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Comparative analyses of the complete mitochondrial genomes of *Cyathostomum pateratum* and *Cyathostomum catinatum* provide new molecular data for the evolution of Cyathostominae nematodes

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Abstract

The parasite *Cyathostomum pateratum*, which occurs in the large intestine of equines, is a common species of the subfamily Cyathostominae. Cyathostominae nematodes are a complex nematode group for which only limited genetic information has been reported. To re-examine the phylogenetic relationships among Cyathostominae nematodes, we sequenced the complete mitochondrial (mt) genome of *Cy. pateratum* and compared it with the mt genome of the congeneric species *Cyathostomum catinatum*. The complete mtDNA sequence of *Cy. pateratum* was 13,822 bp in length, 16 bp shorter than that of *Cy. catinatum*. The mtDNA sequences of both species contained 12 protein-coding genes, two rRNA genes and 22 tRNA genes, and all 36 genes were transcribed in the same direction and in the same strand. Pairwise comparisons of the 12 predicted amino acid sequences between *Cy. catinatum* and *Cy. pateratum* revealed differences of 0.4–3.1%; the least conserved sequence was that of cytochrome *c* oxidase subunit 3 (*cox*3). Phylogenetic analysis of the concatenated amino acid sequences using Bayesian inference and maximum likelihood methods showed that *Cy. catinatum* and *Cy. pateratum* clustered together with very high nodal support, and *Cylicostephanus goldi* was closer to the *Cyathostomum* nematodes than to other Cyathostominae nematodes. The mtDNA sequence of *Cy. pateratum* is reported here for the first time. The study will shed some light on the genetic evolution among parasitic nematodes in *Cyathostomum*.

Introduction

Cyathostominae nematodes, also known as small strongyles of horses, belong to the family Strongylidae. Cyathostomins usually present as a mixed infection and produce symptoms such as weight loss, poor coat, lethargy, anaemia and diarrhoea, even with the high mortality caused by larval stage migration (Lichtenfels *et al*., 2008). Cyathostomins can adapt to different climates and have been reported in horses worldwide (Mfitilodze and Hutchinson, 1990; Smets *et al*., 1999). Their prevalence may be as high as 100%, which can lead to considerable economic damage to horse breeding (Bu *et al*., 2009; Stancampiano *et al*., 2017).

Previous studies on Cyathostominae nematodes have focused mainly on their morphology, life history and epidemiology (Lichtenfels *et al*., 2008; Stancampiano *et al*., 2017), and thus genetic information is limited. Genetic markers, such as the internal transcribed spacer (ITS) of the nuclear ribosomal DNA region and mitochondrial genome sequences, have been used for the genetic classification and evolutionary analysis of cyathostomins (Hung *et al*., 2000; Gao *et al*., 2017a, b).

Mitochondrial (mt) DNA sequences are used widely as genetic markers, not only for population or ecological genetics studies but also for phylogenetic and evolutionary analyses of many kinds of parasites. Cyathostominae nematodes are a complex nematode group, with 51 reported species. The mt genomes of only six Cyathostominae species (*Cylicostephanus goldi*, *Cylicostephanus minutus*, *Cylicocyclus nassatus*, *Cylicocyclus insigne*, *Cyathostomum catinatum* and *Poteriostomum imparidentatum*) have been sequenced to date (Gao *et al*., 2017a, b). *Cyathostomum pateratum* is one of the most common cyathostomins in equine species, but there are insufficient mtDNA sequence data available to determine its phylogenetic relationships with other species.

The aims of this study were to determine the mt genome sequence of *Cy. pateratum*, compare it with the mt genome sequence of *Cy. catinatum* reported previously, and reconstruct the phylogenetic relationships with other Cyathostominae nematodes using the mtDNA sequence data.

Materials and methods

Parasites and isolation of total genomic DNA

Adult *Cy. pateratum* were collected from the large intestine of a naturally infected horse from Daqing City, Heilongjiang Province, China. Individual worms were washed with physiological saline and identified based on their morphological characteristics (Lichtenfels *et al.*, 2008), then stored at −20°C until used.

Total genomic DNA was extracted from individual worms using a TIANamp Genomic DNA Kit (TIANGEN Biotech, Beijing, China). The species was determined by amplifying the ITS sequence of *Cy. pateratum* using primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Gao *et al.*, 2017a). The ITS sequence of *Cy. pateratum* obtained in the present study (GenBank: MG727532) matched the previously reported ITS sequence of *Cy. pateratum* (GenBank: KF850628.1) with 99.9% identity. To better confirm the species, we amplified two genes, *nad*1 and *cox*1, and the obtained sequences matched the previously published *nad*1 (GenBank: KY131816.1) and *cox*1 (GenBank: AF263484.1) sequences with 96.1% and 92.4% identities, respectively.

PCR amplification and sequencing of the mtDNA genome of Cy. pateratum

The complete mtDNA sequence of *Cy. pateratum* was amplified using ten specific primers. The primer sequences and polymerase chain reaction (PCR) cycling conditions were the same as those for *Cy. catinatum* (Gao *et al.*, 2017b). The amplicons were separated on 1% agarose gel, purified using a DNA Gel Extraction Kit (AXYGEN, Suzhou, China), ligated into a pMD-18T plasmid vector (Promega, Madison, Wisconsin, USA), and transformed into DH5α competent cells (BioMed, Beijing, China). The positive recombinant plasmids were sequenced by the Life Technology Company (Shanghai, China). The complete mtDNA sequence was assembled manually and aligned against the mtDNA sequences of other Cyathostominae nematodes by Clustal X (v. 1.83) (Thompson *et al.*, 1997). The secondary structures of transfer RNA (tRNA) genes were predicted using the online program tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE) or manually. The boundaries of protein-coding genes and ribosomal RNA (rRNA) genes, and initiation and termination codons were determined by MEGA 5 (Tamura *et al.*, 2011). The identities were calculated using MegAlign 5.01 (Burland, 2000). The A + T content was determined using DNAStar (v. 5.0) (Burland, 2000). The identities, length and A + T contents of genes of the complete mt genome of *Cy. pateratum* were compared with the mt genomes of *Cy. catinatum* and other nematodes.

Phylogenetic analyses

Phylogenetic trees were constructed based on the concatenated amino acid sequences of the 12 protein-coding mt genes of Strongylidae nematodes available in GenBank. The nematodes belonged to two subfamilies as follows: Cyathostominae (*Cs. goldi* (AP017681), *Cc. insigne* (GQ888712), *Cc. nassatus* (KX819273), *Cy. catinatum* (KY495600), *Cs. minutus* (KY495601) and *P. imparidentatum* (KY495602)), and Strongylinae (*Strongylus equinus* (KM605251), *Strongylus vulgaris* (AP017698), *Triodontophorus brevicauda* (KP191043), *Triodontophorus nipponicus* (KX185155), *Triodontophorus serratus* (KX185154) and *Macropicola ocydromi* (KF361320)), with *Syngamus trachea* (GQ888718) as the outgroup. Bayesian inference and maximum likelihood methods were used to construct the trees as described previously (Gao *et al.*, 2017a). Phylograms were drawn using FigTree v. 1.31 (http://tree.bio.ed.ac.uk/software/figtree/).

Results and discussion

Structure of the complete mt genome of Cy. pateratum and comparisons with Cy. catinatum and other nematodes

The complete mtDNA sequence of *Cy. pateratum* was a typical circular DNA molecule 13,822 bp in length (MG727531.1), which is slightly shorter than that of *Cy. catinatum* (13,838 bp). Both mtDNA sequences contained 36 genes (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *cyt*b, *rrn*L, *rrn*S and 22 tRNAs) and two non-coding regions (long non-coding region (LNCR) and short non-coding region (SNCR)). All 36 genes were transcribed in the same direction in the two genomes, which is the same as other Chromadorea nematodes, such as *Triodontophorus nipponicus* and *Bunostomum phlebotomum* (Gao *et al.*, 2014, 2017), but different for Enoplea nematodes, such as *Trichinella spiralis* and *Trichuris trichiura* (Lavrov and Brown, 2001; Liu *et al.*, 2012b). The A + T content of the mt genome of *Cy. pateratum* was 75.68% (A = 30.42%; T = 45.26%), which is similar to that of the majority of Strongylidae nematodes, such as *Cy. catinatum* (76.12%) and *Strongylus vulgaris* (76.55%), but higher than that of Enoplea nematodes, such as *T. spiralis* (65.2%) and *T. trichiura* (68.1%), and trematodes, such as *Eurytrema pancreaticum* (62.5%) (Lavrov and Brown, 2001; Liu *et al.*, 2012b; Chang *et al.*, 2016; Gao *et al.*, 2017b). The *Cy. pateratum* mtDNA sequence contained 20 intergenic sequences 1–51 bp in length. There was no overlap in the complete *Cy. pateratum* mtDNA sequence, whereas in the complete *Cy. catinatum* mtDNA sequence there was one overlap of 1 bp between *cox*1 and tRNA-Cys. Details of the structure of the *Cy. pateratum* and *Cy. catinatum* mt genes are in table 1.

Comparative analysis between the mt genomes of Cy. pateratum and Cy. catinatum and those of other nematodes

The complete mtDNA nucleotide sequences of *Cy. pateratum* and *Cy. catinatum* shared 94.6% identity. In other congeneric nematode species, such as *B. phlebotomum* China yak isolate and *B. trigonocephalum* sheep isolate, the complete mtDNA nucleotide sequences shared only 85.3% identity, whereas for *Ascaris lumbricoides* and *Ascaris suum* the identity was 98.81% (Liu *et al.*, 2012a; Gao *et al.*, 2014). The predicted concatenated amino acid sequences of the 12 protein-coding genes of *Cy. pateratum* and *Cy. catinatum* differed by 2.2%, which is similar to what was found in other congeneric species, such as *Ancylostoma duodenale* and *Ancylostoma caninum* (4.1%) (Jex *et al.*, 2009). The nucleotide and predicted amino acid sequences of the 12 protein-coding genes of *Cy. pateratum* and *Cy. catinatum* shared 92.2–96.2% and 96.9–99.6% identity, respectively (table 1). The nucleotide and amino acid sequences of *cox*3 were the least conserved among the 12 protein-coding genes. The ATT initiation codon and TAA termination codon were the most frequently used codons in *Cy. pateratum* and *Cy. catinatum* (table 1), and both had an incomplete codon T at the end of the *cox*3 gene, which is consistent with *cox*3 in the *S. equinus* and *Aspiculuris tetraptera* mt genomes (Xu *et al.*, 2015; Wang *et al.*, 2016).

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Table 1. Locations and similarity of the mitochondrial genes of *Cyathostomum pateratum* and *Cyathostomum catinatum*.

Genes	Position 5′ to 3′ (Length, bp)		Initiation codons/Termination codons		Similarity
	Cy. pateraturm	*Cy. catinatum*	*Cy. pateraturm*	*Cy. catinatum*	NS/AAS%
*cox*1	1–1575 (1575)	1–1578 (1578)	ATT/TAA	ATT/TAA	93.9/98.9
tRNA-Cys (C)	1584–1638 (55)	1578–1632 (55)	–	–	–
tRNA-Met (M)	1645–1703 (59)	1643–1701 (59)	–	–	–
tRNA-Asp (D)	1717–1774 (58)	1707–1765 (59)	–	–	–
tRNA-Gly (G)	1777–1832 (56)	1777–1832 (56)	–	–	–
*cox*2	1833–2528 (696)	1833–2528 (696)	ATT/TAA	ATT/TAA	95.1/99.6
tRNA-His (H)	2535–2589 (55)	2534–2587 (54)	–	–	–
*rrn*L	2590–3565 (976)	2588–3563 (976)	–	–	96.8/–
*nad*3	3566–3901 (336)	3564–3899 (336)	ATT/TAG	ATT/TAG	94.9/99.1
*nad*5	3922–5505 (1584)	3917–5500 (1584)	ATT/TAA	ATT/TAA	95.1/98.3
tRNA-Ala (A)	5509–5564 (56)	5504–5559 (56)	–	–	–
LNCR	5565–5837 (273)	5560–5830 (271)	–	–	–
tRNA-Pro (P)	5838–5892(55)	5831–5885 (55)	–	–	–
tRNA-Val (V)	5899–5952 (54)	5897–5950 (54)	–	–	–
*nad*6	5953–6387 (435)	5951–6385 (435)	ATT/TAG	ATT/TAG	92.2/99.3
*nad*4L	6439–6672 (234)	6436–6669 (234)	ATT/TAA	ATT/TAA	96.2/98.7
tRNA-Trp (W)	6690–6746 (57)	6689–6745 (57)	–	–	–
tRNA-Glu (E)	6787–6843 (57)	6787–6843 (57)	–	–	–
*rrn*S	6844–7541 (698)	6844–7551(708)	–	–	98.4/–
tRNA-Ser^{UCN}	7542–7597 (56)	7552–7607 (56)	–	–	–
tRNA-Asn (N)	7598–7654 (57)	7608–7664 (57)	–	–	–
tRNA-Tyr (Y)	7659–7716 (58)	7669–7726 (58)	–	–	–
*nad*1	7717–8589 (873)	7727–8599 (873)	TTG/TAA	ATT/TAA	95.6/99.7
*atp*6	8599–9198 (600)	8610–9209 (600)	ATT/TAA	ATT/TAA	94.7/99.0
tRNA-Lys (K)	9212–9274 (63)	9223–9285 (63)			
tRNA-Leu^{UUR}	9295–9349 (55)	9307–9361 (55)	–	–	–
tRNA-Ser^{AGN}	9350–9401 (52)	9362–9414 (53)	–	–	–
*nad*2	9402–10247 (846)	9415–10260 (846)	TTG/TAA	TTG/TAA	94.6/98.2
tRNA-Ile (I)	10257–10315 (59)	10268–10326 (59)	–	–	–
tRNA-Arg (R)	10329–10383 (55)	10341–10395 (55)	–	–	–
tRNA-Gln (Q)	10390–10444 (55)	10402–10456 (55)	–	–	–
tRNA-Phe (F)	10446–10501 (56)	10458–10513 (56)	–	–	–
*cyt*b	10502–11614 (1113)	10514–11626 (1113)	ATT/TAA	ATT/TAA	95.1/99.2
tRNA-Leu^{CUN}	11630–11684 (55)	11643–11697 (55)	–	–	–
*cox*3	11685–12450 (766)	11698–12463 (766)	ATT/T	ATT/T	92.7/96.9
tRNA-Thr (T)	12451–12505 (55)	12464–12518 (55)	–	–	–
*nad*4	12506–13735 (1230)	12519–13748 (1230)	TTG/TAA	TTG/TAA	94.7/99.3
SNCR	13736–13822 (87)	13749–13838 (90)	–	–	–

The *rrn*L genes of *Cy. pateratum* and *Cy. catinatum* were located between tRNA-His and *nad*3 and both were 976 bp in length. The lengths of the *rrn*S genes differed; 698 bp in *Cy. pateratum* and 708 bp in *Cy. catinatum*. The *rrn*L and *rrn*S genes of *Cy. pateratum* and *Cy. catinatum* varied by 3.2% and 2.6%, respectively. The A + T contents of *rrn*L and *rrn*S in *Cy. pateratum* were 80.64% and 77.65%, and in *Cy. catinatum* 80.84% and 77.26%, respectively (Gao *et al.* 2017b).

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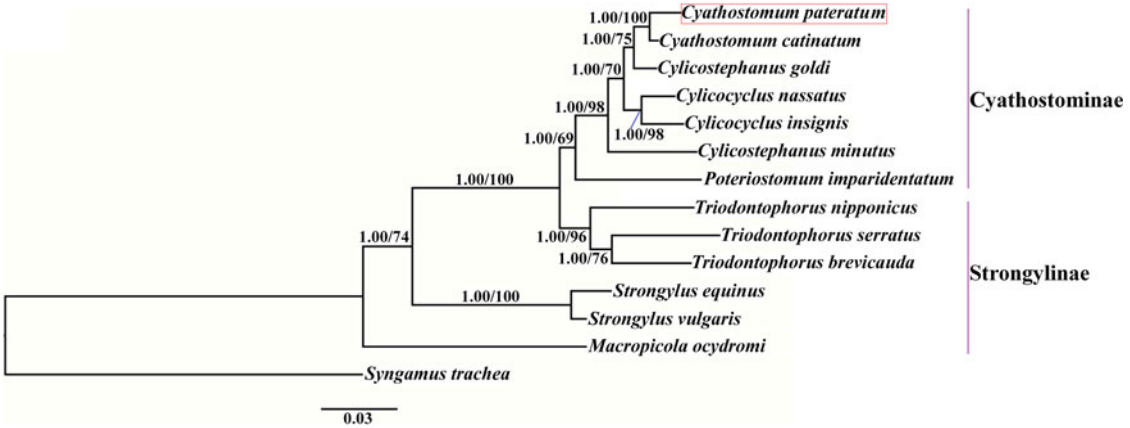

Fig. 1. Phylogenetic relationships among Strongylidae nematodes based on the concatenated amino acid sequences of 12 protein-coding genes by Bayesian inference and maximum likelihood methods. *Syngamus trachea* was used as the outgroup. The red box marks the species that was sequenced in the present study.

The 22 tRNA genes ranged in length from 52 bp (tRNA-Ser^{AGN}) to 63 bp (tRNA-Lys) in the mt genome of *Cy. pateratum*. The predicted secondary structures of the 22 tRNA genes are shown in supplementary fig. S1. The predicted secondary structure of 20 of the tRNAs (excluding two tRNA-Ser) have a DHU (dihydrouridine) arm and a DHU loop, in which the variable TψC arm and variable loop are replaced by a 'TV-replacement loop'. Two tRNA-Ser genes of *Cy. pateratum* mt genome possess a TψC arm and variable loop, but lack the DHU arm and loop, which are common in most other nematode mtDNA sequences (Gao *et al.*, 2017a, b). The two non-coding regions (LNCR and SNCR) in the *Cy. pateratum* mt genome are conserved in the mt genomes of other *Cyathostomum* nematodes (e.g. *Cy. catinatum, Cs. minutus* and *P. imparidentatum*) as well as *A. suum*, *T. serratus* and *T. nipponicus*, but not in other nematodes, such as *T. brevicauda* and *Oxyuris equi*, which have one, three or more non-coding regions in their mt genomes (Liu *et al.*, 2012a; Duan *et al.*, 2015; Zhang *et al.*, 2015; Gao JF *et al.*, 2017; Gao Y *et al.*, 2017b). The LNCRs of *Cy. pateratum* and *Cy. catinatum* were 273 bp and 271 bp in length, and the SNCRs were 87 bp and 90 bp in length, respectively. The non-coding regions in the mt genomes of other nematodes vary greatly; for example, in *Syphacia obvelata*, a non-coding region is 815 bp in length (Wang *et al.*, 2016). The A + T content of the non-coding regions in the *Cy. pateratum* mtDNA sequences was higher than in other regions, namely 82.75% for the SNCR and 86.35% for the LNCR.

Phylogenetic analysis of Cy. pateratum, Cy. catinatum *and other Strongylidae nematodes*

We used Bayesian inference and maximum likelihood methods to construct phylogenetic trees of Strongylidae nematodes based on the concatenated amino acid sequences of the 12 protein-coding mt genes. The topologies of the two trees reconstructed using the two different methods were identical. The phylogenetic trees had two large branches (fig. 1); one branch contained only *M. ocydromi*, a kangaroo parasite, and the other branch contained all the equine parasites. Two *Strongylus* species (*S. equinus*, *S. vulgaris*) clustered together and three *Triodontophorus* species (*T. brevicauda*, *T. nipponicus*, *T. serratus*) clustered together to form clades separate from the Cyathostominae nematodes. *Cyathostomum catinatum* and *Cy. pateratum* formed sister taxa, and congeneric *Cs. goldi* was closer to the two *Cyathostomum* species than to *Cs. minutus*.

Gao *et al.* (2017b) discussed the phylogenetic relationships of 20 Strongyloidea nematodes based on the amino acid sequences of 12 protein-coding genes, which indicated that *Cs. goldi* was closer to *Cy. catinatum* than to other *Cylicostephanus spp*. However, this relationship could not be verified because the necessary biological information was lacking. In this study, *Cy. catinatum*, *Cy. pateratum* and *Cs. goldi* formed a cluster that had high statistical support, although *Cy. pateratum* and *Cy. catinatum* were closer to each other than to *Cs. goldi*. This result is consistent with that of McDonnell *et al.* (2000), who analysed the phylogenetic relationships among Strongylinae and Cyathostominae species using partial mitochondrial gene and rRNA ITS sequences.

Cyathostominae nematodes are a complex nematode group, and the genera of equine strongyles with large subglobular buccal capsules were considered to be ancestral to those with small cylindrical capsules (Lichtenfels, 1987). Hung *et al.* (2000) used ITS sequences to reconstruct systematic relationships of 30 species of equine strongyles, and their molecular data supported this hypothesis. In the present study, the phylogenetic relationships among Strongylidae nematodes obtained using mtDNA data also support this idea. However, mtDNA data are not available for many Cyathostominae nematodes, so more mt genomes need to be sequenced and evolutionary relationships need to be re-examined to discover some complex species.

In conclusion, the complete mtDNA sequence of *Cy. pateratum* is reported here for the first time. Comparative and phylogenetic analyses based on the complete mt genome showed that *Cy. pateratum* is a closely related but distinct species to *Cy. catinatum*.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X18000688

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Conflict of interest. None.

Ethical standards. This study was performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China, and the protocol was reviewed and approved by the Research Ethics Committee of Heilongjiang Bayi Agricultural University, China.

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