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Mitochondrial Genome Haplotype Hypervariation Within the Isopod Parasitic Nematode *Thaumamermis cosgrovei*

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ABSTRACT

Characterization of mitochondrial genomes from individual *Thaumamermis cosgrovei* nematodes, obligate parasites of the isopod *Armadillidium vulgare*, revealed that numerous mtDNA haplotypes, ranging in size from 19 to 34 kb, are maintained in several spatially separated isopod populations. The magnitude and frequency of conspecific mtDNA size variation is unprecedented among all studied size-polymorphic metazoan mitochondrial genomes. To understand the molecular basis of this hypervariation, complete nucleotide sequences of two *T. cosgrovei* mtDNA haplotypes were determined. A hypervariable segment, residing between the *atp6* and *rrnL* genes, contributes exclusively to *T. cosgrovei* mtDNA size variation. Within this region, mtDNA coding genes and putative nonfunctional sequences have accumulated substitutions and are duplicated and rearranged to varying extents. Hypervariation at this level has enabled a first insight into the life history of *T. cosgrovei*. In five *A. vulgare* hosts infected with multiple nematodes, four carried nematodes with identical mtDNA haplotypes, suggesting that hosts may become infected by ingesting a recently hatched egg clutch or become parasitized by individuals from the same brood prior to dispersal of siblings within the soil.

METAZOAN mitochondrial genomes are generally small in size (12–20 kb) and encode 36–37 genes (Boore 1999) whose products contribute to the processes of electron transport, oxidative phosphorylation, and mitochondrial protein synthesis. Much larger genomes (up to 43 kb) have occasionally been described and their expanded sizes are usually the result of duplications of mitochondrial DNA (mtDNA) sequences, rather than increased gene content (Fuller and Zouros 1993; Boore *et al.* 2005; Dellaporta *et al.* 2006). A comprehensive survey of animal mitochondrial DNA size polymorphism revealed that small, incremental mtDNA length variation is common among conspecifics; much of this variation is caused by copy number variation of short, noncoding tandem repeat arrays, often confined to the main replication control region of the molecule (Lunt *et al.* 1998). Larger size variations also occur among animal mtDNAs, but at much lower frequencies (Gach and Brown 1997).

We report here an atypically large and hypervariable mtDNA found in the nematode *Thaumamermis cosgrovei*, an obligate parasite of the terrestrial isopod *Armadillidium vulgare* (Poinar 1981). In a study of >100 *T. cosgrovei* individuals from several geographically isolated populations, we found that this mitochondrial genome is strikingly variable in size (Tang and Hyman 2005); individual nematodes maintain haplotypes ranging from 19 to 34 kb. Surprisingly, no two individuals appear to share the same haplotype, and we have employed the term "hypervariation" to describe this observation. Mitochondrial genome size heteroplasmy within some individuals was also observed. To understand the molecular basis of hypervariation among *T. cosgrovei* mitochondrial genomes, complete nucleotide sequences of two *T. cosgrovei* mtDNA haplotypes have been determined. Our data revealed that *T. cosgrovei* mtDNA can be divided into a constant region and a hypervariable region. It is the hypervariable region that contributes exclusively to *T. cosgrovei* mtDNA size variation, as within this locus, mtDNA coding and putative nonfunctional sequences have been duplicated and rearranged to varying extents. Different copy numbers, sequence rearrangement, and extensive nucleotide substitutions within these repeated elements give rise to size polymorphism and sequence divergence among *T. cosgrovei* mtDNAs.

The extensive and frequent mtDNA size variation in *T. cosgrovei* may also provide the key to a better understanding of its life history. For *T. cosgrovei*, no information is available as to the mechanism of isopod infection and dispersal. Infrequently, a single isopod host is multiply infected with 2–16 juvenile nematodes. Hypervariable mtDNA haplotypes can be used to test whether

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TABLE 1

Primers used in PCR amplification

Primer	Primer sequence
cox1F	5′-CGGTATGGAGACTACCTAGATTCCTACTTG-3′
cox1R	5′-GATCGTCTGTCAATATCCAACCCAACTC-3′
cobF	5′-AATCTGACCTCGATGTTGACTTAA-3′
cobR	5′-TTAAGTCAACATCGAGGTCAGATT-3′
rrnLF	5′-CTTCGATCAATTCCTAACAAGCTGGGTGC-3′
rrnLR	5′-GCACCCAGCTTGTTAGGAATTGATCGAAG-3′
atp6F	5′-TGTAATATTATAGCAGGACATGTG-3′
atp6R	5′-CACATGTCCTGCTATAATATTACA -3′

individuals parasitizing the same host are the result of spatially or temporally separate infection events.

MATERIALS AND METHODS

Nematodes: Isopod hosts were collected from the Botanic Garden on the University of California-Riverside campus and from private yards in Riverside, Highland, and Rancho Cucamonga, California. The isopods were bent backward by hand, splitting the exoskeleton, and submerged into 0.15 M NaCl. Slim white J3 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 was employed to prepare an enriched population of circular molecules from nematodes that was suitable template for rolling circle amplification (RCA) reactions, as described previously (Tang and Hyman 2005). *T. cosgrovei* total genomic DNA was purified from individual nematodes using a mini-lysate procedure (Powers *et al.* 1986) and used for hybridization analysis and as template in conventional PCR amplifications.

PCR amplification: Primer pairs were designed on the basis of conserved nematode cytochrome oxidase subunit 1 (*cox1*), large mitochondrial ribosomal RNA (*rrnL*), cytochrome b (*cob*), and ATP synthase F_0 subunit 6 (*atp6*) gene sequences and are listed in Table 1.

Regions between *rrnL* and *cox1*, *cox1* and *cob*, and *cob* and *atp6* were amplified by long-distance PCR (Expand 20kb^{plus} kit, Roche, Mannheim, Germany). Long-distance PCR amplifications were performed in 50-μl reactions with 38.5 μl sterilized distilled water, 5 μl 10× Expand PCR reaction buffer (with 22.5 mM $MgCl_2$), 2.5 μl 10 mM dNTP, 1 μl of each primer (10 μM), 1 μl DNA template prepared by a mini-lysate procedure (Powers *et al.* 1986), and 1 μl Expand Long Template enzyme mix. Long-distance PCR cycling conditions include 92° for 2 min (initial strand denaturation), followed by 35 cycles that included 92° for 10 sec (denaturation), 56° for 40 sec (primer annealing), and 68° for 10 min (extension). A final elongation step was then conducted at 68° for 7 min.

RCA: RCA reactions were conducted using the TempliPhi kit (Amersham Biosciences, Piscataway, NJ) as previously described (Tang and Hyman 2005).

Restriction enzyme digestion, gel electrophoresis, and Southern blot analysis: Total genomic DNA from individual *T. cosgrovei* nematodes were digested to completion with restriction endonucleases according to manufacturer's instructions. Restriction fragment products were fractionated by agarose gel electrophoresis, visualized by ethidium bromide staining, and then transferred to Immobilon-NY+ membranes (Millipore, Bedford, MA). Digoxigenin-tagged DNA probes were generated by random primed labeling, hybridized to target sequences, immunodetected with antidigoxigenin-AP and Fab fragments and then visualized with the colorimetric substrates nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche, Mannheim, Germany).

Molecular cloning and sequencing: PCR products were cloned into the pGEM-T EASY vector (Promega, Madison, WI) via TA cloning. Targeted mtDNA restriction fragments were excised from agarose gels using the QIAEXII gel extraction kit (QIAGEN, Valencia, CA) and subcloned into the plasmid pCR2.1 (Invitrogen, Carlsbad, CA). Sequencing reactions for recombinant clones were carried out at the University of California-Riverside Institute for Integrative Genome Biology Core Facility on an Applied Biosystems (Foster City, CA) 3730xl automated sequencer.

Sequence analysis: DNA sequence assembly and alignment were conducted using the Accelrys (San Diego) SeqWeb platform v.2 of the GCG package (Madison, WI). Protein and rRNA gene sequences were identified by comparing their similarity to published metazoan mtDNA sequences. Some tRNA genes were recognized by tRNAscan-SE (Lowe and Eddy 1997) and DOGMA algorithms (Wyman *et al.* 2004); others were identified by making visual searches for their anticodon sequences and screened for the potential of adjacent sequences to fold into cloverleaf structures. The secondary structure of rRNA genes was deduced by analogy to other published nematode mitochondrial rRNA gene structures and drawn with the RnaViz program (De Rijk and De Wachter 1997).

Mitochondrial gene rearrangement distance determination: Mitochondrial gene rearrangement distances among different nematodes were determined by GRIMM (Multiple Genome Rearrangements; Tesler 2002). The rearrangement distance between any two genomes is the minimum number of rearrangement steps (including reversals and translocations) required to transform one genome into the other (Sankoff *et al.* 1992).

RESULTS

Physical characteristics of *T. cosgrovei* mtDNA: large size, high frequency of conspecific size variation, and heteroplasmy: Two approaches were used to estimate the sizes of the mtDNA molecules from different individual *T. cosgrovei* nematodes. These included transfer-hybridization experiments with total genomic DNA and restriction enzyme digests of RCA-amplified complete mitochondrial genomes.

T. cosgrovei mtDNA displays a remarkable frequency and extent of length variation, as demonstrated by Southern blot analysis. Figure 1, A and B, depicts representative examples of mtDNA size polymorphism within nine individual *T. cosgrovei* nematodes as revealed by transfer hybridization of an 11-kb digoxigenin-labeled mtDNA segment encompassing *rrnL* to *atp6* (Figure 1C) to *Pst*I- and *Eco*RI-cleaved genomic DNA. *Pst*I is expected to linearize the *T. cosgrovei* mitochondrial genome on the basis of nucleotide sequence (Figure 1C); thus, the size of the hybridized *Pst*I fragment represents the length of the complete mitochondrial genome in each genomic digest. As shown in Figure 1A, the size of the hybridized *Pst*I fragment varies considerably among individuals, indicating mtDNA length heterogeneity within the population. Four of the nine *T. cosgrovei* individuals examined

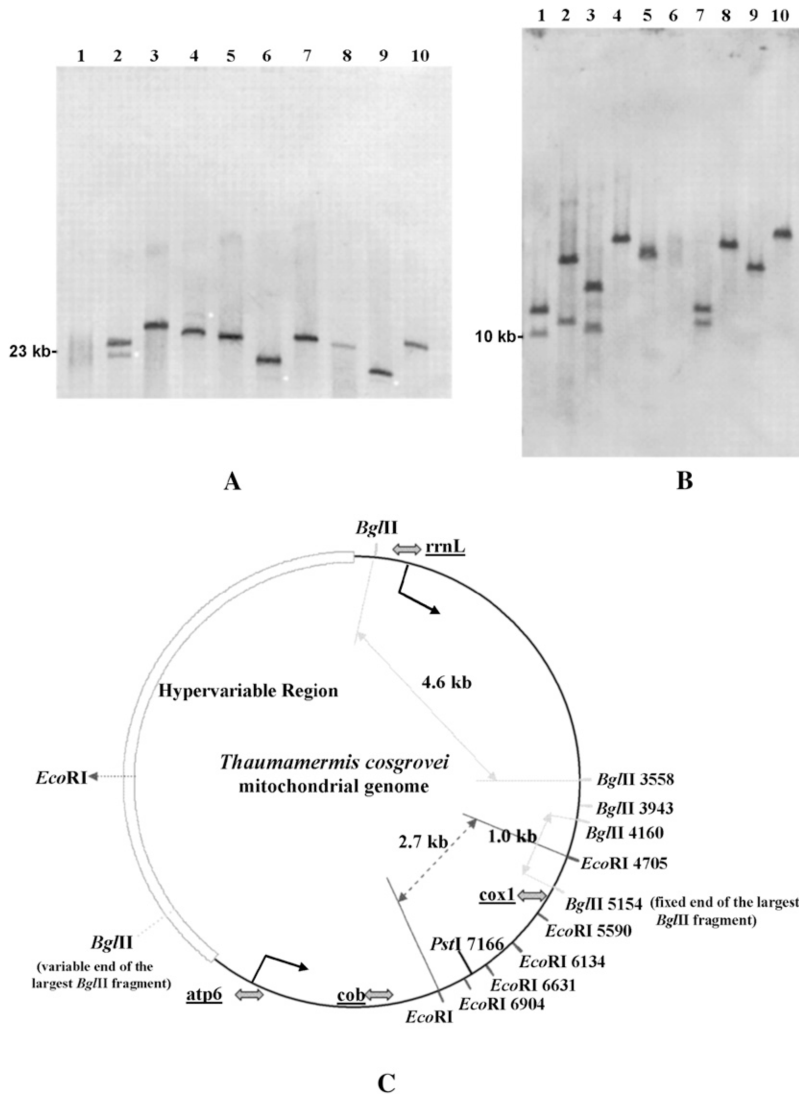

Figure 1.—Hybridization analysis of restriction-enzyme-digested *T. cosgrovei* genomic DNAs from 9 individual and 15 pooled nematodes. (A) Genomic DNA cleaved by *Pst*I. Lane 1, pooled nematodes; lanes 2–10, individuals I–X. (B) Genomic DNA cleaved by *Eco*RI. Lanes 1–5, individuals I–V; lane 6, total DNA from pooled nematodes; lanes 7–10, individuals VI–IX. (C) Restriction map of the *T. cosgrovei* mitochondrial genome based on the complete nucleotide sequence. The mitochondrial segment used as probe in hybridization (*rrnL–atp6*) is delimited by angled arrows; shaded arrows show positions of primers used in long-distance PCR amplification; light arrows delimit the 4.6- and 1.0-kb *Bgl*II fragments shared by all the *T. cosgrovei* individuals in the local University of California-Riverside population; dashed arrow defines a 2.7-kb region that is not expected to be visualized by hybridization in B (see text); small light dots indicate the substoichiometric haplotype in heteroplasmic individuals. All nematodes were obtained from *A. vulgare* hosts collected on the University of California-Riverside campus.

here are heteroplasmic for two distinct size classes of mtDNA (Figure 1A, lanes 2, 4, 6, and 9); typically, in these heteroplasmic individuals, one mtDNA size class is present in substoichiometrical yields. Another restriction enzyme, *Eco*RI, is known to cleave the molecules at six invariant sites located within a 2.7-kb region (between positions 4705 and 7371) (Figure 1C). Comparison of the hybridization profiles of *Pst*I and *Eco*RI digests from the homoplasmic individuals revealed that *Eco*RI might also cleave at one additional position in mtDNAs of some individuals. The size of the hybridized *Eco*RI fragment (Figure 1B, lanes 4, 5, and 8–10) or the sum of the sizes of the two hybridized *Eco*RI fragments (Figure 1B, lanes 1–3 and 7) represents the length of the complete mtDNA circle minus 2.7 kb of the common sequence (the five small bands that have migrated off the bottom of the gel).

The hybridization profiles in Figure 1, A and B, revealed the presence of mtDNA size polymorphism and the numerous haplotypes maintained in the *T. cosgrovei* isolate found on the University of California-Riverside campus. When total cellular DNA prepared from 15 pooled nematodes was cleaved by *Pst*I or *Eco*RI (Figure 1A, lane 1, and Figure 1B, lane 6), distinct bands were not detected; instead, a smear was observed, suggestive of a continuum of mtDNA size variants within the population. Data from *Pst*I and *Eco*RI cleavage were used to estimate the mtDNA size carried within individual nematodes. This analysis revealed that the sizes of their mitochondrial genomes range from 19 to 34 kb within this sampling.

Rolling circle amplification of complete *T. cosgrovei* mitochondrial genomes (Tang and Hyman 2005) provides a more vivid demonstration of mtDNA size variation (Figure 2, A and B). A complex array of restriction products was obtained when the RCA products amplified from the pooled *T. cosgrovei* template were digested with *Bgl*II (Figure 2A, lane 4, and Figure 2B, lane 8). *Bgl*II digestion of RCA products from the eight individual nematodes gave simpler patterns composed of seven to nine visible bands that often represent a subset of the fragments observed when pooled template was

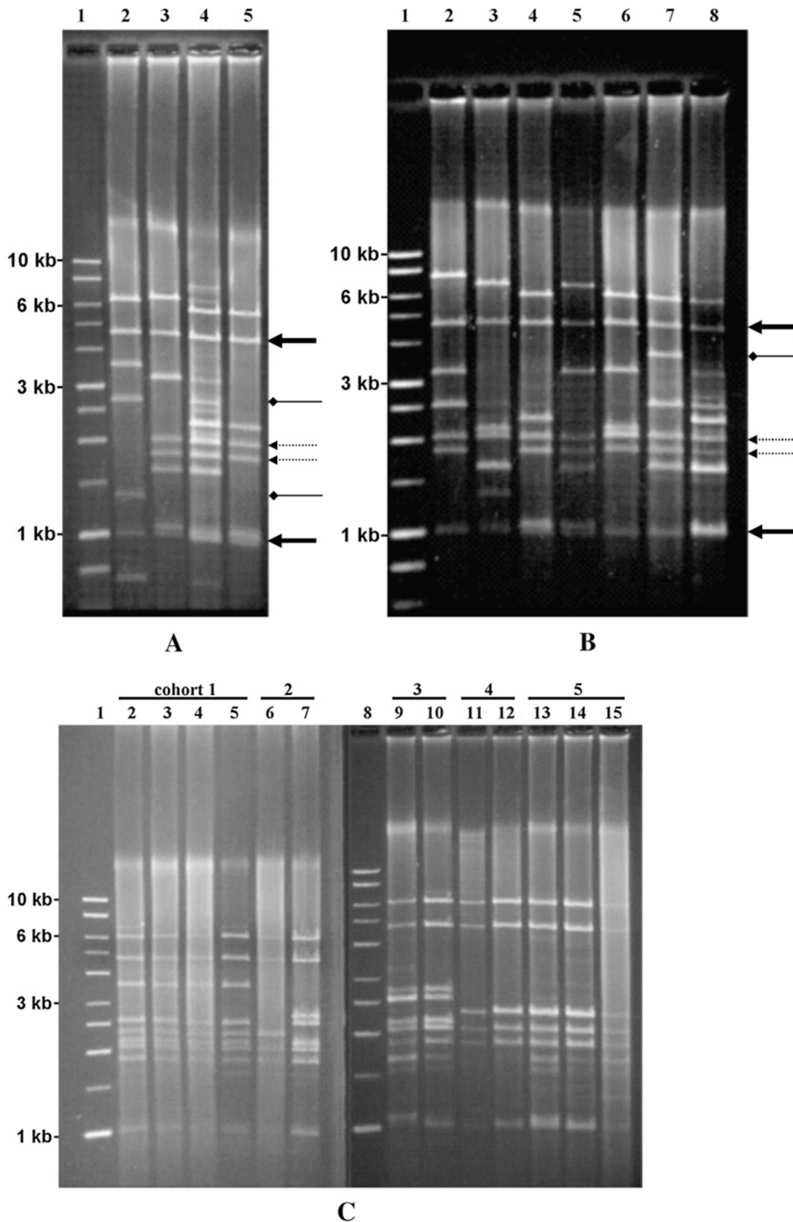

Figure 2.—Rolling circle amplification of *T. cosgrovei* mtDNA from pooled and individual nematodes. Rolling circle amplification reaction products were digested with *Bgl*II and fractionated on a 1.0% agarose gel. Solid arrows designate the 4.6- and 1.0-kb *Bgl*II fragments shared by all *T. cosgrovei* individuals in our local population; dashed arrows indicate *Bgl*II bands that are shared by the majority of individuals; diamond-headed arrows indicate *Bgl*II bands that are infrequently shared among individuals. (A) Lane 1, 1-kb DNA molecular weight marker (Promega); lane 4, *Bgl*II-cleaved RCA product using template from eight pooled nematodes; lanes 2, 3, and 5, *Bgl*II-cleaved RCA product using template from a single nematode; lanes 2 and 5 correspond with mtDNA haplotypes II and I, respectively. (B) Lane 1, 1-kb DNA molecular weight marker; lane 8, *Bgl*II-cleaved RCA product using template from eight pooled nematodes; lanes 2–7, *Bgl*II-cleaved RCA product using template from a single nematode. (C) Characterization of mtDNA structures of nematodes from multiply infected isopod hosts. Lanes 1 and 8, 1-kb DNA molecular weight marker; lanes 2–5 (cohort 1), lanes 9 and 10 (cohort 3), lanes 11 and 12 (cohort 4), and lanes 13–15 (cohort 5) represent four hosts containing cohorts with identical mtDNA structures; lanes 6 and 7 (cohort 2) revealed different mtDNA structures. All nematodes were obtained from *A. vulgare* hosts collected on the University of California-Riverside campus.

employed. On occasion, bands derived from individual haplotypes do not match fragments found in the pool. This is likely the result of sampling a limited number of nematodes to create the mixture. There are only two *Bgl*II restriction products shared by all the individuals sampled, sized at 4.6 and 1 kb. Determination of the complete nucleotide sequence of two haplotypes reveals two additional shared bands of 385 and 217 bp, positioned between *rrnL* and *cox1* (Figure 1C; not visible on the gels displayed in Figure 2). The size and distribution of other *Bgl*II bands differ among individuals. Some additional bands are shared by many of the individuals (Figure 2, dashed arrows), while others may be unique or infrequently shared among individuals (Figure 2, diamond arrowheads).

mtDNA haplotypes are shared by *T. cosgrovei* individuals in the same *A. vulgare* host: Occasionally, individual isopod hosts are multiply infected with J2 stage nematodes. Our surveys have indicated that *A. vulgare* hosts can be infected with 2–16 nematodes. Hypervariation of mtDNA haplotypes allows us to test whether multiple *T. cosgrovei* individuals developing within a single host are the result of temporally or spatially independent infections. Nematodes from multiply infected, individual *A. vulgare* hosts were dissected as a cohort, and the mtDNA haplotype from each individual nematode was determined using RCA and *Bgl*II cleavage. Four of five cohorts (Figure 2C, cohorts 1 and 3–5) reveal identical mtDNA structure whereas differences in mtDNA structure are observed in a fifth group (cohort 2, Figure 2C). Only in multiply infected *A. vulgare* hosts have we found nematodes with shared mtDNA haplotypes.

Two completely characterized *T. cosgrovei* mtDNA haplotypes: genome size and general features: To

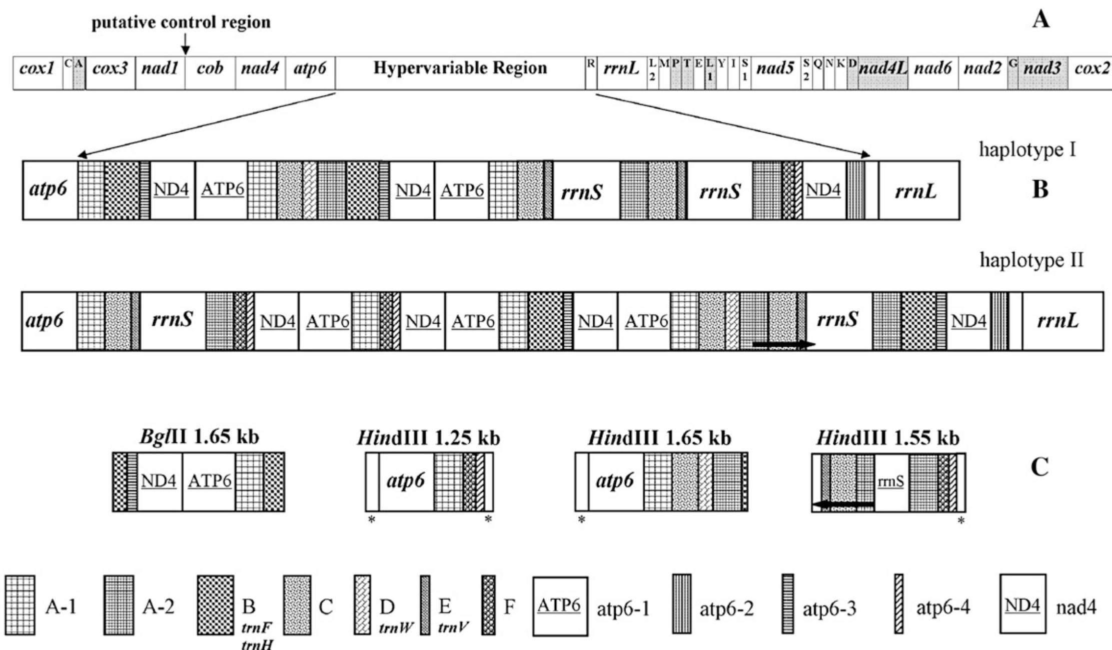

Figure 3.—Transcription organization of two *T. cosgrovei* mtDNA haplotypes. (A) Transcriptional map of *T. cosgrovei* mtDNA common skeleton. (B) Hypervariable regions of haplotypes I and II. (C) Unique segments derived from other hypervariable regions within the haplotype pool. These mtDNA segments were obtained after restriction enzyme digestion and molecular cloning of RCA-amplified mtDNAs using total cellular DNAs from pooled nematodes as template. These fragments did not conform to restriction fragments from the hypervariable region from haplotypes I and II. Although circular, the maps (A) have been displayed in a linear form for ease of comparison. Italized gene names indicate functional, intact gene copies.The tRNA genes are abbreviated using the one-letter amino acid code. L1, L2, S1, and S2 indicate *trnL*(UAA), *trnL*(UAG), *trnS*(UGA), and *trnS*(UCU), respectively. In A, open boxes are genes transcribed from left to right while shaded boxes are genes transcribed from right to left. Repeated units marked with an asterisk in C are partial nad4 repeats. Thick arrows delimit regions with identical nucleotide sequences in direct (→) or reversed (←) orientations.

further understand the molecular basis of hypervariation in *T. cosgrovei* mitochondrial genome size, complete nucleotide sequence was determined for two mtDNA haplotypes. Haplotype I (Figure 2A, lane 5) was chosen because it displays a rather simple *Bgl*II restriction product pattern relative to other haplotypes and all *Bgl*II fragments can be found within the mtDNAs of a majority of the individuals sampled. Haplotype II (Figure 2A, lane 2), in contrast, represents a rather unique architecture; except for the 4.6- and 1-kb shared bands, all other *Bgl*II fragments derived from haplotype II are present infrequently within the population. Both of the chosen haplotypes are also relatively small in size, facilitating their sequencing and assembly. *T. cosgrovei* mitochondrial genome haplotype I is 20,013 bp; haplotype II mtDNA is 21,508 bp in size.

Both *T. cosgrovei* mtDNA haplotypes encode the same set of 36 genes that are typically found in metazoan mtDNA: 12 protein coding genes, two rRNA genes, and 22 tRNA genes. The 36 genes are encoded on both strands (Figure 3A). The ATPase subunit 8 gene is absent from *T. cosgrovei* mitochondrial genomes, as in all other nematode mtDNAs except that of *Trichinella spiralis* (Lavrov and Brown 2001). The mitochondrial gene order of *T. cosgrovei* is unique among known metazoan mitochondrial genomes.

On the basis of the restriction enzyme digestion patterns of RCA products from different *T. cosgrovei* individual nematodes and the complete sequence of the two mtDNA haplotypes, the mtDNA of *T. cosgrovei* can be divided into a constant region and a hypervariable region (Figures 1C and 3). The constant region includes most of the coding genes and is shared among all the haplotypes. The hypervariable region is located between the *atp6* and *rrnL* genes (Figures 1C and 3). The hypervariable segment contains complete *rrnS* gene copies (the only copies of this gene in the mtDNA) and partial, likely nonfunctional copies of some mitochondrial genes (*nad4*, *atp6*, and sometimes *rrnS*), duplicated to different copy numbers, resulting in mtDNA size variation and occasional heteroplasmy.

Nucleotide composition: The A+T content of *T. cosgrovei* mtDNA is 71.36 and 71.33% for haplotypes I and II, respectively. A+T composition is evenly distributed along the genome. Sequence of the constant mtDNA region is composed of 71.72% A+T. The A+T content decreases slightly to 70.79 and 70.81% in the hypervariable regions of haplotypes I and II.

Protein-coding genes: Most mitochondrial protein genes in *T. cosgrovei* are modestly shorter than orthologs in other nematodes except when compared to those of *Xiphinema americanum*, which maintains the smallest

nematode mtDNA described to date (He *et al.* 2005). Eleven protein-coding genes use the standard start codons typical of invertebrate mitochondrial genes (ATT, ATA, ATG, and TTG) as their translation initiation codons, while an ATC codon was predicted to occur at the beginning of the *T. cosgrovei cox2* gene. While not used as an initiation codon in other nematodes studied to date, ATC has been predicted as the start codon for some mitochondrial protein-coding genes in arthropods (Stanton *et al.* 1997 and Kim *et al.* 2006), which are considered close relatives of the nematodes as both are pseudocoelomates (Adoutte *et al.* 1999).

Of the 12 protein coding genes, 10 (*atp6*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad4*, *nad4L*, *nad5*, and *nad6*) employ a complete translation termination triplet codon (either TAA or TAG). Two others (*nad2* and *nad3*) appear to use only a template encoded "T," which can be converted to a complete stop codon TAA by polyadenylation (Ojala *et al.* 1981).

Each amino acid in *T. cosgrovei* mtDNA is represented as either a two- or a four-codon family, except for leucine and serine, which are specified by a combination of two families. Within each codon family, codons with either A or T in their third position are used more frequently. The most abundant acids encoded by the *T. cosgrovei* mitochondrial genome (supplemental Table 1 at http://www.genetics.org/supplemental/) are leucine, serine, phenylalanine, and isoleucine, all of which except serine are nonpolar amino acids. Arginine, cysteine, aspartic acid, and glutamine, the least frequently represented amino acids, are all hydrophilic. These observations are consistent with the fact that the mtDNA-encoded proteins are inner-membrane enzymes involved in oxidative phosphorylation.

Protein-coding gene families: Both *atp6* and *nad4* genes are duplicated in the hypervariable region as nonidentical, putative, nonfunctional, partial copies. Reiterated *atp6* copies are not organized as tandem repeats, but are interspersed between other sequences in the hypervariable region. There are four types of partial *atp6* genes (hereafter designated as atp6-1–atp6-4; intact, functional gene designations are italized while possible pseudogenes are in regular type). The functional *atp6* gene is 534 bp in length and is located just upstream from the hypervariable region in haplotypes I and II (Figure 3). atp6-1 is 529 bp and differs from the intact *atp6* gene at 10 positions, including five nucleotide deletions (supplemental Figure 1A at http://www.genetics.org/supplemental/). Nucleotide sequence divergence between the functional, intact *atp6* gene and the putative, nonfunctional atp6-1 repeat mostly resides in the first 24 bp of the functional *atp6* gene copy. Encoded amino acids conserved among mermithid nematode ATP6 proteins (SPF at positions 7–9, W at position 76) are not found in atp6-1 copies (supplemental Figure 1B at http://www.genetics.org/supplemental/); therefore, atp6-1 is considered a pseudogene. There are two copies of atp6-1 in haplotype I and three copies in haplotype II (Figure 3B). atp6-2 is composed of 217 bp and its sequence is identical to that of the 5′ 217 bp of atp6-1. Only one copy of atp6-2 is found in the hypervariable region of either haplotype I or haplotype II. atp6-3 is 106 bp in length. Four insertions and 18 substitutions (10 transitions and 8 transversions) can be identified when its sequence is aligned with that of the 5′ portion of the intact *atp6* gene, rendering this copy a pseudogene as well. atp6-4, 65 bp in size, presents a further degenerated copy of the *atp6* gene. Its 3′ 32-bp nucleotide sequence is the same as that of atp6-3 while the 5′ 33 bp have undergone substantial substitution (supplemental Figure 1A at http://www.genetics.org/supplemental/).

The intact, functional *nad4* gene copy is located 5′ to the intact *atp6* gene copy within the common skeleton (Figure 3). The *nad4* gene is also duplicated multiple times in the hypervariable region (three times in haplotype I and four times in haplotype II). The duplicated copies are direct, interspersed copies as with the atp6 gene family copies (Figure 3). The putative, nonfunctional nad4 pseudogene copies, 432 bp in length, are truncated and represent only ∼36% of the complete *nad4* gene, which is 1197 bp in size (supplemental Figure 1C at http://www.genetics.org/supplemental/). Several major deletions have occurred in the *nad4* copies. Nucleotide sequences of the nad4 pseudogene copies are almost identical to each other and have not undergone copy-specific changes, as observed among the atp6 pseudogene families.

Ribosomal RNA genes: The relative positions of the two ribosomal RNA genes (*rrnL*, *rrnS*) are not spaced similarly in different *T. cosgrovei* mtDNA haplotypes. There is one large ribosomal RNA gene (*rrnL*) located in the constant region. Two copies of the small ribosomal RNA gene (*rrnS*) reside in the hypervariable region in both haplotypes. In another *T. cosgrovei* mtDNA haplotype analyzed by restriction enzyme mapping, only one copy of *rrnS* resides within the hypervariable region (data not shown).

The predicted *T. cosgrovei rrnS* is 667 bp with a nucleotide composition of T(37.3%) > A(36.9%) > G(14.1%) > C(11.7%) (supplemental Figure 2 at http://www.genetics.org/supplemental/). Its size is similar to that of mitochondrial *rrnS* genes in other nematodes. The *rrnL* gene is predicted to be 821 bp (supplemental Figure 3 at http://www.genetics.org/supplemental/) and the nucleotide composition of this gene is T(39.1%) > A(38.6%) > G(12.2%) > C(10.1%). It is the second smallest of all available nematode *rrnL* gene sequences after that of *X. americanum* (729 bp; He *et al.* 2005).

Transfer RNA genes: Encoded within the *T. cosgrovei* mtDNA is a set of 22 tRNA genes (Figure 3) typical of metazoan mitochondrial genomes. The mt-tRNA genes vary in size from 52 bp (*trnS*-UCU, *trnS*-UGA, *trnQ*, and *trnR*) to 62 bp (*trnH* and *trnV*). Predicted secondary structures of the mt-tRNA genes (supplemental Figure 4 at

TABLE 2

Comparisons among repeated elements (gene families and repeated elements A–F)

Type	Length (bp)	A+T content (%)	Copy no.		Variation among copies (bp)[b]		
			I[a]	II	I	II	I and II
atp6							
atp6-1	529	72	2	3	1	1	2
atp6-2	217	74	1	1	—	—	0
atp6-3	106	69	2	2	0	0	0
atp6-4	65	68	1	2	—	0	0
nad4	432	73	3	4	4	1	5
rrnS	673	74	2	2	0	3	3
A							
A-1	293	68	3	4	1	1	2
A-2	278	69	3	3	0	0	0
B	291	64	2	2	0	1	1
C	258	69	3	3	1	1	1
D	130	72	1	1	—	—	0
E	91	74	2	2	0	0	0
F	174	68	1	2.	—	0	0

[a] Roman numerals I and II refer to haplotypes I and II.
[b] Number of nucleotide differences in base pairs.

http://www.genetics.org/supplemental/) are similar in topology to those found in other nematode mitochondrial genomes.

There are two intact copies of *trnF, trnV,* and *trnH* in both mtDNA haplotypes of *T. cosgrovei*, located within the hypervariable region. Interestingly, phenylalanine, valine, and histidine are not the most frequently used amino acids in *T. cosgrovei* mitochondrial proteome (9.53, 6.12, and 1.89%; supplemental Table 1 at http://www.genetics.org/supplemental/). The presence of multiple copies of the three tRNA genes appears to be a by-product of duplication events, and not due to selective pressure to increase gene numbers to meet the needs of mitochondrial translation.

Noncoding regions in the constant region: There are 749 bp in the constant region (6.1%) unassigned to genes. The longest noncoding region, between *nad1* and *cob* genes and 401 bp in length, is possibly the *T. cosgrovei* mtDNA control region, regulating replication and transcription. It is 73% A+T, slightly elevated relative to the remainder of the mitochondrial genome. This large noncoding region also has the potential to form elaborate stem-loop structures, a common characteristic of control regions (supplemental Figure 5 at http://www.genetics.org/supplemental/).

Hypervariable region in the two fully characterized haplotypes: The hypervariable region reveals a complicated organization; segments within this expanse can be cataloged into nine elements (gene families *atp6*, *nad4*, and *rrnS* as described earlier and the apparent noncoding elements A, B, C, D, E, and F), some of which can be further divided into subtypes (as with atp6-1–atp6-4) (Figure 3 and Table 2). All repeated sequences are arranged as direct repeats, but not positioned as tandem arrays.

Repeated elements A, B, C, D, E, and F vary between 91 to 293 bp in size and range from 64 to 74% in A+T content (Table 2). No significant nucleotide sequence similarities have been detected among these repeated elements nor among any known sequences deposited in the public databases.

There are two subtypes of repeated element A. A-1 is 293 bp in length and is usually adjacent to the 3′-end of the *atp6* gene or its pseudogenes (Figure 3). A-2 is 278 bp and is a truncated form of A-1 in that it does not contain the first 5′ 15 bp present in A-1. Copies of repeated element A exist as direct repeats in the hypervariable region and are separated by other types of repeated sequences. Six and seven copies of repeated element A are identified in *T. cosgrovei* mtDNA haplotypes I and II, respectively.

Repeated element B is 291 bp with 64% A+T content, representing the region of lowest A+T content in the *T. cosgrovei* mitochondrial genome. Functional *trnF* and *trnH* tRNA genes are located in repeated element B. Repeated element C is 258 bp in length and has the potential to encode a polypeptide of 74 amino acids. The element D is 136 bp. The 21 bp at the 3′-end of element D is a duplicated copy of the 3′-end of *rrnS*. *trnW* is encoded within the type D component. The *trnV* gene is situated within element E, which is 91 bp in length. There are two copies of the element E in each haplotype. The element F, 174 bp in length, is present once in haplotype I and twice in haplotype II.

Size variation between haplotypes I and II can be ascribed to the difference in the number of copies of

these repeated elements. There is one extra copy of each of the five repeated elements (atp6-1, atp6-4, nad4, type A-1, and F) in haplotype II and the sum of their size (1593 bp) exactly represents the size difference between mtDNA haplotype I and II.

Other characterized fragments from the hypervariable region: Four additional segments within the hypervariable region have been identified on the basis of restriction enzyme analysis after amplification from template obtained from pooled *T. cosgrovei* nematodes; these cannot be assigned to either haplotype I or haplotype II (Figure 3C). These fragments include 1.65-kb *Bgl*II, 1.65-kb *Hin*dIII, and 1.25-kb *Hin*dIII fragments that are composed of the same set of direct repeated elements detailed above but not syntenic with those in haplotypes I and II. Typically, there is only one copy of the functional *atp6* gene, located in the common skeleton, 5′ of the hypervariable region as found in the two fully characterized haplotypes (Figure 3A). However, a complete *atp6* gene copy can also be embedded within the hypervariable region (as in 1.25- and 1.65-kb *Hin*dIII fragments) of some *T. cosgrovei* mtDNAs. The 1555-bp *Hin*dIII fragment (Figure 3C) contains complete or deleted *rrnS* copies in both direct and inverted orientations relative to element F. The region from nucleotide 628 to 969 is identical to the last 342 bp of *rrnS* 3′ and therefore represents a pseudo-rrnS. DNA sequence from position 449 to 627 in this 1.5-kb element is the 3′ 179 bp of repeated element A and is in inverted direction compared to pseudo-rrnS. Repeated elements C and E are also found in a reverse orientation within this fragment. These observations indicate that additional rearrangements, including inversions, have occurred within the hypervariable region of some mtDNA molecules and contribute to *T. cosgrovei* mtDNA hypervariation.

DISCUSSION

Hypervariability: The magnitude of mtDNA haplotype size variation (19–34 kb) in *T. cosgrovei* is unprecedented among all studied size-polymorphic metazoan mitochondrial genomes. The frequency of intraspecific mtDNA size variation in *T. cosgrovei* individuals is also unique among animal mitochondrial genomes; virtually every *T. cosgrovei* individual present as a single parasite within its *A. vulgare* host carries its own mtDNA haplotype. Only nematodes present as a cohort in multiply infected hosts share a common mtDNA haplotype. We have extended the investigation to three other populations in reproductively isolated locations, including the cities of Riverside, California (∼2 km from the University of California campus), Highland, California (∼24 km north of Riverside), and Rancho Cucamonga, California (∼40 km northwest of the University of California-Riverside campus), and obtained similar results. No common haplotypes among locations were identified; this result stands in stark contrast to what is described for the sea scallop *Placopecten magellanicus*, where ∼50% of the scallops surveyed maintained the most common type in a single population (La Roche *et al.* 1990). Since a common *T. cosgrovei* haplotype was never observed, it is inferred that *T. cosgrovei* mtDNA molecules undergo frequent rearrangement. In another mermithid nematode, *Romanomermis culicivorax*, at least three mtDNA size variants (26, 29, and 32 kb) have been fixed (Powers *et al.* 1986), indicating that extensive mtDNA size polymorphism might be a common trait within this nematode lineage.

It has been proposed that segregation of mitochondrial genome haplotypes is governed by a balance of several factors, including genetic drift during germline vegetative segregation, selection of smaller mtDNA molecules with replicative advantages, and mutation to different mtDNA sizes due to replication errors (Rand and Harrison 1989; Rand 1993). If smaller mtDNAs exhibit prominent selective advantage in replication, it is expected that mitochondrial genomes of shorter contour length would be the most frequent haplotypes in the population and the most prevalent forms within a heteroplasmic individual (Solignac *et al.* 1984; Rand and Harrison 1986). In *T. cosgrovei*, smaller mtDNA haplotypes are often present in substoichiometric yields in most heteroplasmic individuals (Figure 1A, lanes 2, 6, and 9). Moreover, in population surveys cataloguing homoplasmic individuals, intermediate-sized mtDNA haplotypes (Figure 1A, lanes 3–5, 7, 8, and 10) are often observed. This result is consistent with earlier observations in crickets and in *P. magellanicus*, where the most common size class is the one with an intermediate copy number of repeated sequences (Rand and Harrison 1989; Zouros *et al.* 1992). The abundance of these intermediate-sized molecules may represent a balance between replication errors generating expansion of repeats and recombination or slipped-strand mispairing excising some repeat units.

Nematode gene order: Nematode mitochondrial protein-coding and rRNA gene orders are remarkably diverse, and especially so within the Enoplea (supplemental Figure 6 at http://www.genetics.org/supplemental/). The rearrangement distance between any two Enoplean nematodes is no smaller than that between any Enoplean nematode and a Chromadorean nematode, including comparisons within the Mermithidae (supplemental Table 2 at http://www.genetics.org/supplemental/). Nematode phylogenetic affinities based on mitochondrial gene order are misleading because of the high frequencies of gene rearrangement; it is unlikely that gene order can be used as a phylogenetic character with reliable signal for Enoplean nematodes, although further sampling among the Enoplea may be required to verify this premise. Similar conclusions regarding the utility of mtDNA gene order as characters in phylogenetic reconstructions

were drawn from mollusk mtDNA studies (Le *et al.* 2000; Dreyer and Steiner 2004).

Molecular evolution of the hypervariable region: The hypervariable region in *T. cosgrovei* mtDNA is of unparalleled complexity among metazoan mtDNAs, resulting in length polymorphism generating haplotypes that are not shared between individuals and are not duplicated in reproductively isolated populations. The repeated region contains several active and putative pseudogene copies that vary in copy number among different mtDNA haplotypes. These gene copies are not arranged in tandem, a departure from the most common form of animal mtDNA size polymorphism, which is often related to a variable number of tandem repeats (Lunt *et al.* 1998). Interestingly, repeats arranged in an inverted orientation can coexist with direct repeats in the same haplotype. The combination of these features is novel for animal mtDNAs. The brachiopod *Lingula anatine* also has an elaborate mtDNA repeat sequence structure. Nevertheless, the size of repeated segments in *L. anatine* is constant and all repeated elements are organized in the same orientation (Endo *et al.* 2005).

Possible molecular mechanisms involved in generating the repeat region: The most commonly invoked mechanism to explain mtDNA size variation and gene rearrangement is the duplication–random loss model (Moritz *et al.* 1987; Mueller and Boore 2005). In this model, a portion of the genome is duplicated due to replication errors. Those errors may include slipped-strand mispairing (Levinson and Gutman 1987), imprecise replication initiation or termination (Boore and Brown 1998), or illegitimate priming of DNA synthesis by a tRNA at the replication origin (Jacobs *et al.* 1989). After duplication, and depending on the patterns of random repeat copy loss due to mutation in the absence of selective pressure, the original mtDNA gene order is either restored or altered. However, this duplication–random loss model cannot easily explain repeated elements positioned in a nontandem fashion nor in inverted orientation.

Within *T. cosgrovei* mtDNA, repeated sequences are organized as nontandem arrays in the two fully characterized haplotypes and some repeated segments are separated by as much as 7.6 kb (two A elements in haplotype II). Although it is possible for the duplication–random loss model to explain this complicated architecture, this mechanism would require a large number of intermediates after extensive duplication. Moreover, the duplication–random loss model cannot easily account for the presence of inverted repeated units observed in the 1555-bp *Hin*dIII fragment identified from the haplotype pool. Mitochondrial DNA recombination can explain such arrangements if mtDNA is broken and the excised region then is reinserted in the opposite orientation; mtDNA sequence translocation accompanied by inversion could be the end product.

Occurrence of recombination in animal mtDNAs was controversial (Moritz *et al.* 1987). However, a growing literature from physical evidence (Lunt and Hyman 1997; Kajander *et al.* 2000; Ladoukakis and Zouros 2001a; Hoarau *et al.* 2002; Passamonti *et al.* 2003; D'Aurelio *et al.* 2004; Kraytsberg *et al.* 2004; Guo *et al.* 2006), biochemical studies (Thyagarajan *et al.* 1996), and quantitative genetics analysis (Awadalla *et al.* 1999; Ladoukakis and Zouros 2001b; Piganeau *et al.* 2004; Gantenbein *et al.* 2005; Tsaousis *et al.* 2005) from metazoa spanning large taxonomic distances suggests that intramolecular and/or intermolecular recombination can operate on animal mtDNAs. Recombination is now proposed to be a major underlying mechanism that generates highly rearranged mitochondrial genomes in numerous animal lineages (Dowton *et al.* 2003; Miller *et al.* 2004; Mundy and Helbig 2004; Endo *et al.* 2005; Nohara *et al.* 2005; Shao *et al.* 2005). Size polymorphism, heteroplasmy, and mitochondrial genome rearrangement is a common feature among available Enoplean nematode mtDNA complete sequences, including the family Mermithidae, and stands in contrast to the conserved economization and stable gene orders that typify the Chromadorean nematodes (He *et al.* 2005). Duplication, coupled with intra- or intermolecular recombination, would be a plausible and parsimonious explanation for the complicated structure of the hypervariable region and occasional heteroplasmy in *T. cosgrovei* mtDNA and other mermithid nematodes. Such processes must occur frequently to generate and maintain such a rich polymorphism of mtDNAs of vastly different sizes within different *T. cosgrovei* individuals.

Origins and degeneration of repeated elements: Generally, when a gene is duplicated and multiple copies of the same gene coexist in one mitochondrial genome, one or more copies of the repeated gene are expected to evolve under relaxed selection pressure, retaining at least one functional copy. Therefore, sequence divergence among members in the same gene family can suggest the relative ages of the corresponding events.

In *T. cosgrovei* mtDNAs, several gene families and repeated elements within the hypervariable region that contribute to haplotype variation have been defined. For example, *atp6* genes are repeated in four different forms. atp6-1 loci differ from the functional *atp6* copy in only 10 positions (~1.9%), indicating atp6-1 repeats may be products of a recent duplication of authentic *atp6*; atp6-2 repeats match precisely the first 217 bp of atp6-1, suggestive of a recent incomplete duplication of atp6-1. However, considerable degeneration must have occurred to convert the authentic *atp6* gene, or atp6-1, into atp6-3 and atp6-4 repeats (79 and 83% similar to corresponding sites in *atp6*; supplemental Figure 1A at http://www.genetics.org/supplemental/). The *nad4* gene and nad4 repeat copies display an even lower level of nucleotide similarity at 68.3% (supplemental Figure 1C at http://www.genetics.org/supplemental/). With such substantial divergences, we may infer that the very first duplication of *nad4* and *atp6* genes to yield the nad4

and the atp6-3 and atp6-4 pseudogene copies are earlier events. atp6-4 is more similar to atp6-3 than to atp6, atp6-1, or atp6-2, implying that atp6-4 might be the consequence of amplification of atp6-3 and further deterioration. A+T content of other repeated elements is similar to that of the constant region (64–74% *vs.* 72%), suggesting that these repeats might also have originated from within the mitochondrial genome but decayed beyond recognition. If so, the degree of substitution indicates that these duplication events were also quite early.

Low-level divergence between copies of the same repeated element in current haplotypes: Although substantial divergences have been identified between functional *atp6*, *nad4* genes, and their putative pseudogene forms, sequence conservation is found at levels >99% among different copies of the same gene family members (*e.g.*, all atp6-1 and nad4 copies) in *T. cosgrovei* mtDNA (Table 2). This is true for both coding and noncoding regions of the hypervariable segment. Such a high level of nucleotide conservation among repeats could be explained by two possible mechanisms: either these major duplications have occurred recently or concerted evolution mechanisms may be active to homogenize repeats, as suggested for *R. culicivorax* mtDNA (Hyman and Azevedo 1996). In *T. cosgrovei*, because virtually every individual nematode carries a different mtDNA haplotype and new haplotypes are generated frequently, recent duplications appear to be the more plausible explanation for the low-level divergence between copies of the same repeat unit.

In addition, we have sequenced 4-kb PCR products from the common skeleton using templates derived from both pooled nematodes and from *T. cosgrovei* individuals. This expanse encompasses five protein-coding genes. Greater than 99.5% sequence identity was found among all these segments (data not shown). This evidence indicates that strong selection is acting on the common skeleton to maintain the integrity and function of major coding genes.

Factors that may contribute to hypervariation and unique gene order: Previous research suggests that there may be a link between compact mitochondrial genomes and metabolic efficiency (Selosse *et al.* 2001). In human cell lines, high levels of mitochondrial duplications lead to measurable reduction in respiratory chain efficiency (Holt *et al.* 1997). *T. cosgrovei*, as well as other mermithid nematodes, are obligate parasites of invertebrates and have low metabolic requirements in their postparasitic stage (Nickle 1972). Such life-history traits may be a factor in the evolution of the enlarged and hypervariable *T. cosgrovei* mitochondrial genome.

An accelerated rate of mtDNA rearrangement associated with a parasitic life style has been proposed (Dowton and Austin 1995; Castro *et al.* 2002). Factors suggested to impact rearrangements within parasite mitochondrial genomes include elevated speciation rates, increased rates of mutagen flux, and deficiency in mtDNA repair (Castro *et al.* 2002).

Another possible factor in generating unique parasite mitochondrial genome architectures could involve horizontal gene transfer between the host and the parasite organelle genomes (Davis and Wurdack 2004; Andersson 2005). *A. vulgare*, the host of *T. cosgrovei*, also maintains an extremely unusual mitochondrial genome; the host mtDNA is structurally polymorphic, composed of 14-kb monomer and 28-kb dimeric circular molecules (Raimond *et al.* 1999). However, mitochondrial gene order is not available for *A. vulgare* and it is not yet possible to determine if there were gene transfers between mitochondrial genomes in this host–parasite interface.

Mitochondrial DNA strand nucleotide composition bias (Crozier and Crozier 1993) can influence the success of rearrangement by placing genes in an unfamiliar, A+T-rich context. Analysis of nucleotide composition in the *T. cosgrovei* mitochondrial genome reveals no strand bias (data not presented), indicating that this mtDNA may be tolerant of a wide ensemble of events.

Using mtDNA haplotypes to study the mode of isopod infection by *T. cosgrovei*: The life history of *T. cosgrovei* has not been studied and little is known about how *A. vulgare* becomes infected by nematodes. There are two general routes for multiple nematode infection. One possible mechanism involves host ingestion of an egg clutch containing J1 stage juvenile nematodes (passive infection, as was described for two other mermithid nematodes, Mermis and Pheromermis; Kaiser 1991); a second pathway to parasitism suggests that hatched, multiple J2 stage infectious juveniles independently infect a single host, perhaps separated in a temporal and spatial sense. The second mode is termed active infection and is characteristic of all other mermithid genera with which infectivity has been studied (Kaiser 1991). The extensive hypervariation of mtDNA haplotypes in *T. cosgrovei* now leads to an answer to the question of whether multiple *T. cosgrovei* individuals developing within a single host are the result of independent parasitism by infectious juveniles that may represent different maternal lineages. Identical mtDNA haplotypes in four of five cohorts (Figure 2C) suggest that most often (i) isopod hosts ingest *T. cosgrovei* eggs carrying juveniles from a single maternal source and genetically related *T. cosgrovei* individuals develop within the host or (ii) these parasites, derived from the same maternal lineage, enter the isopod at an early preparasitic stage just after hatching but before they disperse to different localities in the environment. The difference in mtDNA haplotypes among individuals of one cohort suggests that, on less-frequent occasions, (i) mixed cohorts can be the result of parasitism by a single, heteroplasmic matrilineal lineage or (ii) juvenile *T. cosgrovei* nematodes derived from different lineages may independently infect isopods by direct penetration. However, the fluidity of *T. cosgrovei* mtDNA architecture may suggest that the mixed haplotype

cohort could instead be due to *de novo* changes after infection.

Conclusions: The *T. cosgrovei* mitochondrial genome displays several features that are unusual among metazoan mtDNA architectures. These distinctive features include (i) frequent and large-scale intraspecific mtDNA size variation (hypervariation); (ii) extensive duplication of coding genes (*atp6*, *nad4*, *rrnS*, *trnF*, *trnV*, and *trnH*); (iii) remnants of ancient duplications (*e.g.*, *nad4* and *atp6* genes and their pseudogene copies), as well as indications of recent DNA amplifications (*e.g.*, low-level divergence among nad4 pseudogene copies); and (iv) a unique metazoan mtDNA gene order. The two *T. cosgrovei* mtDNA mitochondrial genomes analyzed in this study provide the first record of multiple intraspecific mtDNA haplotypes with large-scale size variation that has been fully characterized at the nucleotide level. The nontandem arrangement of repeated units in these two haplotypes and the discovery of occasional inversion of some duplicated segments in the haplotype pool suggest that duplication, coupled with recombination, could be an underlying mechanism to generate *T. cosgrovei* mtDNA size polymorphism and occasional heteroplasmy. These processes must occur very frequently, possibly in real time, to generate and maintain such a rich polymorphism. Full characterization of additional *T. cosgrovei* mtDNA haplotypes may be helpful in providing additional insights into the molecular evolutionary history of *T. cosgrovei* mitochondrial genomes.

The high frequency of size variation makes *T. cosgrovei* an excellent model system for categorizing genetic variation among intraspecific individuals, for investigating the nature and frequencies of mtDNA mutations, for studying life histories and evolutionary origins of mermithid species, and for examining the mechanisms for mitochondrial gene rearrangements and patterns of non-Mendelian inheritance. This system may also offer the opportunity for "real time" observation of sequence trafficking that contributes to hypervariation while maintaining the integrity of the common skeleton.

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