Journal of Helminthology

cambridge.org/jhl

Characterization of the complete mitochondrial genome sequence of *Tracheophilus cymbius* (Digenea), the first representative from the family Cyclocoelidae

Y. Li, X.X. Ma, Q.B. Lv, Y. Hu, H.Y. Qiu, Q.C. Chang and C.R. Wang

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province 163319, PR China

Research Paper

Cite this article: Li Y, Ma XX, Lv QB, Hu Y, Qiu HY, Chang QC, Wang CR (2020). Characterization of the complete mitochondrial genome sequence of *Tracheophilus cymbius* (Digenea), the first representative from the family Cyclocoelidae. *Journal of Helminthology* **94**, e101, 1–7. https://doi.org/10.1017/S0022149X19000932

Received: 11 June 2019
Revised: 21 September 2019
Accepted: 23 September 2019

Key words:
Tracheophilus cymbius; Cyclocoelidae; complete mitochondrial genome; phylogenetic analysis

Author for correspondence:
C.R. Wang, E-mail: chunrenwang@126.com

Abstract

Tracheophilus cymbius (Trematoda: Cyclocoelidae) is a common tracheal fluke of waterfowl, causing serious loss in the poultry industry. However, taxonomic identification of *T. cymbius* remains controversial and confused. Mitochondrial (mt) genomes can provide genetic markers for the identification of closely related species. We determined the mt genome of *T. cymbius* and reconstructed phylogenies with other trematodes. The *T. cymbius* mt genome is 13,760 bp in size, and contains 12 protein-coding genes (*cox* 1–3, *nad* 1–6, *nad* 4L, *cyt* b and *atp* 6), 22 transfer RNA (tRNA) genes, two ribosomal RNA genes and one non-coding region. All are transcribed in the same direction. The A + T content is 62.82%. ATG and TAG are the most common initiation and termination codons, respectively. Phylogenetic analyses of concatenated nucleotide sequences show *T. cymbius* grouping in suborder Echinostomata, and clustering together, with high statistical support, as a sister taxon with *Echinochasmus japonicus* (Echinochasmidae), the two forming a distinct branch rooted to the ancestor of all Echinostomatidae and Fasciolidae species. This is the first report of the *T. cymbius* mt genome, and the first reported mt genome within the family Cyclocoelidae. These data will provide a significant resource of molecular markers for studying the taxonomy, population genetics and systematics of trematodes.

Introduction

Tracheophilus cymbius (Diesing, 1850) Skrjabin, 1913, belongs to the family Cyclocoelidae (Stossich, 1902), and is parasitic in the trachea of ducks and other waterfowl, often causing mild clinical symptoms, including cough, malnutrition, weight loss and dyspnoea, but sometimes even causing death (Bisseru, 1957; Tang & Tang, 1978; Scott *et al.*, 1982). *Tracheophilus cymbius* have been reported in Asia, Europe, America and Africa (Bisseru, 1957; Gu *et al.*, 1973; Tang & Tang, 1978; Scott *et al.*, 1982; Wang *et al.*, 2004). *Tracheophilus cymbius*-infected domestic ducks and wild aquatic birds have also been widely recorded in China, including Fujian, Hebei, Ningxia, Jiangxi and Heilongjiang Provinces (Gu *et al.*, 1973; Tang & Tang, 1978; Wang *et al.*, 2004). The prevalence of *T. cymbius* in domestic ducks ranges from 5.8% to 30% in Fujian Province, and from 2.6% to 37.8% in wild aquatic birds in Bai-Yang-Dian Lake, Hebei Province (Gu *et al.*, 1973; Tang & Tang, 1978). *Tracheophilus cymbius* is particularly common and prevalent in domestic ducks in Heilongjiang Province, causing severe clinical symptomology. Our research shows 5.4% (12/222) *T. cymbius* prevalence in Heilongjiang Province in 2003, and 22.3% (21/94) in 2017, with an infection intensity of 1–8 (Wang *et al.*, unpublished data), causing significant economic loss to the duck industry. Therefore, effective control and prevention of this trematode is important to the poultry industry.

Traditional approaches for the identification and differentiation of parasites based on morphological features have long been used worldwide. However, morphological approaches have limitations in identifying and distinguishing closely related species (McManus & Bowles, 1996). Although the family Cyclocoelidae was first named over a century ago, the status of the family and several cyclocoelid species remains controversial and uncertain. This is particularly the case with the important tracheal fluke *T. cymbius* Skrjabin (1913), considering that *Tracheophilus* Skrjabin and *Typhlocoelum* Stossich were classified into two different genera based on testes shape (Skrjabin, 1913; Bisseru, 1957). However, Joyeux & Baer (1927) recognized *Tracheophilus* Skrjabin and *Typhlutimum* Witenberg as synonyms of *Typhlocoelum* Stossich, 1902, and the two species were combined into the same genus, namely *Typhlocoelum cymbius* (Diesing, 1850) (syn. *Tracheophilus sisowi* Skrjabin, 1913, *Typhlocoelum sisowi* Skrjabin, 1913) and *Typhlocoelum cucumerinum* (Rudolphi, 1809) (Joyeux & Baer, 1927; Bisseru, 1957). Tang & Tang (1978) reported that *T. cymbius* and *T. sisowi* should be considered the same species based on life cycle, in spite of the two trematodes having one key difference in morphological

CAMBRIDGE UNIVERSITY PRESS

features (*T. cymbius* with the posterior vitelline united, versus *T. sisowi* with a separated posterior vitelline) (Tang & Tang, 1978). Scott *et al.* (1982) used *Typhlocoelum cucumerinum sisowi* (Skrjabin, 1913) and *Typhlocoelum cucumerinum* (Rudolphi, 1809) metacercariae, identified by morphological observation, to infect wild waterfowl, and the results suggested that the two parasites are not separate species, and rather should be considered two separate sub-species (Scott *et al.*, 1982).

Mitochondrial (mt) genome and nuclear ribosomal DNA (rDNA) sequences can effectively identify species of the parasite (Wang *et al.*, 2011; Gao *et al.*, 2017). However, only a partial *Typhlocoelum* sp. 28S ribosomal RNA (rRNA) sequence (KT956960) is currently available in GenBank, and no complete mt genome data for any parasite belonging to the family Cyclocoelidae is available. This is a crucial limitation into investigations of the systematics and phylogeny of this family.

Thus, the purpose of our study was to determine the complete *T. cymbius* mt genome, and to analyse the phylogenetic relationships of *T. cymbius* with other trematodes based on a dataset consisting of the concatenation of 12 protein-coding gene nucleotide sequences. The results will help resolve issues of taxonomy, population genetics and systematics within and beyond the fluke family Cyclocoelidae.

Materials and methods

Parasite and DNA extraction

Adult *T. cymbius* flukes were collected from the trachea of naturally infected ducks in Daqing, Heilongjiang Province, China. Specimens were washed in physiological saline, and then morphologically identified to the species level (Tang & Tang, 1978). Next, the flukes were fixed in 70% ethanol and stored at −20°C until further use. Total genomic DNA was extracted from individual worms with a TIANamp Genomic DNA Kit (Tiangen, Beijing, China), according to the manufacturer's protocol.

Amplification and sequence analysis

The entire mt genome of a single *T. cymbius* specimen was amplified in seven overlapping fragments using primers (supplementary table S1) designed from relatively conserved regions of the mt genome nucleotide sequences of closely related species. Polymerase chain reaction (PCR) cycling conditions used to amplify the *T. cymbius* mtDNA genome were based on a previous report (Li *et al.*, 2019). PCR products were sent to Sangon Biotech Company (Shanghai, China) for sequencing in both directions using the same primers.

Sequences were assembled and aligned manually against the complete *Fasciola gigantica* (NC024025) and *Fasciola hepatica* (NC002546) mt genome sequences to identify gene boundaries using the program DNAStar v. 5.0 (Burland, 2000). Each protein-coding gene was translated into amino acid sequences using the trematode mt genetic code in MEGA X (Kumar *et al.*, 2018). The secondary structures of the 22 predicted tRNA genes were estimated using the online program ARWEN (http://130.235.46.10/ARWEN/), and/or by visual identification combined with manual proofreading. The rRNA genes were identified by comparison with the mt genomes of closely related species.

Using the individual protein-coding genes of *T. cymbius*, *Echinochasmus japonicus*, *F. hepatica* and *Echinostoma miyagawai*, all members of Echinostomata, comparisons were made based on complete mt genome size, gene arrangement, A + T content, AT/GC-skew and nucleotide and amino acid sequence differences. Nucleotide and amino acid sequences differences were calculated using DNAStar v. 5.0 (Burland, 2000). The AT-skew and GC-skew values in both coding and non-coding regions (NCRs) were calculated using the following equations: AT-skew = (A − T)/(A + T), and GC-skew = (G − C)/(G + C).

Phylogenetic analyses

Nucleotide sequences from the 12 protein-coding genes in the *T. cymbius* mt genome were concatenated and aligned with those from published trematodes mt genomes using MEGA X (Kumar *et al.*, 2018). We used the Gblocks online server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) to exclude ambiguously aligned regions from the multiple nucleotide sequence alignment, specifying the 'less stringent' selection option (Castresana, 2000). We used 38 trematode mt genomes available in GenBank, in addition to our *T. cymbius* data, to create our phylogenetic datasets (see supplementary table S2). *Gyrodactylus salaris* (NC008815) was included as an outgroup. Phylogenetic trees were reconstructed using the Bayesian inference (BI) and maximum likelihood (ML) methods on the concatenated nucleotides sequences of the 12 protein-coding genes.

BI was performed using the mixed model in MrBayes v. 3.1.1 (Ronquist & Huelsenbeck, 2003) with 1,000,000 metropolis-coupled Markov chain Monte Carlo generations (Ronquist & Huelsenbeck, 2003). The first 250 trees were omitted as burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities. We used PAUP v. 4.0 Beta 10 (Swofford, 2002), with 100 random addition searches and tree-bisection–reconnection branch swapping, for the nucleotide ML analysis. ML bootstrap reliability estimates were calculated from 100 bootstrap replicates with ten random additions per replicate, also using PAUP (Swofford, 2002). Phylograms were drawn using Tree View v. 1.65 (Page, 1996) for all inferences.

Results and discussion

Tracheophilus cymbius mtDNA features

The whole *T. cymbius* mt genome (GenBank accession code MK355447) is a typical circular mtDNA molecule 13,760 bp in size (supplementary fig. S1). The mt genome contains 36 genes: 12 protein-coding genes (*cox* 1–3, *nad* 1–6, *nad* 4L, *atp* 6 and *cyt* b), 22 tRNA genes, two rRNA genes (*rrn* L and *rrn* S) plus one NCR (table 1 and supplementary fig. S1). All genes are transcribed in the anticlockwise direction. The *T. cymbius* mt genome gene arrangement is identical to those of *F. hepatica*, *Clinostomum complanatum* and some Opisthorchiidae species (*Opisthorchis felineus*, *Metorchis orientalis* and *Clonorchis sinensis*) (Le *et al.*, 2001; Cai *et al.*, 2012; Chen *et al.*, 2016; Na *et al.*, 2016). The entire *T. cymbius* mt genome nucleotide composition is biased toward A and T, with an overall A + T content of 62.82% (table 2). There is a very low base C content (9.75%) in the *T. cymbius* mt genome. Furthermore, a 40 bp overlap between the *nad* 4L and *nad* 4 genes exists in *T. cymbius* mtDNA, which is consistent with most flukes, but longer than those of *Schistosoma haematobium* (28 bp) and *Schistosoma japonicm* (37 bp), and shorter than that of *Schistosoma mekongi* (64 bp) (Le *et al.*, 2001, 2016; Littlewood *et al.*, 2006; Cai *et al.*, 2012; Liu *et al.*, 2016).

https://doi.org/10.1017/S0022149X19000932 Published online by Cambridge University Press

Table 1. Mitochondrial genome organization of *Tracheophilus cymbius*.

	Positions		Size		Codons	
Gene/region	Start	End	No. of nt	No. of aa	Initiation	Termination
cox 3	1	645	645	214	ATG	TAG
trn H	655	730	76			
cyt b	734	1849	1116	371	ATG	TAG
nad 4L	1860	2129	270	89	ATG	TAG
nad 4	2090	3382	1293	430	GTG	TAA
trn Q	3388	3450	63			
trn F	3457	3522	66			
trn M	3536	3602	67			
atp 6	3606	4121	516	171	ATG	TAG
nad 2	4140	5018	879	292	ATG	TAG
trn V	5024	5092	69			
trn A	5106	5170	65			
trn D	5172	5241	70			
nad 1	5242	6144	903	300	GTG	TAG
trn N	6165	6234	70			
trn P	6252	6315	64			
trn I	6326	6392	67			
trn K	6414	6481	68			
nad 3	6482	6835	354	117	GTG	TAG
trn S1 (GCU)	6847	6911	65			
trn W	6919	6985	67			
cox 1	6993	8531	1539	512	ATG	TAG
trn T	8567	8630	64			
rrn L	8631	9615	985			
trn C	9616	9687	72			
rrn S	9688	10,447	760			
cox 2	10,448	11,044	597	198	ATG	TAG
nad 6	11,055	11,510	456	151	ATG	TAG
trn Y	11,511	11,574	64			
trn L1 (UAG)	11,586	11,650	65			
trn S2 (UGA)	11,647	11,715	69			
trn L2 (UAA)	11,727	11,791	65			
trn R	11,809	11,874	66			
nad 5	11,875	13,458	1584	527	GTG	TAG
trn E	13,481	13,545	65			
trn G	13,550	13,618	69			
NCR	13,619	13,760	142			

aa, amino acid; nt, nucleotide.

The lengths of the 12 protein-coding *T. cymbius* mtDNA genes arrange in the order *nad* 5 > *cox* 1 > *nad* 4 > *cyt* b > *nad* 1 > *nad* 2 > *cox* 3 > *cox* 2 > *atp* 6 > *nad* 6 > *nad* 3 > *nad* 4L. A total of 3372 amino acids are encoded in the *T. cymbius* mt genome (table 2). Four genes (*nad* 1, *nad* 3, *nad* 4 and *nad* 5) use GTG as a start codon, eight genes (*atp* 6, *cyt* b, *cox* 1, *cox* 2, *cox* 3, *nad* 2, *nad* 4L and *nad* 6) use ATG as a start codon. Additionally, all genes have complete TAG termination codons (table 1). The most

https://doi.org/10.1017/S0022149X19000932 Published online by Cambridge University Press

Table 2. Comparison of complete mtDNA among *Tracheophilus cymbius* and other Echinostomata species.

	No. aa					No. nt (bp)				
Genes	*T. c*	*Es. m*	*Ec. j*	*F. h*	aa s%	*T. c*	*Es. m*	*Ec. j*	*F. h*	nt s%
cox 3	214	216	215	213	57.3–65.3	645	651	648	642	65.4–70.8
cyt b	371	369	371	370	78.4–82.9	1116	1110	1116	1113	76.1–78.6
nad 4L	89	90	89	90	71.9–77.5	270	273	270	273	74.4–79.3
nad 4	430	427	427	423	52.5–62.7	1293	1284	1284	1272	62.3–67.9
atp 6	171	172	172	172	61.4–67.4	516	519	519	519	67.4–72.4
nad 2	292	289	293	288	56.6–61.5	879	870	882	867	65.3–68.8
nad 1	300	300	301	300	70.3–76.3	903	903	906	903	48.7–76.5
nad 3	117	118	118	118	67.8–77.1	354	357	357	357	72.0–76.8
cox 1	512	512	512	510	77.1–82.9	1539	1539	1539	1533	74.5–81.6
cox 2	198	202	198	200	55.1–66.5	597	609	597	603	65.3–70.5
nad 6	151	150	149	150	57.0–62.0	456	453	450	453	62.6–70.4
nad 5	527	521	524	522	59.7–69.1	1584	1563	1575	1569	59.7–69.1
Total AA	3372	3366	3369	3356	64.0–69.9					
Total size (bp)						13,770	14,413	15,865	14,462	67.6–72.0%
A + T %						62.82	65.30	61.48	62.18	

T. c, *Tracheophilus cymbius*; *Es. m*, *Echinostoma miyagawai*; *Ec. j*, *Echinochasmus japonicus*; *F. h*, *Fasciola hepatica*; nt s, nucleotides similarity; aa s, amino acid similarity.

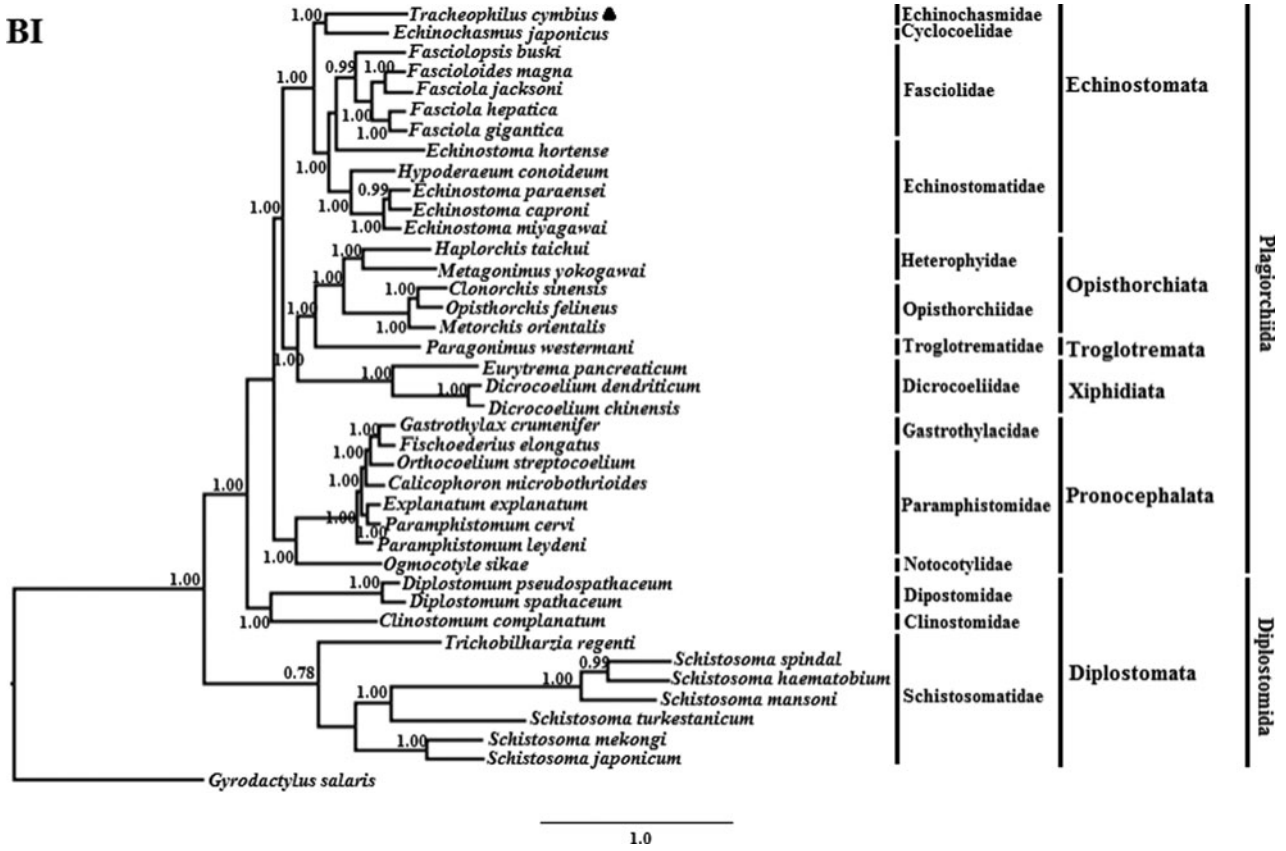

Fig. 1. Genetic relationships of *Tracheophilus cymbius* with other representative trematodes based on mitochondrial nucleotide data. Phylogenetic analysis based on the concatenated mitochondrial sequence data representing 12 protein-coding genes was conducted using Bayesian inference (BI), with *Gyrodactylus salaris* as the outgroup.

https://doi.org/10.1017/S0022149X19000932 Published online by Cambridge University Press

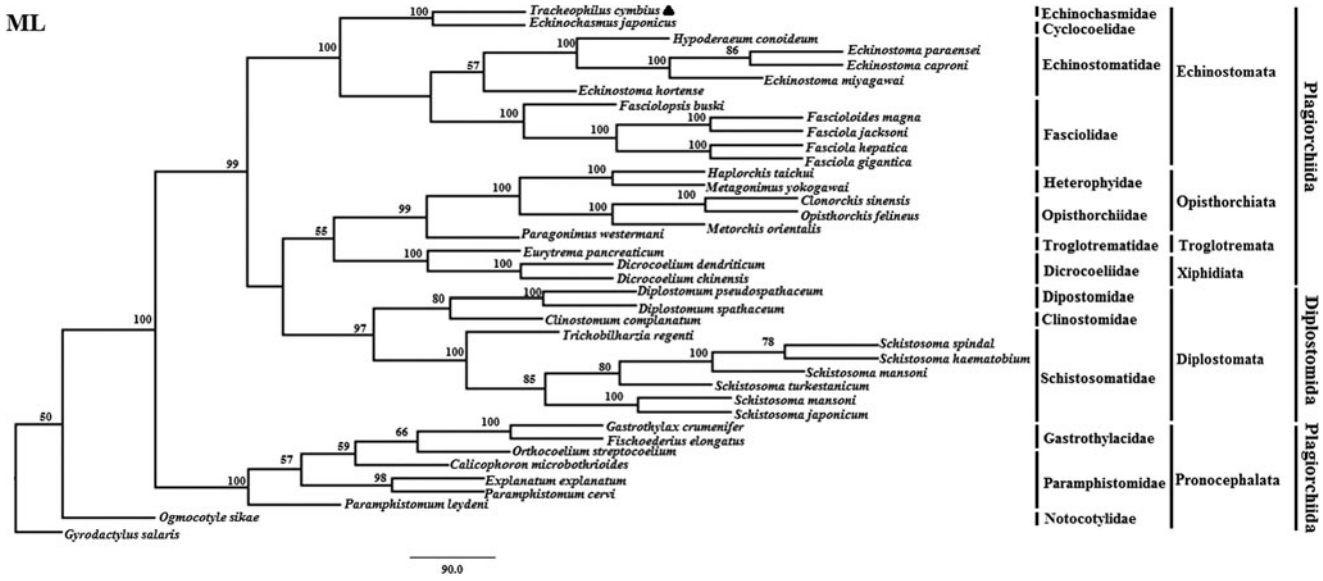

Fig. 2. Genetic relationships of *Tracheophilus cymbius* with other representative trematodes based on mitochondrial nucleotide data. Phylogenetic analysis based on the concatenated mitochondrial sequence data representing 12 protein-coding genes was conducted using maximum likelihood (ML), with *Gyrodactylus salaris* as the outgroup.

frequent codon is TTT (Phe) in the 12 protein coding genes, with a frequency of 12.5%. The least frequent codons are ACC (Thr), CGA (Arg) and AGC (Ser), all with frequencies of 0.12% (supplementary table S3).

The *T. cymbius rrn* L gene is located between *trn* T and *trn* C, and *rrn* S is located between *trn* C and *cox* 2. The lengths of the *rrn* S and *rrn* L genes are 760 bp and 985 bp, respectively (table 1). The A + T contents of the *rrn* L and *rrn* S are 61.93% and 59.61%, respectively. A total of 22 tRNA genes were identified in the *T. cymbius* mt genome, ranging from 63 bp to 76 bp in length (table 1). Twenty-one of the tRNA gene products fold into a predicted secondary structure with the conventional clover-leaf shape. The remaining *trn* S1 transcript has unpaired D-arms replaced by 7-bp loops (supplementary fig. S2). This is different than *Echinostoma hortense*, *Es. miyagawai* and *F. hepatica*, in which the two *trn* S transcripts both have unpaired D-arms (Le *et al.*, 2001; Liu *et al.*, 2016; Li *et al.*, 2019). The *T. cymbius* mt genome NCR is located between *trn* G and *cox* 3, with a length of 142 bp (table 1).

Comparative mt genome analysis: T. cymbius and other closely related species

The complete *T. cymbius* mt genome is shorter than that in *Ec. japonicus* (15,865 bp), *F. hepatica* (14,462 bp) and *Es. miyagawai* (14,413 bp) (table 2). This length discrepancy is mainly due to the NCR lengths: 152 bp in *T. cymbius*, 931 bp in *Es. miyagawai*, 2342 bp in *Ec. Japonicus* and 806 bp in *F. hepatica*. Full-length mt genome nucleotide sequence identities range from 67.6% to 72.0% among the four Echinostomata trematodes (*T. cymbius*, *Ec. japonicus*, *F. hepatica* and *Es. miyagawai*) (table 2). Among them, the highest full-length nucleotide sequence identity (69.1%) is between *T. cymbius* and *F. hepatica* (70.7% nucleotide identity and 64.8% amino acid identity in the 12 protein-coding gene sequences) (supplementary table S4). The total A + T content (62.82%) is slightly higher than those of *Ec. japonicus* (61.48%) and *F. hepatica* (62.18%), but lower than that of *Es. miyagawai* (65.3%). ATG and TAG are the most frequently used initiation and termination codons, respectively, which is the case in many other trematodes, including *F. hepatica*, *Es. miyagawai* and *Ec. japonicus* (Le *et al.*, 2001, 2016).

The AT/GC-skews in each mt genome gene or region of the four Echinostomata trematodes are listed in supplementary table S5. AT-skew values of the four trematodes are generally negative, and the majority of GC-skew values are positive. However, the AT-skew value for the *T. cymbius* NCR (−0.500) is lower than the other three (*Ec. japonicus*, *F. hepatica* and *Es. miyagawai*), which range from −0.386 to −0.214. GC-skew values in the four species have a large range of variation, with the lowest (0.238) in *Ec. japonicus* and the highest (0.676) in *T. cymbius*. Although some genes in *T. cymbius* and *Ec. japonicus* possess different skew values, the overall pattern of nucleotide skew in these two species is quite close.

Phylogenetic analyses

Phylogenetic analyses of the nucleotide sequences of the 12 concatenated coding regions from 39 trematode mt genomes were performed using two approaches (BI and ML).

The phylogenetic results of the two methods are somewhat different (See figs 1 and 2). The BI phylogenetic tree splits into two large clades. One clade contains seven members of Schistosomatidae, and the other clade contains 32 members of another 15 families (fig. 1). Interestingly, although the families Dipostomidae and Clinotomidae are both traditionally placed in the suborder Diplostomata, these species did not group with Schistosomatidae, which is also traditionally placed in the order Diplostomida. Rather, these species cluster basal within the order Plagiorchiida, paraphyletic to Diplostomida. This result is consistent with a previous study using mt sequences and ultra-conserved genomic elements to analyse the validity of the Diplostomoidea and Diplostomida (Locke *et al.*, 2018). However,

https://doi.org/10.1017/S0022149X19000932 Published online by Cambridge University Press

our result is inconsistent with another earlier study using nuclear small-subunit ribosomal and large-subunit ribosomal DNA sequences to analyse the phylogeny of Digeneans (Olson *et al.*, 2003). In the non-Diplostomata clade of our BI tree, trematodes of the suborder Pronocephalata form a clade basal to all the other suborders (just more recent than the aberrant Dipostomidae and Clinotomidae clade). In the Echinostomata branch, all of the Echinostomatidae species cluster together within the Echinostomatidae clade, except *Es. hortense*. *Echinostoma hortense* clusters with the *Fasciola*, *Fascioloides* and *Fasciolopsis* species used in our study. The result is consistent with a recent study by Li *et al.* (2019), who used mtDNA sequences to analyse the phylogenetic relationships of *Es. miyagawai* with other species of Echinostomata (Li *et al.*, 2019).

The ML phylogenetic (fig. 2) tree differs from the BI phylogenetic tree in a couple of key respects. Suborders Echinostomata, Opisthorchiata, Troglotremata, Xiphidiata and Pronocephalata form independent, monophyletic groups in both phylogenetic trees. Furthermore, the family Schistosomatidae forms a monophyletic clade in both phylogenetic trees, yet the suborder Diplostomata (Dipostomidae + Clinostomidae + Schistosomatidae) is an independent monophyletic clade in the ML tree and paraphyletic in the BI tree. Both methods' trees confidentially group *T. cymbius* and *Ec. japonicus* as sister taxa with high support values (BI 1.0, ML 100%), basal to the other Echinosomata. Both phylogenies show *T. cymbius* to be most closely related to Echinochasmidae, with a common ancestor basal to a clade containing Echinostomatidae and Fasciolidae. This is inconsistent with a previous study using the nuclear 28S rRNA gene (Tkach *et al.*, 2016) in which the Cyclocoelidae are more closely related to Echinostomatidae and Fasciolidae than to Echinochasmidae. Inconsistency between nuclear and mt phylogenies is not uncommon though, perhaps here due to a lower phylogenetic signal/noise ratio in the mt genomes of digeneans than in nuclear genomes, aggravated by the effect of incomplete taxon sampling (Philippe *et al.*, 2011; Locke *et al.*, 2018).

In conclusion, the *T. cymbius* complete mtDNA genome sequence has been determined and reported for the first time. This represents the first mt genome available from any member of the family Cyclocoelidae. These data will provide novel mtDNA markers for studying the molecular epidemiology and population genetics of Cyclocoelidae and other trematodes.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19000932.

Acknowledgements. The authors would like to thank Mr. Zhong Fu Wang who helped in the collection of trematodes, and Mr. Steven M. Thompson, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of drafts of this manuscript.

Financial support. This work was supported by a grant from the National Natural Science Foundation of China (grant number 31972703) and the National Key Research and Development Program of China (grant number 2017YFD0501300).

Conflicts 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.

References

Bisseru B (1957) On three known trematodes from African birds, with notes on the genera *Typhlocoelum*, *Paryphostomum* and *Petasiger*. *Journal of Helminthology* **31**, 173–186.

Burland TG (2000) DNASTAR's Lasergene sequence analysis software. *Methods in Molecular Biology* **132**, 71–91.

Cai XQ, Liu GH, Song HQ, *et al.* (2012) Sequences and gene organization of the mitochondrial genomes of the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* (Trematoda). *Parasitology Research* **110**, 235–243.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**, 540–552.

Chen L, Feng Y, Chen HM, *et al.* (2016) Complete mitochondrial genome analysis of *Clinostomum complanatum*, and its comparison with selected digeneans. *Parasitology Research* **115**, 3249–3256.

Gao Y, Zhang Y, Yang X, *et al.* (2017) Mitochondrial DNA evidence supports the hypothesis that *Triodontophorus* species belong to cyathostominae. *Frontiers in Microbiology* **8**, 1444.

Gu CD, Pan CN, Qiu ZZ, *et al.* (1973) A survey on the helminthes of birds from Bai-Yang-Dian lake, HeBei Province, China. 2. Trematoda. *ACTA Zoologica Sinica* **19**, 130–148.

Joyeux C and Baer JG (1927) Note sur les Cyclocoelidae (Trematodes). *Bulletin de la Société Zoologique de France* **52**, 416–434.

Kumar S, Stecher G, Li M, *et al.* (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547–1549.

Le TH, Blair D, McManus DP, *et al.* (2001) Complete DNA sequence and gene organization of the mitochondrial genome of the liver fluke, *Fasciola hepatica* L. (Platyhelminthes; Trematoda). *Parasitology* **123**(Pt 6), 609–621.

Le TH, Nguyen NTB , Nguyen KT, *et al.* (2016) A complete mitochondrial genome from *Echinochasmus japonicus*, supports the elevation of Echinochasminae Odhner, 1910 to family rank (Trematoda: Platyhelminthes). *Infection, Genetics and Evolution* **45**, 369–377.

Littlewood DT, Lockyer AE, Webster BL, *et al.* (2006) The complete mitochondrial genomes of *Schistosoma haematobium* and *Schistosoma spindale* and the evolutionary history of mitochondrial genome changes among parasitic flatworms. *Molecular Phylogenetics and Evolution* **39**, 452–467.

Liu ZX, Zhang Y, Liu YT, *et al.* (2016) Complete mitochondrial genome of *Echinostoma hortense* (Digenea: Echinostomatidae). *Korean Journal of Parasitology* **54**, 173–179.

Li Y, Qiu YY, Zeng MH, *et al.* (2019) The complete mitochondrial genome of *Echinostoma miyagawai*: comparisons with other closely related species and phylogenetic implications. *Infection, Genetics and Evolution*. doi:10.1016/j.meegid.2019.103961

Locke SA, Van Dam A, Caffara M, *et al.* (2018) Validity of the Diplostomoidea and Diplostomida (Digenea, Platyhelminthes) upheld in phylogenomic analysis. *International Journal for Parasitology* **48**(13), 1043–1059.

McManus DP and Bowles J (1996) Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *International Journal for Parasitology* **26**, 687–704.

Na L, Gao JF, Liu GH, *et al.* (2016) The complete mitochondrial genome of *Metorchis orientalis*, (Trematoda: Opisthorchiidae): comparison with other closely related species and phylogenetic implications. *Infection, Genetics and Evolution* **39**, 45–50.

Olson PD, Cribb TH, Tkach VV, *et al.* (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology* **33**, 733–755.

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.

Philippe H, Brinkmann H, Lavrov DV, *et al.* (2011) Resolving difficult phylogenetic questions: why more sequences are not enough. *PLoS Biology* **9**, e1000602.

Ronquist F and Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Scott ME, Rau ME, McLaughlin JD, *et al.* (1982) Comparative migration, growth and development of *Typhlocoelum cucumerinum sisowi* in dabbling ducks and *Typhlocoelum cucumerinum cucumerinum* in diving ducks. *Parasitology* **84**, 18.

Skrjabin KI (1913) Tracheophilus sisowi n.g., n.sp. *Zentralblatt für Bakteriologie, Parasitenkunde, lnfektionskrankheiten und Hygiene Abt. I, Originale* **69**, 90–94.

Swofford DL (2002) *PAUP*: phylogenetic analysis using parsimony (and other methods)*. Version 4.0b10. Sunderland, MA, Sinauer Associates.

Tang CT and Tang C (1978) Studies on trematodes of the family Cyclocoelidae of Fujian with further remarks on the life history and epidemiology of *Tracheophilus cymbius* (Dies., 1850) Skrjabin, 1913. *ACTA Zoologica Sinica* **24**, 91–101 (in Chinese).

Tkach VV, Kudlai O, Kostadinova A, *et al.* (2016) Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). *International Journal for Parasitology* **46**, 171–185.

Wang CR, Qiu JH, Wang ZF, *et al.* (2004) *Tracheophilus cymbius* were found in ducks in Heilongjiang province. *Heilongjiang Animal Science and Veterinary Medicine* **9**, 95–95.

Wang Y, Wang CR, Zhao GH, *et al.* (2011) The complete mitochondrial genome of *Orientobilharzia turkestanicum* supports its affinity with African *Schistosoma spp*. *Infection, Genetics and Evolution* **11**, 1964–1970.

https://doi.org/10.1017/S0022149X19000932 Published online by Cambridge University Press