

Genome skimming reveals a multipartite mitochondrial genome in the parasitic nematode *Diectophyme renale*

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Abstract

Background

Diectophyme renale, known as the “giant kidney worm,” is a parasitic nematode of significant medical and biological importance due to its large size and complex interactions with various mammals, including humans, causing dioctophymiasis. Despite its importance, genomic information for *D. renale* is limited. Only partial sequences of mitochondrial genes and 18S rRNA have been reported, classifying it within Clade I nematodes, alongside *Trichuris* and *Trichinella*. Mitochondrial genomes are valuable for phylogenetic, biogeographic, and population studies. The nematode mitochondrial genome is typically circular, encoding 12 protein-coding genes, 22 tRNAs, and two rRNAs, although some species exhibit multipartite mitogenomes. Given the lack of comprehensive genomic data, this study aims to sequence the mitogenome of *D. renale* using genome skimming and long-read sequencing, and to perform comparative analyses to assess its structure, composition, and phylogenetic relationships within Clade I nematodes, considering the influence of its unique life history traits.

Results

We found that the single circular mitochondrial chromosome typically found in animals has evolved into a multipartite organization in *Diectophyme renale*, consisting of eleven minichromosomes, each containing a single protein-coding gene and an associated non-coding region. While multipartite mitogenomes have been reported in other metazoan lineages, this architecture, strongly supported by long-read sequencing from independent library constructions, represents a novel mitogenome structure within nematodes. Comparative analyses indicate that mitochondrial protein-coding sequences in *D. renale* cluster phylogenetically with those of vertebrate-parasitic nematodes within Clade I, whereas the structural fragmentation of the mitogenome appears to have evolved independently.

Conclusions

Although the functional implications of a multipartite mitogenome remain unknown, future work should examine whether specific life-history traits or selective pressures associated with parasitism may have contributed to its emergence. We also present a long-read genome-skimming workflow suitable for non-model organisms, offering a strategy to expand mitogenomic resources in taxonomic groups that remain markedly underrepresented.

Background

The parasitic helminth *Diectophyme renale* (Goeze, 1782) (Nematoda, Dioctophymidae), known as the “giant kidney worm,” is a parasitic nematode of great significance in both medical and biological fields due to its impressive size and its complex relationship with various mammals, including humans. It is the causative agent of dioctophymiasis [1]. This parasite belongs to the Order Dioctophymatida and the Family Dioctophymatidae, and it is recognized as the largest parasitic nematode affecting mammals. Females can reach dimensions of up to 103 cm in length and 1.2 cm in width, while males measure up to 45 cm in length and 0.5 cm in width [1]. It affects various domestic and wild mammals, with carnivores of the Mustelidae family being the most susceptible, followed by canids. Felines, ruminants, and equines are more rarely affected [2]. This parasitosis has a worldwide distribution, with the possible exception of Africa and Oceania [3]. In the Americas, it has been reported in Canada, the United States, Brazil, Paraguay, Uruguay, and Argentina [4]. Naturally occurring life cycle affects wild canines like maned wolves (*Chrysocyon brachyurus*) inhabiting riparian areas but it also affects domestic dogs (*Canis lupus familiaris*) with high prevalence. With a complex life cycle, this parasite requires multiple hosts to complete its development. Eggs produced by mature females in the kidney of a definitive host (usually carnivores) are excreted through urine, thereby contaminating aquatic environments. There, they are ingested by an intermediate host, mainly *Lumbriculus variegatus*, a freshwater oligochaete, in which they develop to the L3 larval stage. Subsequently, these parasites can be transferred to paratenic hosts such as fish, amphibians, or even reptiles through predation. Mammals, the definitive hosts, acquire the infection by drinking contaminated water or consuming infected paratenic hosts. Within mammalian tissues, the parasite completes its development, reaching the final larval stages and sexual maturity in the kidneys, usually the right one [5].

Currently, there is no effective treatment for *D. renale* infection, therefore, one of the main concerns is the search for new proteins that could serve as therapeutic targets. In this regard, proteins from the *D. renale* pseudo coelomic fluid have been described with functional roles in lipid binding properties and lipid mass distribution, making them new treatment candidates [6, 7]. Another priority for controlling the disease focuses on prevention and early diagnosis. In this regard, molecular markers were investigated for the development of ultra-sensitive assays [8]. However, the limited genomic information and the apparent divergence of *D. renale* from other nematodes make progress in this area difficult. In general terms, the recent advancement of x-omics technologies and computational power has enabled the acquisition of a large amount of genomic data. In consequence, various initiatives have emerged with the aim of expanding research on more distant species. Regarding helminth parasites, the WormBase ParaSite initiative has been developed to facilitate the completion of the phylogenetic landscape surrounding the high-quality reference genomes of current helminths and to identify key differences among parasite lineages [9]. Even within these databases, and despite its importance in animal health and its zoonotic potential, there is no complete genomic information available for *D. renale*. The available *D. renale* 18S rRNA sequence information was included in a nematode evolutionary analysis performed by Blaxter et al. [10]. According to this analysis *D. renale* is classified within Clade I, which includes other better-studied parasitic nematodes of vertebrates, such as those belonging to the genera *Trichuris* and *Trichinella*. Also, the study of mitochondrial DNA (mtDNA) has been extensively used to analyze phylogeny, biogeography, systematics, population genetics, and other areas of great relevance in nematodes [11]. Particularly, partial *D. renale* mtDNA genes COX1 and ND4 were described previously by us and confirm the *D. renale* Clade I classification [8, 12]. However, considering the total number of complete mitochondrial genomes available, nematodes are the least represented organisms [13].

At present, there are approximately 250 nematode mitogenomes available, and only 27 belong to nematode Clade I [14, 15]. Generally, mtDNA consists of a circular DNA, which encodes ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and a subset of proteins-coding genes (PCG) with specific mitochondrial functions [16]. These genes usually undergo polycistronic transcription before they are split into individual transcripts [17]. Unlike eukaryotic nuclear genes, PCGs do not contain their own regulatory sequences but contain non-coding regions (NCRs) that are found across species and have been proposed to function as a start site for DNA replication and transcription. Also, species-specific NCRs have been identified across species [18]. Generally, mitochondrial genomes of nematodes are similar to those of other animals, consisting of a single circular chromosome that ranges in size from 12.6 kb to (*Xiphinema americanum*) to 26 kb (*Romanomermis culicivorax*), with structural variations described in some species, such as *Globodera pallida*, which contains a multipartite mitogenome. In general, nematode mitogenomes contain 12 PCGs with the *atp8* gene usually missing, 22 transfer RNA genes (tRNAs), and 2 ribosomal RNA genes (rRNA). Similar to other metazoans, the genome size variation has been attributed to hypervariable noncoding, often repetitive, regions [19]. Given the lack of genomic information and the usefulness of mitogenomes for phylogenetic and population studies, we set out to obtain the mitogenome of *D. renale* using a genome skimming strategy. We hypothesized that the particular *D. renale* life trait would have an impact on the composition and structure of its mitogenome. We utilized long read sequencing and comparative mitogenome analysis focused on phylogenetics and amino acid identity to compare how these factors were associated with the Clade I trait characteristics.

Methods

Parasite material

The specimen of *Diectophyme renale* analyzed in this study corresponds to an adult male that was donated from a local veterinary surgeon after standard treatment of a naturally infected dog (*Canis lupus familiaris*) from the surrounding area of the city of La Plata, Buenos Aires, Argentina. No animal experimentation was undertaken in this study. No euthanasia or animal sacrifice was performed in this study. The domestic dog involved in this study was not subjected to any experimental procedures. The dog owner voluntarily requested medical attention. The *D. renale* infection was diagnosed and the dog was treated. In private practice, dogs that are found to be positive for diectophymosis by urine sedimentation and ultrasonography are usually treated by nephrectomy of the (non-functional) infected kidney carried out by veterinary surgeons using standard anaesthetic, surgical, and recovery procedures appropriate for the condition. We have established contact with a network of Veterinary Clinics in the area to ask for the worms that are removed after surgery. It is important to note that if worms are not claimed for research they are discarded.

The sample was collected in November 2020 and preserved in DESS solution at -20°C until total DNA extraction. DESS solution, composed of dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), and saturated sodium chloride (NaCl), is particularly suitable for long-term preservation of nematode samples, maintaining both morphological and molecular integrity and enabling subsequent molecular analyses.

Total DNA extraction

Prior to high-molecular-weight genomic DNA (gDNA) extraction, the sample was washed three times with 1X PBS to remove the DESS solution. Additionally, it was ground in liquid nitrogen to pulverize the tissue and improve yield. Genomic DNA was then extracted using the Magnetic Animal Tissue Genomic DNA Kit (TIANGEN) following the manufacturer's instructions. This extraction method is based on the reversible binding of gDNA to the surface of magnetic beads under high-salt conditions in the presence of ethanol, with subsequent separation using a magnetic rack. This gDNA extraction procedure is recommended for applications such as high-throughput sequencing due to the purity and speed with which gDNA can be obtained. The quality of the extracted gDNA (concentration and integrity) was assessed prior to downstream sequencing using Oxford Nanopore Technologies (ONT). First, the presence of high-molecular-weight fragments (> 40 kb) was confirmed by electrophoresis on a 0.8% agarose gel in TBE buffer, using the Quick Load 1 kb Extend DNA Ladder (NEB) as a marker. Subsequently, gDNA purity was determined by Nanodrop absorbance: 260/280 nm and 260/230 nm ratios and by Qubit Broad Range assay (ThermoFisher). Samples were sequenced if the ratio between Nanodrop concentration / Qubit concentration did not exceed 2.5 and if 260/230 nm and 260/280 nm ratios were > 1.8 .

Library Construction and Long read sequencing

Starting from 1 μg of high-molecular-weight genomic DNA (gDNA), the sequencing library was prepared using the Ligation Sequencing DNA Kit v14 (SQK-LSK114; Oxford Nanopore Technologies) following the manufacturer's protocol. This protocol requires an enzyme module provided by NEB (NEBNext Companion Module for Oxford Nanopore Technologies® Ligation Sequencing). Briefly, the procedure involves end-repair and preparation of the gDNA ends, followed by ligation of sequencing adapters that enable the gDNA molecules to pass through the nanopores present in the flow cell. Library preparation also includes magnetic bead-based purification at both steps. The resulting gDNA library was quantified using the Qubit High Sensitivity assay (ThermoFisher), and approximately 300 ng of library (the amount recommended by the manufacturer for fragments > 10 kb) was loaded onto the PromethION flow cell. Data acquisition and real-time fast basecalling were performed using the MinKNOW software installed on the computer connected to the sequencer. For comparative purposes, a sequencing library was prepared using the Rapid Sequencing Kit SQK-RBK114 v14 (Oxford Nanopore Technologies) starting from 200 ng of high-molecular-weight genomic DNA (gDNA) from the same sample according to the manufacturer's instructions. This kit employs a transposase-based fragmentation and adapter attachment system, which simultaneously fragments the gDNA and attaches sequencing adapters in a single step, eliminating the need for separate end-repair or ligation reactions. Library preparation also involves a brief magnetic bead-based clean-up to remove excess reagents and small DNA fragments. The prepared library was quantified using the Qubit High Sensitivity assay (ThermoFisher), and was loaded onto the PromethION flow cell. Data acquisition and real-time fast basecalling were carried out using the MinKNOW software installed on the sequencing computer.

Bioinformatic analysis and preliminary assembly of *D. renale* mtDNA

Raw nanopore sequencing reads obtained from the sequenced library prepared using the Ligation Sequencing Kit were basecalled with Super Accurate algorithm (SUP, Dorado software) and subjected to quality control using NanoPlot to assess read length distribution and quality metrics. Total raw reads obtained (1,073,079) were first filtered using DIAMOND BLASTX against a database of Clade I mitochondrial proteins (Supplementary files), with the following parameters:

`--query --> ncode5 --long --reads --tra --sensitive. Then,  $fi < eredreads were assem \leq du \sin gFlyev2.9.5 with option so$`
`--nano-raw --meta --keep-haplotypes --min-overlap 4000`. The initial assembly was polished through three iterations of Racon, producing a draft assembly containing all protein-coding genes (PCGs) and identifying the presence of non-coding regions (NCRs). At this stage, NCRs were labeled but not annotated. A refined filtering step was performed using DIAMOND BLASTX against Diectophyme renale mitochondrial proteins derived from the draft assembly, with parameters: --query --> ncode5 --long --reads --sensitive -F15. This step yielded a total of 8,917 reads, which were subsequently used for the multipartite assembly and further curation of mitochondrial genomic regions.`

Final multipartite *D. renale* mtDNA

The number of reads containing PCGs was identified, and their frequency was analyzed using a t-test in R. For each set of PCG-associated reads, an independent assembly was performed using Flye v2.9.5 with the same optimized parameters: `--nano-raw --meta --keep-haplotypes --min-overlap 3800`. The final multipartite mitochondrial DNA (smtDNA I to XII) assemblies, where each molecule corresponds to a distinct PCG-associated genomic element plus 1 NCR. Each assembly was used as a reference to remap the reads previously identified as mitochondrial with minimap2. The final *D. renale* mitogenome sequences are available at GenBank under the accession numbers: PX715184-PX715194.

Comparative analyses of the multipartite *D. renale* mtDNA

Pairwise comparisons were performed using Minimap2 with the parameter `--map-ont`, and alignments were processed and visualized with pafR to detect NCRs present across the multipartite mtDNAs. For each molecule, the intergenic region was extracted from one base downstream of the terminal gene to one base upstream of the subsequent gene. These extracted NCR sequences were aligned using Clustal Omega implemented in MEGA11. Terminal alignment positions lacking 100% base coverage across all sequences were trimmed to ensure positional consistency and remove alignment artifacts. A WebLogo representation was then generated to visualize sequence conservation patterns, and the proportion of variable nucleotide positions was quantified relative to the total NCR length to assess variability and conservation among mtDNA molecules.

Transposase long sequencing dataset comparison

Quality control of the raw reads obtained from the sequenced library prepared using the Rapid Sequencing Kit (using a transposase to link sequencing adaptors to each DNA molecule) was performed using NanoPlot to assess read length distribution and quality, followed by MultiQC for integrated visualization and reporting. Filtered reads were identified using DIAMOND BLASTX against *Diectophyme renale* mitochondrial protein sequences, employing the parameters `--query-gencode 5 --long-reads --sensitive` to ensure high-sensitivity detection of mitochondrial reads. To evaluate sequence correspondence between datasets, Minimap2 alignments (`--map-ont`) were conducted to compare the rapid reads against the multipartite mitochondrial assemblies, and the results were further validated through DIAMOND BLASTX comparisons. Specifically, reads from the ligation libraries were compared to those from the rapid sequencing libraries by CD-HIT. Clustering analyses were carried out using the parameters `-c 0.9 -n 5` to cluster identical reads and assess sequence identity with more than 85% identity between datasets [20]. Cluster composition and similarity distributions were summarized and visualized in R using ggplot2, allowing for quantitative comparison of read overlap and dataset complexity between the rapid and ligation sequencing approaches.

Mitogenome annotation

Prior to the final annotation of the mitochondrial assembly, a manual curation process was carried out. To do this, the mitochondrial reads were aligned to the mitochondrial assembly (previously *de novo* assembled using those same mitochondrial reads) using Minimap2 [21] in the ‘`--map-ont`’ mode. To visualize and explore the genomic data, IGV (Integrative Genomics Viewer) v2.19.2 [22] was used with default parameters, except: Translation table = Invertebrate Mitochondrial. Annotation of the mitochondrial assembly of *D. renale* was obtained by the MITOS2 tool [23] implemented on the European Galaxy web server [24]. Default parameters were applied except for: (i) Genetic code = Invertebrate (5), and (ii) Reference data = RefSeq89 Metazoa. Using this approach, protein-coding genes (PCGs), transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs) were annotated. Manual curation was performed for COX1 and ND3/ND4L stop codons and trnY since software MITOS2 was unable to automatically annotate these genes. Nucleotide composition biases in the protein-coding gene sequences obtained were analyzed using a similar approach to that described in [11]. Non-coding regions (NCRs) were identified in the mitochondrial assembly by analyzing the flanking regions of the protein-coding genes.

Comparative genomics

The annotated mitochondrial protein-coding genes of *Diectophyme renale* were characterized *in silico* through comparative genomics with nematode species belonging to Clade I. For this purpose, all Clade I nematode species with available reference mitochondrial amino acid sequences in NCBI [15] were selected. The species names and corresponding GenBank accession codes are shown in Supplementary files. To compare the amino acid sequence identity and coverage between *D. renale* and their corresponding orthologs in Clade I species, BLASTP [25] was used, available at [26], with default parameters. Only for the ND4L gene was the parameter ‘`word size = 3`’ applied.

Phylogenetic analyses

To perform multiple sequence alignments prior to phylogenetic tree construction, ClustalW [27] implemented on the European Galaxy web server [24] was used with default parameters except for: 'Data type = protein sequence, Algorithm for the alignment guide tree = Slow, Output tree = Nexus, Output alignment format = FASTA format, and Output order = aligned'. The amino acid sequences used included those obtained in this study for *D. renale* as well as the reference amino acid sequences corresponding to all Clade I parasitic nematodes available mitogenomes. Phylogenetic trees were constructed using both the COX1 amino acid sequences and the concatenated amino acid sequences of all mitochondrial genes (complete mitogenomes). To construct the phylogenetic trees using the Maximum Likelihood (ML) method, the IQ-TREE tool [28] implemented on the European Galaxy web server was employed. Default parameters were used except for: i) Sequence type = AA, ii) AA model = Mitochondrial, iii) Bootstrap parameters: {Ultrafast bootstrap [Bootstrap replicates = 100,000]}. For visualization and editing of the phylogenetic trees, the online software iTOL v6.6 [29] was used.

Results

Genome size and composition

The complete workflow implemented for *D. renale* genome skimming is shown in Fig. 1. *D. renale* DNA sequences were assembled, and 46,169-pb mtDNA was obtained, which was assembled into several small mtDNAs with a size of ~ 4Kb and named "small mitochondrial DNA" smtDNA I to XI (Fig. 2). A total of 36 genes were encoded in the whole mtDNA, including 12 protein-encoding genes (PCG), 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes. PCG are: one ATP synthase subunit gene (ATP6), three cytochrome C oxidase subunits genes (COX1, COX2 and COX3), one cytochrome B gene (CYTB), seven nicotinamide adenine dinucleotide (NADH) dehydrogenase subunits genes (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6), two rRNAs (rrnL and rrnS) and 22 tRNAs. Also, non-coding regions (NCR) were found in each smtDNA with an average length of 2,589-pb (Table 1).

Table 1
Dioctophyme renale mitochondrial assembly. Protein coding genes, non-coding regions lengths and number of reads are shown.

Name	Total length (bp)	Protein coding gene	Number of reads	N50	Non coding region	NCR length (bp)
smtDNA I	4,328	ATP6	579	4317	NCR1	2578
smtDNA II	4,640	COX1	945	4318	NCR2	1660
smtDNA III	3,834	COX2	539	3843	NCR3	2866
smtDNA IV	4,100	COX3	689	4078	NCR4	2730
smtDNA V	4,522	CYTB	1826	4740	NCR5	2205
smtDNA VI	3,972	NAD1	799	3953	NCR6	2854
smtDNA VII	4,031	NAD2	800	4019	NCR7	2942
smtDNA VIII	4,127	NAD3 and NAD4L	435	4110	NCR8	2830
smtDNA IX	4,108	NAD4	701	4086	NCR9	2804
smtDNA X	4,403	NAD5	1017	4355	NCR10	2760
smtDNA XI	4,104	NAD6	587	4092	NCR11	2245
Total mitogenome length (bp)	46,169		Total reads 8917		Average NCR length (bp)	2589

We found that the single circular mitochondrial chromosome typically found in animals has evolved into a multipartite organization in *D. renale*, consisting of eleven minichromosomes, each containing a single protein-coding gene and an associated non-coding region (Fig. 2, Supplementary Figures). Among the total reads associated with the *D. renale* mitogenome, we identified a subset containing more than one PCG. Frequency analysis indicated that such reads represent a significant minor fraction compared with those containing a single PCG (t-test, $p < 0.01$) (Supplementary Figures). To confirm that this particular assembly was indeed the *D. renale* mtDNA, the mitogenome was analyzed for several characteristics including exhaustive annotation, bp length of each gene, %GC and %AT composition of each PCG sequence and comparison with available mitochondrial reference sequences. Also, an independent sequencing library using transposase instead of ligase was made to corroborate the multipartite *D. renale* mtDNA.

Protein-encoding genes in the mtDNA of *D. renale*

In agreement with the reported Clade I nematode mitogenome PCG composition twelve PCGs were annotated in the *D. renale* mtDNA. All PCGs had a complete initiation codon and termination codon. Of the 12 PCGs obtained, 10 were described for the first time in this study. The only two genes for which partial information was previously available are COX1 and ND4, these two genes were compared with the sequences reported from the population genetics analysis of *D. renale* isolates obtained previously by us [8]. The identity percentages obtained for COX1 were 98.6 to 100% and the identity percentages obtained for ND4 values ranging from 97.46% to 100%. The 12 PCG were compared to proteins found in Clade I species. The PCGs exhibit variable but consistent (from 27.7% to 65%) sequence identity when compared to orthologous proteins from species within Clade I nematodes. The higher amino acid conservation among Clade I species was found in COX1. In general, the aminoacidic sequences of PCGs from *D. renale* showed higher conservation with species from *Trichuris* than other Clade I nematodes (Fig. 3). Then, the GC nucleotide skews ($GC\ skew = (G - C) / (G + C)$) were calculated for each PCG, and each codon position of each PCG. The A + T content of each smtDNA molecule ranged from 59 to 66%, indicating a significant AT bias and the lowest GC skews (Table 2) as previously reported in animal parasites and animal-associated taxa [19].

Table 2
Analysis of nucleotide composition bias in protein-coding genes (PCG). AT Skew = $(A\% - T\%)/(A\% + T\%)$ and GC Skew = $(G\% - C\%)/(G\% + C\%)$.

Nucleotide composition								
PCG	%A	%T	%G	%C	%A + T	%G + C	AT skew	GC skew
ATP6	19.2	41.4	27.7	11.7	60.6	39.4	-0.37	0.40
COX1	20.9	39.0	22.0	18.2	59.9	40.1	-0.30	0.10
COX2	19.7	39.5	25.0	15.8	59.2	40.8	-0.33	0.23
COX3	18.2	40.7	24.4	16.7	58.9	41.1	-0.38	0.19
COB	19.4	36.6	21.7	22.3	56.0	44.0	-0.31	-0.01
ND1	17.5	41.1	27.2	14.1	58.7	41.3	-0.40	0.32
ND2	32.2	29.5	18.1	20.2	61.6	38.4	0.04	-0.05
ND3	20.2	43.5	25.9	10.5	63.6	36.4	-0.37	0.42
ND4L	36.9	29.4	24.2	9.5	66.3	33.7	0.11	0.44
ND4	34.3	29.7	16.0	20.1	64.0	36.0	0.07	-0.11
ND5	32.5	28.3	19.3	19.8	60.9	39.1	0.07	-0.01
ND6	16.1	43.7	23.4	16.8	59.8	40.2	-0.46	0.16

D. renale mitochondrial non-coding genes and repetitive regions

A total of 22 tRNAs with a length range of 52–70 bp were identified, 2 (*trnS* and *trnL*) of which were two copies in different smtDNAs and had different secondary structures (Fig. 4). The rRNA genes in the mtDNAs of *D. renale*, *rrnL* and *rrnS*, were 782 and 663-bp in average, respectively, similar to the rRNA lengths of previously reported rRNA from parasite nematodes (Supplementary Files, Supplementary figures). Also, the mtDNA of *D. renale* contains 11 non-coding regions (NCRs), one per smtDNA and with variable sizes, ranging in length from 1660 to 2942 bp with average length of 2589-pb (Table 1, Supplementary files). These NCR copies exhibit a high degree of sequence conservation, with pairwise identities ranging from 99.5% to 99.9%. (Fig. 5). Moreover, each NCR has mitochondrial sequences with identity to replication origin (Supplementary files). To verify the *D. renale* mitogenome multipartite structure organization, a DNA sequencing library was constructed by Oxford Nanopore Rapid Sequencing kit, which uses transposase instead of a ligase to bind adapters to the DNA molecules (named here “Rapid”). More than 90% of identity between reads from both libraries was found (Fig. 6).

Phylogenetic analysis

The phylogenetic tree was constructed based on the 12 concatenated amino acid sequences from PCGs of *D. renale*. All Clade I parasitic nematode species for which reference mitogenomes were available were included. The *C. elegans* concatenated PCGs were included as an outgroup (Fig. 7). The phylogenetic placement of *D. renale* in the reconstructed tree is congruent with previous reports based on nuclear 18S rRNA genes [10], supporting its classification within the Clade I taxonomic group. The tree topology shows that *D. renale* is unique in its branch, with the closest Order group being *Trichinellida* which includes all parasitic nematode species found in vertebrate animals. Conversely, the most distant Order groups are Mermithida and Dorylaimida, these groups include parasitic nematode species from invertebrate animals and plants, respectively.

Discussion

Mitogenomes are expected to consist of single, circular DNA molecules. However, significant variations in genome size, structure and gene content exists among different eukaryote lineages [30]. Particularly interesting are multipartite or fragmented genomes whereby either several different circular (i.e., minicircles) or linear DNAs encode the organelle gene set. Unlike mtDNA gene content, mtDNA organization and gene order have been given less attention. Although most studied organisms harbor a circular mtDNA, multipartite mitogenomes were already known from four lineages of bilateral animals. Some organisms, such as medusozoan, harbor linear mtDNA [31], and in other metazoans (such as parasitic nematodes, endoparasitic myxozoa and ectoparasite lice) the mtDNA is fragmented into groups of circular chromosomes that co-segregate across generations [32, 33]. The function and evolutionary significance of multipartite mitochondria remain poorly understood, as research has predominantly focused on mammalian mitochondria. Mammal mtDNA is transcribed in strand-specific polycistron, nearly at the length of the entire linearized mtDNA molecule; such long precursor transcripts are, in turn, cleaved into mature, individual mRNA molecules [34]. Therefore, gene order rearrangements within a given polycistron are not expected to affect the local transcriptional regulation. But studies of mtDNA structure in other metazoans revealed exceedingly more mtDNA gene order variability in phyla such as *Cnidaria*, *Annelida*, and *Porifera* as compared to other metazoan phyla; furthermore, more than 70% of the studied *Mollusca* and *Annelida* possess unique mtDNA gene orders [35]. The analysis of mtDNA-derived nascent RNA transcription in diverse species provides insights on how mitochondrial gene order affects regulation of expression. Human and mouse mitochondrial genomes displayed light strand and heavy strand polycistronic transcripts but *Drosophila melanogaster* had multiple initiation and termination sites and *Caenorhabditis elegans* had only a single heavy strand initiation and termination site. The latter two invertebrate organisms vary in gene order which may indicate that mitogenome gene order affects transcriptional patterns [36].

This study incorporates a previously undescribed species possessing a multipartite mitogenome into the tree of life. *D. renale* is the new parasitic nematode species in the multipartite mtDNA category with the higher number of molecules already described in its lineage, reaching 11 small circular mitochondrial genomes with one PCG per molecule. A similar kind of structure was only found previously in *Pediculus humanus* (see Fig. 8 adapted from reference [37]). It is proposed that when mitochondrial chromosomes of different sizes co-exist, the selection pressure may favor small ones over the typical large mitogenomes. Thus, fragmentation of mitogenomes might be one of strategies of size-reducing for streamlining mitochondrial chromosomes and small mitochondrial chromosomes may have selective advantages over the typical large ones [37]. Indeed, in cultured human cells increased mitochondrial fragmentation is observed when more than one mitochondrial molecule co-exists [38]. Also, fragmentation of mitochondrial genomes has been previously linked to increased sequence divergence and rearrangement rates. Recombination between mitochondrial minichromosomes could increase sequence variation in mitochondrial genes and it could affect the regulation of mitochondrial DNA transcription and replication [39]. Increased sequence variation might have been an advantage to the ancestor of *D. renale* when they were adapting to a new organ like kidney where particularly metabolism requirements are needed. The fragmentation of the mitogenome may be positively selected during the parasite's adaptation to vertebrate definitive hosts.

Regarding non-coding regions, the NCR (D-loop) serves as both a DNA replication origin and a transcription start site in human mtDNA. In addition, mtDNA replication starts with a short RNA transcript as a primer that is also derived from the D-loop, indicating a coupling between DNA replication and transcription. Regarding 3'UTR regulation, unlike nuclear genes, the 3' UTR from mitochondrial genes is a short stretch of RNA [40]. In nematodes, the NCR sequences show divergence among species [41]. In the genus *Caenorhabditis*, some species contain NCR sequences with numerous tandem and inverted repeats. Particularly, the mtDNA from the *Caenorhabditis elegans* has NCR which is approximately 100 bp in length and is located between the ND4 and COX1 genes. Also, *Pristionchus pacificus*, which belongs to the same Clade V as *Caenorhabditis* species, possesses a comparable sequence, albeit with a reduced length of 51 bp [42]. Although mitochondrial NCRs were reported long ago to be variable in most species, their function remains largely unknown [43]. As in the previously mentioned nematode species, *D. renale* possesses NCR sequences that are unique in the tree of life with particular structure and sequence. The NCR sequence found in this work are the long ones already described, the position of each NCR flanking PCGs but not in other positions suggests that may serve as a regulation site for replication and/or transcription. Unlike mtDNAs structure, coding genes are sequence conserved in *D. renale*. The mitogenome encodes 12 protein, two rRNAs (12S and 16S) and 22 tRNAs, all of which are presumably controlled by an NCR region. The nucleotide and amino acid sequences indicate that *D. renale* is closely related to nematode parasites from vertebrate hosts such as *Trichuris* and *Trichinella* and more divergent were *Romanomermis* species that present a mitochondrial structure consisting in three repeat-containing regions where the ND3 gene is found in association with multiple tRNAs, two of which occur in tandem, while another is located elsewhere in the genome [44, 45]. Further investigation of a larger number of parasitic nematode mitogenomes would be of interest to elucidate whether structural variation in their mitogenomes correlates with differences in their life-history characteristics.

Conclusion

Our study reveals that the mitochondrial genome of *D. renale* is organized into eleven minichromosomes, each carrying a single protein-coding gene and an associated non-coding region. This multipartite organization represents a novel mitogenome structure within nematodes diverging from typical single circular mitochondrial chromosomes found in most animals. Future work should explore the functional implications of this mitochondrial structure in *D. renale* biology. Our study also describes a long-read genome-skimming workflow suitable for non-model organisms, providing a strategy to expand mitogenomic resources in underrepresented taxonomic groups.

Declarations

Availability of data and materials: The datasets generated and/or analysed during the study are available at GenBank under accession numbers: PX715184-PX715194.

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: AB and NM contributed equally to this work and shared first authorship. AB genome assembly, genome analysis, MITOS2 analysis, gene prediction, gene annotation, phylogeny, and comparative genome analyses, data interpretation and drafting of the paper; NM DNA extraction, QC analysis, library construction, raw data interpretation, comparative genomics, drafting of the paper, MLI, IS, long read sequencing implementation, LFA, molecular marker validation, TF-S data analysis, GF and LK design and concept of the study, writing of the final manuscript. All of the authors read and approved the final manuscript.

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Figures

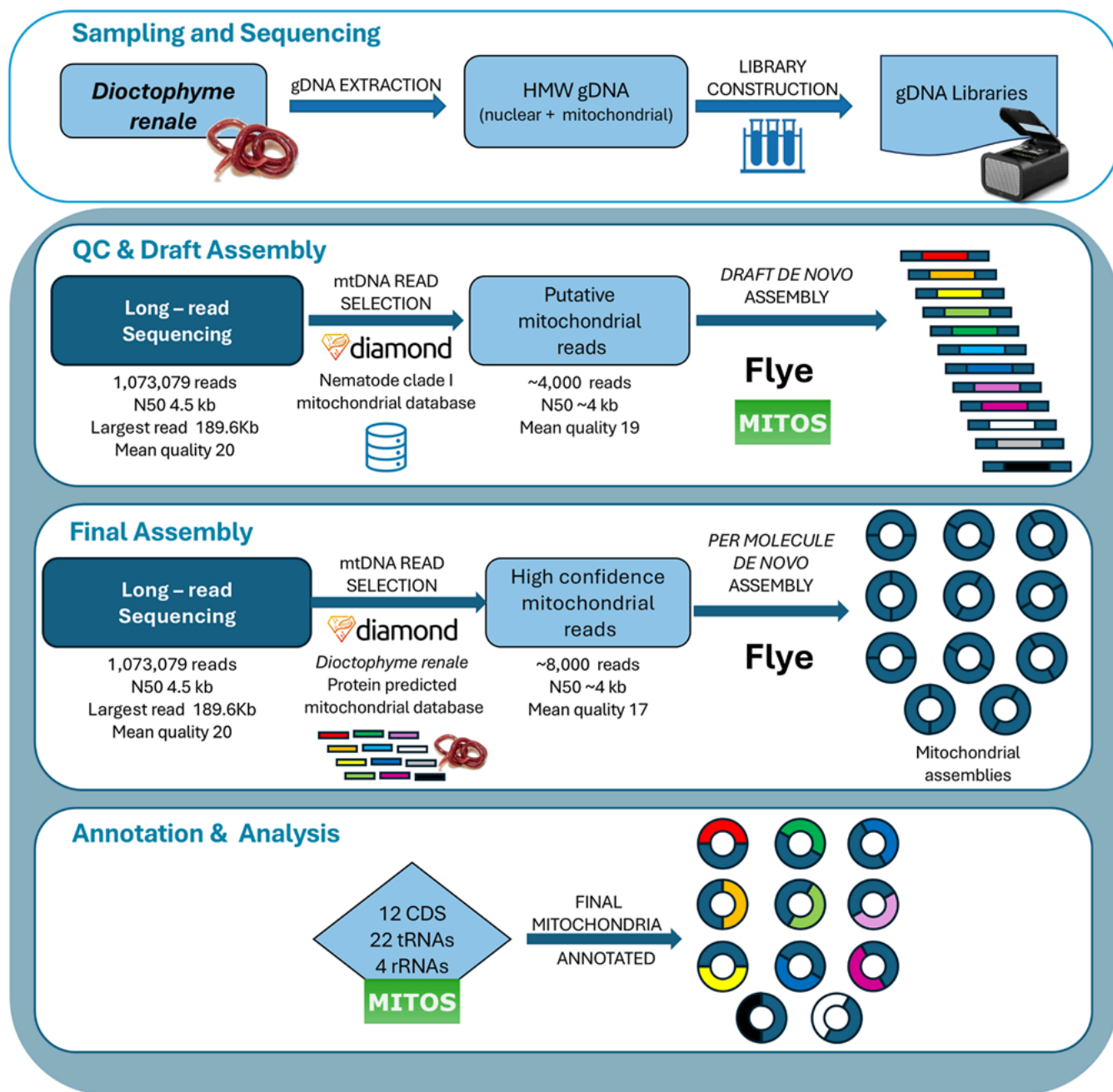


Figure 1

Genome skimming workflow for *Diotrophyme renale* mitogenome assembly and annotation.



Figure 2

Annotation and structure of small circular mitochondrial genomes in *Diectophyme renale*. Protein coding genes, non coding genes and non coding regions were identified using different colours. The built-in dark grey histogram show the GC content of the genome, and the coverage in light blue

Mitochondrial coding genes	COX1	63.2	63.4	63.7	63.9	63.9	62.5	62.3	63.3	62.1	62.9	62.5	62.5	62.7	64.7	63.0
	COX2	38.4	41.6	39.5	39.7	39.6	44.9	46.0	42.3	45.4	45.6	46.1	45.1	46.1	43.2	40.8
	COX3	44.7	48.4	46.5	43.0	50.5	38.9	37.9	38.7	38.2	38.2	38.1	38.8	38.1	40.6	39.7
	ATP6	44.6	41.0	44.0	41.1	45.8	48.0	47.8	44.3	46.0	48.7	48.0	45.6	48.3	38.0	43.0
	NAD1	42.9	42.3	43.3	42.7	43.0	44.1	43.6	44.2	44.4	43.6	43.6	43.5	42.7	41.4	40.9
	NAD2	34.0	31.8	31.6	31.9	31.6	32.9	33.8	31.3	32.5	34.1	32.4	34.2	33.5	32.2	31.7
	NAD3	36.9	40.7	40.6	41.9	40.6	41.4	40.4	46.8	41.4	40.2	40.4	40.4	40.4	41.6	53.1
	NAD4	37.2	37.2	35.4	41.0	35.2	40.1	38.4	37.5	39.3	41.0	36.7	39.3	42.3	37.3	38.2
	NAD4L	37.5	35.1	34.2	43.8	33.3	37.0	38.3	39.8	39.5	38.3	39.5	38.3	39.7	38.9	35.0
	NAD5	40.0	38.0	34.5	36.1	37.1	38.5	38.8	39.6	35.6	38.8	39.5	37.2	38.1	37.3	36.9
	NAD6	44.9	27.7	34.2	34.7	33.1	34.6	29.6	30.2	32.0	31.6	32.4	31.8	31.8	32.9	38.0
	COB	47.6	49.4	46.6	49.6	48.4	48.4	46.9	45.5	47.6	48.5	46.9	46.4	46.4	47.7	50.1
Species		Trichuris trichiura	Trichuris suis	Trichuris ovis	Trichuris muris	Trichuris discolor	Trichuris zimbabwensis	Trichinella spiralis	Trichinella pseudospiralis	Trichinella papuae	Trichinella nelsoni	Trichinella nativa	Trichinella murrelli	Trichinella britovi	Eucoleus annulatus	Aonchotheca putorii

Figure 3

Conservation of mitochondrial protein coding genes (PCGs) between *Diectophyme renale* and Clade I nematodes. Heatmap showing the amino acid sequence identity of 12 mitochondrial PCGs (COX1, COX2, COX3, ATP6, NAD1–NAD6, NAD4L, and CYTB) between *D.renale* and Clade I nematodes. Pairwise identities were calculated using BLASTP. Warmer colors indicate higher identity, cooler colors indicate lower identity, and numbers show exact percentages. The sequences used are reference amino acid sequences from species of the order Trichinellida.

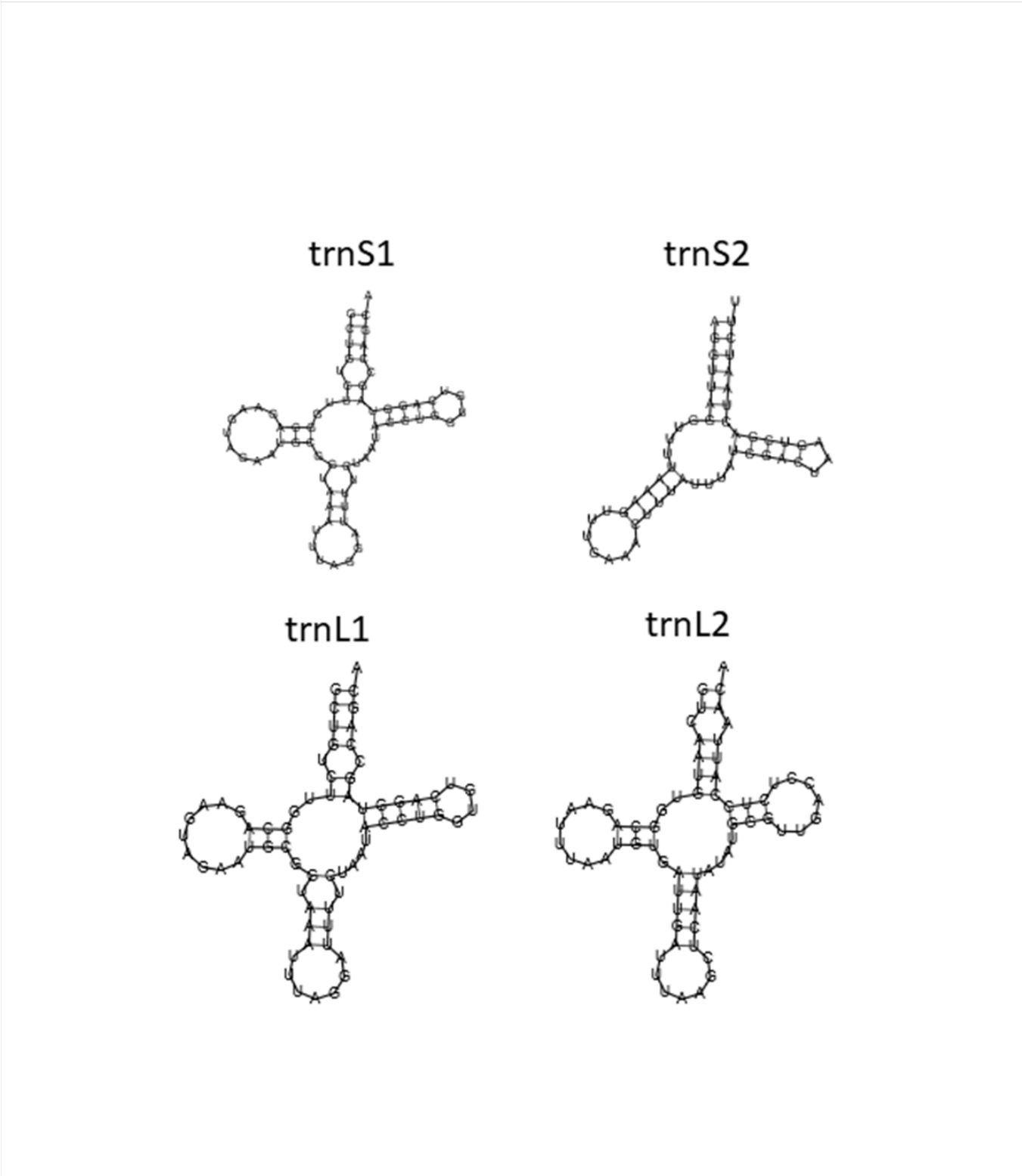


Figure 4

The secondary structures of duplicated *Diectophyme renale* mitochondrial tRNAs with typical clover structures obtained by MitoS2. The genes trnS2 and trnL1 were found in smtDNA_VIII. The genes trnS1 and trnL2 were found in smtDNA_I and smtDNA_III, respectively.

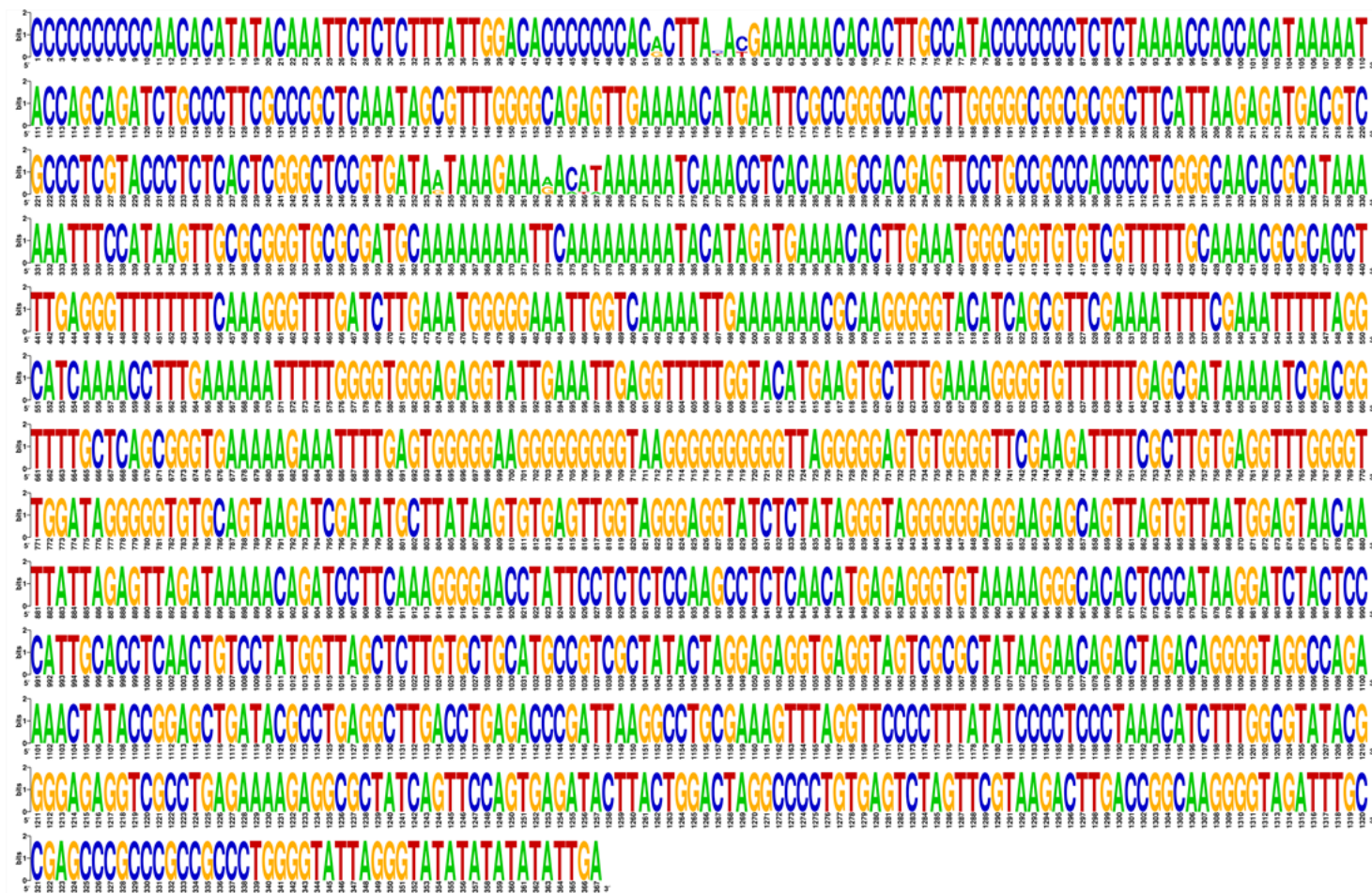


Figure 5

Conservation of non-coding regions (NCRs). A multiple sequence alignment of 11 NCRs (total length: 2,349 nucleotides) was generated using MUSCLE, and sequence conservation was visualized with WebLogo. The logo shows two regions (positions 52, 57, 59, 60 and 263, 265, 266, 267) that exhibit lower conservation among the analyzed sequences.

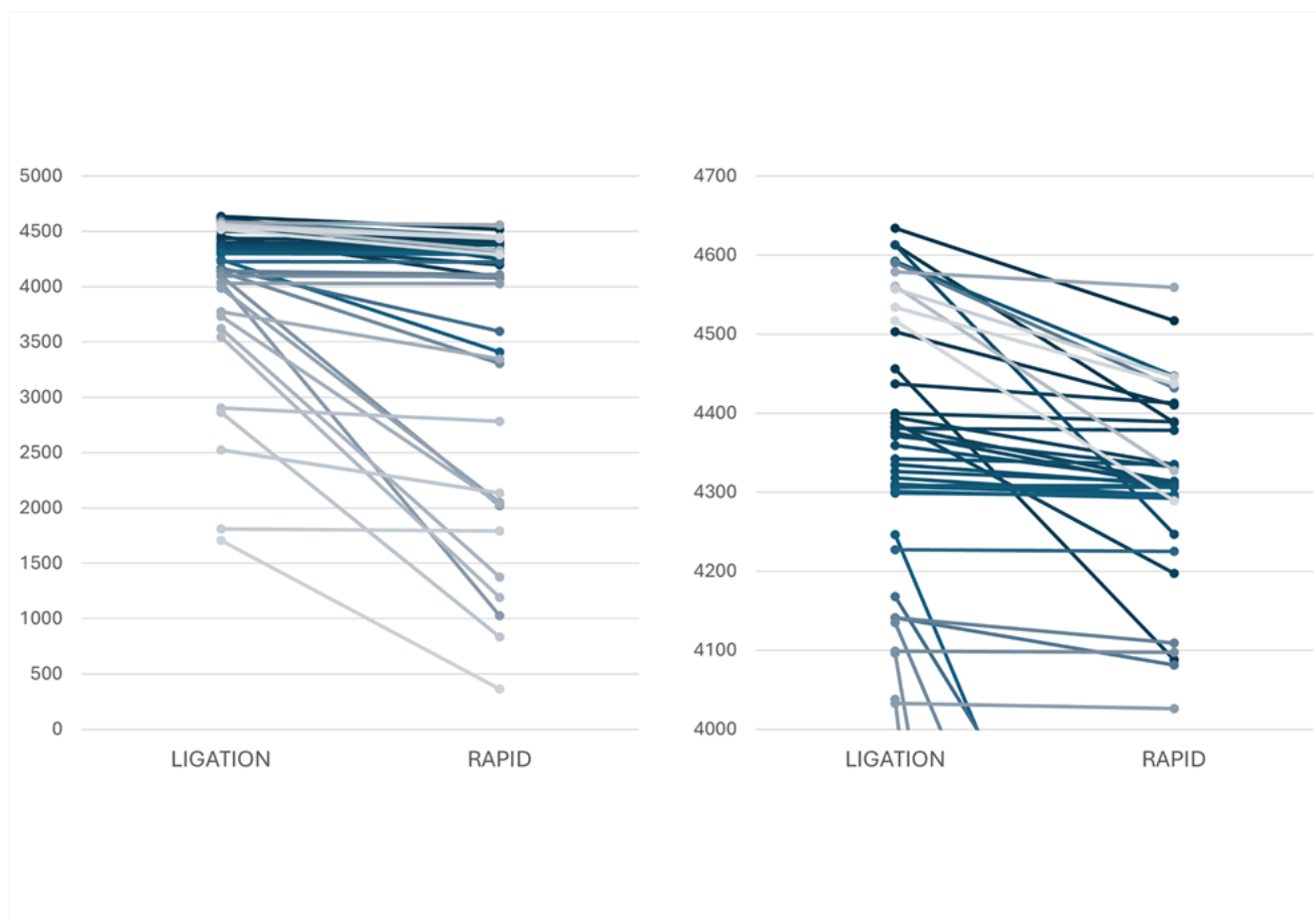


Figure 6

Nucleotide identity between sequences reads from two sequencing libraries. Reads from “ligation library” were pairwise aligned to each “rapid library” by CDHIT analysis (see Methods). Plots showed lines between identical reads from both libraries. Left: whole nucleotide length for smtDNA_II molecule. Right: zoom on range 4000 to 4700-pb.

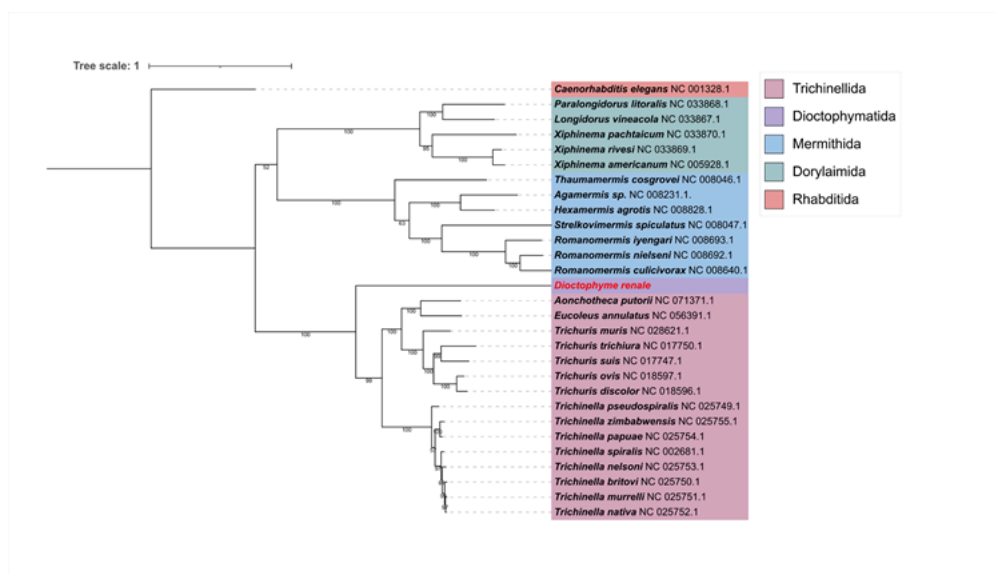


Figure 7

Phylogenetic tree inferred from concatenated mitochondrial *Diectophyme renale* PCGs. Phylogenetic tree constructed from the concatenated alignment of the 12 proteins encoded by the mitochondrial genome from *D. renale*, with the corresponding sequences from all Clade I reference mitogenomes. Multiple alignment was performed with MAFFT, yielding a total of 3032 positions. Maximum likelihood phylogenetic reconstruction was performed using the mtMet+F+I+G4 model and 100,000 ultra-fast bootstrap replicates to evaluate nodal support. The nematode Order was indicated in different colors.

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Figure 8

Figure 8. The architecture of mitochondrial chromosomes in bilateral animals. The figure was adapted from reference [37]. The expected mitochondrial chromosome is represented as a single red color circle whereas multipartite chromosomes are represented as black small circles. *Diectophyme renale* mitogenome is represented in red. Numbers in brackets indicate the number of species for which entire genome sequences are known. Stars indicate partial mitochondrial genomes. Phylogeny adapted from the Tree of Life Web Project [46].

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