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Nematode Mitochondrial DNA: Anomalies and Applications[1]

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Abstract: The mitochondrial genome of animal cells is currently under intense investigation by molecular, evolutionary, and population biologists. This review summarizes the available information on the molecular biology of nematode mitochondrial DNA and explains how the fundamental knowledge obtained from these basic studies may be applied to nematode systematics, evolution, and diagnostics.

Key words: diagnostics, gene amplification, mitochondrial DNA, systematics.

Nematodes represent a cosmopolitan group of organisms occupying virtually every conceivable habitat. Many species are obligate parasites of plants or animals; as such they cause a wide variety of pathogenic conditions. The economic importance of infectious nematodes to agriculture and to veterinary and clinical medicine has engendered a rich history of experimental investigation. These research directions have traditionally focused upon solving problems in disease prevention and treatment of infected hosts (30).

Within the past decade, a renewed emphasis on understanding the basic biology of nematodes has emerged. Contributing to these advances is the application of molecular genetics to nematological investigation. Until recently, molecular biology of nematodes has been confined to *Caenorhabditis elegans,* originally recognized by Brenner (7) as a useful model to examine metazoan development. *C. elegans* has since proven to be an exceptional experimental system, accommodating such diverse fields as microanatomy, biochemistry, cellular and molecular biology, and developmental genetics (33). Much of the fundamental information garnered from studies on *C. elegans* will most certainly be applicable to other nematodes as well.

The first unified discipline arising from molecular approaches to non-free living nematodes concerns the characterization of their mitochondrial genomes. Because of its abundance within eukaryotic cells, simple genetic constitution, and relevance to population and evolutionary biology (3), mitochondrial DNA (mtDNA) was an obvious object of study for molecular geneticists interested in venturing into nematology. The purpose of this brief review is to convey the excitement being generated within this emerging field and to summarize the available information on the molecular biology of nematode mtDNA, including several surprising discoveries currently exclusive to nematode mitochondrial genomes. In addition, I hope to convey how the knowledge obtained from these basic studies can be directly applied to the venerable fields of nematode identification, systematics, evolution, and pathogen treatment.

A Short Primer on Animal mtDNA

Mitochondria and chloroplasts contain double-stranded DNA molecules that typically comprise 1–10% of the total genetic material found within eukaryotic cells. Cytoplasmic DNA is distinct from bulk nuclear DNA in that the molecules are significantly smaller than eukaryotic chromosomes and encode a limited number of genes necessary, but not sufficient, for organelle function. Additional genes required for organelle biogenesis are located within nuclear DNA. Because of the small physical size and elevated cellular copy number, organelle genomes can be easily purified and characterized at the molecular level.

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Animal mtDNA has now been characterized from a diverse set of vertebrate and invertebrate taxa. Despite large evolutionary distances, striking conservation is observed with respect to circular structure, genome size, and gene content (8,22). MtDNA varies only slightly in molecular size, generally ranging from 14 to 19 kilobase pairs (kb). Based on contour length as observed in the electron microscope, animal mtDNA is often described as a "five-micron circle."

The same set of genes are encoded in almost all animal mitochondrial genomes, irrespective of the source. This includes two ribosomal RNA (rRNA) genes and information for 22 transfer RNA (tRNA) molecules which are part of an organelle-specific protein synthesis (translation) system. Each mitochondrial genome contains 13 structural (protein coding) genes whose products contribute to the electron transport chain and oxidative phosphorylation (ATPase) complexes. In short, the genetic content of mtDNA provides information necessary for the assembly of a functional, respiring organelle. In addition to mitochondrial genes, each molecule contains a control region responsible for the regulation of mtDNA replication and RNA synthesis (transcription). Slight differences in control region length are responsible for the small variations in mitochondrial genome size observed within different organisms (22).

Animal cell mtDNA is highly economized. Unlike their nuclear counterparts, mitochondrial genes are not split by introns (noncoding DNA segments) nor separated from each other by lengthy stretches of spacer DNA sequences. Informational sequences within mtDNA adjoin each other and may even share nucleotides, creating "overlapping" genes.

In many organisms, mtDNA is evolving at an apparent rate 10–100 times that of single copy nuclear DNA (9). It is this unusual finding that has become exceedingly attractive to evolution and population biologists. Divergence in mtDNA nucleotide sequences may accumulate during a relatively short evolutionary time window, a period in which traditional genetic and morphological markers would remain unchanged. Therefore, mtDNA analysis has recently become a powerful method for studies concerning population dynamics and evolution (3,22).

Molecular Biology of Nematode mtDNA

The available information regarding the physical and genetic structure of nematode mtDNA will be compared with the "standard" five-micron circular animal mitochondrial genome. To date, mtDNA from eight different *Meloidogyne* spp. and host-races (17,24) and two *Heterodera* spp. (25) have been partially characterized at the molecular level, whereas the mitochondrial genomes from *Romanomermis culicivorax* (16,23), *Ascaris suum* (32), and *C. elegans* (32) have been analyzed in greater detail.

Identification and initial characterization: Nematode mtDNA was first detected in *Ascaris* eggs. Equilibrium centrifugation of DNA prepared from these cells revealed a minor component exhibiting a buoyant density different from that of bulk chromosomal DNA. The assumed mitochondrial origin of this material (6) was verified by demonstration that this same DNA species was enriched in purified mitochondrial fractions and occurred as five-micron circular molecules (11,28). MtDNA levels were elevated in aerobic eggs but were appreciably lower in cell types with reduced respiratory activity or mitochondria populations, such as anaerobic body wall muscle tissue and spermatozoa (19,26). At the molecular level, the *Ascaris* mitochondrial genome remains the best characterized nematode mtDNA, as detailed in the following sections.

Diversity of nematode mtDNA: Table 1 lists reported several molecular size estimates for nematode mtDNA. These lengths range from an unusually small 13.8 kb for *C. elegans* mtDNA (D. Wolstenholme, pers. comm.) to a remarkably large 26–32 kb for the *R. culicivorax* mitochondrial genome (16,23). This wide variation in mtDNA sizes

TABLE 1. Nematode mitochondrial DNA size diversity.

Nematode	mtDNA size†	Comment	Reference
Romanomermis culicivorax	26–32‡	Tandem and unlinked repeated sequences	Powers et al. (23); Beck and Hyman (4)
Heterodera glycines	22.5	—	Radice et al. (25)
Heterodera schachtii	23.0	—	Radice et al. (25)
Meloidogyne spp.	20–23	Size variation among species	Powers et al. (24); Hyman et al. (17)
Ascaris suum	14.3	ATPase8 absent; small tRNA genes	Wolstenholme et al. (32)
Caenorhabditis elegans	13.8	ATPase8 absent; small tRNA genes	D. Wolstenholme, pers. comm.

† mtDNA sizes are listed in kilobases (kb).
‡ Several size polymorphisms present in laboratory population.

represents the first of several departures from typical animal mtDNA.

The molecular basis of mtDNA size diversity among different nematodes is now partially understood. The small size of the *A. suum* and *C. elegans* mitochondrial genome (32; D. Wolstenholme, pers. comm.) is primarily due to the absence of the entire gene encoding the ATPase8 polypeptide (Fig. 1), a coding sequence present within all other animal mtDNA studied to date. Whether this gene resides within nuclear DNA or is entirely dispensable for catalytic activity of the *A. suum* and *C. elegans* ATPase complex is not known.

R. culicivorax mtDNA is 8–14 kb larger than standard animal mitochondrial genomes. A recent discovery revealed that *R. culicivorax* mtDNA contains reiterated DNA sequences (16). The amplified DNA segments are present as tandem duplications (stretches of identical nucleotide sequence placed adjacent to each other within the mtDNA). Several replicas of this same DNA sequence are also distributed elsewhere in the mitochondrial genome. These "dispersed" copies are present in an inverted orientation relative to the tandemly repeated units. This organization suggests that the original amplification event produced numerous copies, some of which became displaced by genetic rearrangement, possibly involving sequence inversion. Sequence amplification resulting in tandem

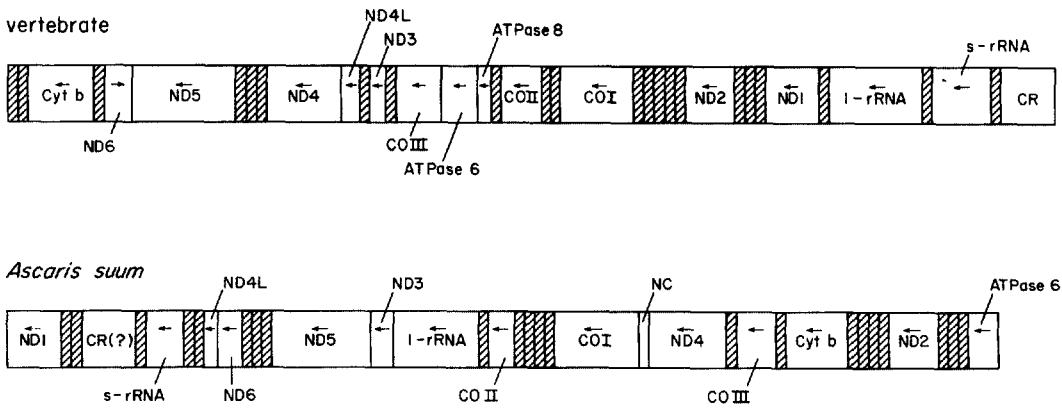

FIG. 1. Genetic organization of vertebrate and *Ascaris* mtDNA. Striped regions represent 22 individual tRNA genes, which are not specifically identified. Arrows represent direction of transcription. COI–COIII, genes for subunits I, II, and III of the cytochrome *c* oxidase complex; ND1–ND6, genes for subunits 1 through 6 of the NADH dehydrogenase complex; Cyt b, gene encoding the apoenzyme of cytochrome *b*; s-rRNA and l-rRNA, genes for the small and large ribosomal RNA; NC, noncoding DNA region. This composite figure has been adapted from Bibb et al. (5), Moritz et al. (22), and Wolstenholme et al. (31).

duplications has been observed within several vertebrate mitochondrial genomes (18,20,21,29) and in scallop mtDNA (27); however, the occurrence of both tandem and dispersed repeats within the same mitochondrial genome has not been observed in other animal mtDNA. The size variation observed among *R. culicivorax* mitochondrial genomes (26–32 kb) is due to the precise copy number of the repeated segments found in each molecule (16). If only one copy of this amplified DNA segment is necessary for proper mtDNA function, the information content of this organelle genome is approximately 17 kb, well within the size range of typical animal mtDNA. Therefore, the unusually large size does not represent increased genetic coding capacity of the *R. culicivorax* mitochondrial genome.

Gene order: The placement of genes within vertebrate mtDNA is highly conserved. Based on the complete nucleotide sequences determined for the human (1), bovine (1), and mouse (5) mitochondrial genomes, the order of the protein coding and rRNA genes is displayed in Figure 1. Punctuating these major genes are 22 tRNA-encoding sequences whose position is also invariant among vertebrate mtDNA. The replication and transcription control region resides between Cyt b and the small rRNA gene.

In contrast to the conservation of gene order among vertebrate mtDNA, gene arrangement varies among the few invertebrate mitochondrial genomes that have been examined (31). In turn, each differs from vertebrate mtDNA. Among nematodes, complete nucleotide sequence information is available for *A. suum* and *C. elegans* mtDNA (32; D. Wolstenholme, pers. comm.). The gene order within *Ascaris* mtDNA is compared with the vertebrate mitochondrial genome in Figure 1. As within vertebrate mtDNA, tRNA genes also lie between these protein coding and rRNA genes; however, the position of the tRNA genes differs from corresponding locations within vertebrate mtDNA.

Limited information on gene mapping within *R. culicivorax* mtDNA is also available. The COI and COII genes are separated by approximately 8 kb (16). This result was unexpected because within vertebrate and invertebrate mitochondrial genomes, including those of *A. suum* and *C. elegans* (32), COI and COII are adjacent protein coding sequences (Fig. 1). It is possible that the genetic rearrangements involved in establishing the distribution of the previously described reiterated sequences within this mtDNA also may have played a role in separating these two genes. Based on this result, the gene organization within *R. culicivorax* mtDNA differs from *A. suum* and *C. elegans*, as well as from other invertebrates.

Other anomalies: Another surprise emerging from studies of nematode mtDNA is the structure of the organelle-specific tRNA molecules, as deduced from the nucleotide sequence of the *A. suum* and *C. elegans* mitochondrial genomes (32). The secondary structure of prokaryotic, eukaryotic, and organelle tRNA is a universal, four-armed cloverleaf configuration. Mitochondrial tRNA from *C. elegans* and *A. suum* appear to be missing one complete arm. This unique structural feature has been deduced from mtDNA gene sequences; verification awaits isolation and analysis of the actual tRNA molecules. If this novel conformation remains correct, our perception of the architecture for the mitochondrial translational machinery in these two nematodes must necessarily change. It will be of utmost interest to examine whether this unusual tRNA structure is conserved among other nematode mitochondrial genetic systems.

Applications of mtDNA to Nematological Problems

Molecular diagnostics: Several features unique to mtDNA make it an obvious tool for nematode molecular diagnostics. Because it is often present in hundreds of copies in each cell, mtDNA can be obtained in preparative yields. The elevated cellular copy number is also easily detected in DNA–DNA hybridization assays. The

rapid evolution rate of mtDNA (9) provides a taxonomic fingerprint that may discriminate among closely related isolates. As mtDNA is maternally inherited and does not recombine at detectable levels (12), genetic changes can appear only within descendents of a mutant female. This provides a convenient tool for relating individuals to one another.

Two general types of approaches have been used in the development of mtDNA-based assays for the detection and identification of nematode populations. The first strategy involves DNA–DNA hybridization using labeled mtDNA to detect the presence of related mitochondrial genomes in exudates prepared from infected host tissue. The molecular basis of nucleic acid hybridization as a diagnostic tool lies in the complementarity of nucleotides in double-stranded DNA that form precise hydrogen bonds which hold the two strands together. These bonds can be broken at elevated temperatures, thereby denaturing the DNA into single strands. Upon subsequent cooling, the single strands can then hybridize or anneal with each other via hydrogen bonding to regenerate the double-stranded form. In molecular diagnostic assays, specific hybridization probes are generated by isolating a fragment of DNA (e.g., mtDNA) from the target organism. This DNA is labeled in vitro with radioactive compounds or biotinylated nucleotides. Once labeled and denatured, the probe is able to bind to any single-stranded molecule that shares nucleotide sequence similarity. DNA preparations to be "hybridized" may be derived from crude lysates containing mixtures of cell types. The DNA within these preparations is denatured and bound to a nitrocellulose filter. By incubating the labeled probe with filter-bound DNA, the presence of nematode mtDNA can be unambiguously detected. The specificity of nucleotide complementarity between two strands of a double helix permits DNA chains of similar sequence, but not divergent DNA, to anneal in hybridization assays conducted under the appropriate experimental conditions. Even though this strategy requires technical expertise, it is well suited to field testing.

Meloidogyne mtDNA has been detected in the presence of unfamiliar DNA derived from infected host tissue and other contaminating organisms using a simple spot-blot procedure (17,24). Hybridization signals can be obtained from a single egg, larva, or adult (17,23) because of the high cellular copy number of the target mtDNA. Lastly, several hundred samples can be simultaneously processed.

The second strategy involves direct physical characterization of nematode mtDNA by restriction enzyme analysis. Restriction endonucleases are enzymes that recognize a specific nucleotide sequence within the DNA and make two cuts, one in each strand. The DNA is thus cleaved into smaller fragments of specific sizes. Single base pair changes within the DNA may create or eliminate specific target sites for cleavage by restriction enzymes. Such changes are reflected in the sizes of DNA segments produced by the restriction enzymes. When fractionated according to size by gel electrophoresis, the patterns of these mtDNA restriction fragments may act as diagnostic physical markers for closely related nematode isolates. Divergence in mtDNA derived from *M. chitwoodi* and *M. hapla,* as assessed by restriction enzyme analysis, is displayed in Figure 2.

Restriction fragment length polymorphism (RFLP) within nematodes was first discovered in the repetitive component of total cellular DNA prepared from two different strains of *C. elegans* (variants Bristol and Bergerac) and in *C. briggsae* (15). Restriction analysis of *Trichinella, Romanomermis, Steinernema* (syn. *Neoplectana*) (13), and several *Meloidogyne* spp. (13,14) cellular DNA also revealed striking polymorphism. The differences in DNA fragmentation patterns observed among these isolates could not be assigned to either reiterated nuclear DNA sequences or to mtDNA, as both were present in these types of preparations. RFLP within repeated chromosomal DNA or mtDNA are easily visualized by gel electrophoresis, and either

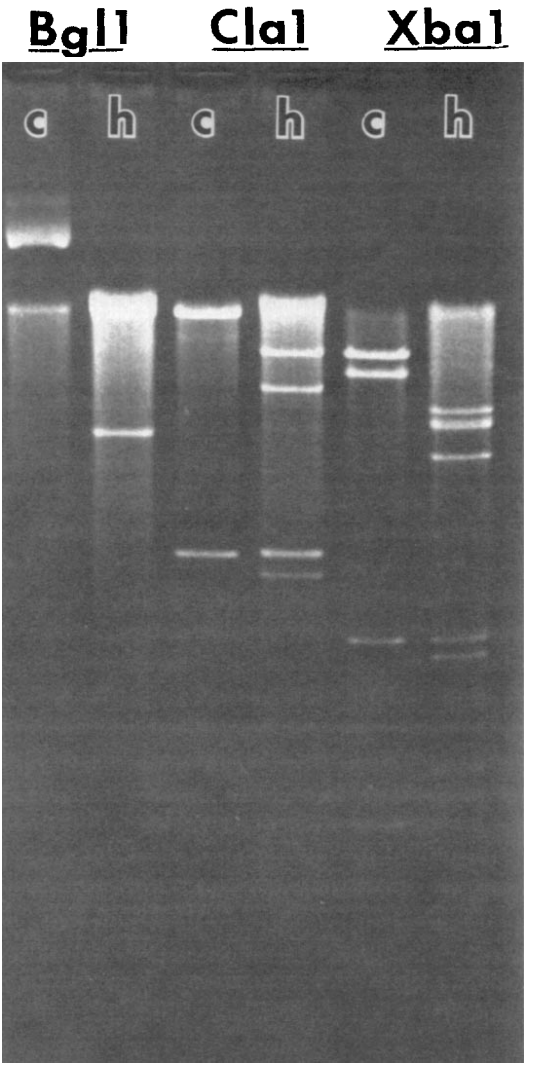

FIG. 2. Divergence of *Meloidogyne* spp. mtDNA as revealed by restriction enzyme analysis. MtDNA was prepared from either *M. chitwoodi* (c) or *M. hapla* (h) and cleaved in independent reactions with the restriction enzymes *Bgl*I, *Cla*I, or *Xba*I. Sequence divergence among the restriction sites for each mtDNA generates a different spectrum of polymorphic DNA restriction fragment products that is diagnostic for each of the two species. (Photograph provided by J. Peloquin and B. Hyman.)

would provide convenient markers for identification and discrimination among nematodes. The decided advantages of employing mtDNA for these purposes include its cellular copy number, which is often elevated above that of repeated nuclear DNA segments, and its rapid rate of evolution (9), which increases the probability of RFLP occurring among closely related species.

Fragment length polymorphism within nematode mtDNA was first discovered in *Meloidogyne* spp. (24). Diagnostic restriction fragment patterns were observed among *M. incognita, M. javanica, M. hapla,* and *M. arenaria,* with *M. hapla* mtDNA exhibiting the most divergence among these mitochondrial genomes. Consistent with restriction enzyme analysis, DNA hybridization assays revealed that *M. incognita* mtDNA shared sequence similarity with *M. arenaria* and *M. javanica* mitochondrial genomes, but not with *M. hapla* mtDNA (24). Recently, similar experiments have demonstrated that the mtDNA derived from *M. hapla* and *M. chitwoodi* do not share significant amounts of nucleotide sequence similarity (17). This result may be of immediate utility to the wheat and potato industries in the Pacific Northwest, where *M. hapla* and *M. chitwoodi* are sympatric and precise identification among these species is crucial for implementation of proper crop rotation regimes. MtDNA-based molecular diagnostics is also under development for the detection of several soybean and sugarbeet cyst nematodes (4).

Molecular evolution and systematics: Because mitochondrial genomes are easily characterized, subject to rapid mutation rates (9), composed mostly of coding sequences, and uniparentally transmitted by maternal inheritance, mtDNA has recently become an important tool for addressing problems in population genetics, phylogenetic relationships, and evolutionary biology (3,22). MtDNA has been used as a marker for founder events, species history, hybrid zone dynamics, and female gene flow.

MtDNA analysis was first used to study genetic variation and population structure within the genus *Peromyscus* (the deer mouse) (2). These experiments revealed that conspecific populations may have arisen from either a single female or small group of females that carried a progenitor mitochondrial genome. Cann et al. (10) have employed similar strategies to inves-

tigate the evolution of present-day human populations. Analysis of polymorphic restriction enzyme cleavage sites within mtDNA derived from 147 individuals (representing five geographical locations) suggests that all human mtDNA may stem from a common African source, such as a single female that lived some 200,000 years ago.

Limited information is available regarding application of mtDNA analysis to nematode evolutionary biology. Multiple restriction site polymorphisms were observed within the mitochondrial genomes of five *Meloidogyne* spp. (17,24). Host-race separation, as compared to speciation, appears to be a recent event, as judged by the relative absence of polymorphism found within mtDNA derived from *M. incognita* races 1, 3, and 4 (24). Estimation of nucleotide substitution rates and divergence time between isolates will require a more quantitative treatment of *Meloidogyne* mtDNA analysis by applying a wider spectrum of restriction enzymes to mtDNA preparations isolated from several geographically separated populations.

Divergence time between *Heterodera glycines* and *H. schachtii,* two cyst nematode sibling species, has recently been estimated using mtDNA restriction enzyme analysis (25). Assuming that nematode populations analyzed in these experiments were subject to similar selective pressures and that nucleotide substitution rates for nematode mtDNA reflect those measured for unrelated taxa, it is estimated that *H. glycines* and *H. schachtii* diverged 7.3–14.5 million years ago. This indicates that speciation has been established over a lengthy time period.

Perspectives and Future Prospects

Investigation into the diversity of animal cell mitochondrial genomes has generated an important instrument for examining population dynamics and phylogenetic relationships. Several powerful methods using mtDNA for detection and identification of closely related species have been developed. Numerous studies covering a wide variety of organisms have benefited from mtDNA characterization (3,22). Only recently have these approaches been used to address related problems in nematology. The initial reports in these areas have demonstrated that the theory and practice of mtDNA analysis can easily be extended to nematode systems.

Characterization of gene order within mtDNA may provide another strategy for studying the evolutionary history and phylogenetic relationships of nematodes. The Nematoda has traditionally been divided into two main classes, the Secernentea and Adenophorea. Of the three nematodes whose mitochondrial gene organization has now been analyzed, *A. suum* and *C. elegans* reside among the Secernentea and contain mtDNA exhibiting identical gene order (32; D. Wolstenholme, pers. comm.). In contrast, *R. culicivorax* is in the Adenophorea and exhibits a mitochondrial gene order different from that of *A. suum* and *C. elegans* (16). Given the substantial differences in the physical and genetic constitution of mitochondrial genomes derived from this limited sampling of nematode classes, further studies on the organization of nematode mtDNA may illustrate the utility of genome rearrangements as an important tool in nematode systematics.

Less obvious is the potential for the direct application of several anomalies now described for nematode mitochondrial genetic systems. The likelihood that genome rearrangements may become useful tools for taxonomic classification has already been broached. The novel tRNA genes found in *A. suum* and *C. elegans* mtDNA (32) raises the intriguing possibility that the organelle-specific translation machinery of some parasitic nematodes may be decidedly different from that of its plant or animal host. If so, nematicidal compounds that interfere with nematode mitochondrial gene expression (and therefore respiration) while leaving host cells unaffected might be developed.

An impressive amount of information about the molecular genetics of nematode mitochondrial genomes has been accu-

mulated in the last few years. Based on the studies summarized here, nematode mtDNA analysis should continue to provide an important resource for investigators interested in systematics, evolutionary biology, and fundamental molecular genetics.

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