction (PCR) amplification of the complete mitochondrial genomes of a nemertean and a sipunculan worm in one piece using a recently published two-polymerase protocol for long and accurate DNA amplification. Successful amplification was achieved from nanogram quantities of both purified mitochondrial DNA (nemertean) and crude total DNA (sipunculan). This technique allows the rapid generation of sufficient quantities of entire mitochondrial DNAs for cloning and restriction fragment length polymorphism (RFLP) analyses, and thus will facilitate comparative studies of metazoan mitochondrial genomes.

### Introduction

The polymerase chain reaction has revolutionized comparative analyses of mitochondrial DNA (mtDNA), allowing the rapid accumulation of RFLP and sequence data from fragments of the genome. These data are useful for assessing intraspecific variation and for inferring relationships among animals at many taxonomic levels (Simon et al., 1994; Dowling et al., 1996). Comparisons of entire mtDNA sequences among major metazoan groups are beginning to reveal characters (e.g., gene arrangements) that are informative for assessing relationships among phyla and classes (Smith et al., 1993; Boore and Brown, 1994; Boore et al., 1995). However, the utility of PCR for this purpose has been limited

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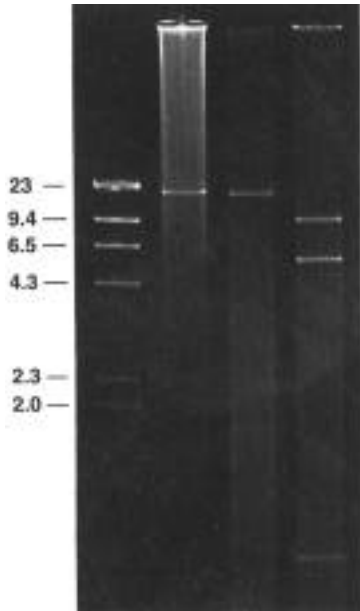
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because routine, high-yield amplification of DNA fragments longer than 5 kilobase pairs was not possible until recently (Barnes, 1994). Comparative sequence analyses of entire mtDNA typically require purification of mtDNA from tissues for subsequent restriction site analysis, cloning, or Southern analysis. The purification procedure is time-consuming and frequently requires optimization for each new taxon and pooling of individuals to obtain sufficient quantities of tissue (Dowling et al., 1990). Here we report the first successful PCR amplification of entire mtDNAs from representatives of two invertebrate metazoan phyla (Nemertea, Sipuncula) from purified mtDNA and from total DNA preparations, following a recently published protocol for obtaining long and accurate PCR amplification (LA-PCR) that uses a combination of two polymerases (Barnes, 1994). Previously, only complete human mtDNAs have been amplified in one piece using an alternative two-enzyme technique (Cheng et al., 1994a). Entire mtDNAs have been successfully amplified in two or more overlapping pieces (e.g., Torroni et al., 1992; Roehrdanz, 1995), but amplification of complete mtDNAs in one piece obviates the need for multiple primer sets and additional reactions.

### Results and Discussion

We have successfully amplified entire mtDNAs from CsCl-purified mtDNA and from both CsCl-purified and crude total DNA preparations using taxon-specific primers. The full-length products for the sipunculan and nemertean are shown in Figure 1, lanes 2 and 3, respectively. We used primer pairs derived from the *ND1* gene sequence for the nemertean and from the *C01* gene sequence for the sipunculan. The primer sequences were as follows: LND1-LA (31-mer) 5'-GCA TCC GCT AAC GGC TGT GGA ACA CCA ATA A-3', HND1-LA (32-mer) 5'-TTA ATG TTT ATG GGA CGT TAG TTG CGG GTT GG-3', LCO-LA (26-mer) 5'-CTA TAT AAG AGG AAG GAG TCA GTT CC-3', HCO-LA (25-mer) 5'-GTG GAC AAG GTT TCT GAA TAT TCC G-3'. There



**Figure 1.** Electrophoretic analysis of nemertean and sipunculan LA-PCR products and an *EcoRI* restriction endonuclease digest of the nemertean PCR product in a 1% agarose gel, after staining with ethidium bromide. Lane 1, DNA restricted with Hind III. Numbers correspond to fragment sizes in kilobase pairs. Lane 2, *Phascolopsis gouldii* LA-PCR product. Lane 3, *Cerebratulus lacteus* LA-PCR product. A small quantity of nonspecific product (< 9.4 kb in length) is present in lanes 2 and 3. Lane 4, aliquot of *C. lacteus* product digested with *EcoRI*. The arrowhead indicates a 1.1-kb restriction fragment. The pattern in lane 4 corresponds to that obtained by digesting purified mtDNA (data not shown). The DNA samples (7  $\mu$ l of a 50- $\mu$ l LA-PCR reaction for lanes 2 and 3) were electrophoresed at 100 V in 1 x TBE buffer until the bromophenol blue marker dye migrated to the end of the 14-cm long horizontal gel.

were no apparent differences in electrophoretic banding patterns or product yields when primers 25 nucleotides in length were used for the nemertean (data not shown).

We employed hot starts using wax beads to limit the possible formation of shorter nonspecific products that might have a higher amplification rate than the specific product (M. Frohlich, personal communication; Cheng et al., 1994b). However, a detectable amount of nonspecific product was obtained in some reactions (Figure 1). Nonspecific products often can be reduced or eliminated by decreasing either the number of amplification cycles or the amount of starting template (unpublished observations; see Roux, 1995, for standard PCR). In all amplification reactions the duration of the denaturation step was 3 to 5 seconds to limit possible heat-

induced depurination or deamination of the template DNA (see Barnes, 1994, and Cheng et al., 1994b). Heat-induced damage to DNA presumably inhibits strand extension and thus long-PCR (see Barnes 1994).

The identity of the amplified products as mtDNA was determined by comparing restriction fragment patterns with those obtained from purified mtDNA (nemertean; Figure 1, lane 4) and by sequencing the cloned PCR products (nemertean and sipunculan; data not shown). For successful cloning of long PCR products, we found it necessary to completely remove the polymerases by phenol-chloroform extraction or gel purification prior to restriction enzyme digestion. We speculate that residual KlenTaq activity may fill in overhangs or that the 3'  $\rightarrow$  5' exonuclease activity of *Pfu* may degrade the protruding 3' termini of the DNA after digestion, thereby greatly reducing the efficiency of ligation of the long-PCR product to the cloning vector (see also Crowe et al., 1991; Stewart et al., 1995).

Amplification of the entire mtDNA eliminates the time-consuming and variably successful mtDNA purification procedure, allows quantities of mtDNA necessary for cloning and RFLP analysis to be generated from nanogram amounts of total DNA, and should make it possible to obtain entire mtDNAs from single individuals of very small metazoans, including meiofauna. This methodology will greatly facilitate comparative analyses of gene arrangements in metazoan mitochondrial genomes, which promise to contribute additional characters useful for inferring high-level relationships. As previously suggested (Cheng et al., 1994a; Dowling et al., 1996), long PCR will also enhance the study of intraspecific variation by RFLP analyses.

### Experimental Procedures

DNA amplifications were carried out following the LA-PCR protocol described by Barnes (1994), using minor modifications suggested by Dr. Michael Frohlich (personal communication). Each 50- $\mu$ l reaction contained 50 mM Tris-HCl, pH 9.1, 3.5 mM MgCl<sub>2</sub>, 16 mM (NH<sub>2</sub>)<sub>2</sub>SO<sub>4</sub>, 150  $\mu$ g/ml bovine serum albumin (BSA), 300  $\mu$ M of each dNTP, 0.1  $\mu$ M of each primer, 14 KlenTaq LA units (Ab Peptides, Inc.), and 0.09 units of *Pfu* (Stratagene). We added 6.25  $\mu$ l of *Pfu* to 100  $\mu$ l of KlenTaq LA and used 0.6  $\mu$ l of this enzyme mix per 50- $\mu$ l reaction (M. Frohlich, personal communication). The 3' exonuclease activity of *Pfu* polymerase removes 3' mismatched bases that would otherwise inhibit further

strand extension by Klentaq LA (Barnes, 1994; Cheng et al., 1994b). Hot starts using wax beads (PCR Gems, Perkin-Elmer) were employed in all cases (M. Frohlich, personal communication; Chou et al., 1992; Chong et al., 1994b). Only buffer, enzyme, and template were added above the wax barrier. The mtDNA of the nemertean *Cerebratulus lacteus* was amplified from both purified mtDNA (less than 2 ng; Figure 1) and 250 ng of CsCl-purified total DNA (data not shown). The mtDNA of the sipunculan *Phascolopsis gouldii* was amplified from approximately 10 to 80 ng of CsCl-purified DNA (data not shown) and from 150 ng of a crude total DNA preparation (Figure 1; CTAB extraction, Saghai-Marooof et al., 1984). We used a Perkin-Elmer Cetus 9600 thermal cycler for all reactions. The conditions used were 94°C for three to five seconds for denaturation and 68°C for 15 minutes for extension. The two-step profile was repeated for 30 to 35 cycles. Amplification products were phenol-chloroform extracted and digested with *EcoRI* according to routine procedures outlined in Sambrook et al. (1989). *EcoRI* fragments were cloned into BlueScript KS<sup>+</sup>, (Stratagene, Inc.), following standard protocols (Sambrook et al., 1989). Dideoxy chain-termination sequencing of plasmid DNA was performed as described in the Sequenase handbook (U.S. Biochemical).

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