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BRIEF COMMUNICATION

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Both-strand gene coding in a plastome-like mitogenome of an enoplid nematode

Olga V. Nikolaeva[1] | Leonid Yu Rusin[2] | Kirill V. Mikhailov[1,2] | Vladimir V. Aleoshin[1,2] | Paul De Ley[3]

[1]Belozersky Institute of Physicochemical Biology, Lomonosov Moscow State University, Moscow, Russia

[2]Institute for Information Transmission Problems (Kharkevich Institute), Russian Academy of Sciences, Moscow, Russia

[3]Department of Entomology, Plant Pathology & Weed Science, New Mexico State University, Las Cruces, New Mexico, USA

Correspondence
Vladimir V. Aleoshin, Belozersky Institute of Physicochemical Biology, Lomonosov Moscow State University, Moscow, Russia.
Email: aleshin@genebee.msu.su

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Abstract

The phylum Nematoda remains very poorly sampled for mtDNA, with a strong bias toward parasitic, economically important or model species of the Chromadoria lineage. Most chromadorian mitogenomes share a specific order of genes encoded on one mtDNA strand. However, the few sequenced representatives of the Dorylaimia lineage exhibit a variable order of mtDNA genes encoded on both strands. While the ancestral arrangement of nematode mitogenome remains undefined, no evidence has been reported for Enoplia, the phylum's third early divergent major lineage. We describe the first mitogenome of an enoplian nematode, *Campydora demonstrans*, and contend that the complete 37-gene repertoire and both-strand gene encoding are ancestral states preserved in Enoplia and Dorylaimia versus the derived mitogenome arrangement in some Chromadoria. The *C. demonstrans* mitogenome is 17,018 bp in size and contains a noncoding perfect inverted repeat with 2013 bp-long arms, subdividing the mitogenome into two coding regions. This mtDNA arrangement is very rare among animals and instead resembles that of chloroplast genomes in land plants. Our report broadens mtDNA taxonomic sampling of the phylum Nematoda and adds support to the applicability of *cox1* gene as a phylogenetic marker for establishing nematode relationships within higher taxa.

KEYWORDS
Campydora, *cox1*, Enoplia, Nematoda, perfect inverted repeat

1 | INTRODUCTION

In the phylum Nematoda, mitogenomes vary considerably between major taxa with respect to gene order and orientation. Among approximately 500 nematode mitogenomes deposited in GenBank by early 2023, the majority exhibit a similar arrangement of same strand-encoded genes due to sampling bias towards the parasitic, economically important and model species of Rhabditida (*sensu lato*; De Ley & Blaxter, 2004), a sublineage of the Chromadoria major clade. Chromadorian sampling beyond Rhabditida is limited to two *Plectus* species, whose genes are arranged in a different order and encoded on both mtDNA strands (Kim et al., 2017). Both-strand mt gene encoding is also known for the few sampled members of Dorylaimia (Hyman et al., 2011; Kern et al., 2020). Meanwhile, no mtDNA data has been reported for Enoplia, the phylum's third major lineage.

Since the description of the genus *Campydora* (Cobb, 1920), its systematic classification has long remained a matter of debate due to a unique combination of characters (Mullin et al., 2003). The genus was erected with the single species *Campydora demonstrans* originally placed in the order Anaxonchia, along with a few other taxa, most of which were subsequently relocated between the orders Enoplida and Chromadorida (Hansson, 1998). Thorne (1935) assigned it within the Campydorinae subfamily, subsumed afterwards into the family Leptonchidae within the order Dorylaimida (Thorne, 1939). Another

view, grounded in the pharyngeal and amphid morphology, supported the position of Campydorinae within Enoplida (Siddiqi, 1983), eventually identifying the morphology-based alternatives to place the *Campydora* genus either within Dorylaimida or Enoplida. The affinity of *C. demonstrans* within the order Enoplida has been validated in SSU rDNA-based phylogenetic analyses (Mullin et al., 2003; van Megen et al., 2009), including a large-scale Bayesian assay of >200 taxa (Ahmed & Holovachov, 2021).

2 | RESULTS AND DISCUSSION

The integrity of total DNA extracted from a formaldehyde-fixed and long-stored sample was sufficient for generating a good-quality NGS library and consistent mtDNA assembly (per-base coverage 455.06, SD 116.25; Supplementary Material 1). The mitochondrial genome of *Campydora demonstrans* (GenBank OP163863) constitutes a single, circular DNA molecule of 17,018 bp in size, containing 37 genes (2 rRNAs, 22 tRNAs and 13 proteins; Figure 1). The sample's taxonomic identity was verified in phylogenetic analyses of the cox1 (Figure 2) and nuclear rRNA (Figures S1 and S2) genes.

Both-strand orientation of mitochondrial genes is shared by this member of Enoplia with species of Dorylaimia and non-rhabditid Chromadoria (plectids), as well as four species of the outgroup phylum Nematomorpha (Mikhailov et al., 2019), thus being likely to represent an ancestral state for Nematoda and suggesting late acquisition of one-strand orientation by Rhabditida. The *atp8* gene present in *Campydora demonstrans* is commonly found in other metazoan mitogenomes (with the rare exception of parasitic neodermatan platyhelminths; Egger et al., 2017) but is typically absent from the nematode mitogenomes sampled to date (except for *Trichinella* spp. and *Trichuris* spp. of the dorylaimian Trichinellida lineage: Hawash et al., 2015; Lavrov & Brown, 2001; Liu et al. Liu et al. 2012a, 2012b, 2013; Mohandas et al., 2014). Although the short length and high variability of *atp8* might have resulted in overlooking this gene from some nematode mitogenomes, its observed taxonomic distribution suggests that the complete 37-gene repertoire is ancestral for Nematoda. *C. demonstrans* differs in the mt gene arrangement from all other nematodes sampled to date, which can be expected from its distant relationship to Dorylaimia and Chromadoria, as well as the lack of conservation of gene order observed from some dorylaimian lineages (Hyman et al., 2011).

Among the 13 protein-coding genes, six (*atp8*, *cox1*, *nad1*, *nad2*, *nad3*, *nad6*) use ATT as a start codon, three (*atp6*, *cytb*, *nad5*) – ATG, three (*cox2*, *cox3*, *nad4l*) – TTA and one (*nad4*) – ATA; 12 genes use TAA as a stop codon, while *nad6* terminates at TAG. The TAA stop codon in *cox3* is completed by polyadenylation of mRNA. Overall mitogenome A + T content is 66%. Protein-coding gene A + T content is 69%, 63%–in tRNAs, 65%–in rRNAs, whereas 60% – in noncoding regions. The mitogenome includes two long noncoding regions, each containing a 2,013 bp-long arm of a perfect inverted repeat, that subdivide circular mtDNA into two coding regions of 4156 and 8825 bp in length, containing 15 and 22 genes, respectively. Long inverted repeats are typical of chloroplast genomes (plastomes) of land plants. The plastome repeats, however, are gene-coding, with the two repeat arms (approximately 25 kb each) comprising mirror images of one another in terms of gene complement (with a few exceptions; Shaw et al., 2007). Chloroplast DNA is able to exist in two structural haplotypes differing in the orientation of single-copy regions (Palmer, 1983; Wang & Lanfear, 2019). By this analogy, we assume that *Campydora demonstrans* mtDNA may as well maintain two "flip–flop" alternatives in vivo, as described by the assemblies in

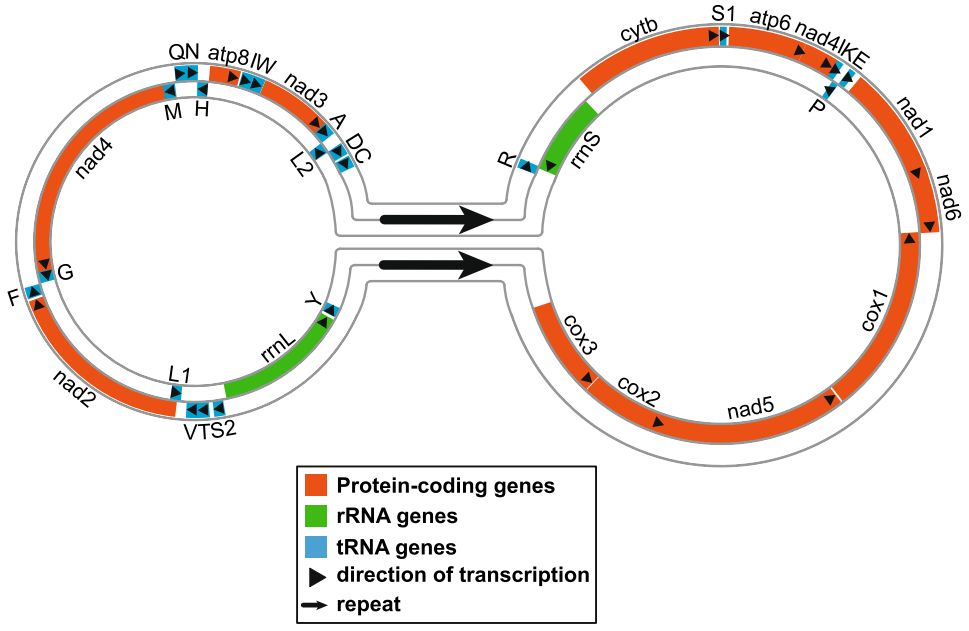

FIGURE 1 Circular map of *Campydora demonstrans* mitogenome.

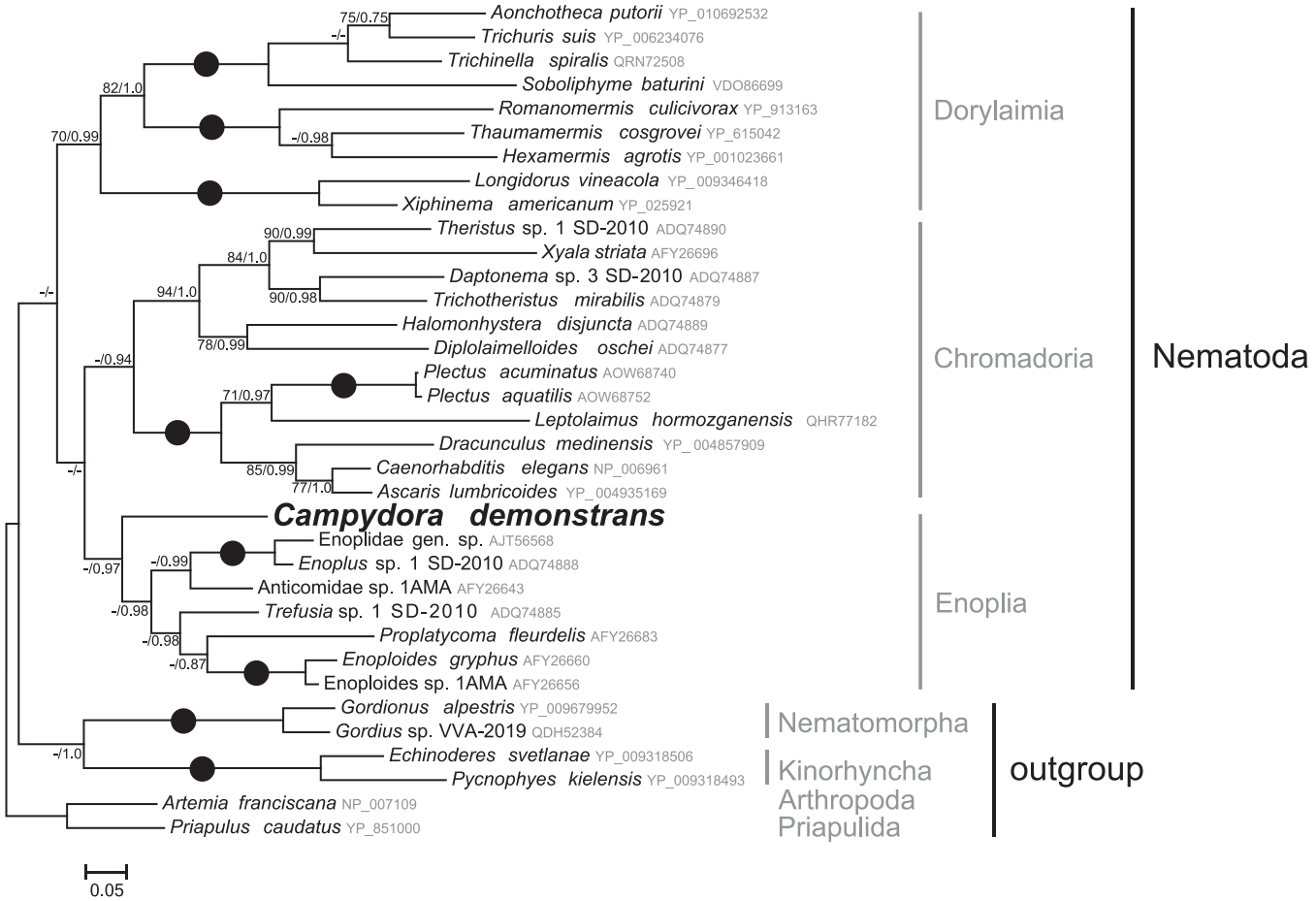

FIGURE 2 Bayesian tree of Nematoda based on cox1 amino acid data. Node labels contain Bayesian posterior probabilities (right) and maximum likelihood bootstrap support values (left). Values < 70/0.7 replaced with dashes (–). Bipartitions with 100/1.0 support are marked with black dots. GenBank accession IDs for species are shaded in gray.

Figure 1 and Figure S3. We screened all animal mitogenomes in Refseq (>12,200 by the date of analysis) and found that only two exhibit perfect repeats with >1000 bp-long arms–the mermithid *Hexamermis agrotis* (Dorylaimia) and the legless lizard *Anguis graeca*. Unlike in *C. demonstrans*, these repeats are gene-coding. Thus, *H. agrotis* possesses a nested pair of inverted repeats (1268 and 1661 bp in size) in the control region, producing duplicated *atp6* and *trnS* genes; intervening regions remain noncoding. In *A. graeca*, the 1326 bp-long repeat arms contain six genes, whereas the 1,719 bp-long intervening region contains three genes. This lizard species has acquired the duplication relatively recently, since its close relatives (*A. fragilis*, *A. veronensis*, *A. cephallonica* and *A. colchica*) lack the repeat and have a ~2000 bp-shorter mitogenome. The A + T content in the repeats of *H. agrotis* and *A. graeca* matches its whole-mitogenome average (78% and 55%, correspondingly), whereas the repeat in *C. demonstrans* exhibits lower A + T content than its mitogenome average, thereby suggesting that different mechanisms and functional roles may underlie these repeats' emergence and conservation.

Bayesian phylogenetic analysis of the cox1 data set containing *Campydora demonstrans* infers monophyly of the three major nematode lineages (Enoplia, Dorylaimia and Chromadoria) with support 0.94–0.99, albeit with root trichotomy remaining unresolved (Figure 2). *C. demonstrans* groups with other members of Enoplia, in accord with earlier dedicated SSU rDNA-based analyses (Mullin et al., 2003; van Megen et al., 2009). The *cox1* gene is commonly used today as a primary DNA barcode for the purposes of biodiversity profiling and species delimitation in animals (Ratnasingham & Hebert, 2007) and has achieved high taxonomic coverage in public databases. Although its applicability as a universal, phylum-level barcode in nematodes has inherent limitations in designing robust PCR primer pairs (e.g. Bik et al., 2010), it has been proved efficient for putative species delimitation (Gonçalves et al., 2021), as well as phylogenetic reconstruction (Chan et al., 2020) within the currently sampled nematode mt marker diversity of almost exclusively rhabditid taxa. Our report elicits good suitability of *cox1* for inferring nematode taxonomic relationships within all three major lineages, substantiating its wider application in basic and applied nematology in studies leveraging genomic and/or environmental metagenomic approaches (e.g. D'haeseleer et al., 2017; Woehle et al., 2018), along with mt rDNA markers (Chan et al., 2020) and the nuclear LSU rDNA D2D3 region (De Ley et al., 2005).

3 | MATERIALS AND METHODS

3.1 | Material sampling and DNA extraction

Live individuals of *Campydora demonstrans* were extracted with Baermann funnel from soil samples collected near Trebejov, Košice Region, Slovakia (September 1997), fixed with hot TAF (4% formaldehyde-containing fixative; Courtney et al., 1955), stored in glycerin and re-transferred in TAF after 2 years (material identified and kindly provided by Prof. Dieter Sturhan). Total DNA was extracted from specimens pre-washed in deionized water for 1 h, using standard worm lysis solution and a protocol (Williams et al., 1992; 10 min 70°C, 1 h 65°C, 10 min 95°C), and stored at −20°C for ~20 years until NGS library preparation.

3.2 | DNA sequencing, assembly and annotation

A total DNA library was prepared using Accel-NGS chemistry and sequenced on an Illumina HiSeq 4000 platform by following the manufacturers' protocols to generate 43.2M paired-end reads. Illumina reads were processed with Trimmomatic (Bolger et al., 2014) tools to remove adapter sequences and assembled using SPAdes (Bankevich et al., 2012). Contigs corresponding to rDNA were extracted from the SPAdes assemblies using BLAST (Altschul, 1997). The rDNA-containing contigs were merged by overlapping, and the resulting consensus was error checked by read mapping with Bowtie2 (Langmead & Salzberg, 2012) and the mapping inspection in Tablet (Milne et al., 2013). The assembled rDNA sequence of *Campydora demonstrans* includes the nuclear SSU, 5.8 and LSU rDNA genes and is available at NCBI GenBank under accession ID OR852751. Mitogenome was assembled with NOVOPlasty (Dierckxsens et al., 2016) and annotated with the MITOS Web Server (Bernt et al., 2013), followed by manual annotation correction. The mitogenome sequence was examined for errors by read mapping with Bowtie2 and the mapping inspection in Tablet. Repeat finding was conducted on a complete (12,200 total) set of RefSeq metazoan mitogenomes using the EMBOSS *palindrome* utility (Rice et al., 2000). The fully assembled mitochondrial genome of *Campydora demonstrans* is available at NCBI GenBank under accession ID OP163863; raw NGS sequence reads are deposited under NCBI BioProject PRJNA939523, SRA record SRR23640105.

3.3 | Phylogenetic analysis

Bayesian phylogenetic reconstruction was conducted with MrBayes 3.2.6 (Ronquist et al., 2012) under the GTR + Γ4 + I model, 4 runs of 5M chain generations with 50% burn-in, for cox1 amino acid sequences of *Campydora demonstrans*, 28 other nematode species representative of three major nematode lineages (Dorylaimia, Chromadoria and Enoplia), as well as six outgroup species. Enoplian sequences from Bik et al. (2010) were omitted from selection as not overlapping with the data set's longer N-terminal sequences (Derycke et al., 2010; Martínez-Arce et al., 2020). Maximum likelihood standard bootstrap support values were estimated in 100 replicates for the fixed Bayesian topology under the GTR20 + F + G4 model using IQ-TREE (Nguyen et al., 2015).

4 | CONCLUSIONS

The mitogenome of *Campydora demonstrans* broadens the mtDNA taxonomic sampling of the phylum Nematoda to include a member of Enoplia, the third major nematode lineage. Its distinctive feature is a plastome-like organization, rare among the animals, with two control regions containing arms of a perfect inverted repeat, which subdivides circular mtDNA into two coding parts and potentially folds into a control secondary structure. The mechanisms of this repeat's emergence and conservation are pending further research. Our phylogenetic analysis supports *cox1* gene applicability to establish phylogenetic relationships within major nematode groups. Mitogenomic approaches are more likely to guarantee success in obtaining *cox1* sequences from nematodes that failed to amplify with PCR primers tested in previous studies.

AUTHOR CONTRIBUTIONS

Olga V. Nikolaeva performed most of the computations, analyzed the data and drafted the manuscript. Leonid Yu. Rusin, Kirill V. Mikhailov and Vladimir V. Aleoshin performed additional computations, analyzed the data and wrote the manuscript. Paul De Ley designed and supervised the research. All authors have read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The originally obtained, fully assembled mitochondrial genome and nuclear rDNA operon of *Campydora demonstrans* are available at NCBI GenBank under accession IDs OP163863 and OR852751, respectively; raw NGS sequence reads are deposited under NCBI BioProject PRJNA939523, SRA record SRR23640105 (www.ncbi.nlm.nih.gov).

ORCID

Leonid Yu Rusin http://orcid.org/0000-0003-2424-298X
Vladimir V. Aleoshin http://orcid.org/0000-0002-3299-9950

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