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HELMINTHOLOGY - SHORT COMMUNICATION

Characterization of the complete mitochondrial genome of the echinostome *Echinostoma miyagawai* and phylogenetic implications

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Abstract

Echinostomes are important intestinal foodborne parasites. Despite their significance as pathogens, characterization of the molecular biology and phylogenetics of these parasites are limited. In the present study, we determined the entire mitochondrial (mt) genome of the echinostome *Echinostoma miyagawai* (Hunan isolate) and examined the phylogenetic relationship with selected members of the suborder Echinostomata. The complete mt genome of *E. miyagawai* (Hunan isolate) was 14,468 bp in size. This circular mt genome contained 12 protein-coding genes, 22 transfer RNA genes, two ribosomal RNA genes, and one non-coding region. The gene order and genomic content were identical with its congeners. Phylogenetic analyses (maximum parsimony, maximum likelihood, and Bayesian inference) based on the concatenated amino acid sequences of 12 protein-coding genes strongly supported monophyly for the genus *Echinostoma*; however, they rejected monophyly for the family Echinostomatidae and the genus *Fasciola*. The mt genomic data described in this study provides useful genetic markers for studying the population genetics, molecular biology, and phylogenetics of these echinostomes.

Keywords Echinostome · Mitochondrial genome · Mitochondrial DNA · Phylogenetic analyses

Introduction

Echinostomiasis is a foodborne parasitic disease caused by intestinal trematodes belonging to the family Echinostomatidae or echinostomes (Graczyk and Fried 1998). To date, human echinostomiasis is endemic to Southeast Asia and the Far East, such as China, India, Indonesia, Korea, Lao PDR, Malaysia, Nepal, the Philippines, and Thailand (Toledo and Esteban 2016). Importantly, recent epidemiological datasets have suggested that the at-risk population and the disease distribution are expanding and changing with respect to factors that include international markets, international tourism development, and changes in eating habits (Toledo and Esteban 2016). Humans can become infected with echinostomes by ingesting raw or insufficiently cooked molluscs, fish, crustaceans, and amphibians (Shin et al. 2008). Although echinostomiasis has major implications for public health, it has been neglected for years.

The classification of echinostomes has been controversial due to the morphological similarity to other echinostome species, particularly within the genus *Echinostoma*, which includes nominal species and '*revolutum*' species groups (Georgieva et al. 2014; Toledo et al. 2014). Molecular tools that employ molecular markers, particularly mitochondrial (mt) DNA and internally transcribed rDNA spacers (ITS), are valuable alternatives to overcome this limitation and have been widely used to identify and differentiate *Echinostoma* species (Noikong et al. 2014; Nagataki et al. 2015; Buddhachat and

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Chontananarth 2019; Mohanta et al. 2019). Previous studies using mt *cox*1, *nad*1, and ITS sequences have shown that the genus *Echinostoma* forms a monophyletic clade (Kostadinova et al. 2003; Detwiler et al. 2010; Saijuntha et al. 2011). However, single-gene molecular markers are not always suitable for resolving the phylogenetic relationships within the subclass Digenea trematodes due to the small amount of information. Because it contains a number of highly unique phylogenetic signals, the mt genome (containing 36–37 genes) is a suitable molecular resource to address questions in systematics and phylogenetics of many trematodes (Le et al. 2000; Ma et al. 2016; Na et al. 2016; Ma et al. 2017; Zhang et al. 2018). There are more than 120 nominal species in the genus *Echinostoma* (Kostadinova et al. 2003), but the complete mt genomes of only four echinostomes (including *E. miyagawai* Heilongjiang isolate) have been sequenced. Moreover, these mt genomes of echinostomes have not yet been characterized.

The objectives of the present study were to sequence the entire mt genome of echinostome *E. miyagawai* (Hunan isolate) and then test the hypothesis that *Echinostoma* is a monophyletic taxon in a phylogenetic analysis of mt genomic datasets.

Materials and methods

Ethics approval and consent to participate

All procedures involving animals in the present study were approved, and this study was approved by the Animal Ethics Committee of Hunan Agricultural University (No. 43321503).

Specimens and DNA extraction

An adult specimen of *E. miyagawai* (Hunan isolate) was collected from the intestinal tract of a chicken in Hunan province, China. The trematode sample was washed separately in physiological saline, identified morphologically (Platt and Zelmer 2016), fixed in 70% (v/v) ethanol, and stored at − 80 °C. Total genomic DNA was extracted separately from these samples using sodium dodecyl sulphate/proteinase K treatment and spin column purification (Wizard® SV Genomic DNA Purification System, Promega). These specimens were further identified as *E. miyagawai* (Hunan isolate) based on PCR-based sequencing of the mt *cox*1 gene and the ITS-2 rDNA region using a previously established method (Detwiler et al. 2010).

Long-range PCR amplification and sequencing

The primers (Table 1) were designed to conserved regions of the mt genomic sequences of *E. miyagawai* (Heilongjiang isolate; GenBank accession no. NC_039532) and *Echinostoma paraensei* (GenBank accession no. KT008005). The complete mt genome of *E. miyagawai* (Hunan isolate) was amplified by long-PCR as six overlapping amplicons located between *cyt*b and *nad*2 (~ 3.2 kb), between *nad*2 and *nad*1 (~ 1.4 kb), between *nad*1 and *rrn*S (~ 4.0 kb), between *rrn*S and *nad*5 (~ 2.3 kb), between *nad*5 and *cox*3 (~ 4.5 kb), and between *cox*3 and *cyt*b (~ 1.0 kb).

Each long-range PCR reaction was conducted in a total volume of 50 μl and contained 25 μl PrimeStar Max DNA polymerase premix (Takara, Dalian, China), 21 μl nuclease-free water (TransGen Biotech, Beijing, China), 1 μl of each primer (synthesized in Sangon Biotech, Shanghai, China) and 2 μl DNA template. The thermocycler (Biometra, Göttingen, Germany) PCR conditions were as follows: 94 °C for 2 min (initial denaturation), then 94 °C for 30 s (denaturation), 50–58 °C for 45 s (annealing) and 66 °C for 5 min (extension) for 9 cycles, followed by 94 °C denaturation for 2 min, 21 cycles of denaturation at 94 °C for 30 s, annealing at 50–58 °C for 45 s, and extension at 66 °C for 5 min, followed by a final extension of 10 min at 68 °C. Samples without genomic DNA (negative control) were included in each experiment. Each reaction (2 μl) was detected by 1.5% agarose gel electrophoresis to validate amplification efficiency. The PCR products were then sent to Sangon Biotechnology Company (Shanghai, China) for sequencing from both directions.

Assembly, annotation, and bioinformatics analysis

The mt genomic sequences of *E. miyagawai* (Hunan isolate) were assembled manually and aligned using the complete mt genomic sequence of *E. miyagawai* (Heilongjiang isolate) (GenBank accession no. NC_039532) using the computer program MAFFT 7.122 (Katoh and Standley 2013) to define gene boundaries. Open reading frames (ORFs) were analysed by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) using the trematode mt genetic code and compared with that of *E. miyagawai* (Heilongjiang isolate). The translation initiation and termination codons were identified based on comparison with these sequences. The tRNA genes were identified using the program tRNAscan-SE (Lowe and Eddy 1997). The two rRNA genes were predicted by comparison with that of *E. miyagawai* (Heilongjiang isolate).

Phylogenetic analysis

All of the mt genomic sequences of the suborder Echinostomata, along with selected *Opisthorchis viverrini* sequences (GenBank accession no. JF739555) (Cai et al. 2012), were obtained from GenBank and combined for phylogenetic analysis. All of the inferred amino acid sequences were

Table 1 Sequences of primers used to amplify PCR fragments from *E. miyagawai* from Hunan province, China

Primer	Sequence (5′ to 3′)	Region	Amplicon size (bp)
EMI1F	TTGGGGTTTGAAATGTTGGA	*cyt*b-*nad*2	~3200
EMI1R	AGCAACTCCCCCCTAAGAATC		
EMI2F	GTTTGGGGGATGATGATAGT	*nad*2-*nad*1	~1400
EMI2R	AGAACGAACACACCTGAGCA		
EMI3F	ATAAGTATGCTTTGCTCAGGTGTG	*nad*1-*rrn*S	~4000
EMI3R	ACTGTCTCTTACGATACACACTCC		
EMI4F	TATCGTAAGAGACAGTCCGCT	*rrn*S-*nad*5	~2300
EMI4R	ACCAGTGTAGAAGAATGAACCA		
EMI5F	ATGTTGGGGGAGTTGATACG	*nad*5-*cox*3	~4600
EMI5R	AAAACAACCTAAAAGAGGCAAT		
EMI6F	TCGTTGGTATCTCTTGGCT	*cox*3-*cyt*b	~1000
EMI6R	TCCCCCAACCACAAACTTA		

Forward (F) and reverse (R) primers used for quantitative PCR

separately aligned using MAFFT 7.122 and then concatenated. Ambiguous regions of the alignment were excluded using Gblocks 0.91b (Talavera and Castresana 2007) with the default settings for less strict conservation of flanking positions. Phylogenetic analysis using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) was conducted as described previously (Swofford 2002; Guindon and Gascuel 2003; Ronquist and Huelsenbeck 2003). Phylograms were drawn by FigTree v. 1.42 (http://tree.bio.ed.ac.uk/software/figtree).

Results and discussion

Identity of *E. miyagawai*

The mt *cox*1 sequence of *E. miyagawai* (Hunan isolate) was 100% identical to the previously reported *cox*1 sequence of *E. miyagawai* from Thailand (GenBank accession no. KP455515). Furthermore, the ITS-2 sequences of *E. miyagawai* specimen (Hunan isolate) was 99.5% similar to previously published ITS-2 sequences of duck *E. miyagawai* in the Heilongjiang province, China (GenBank accession no. MH796365).

General features of the mt genome of *E. miyagawai*

The complete mt genome of *E. miyagawai* (Hunan isolate) (GenBank accession no. MN116740) was 14,468 bp in size. The circular mt genome contained 12 protein-coding genes (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, and *cyt*b), 22 tRNA genes, two rRNA genes, and one *A* + *T* region or non-coding region (NCR) (Fig. 1; Table 2). The gene order and genomic content were identical to its congeners, such as *E. paraensei* and *Echinostoma caproni*. The nucleotide composition of the *E. miyagawai* (Hunan isolate) mt genome is *A* = 2918 (20.2%), *T* = 6573 (45.4%), *G* = 3448 (23.8%), and *C* = 1529 (10.6%). The *A* + *T* content was 65.6% for *E. miyagawai*, which is consistent with the mt genomes of other echinostomes (*E. paraensei* and *E. caproni*).

Annotation

As shown in Table 2, the predicted start and stop codons for the 12 protein-coding genes of *E. miyagawai* (Hunan isolate) were compared with those of the *E. miyagawai* (Heilongjiang isolate) mt genome. In the *E. miyagawai* (Hunan isolate) mt genome, nine genes (*cox*3, *cyt*b, *nad*4L, *nad*4, *atp*6, *nad*2, *nad*3, *cox*2, and *nad*6) started with ATG and three genes (*nad*1, *cox*1, and *nad*5) started with GTG. All of the genes had complete termination codons; ten genes (*cyt*b, *nad*4L, *nad*4, *atp*6, *nad*2, *nad*1, *nad*3, *cox*2, *nad*6, and *nad*5) used TAG and two genes (*cox*3 and *cox*1) used TAA, respectively. The *rrn*L gene was located between tRNA-Thr and tRNA-Cys, and the *rrn*S gene was located between tRNA-Cys and *cox*2. The lengths of the *rrn*L and *rrn*S genes for *E. miyagawai* (Hunan isolate) were 975 bp and 749 bp, respectively (Table 2). The *A* + *T* content of the *rrn*L and *rrn*S for *E. miyagawai* (Hunan isolate) are 65.1% and 61.9%, respectively. A total of 22 tRNA sequences (ranging from 60 to 70 nucleotides in size) were identified in the mt genome of *E. miyagawai* (Hunan isolate). There was one non-coding region (or *A* + *T*-rich region) in the mt genome of *E. miyagawai* (Hunan isolate), which was located between the tRNA-Glu and *cox*3 (Fig. 1; Table 2). The size of non-coding region was 990 bp and had an *A* + *T* content of 59.4%. These results are consistent with those of *E. paraensei* and *E. caproni.*

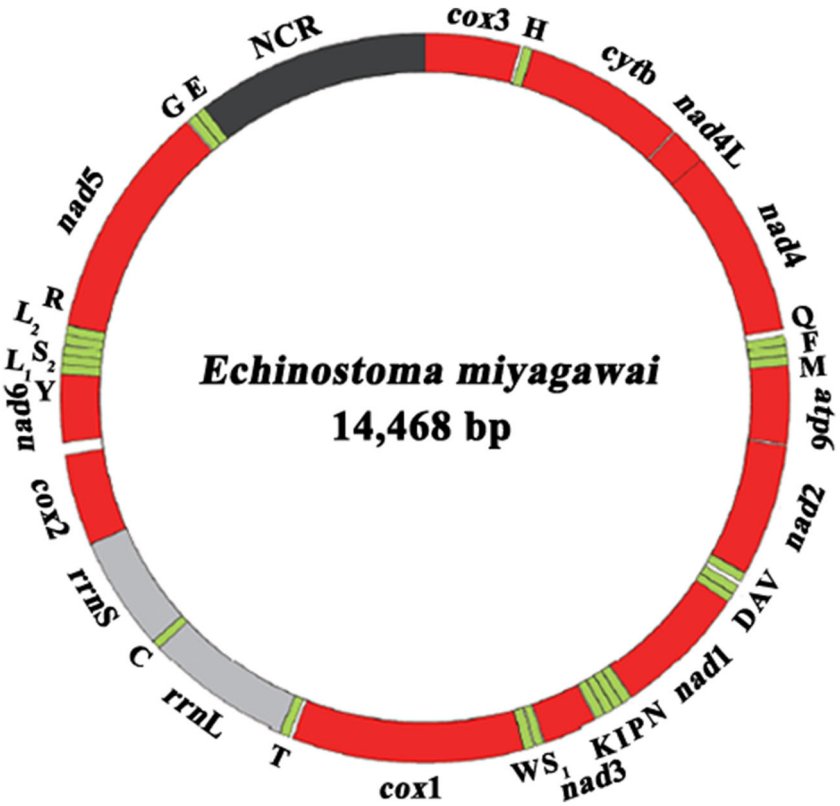

Fig. 1 Arrangement of the mitochondrial genome of *Echinostoma miyagawai*. The scales are similar. All of the genes were transcribed in the clockwise direction, using standard nomenclature. The 22 tRNA genes are represented by the one-letter code for the corresponding amino acid, with numerals differentiating each of the two leucine- and serine-specific tRNAs (L1 and L2 for codon families CUN and UUR, respectively; S1 and S2 for codon families AGN and UCN, respectively). "NCR" refers to non-coding region

Comparative analyses between *E. miyagawai* Hunan and Heilongjiang isolates

The complete mt genome of *E. miyagawai* (Hunan isolate) was slightly longer (52 bp) than that of *E. miyagawai* (Heilongjiang isolate). The sequence difference across the complete mt genome (except for the non-coding region) was 1.3%. Comparison of the 12 protein-coding genes revealed sequence divergences at both the nucleotide (0.3–3.8%) and amino acid (0–18.5%) levels. The magnitude of nucleotide sequence variation in each gene ranged from 0.2 (*cox*2) to 3.8% (*cox*3). The amino acid sequence differences ranged from 0 to 18.5%, with NAD1 being the most conserved protein and COX3 the least conserved. For the 22 transfer RNA genes, the sequence variation was 0–30.6% (tRNA-Thr). The sequence divergences for the *rrn*L and *rrn*S genes were 3.8% and 1.1%, respectively. The sequence divergence for the non-coding region was 17.8% between the *E. miyagawai* Hunan and Heilongjiang isolates.

Phylogenetic analysis

Phylogenetic analysis of the concatenated amino acid sequence data for all 12 protein-coding genes showed that *Isthmiophora hortense* (family Echinostomatidae) was more closely related to the Fasciolidae than to the other members of the Echinostomatidae. This suggested that Echinostomatidae was paraphyletic, which was strongly supported by ML and BI analyses (Bf = 100; Bpp = 1.0) and weakly supported by MP analysis (Bf = 64) (Fig. 2). Within *Echinostoma*, four *Echinostoma* isolates grouped together with high statistical support in all of the three phylogenetic analyses in this study (Bf = 100; Bpp = 1.0) (Fig. 2). These results strongly supported monophyly for the genus *Echinostoma*, which is consistent with previous studies that used the *cox*1, *nad*1, and ITS sequences (Kostadinova et al. 2003; Detwiler et al. 2010; Saijuntha et al. 2011). In addition, phylogenetic analyses of the mt *cox*1 sequence data revealed support for the monophyly of the genus *Echinostoma* (Supplementary material S1). Within Fasciolidae, *Fasciola jacksoni* and *Fascioloides magna* strongly clustered together (Bf = 100; Bpp = 1.0), but excluded *Fasciola gigantica* and *Fasciola hepatica* (Fig. 2), rejecting monophyly of the genus *Fasciola.*

Significance and implications

Human echinostomiasis is a foodborne parasitic disease that is caused by intestinal trematodes belonging to the family Echinostomatidae. The clinical signs are related to parasite load. Abdominal pain, fatigue, diarrhoea, and weight loss are the most common clinical symptoms in human echinostomiasis (Graczyk and Fried 1998). However, severe infections are associated with eosinophilia, watery diarrhoea, oedema, headache, nausea and vomiting, anaemia, and

Table 2 The features of the mitochondrial genomes of *E. miyagawai* from Hunan (EMHN) and Heilongjiang (EMHLJ) provinces, China

Gene/region	Positions and size (bp)		Initiation and termination codons		Anticodon	Intergenic nucleotides
	EMHN	EMHLJ	EMHN	EMHLJ	EMHN/EMHLJ	EMHN/EMHLJ
*cox*3	1–645 (645)	1–651 (651)	ATG/TAA	ATG/TAA		0/0
tRNA-His (H)	649–713 (65)	655–720 (66)			GTG	+ 3/+ 3
*cyt*b	716–1825 (1110)	723–1832 (1110)	ATG/TAG	ATG/TAG		+ 2/+ 2
*nad*4L	1826–2098 (273)	1833–2105 (273)	ATG/TAG	ATG/TAG		0/0
*nad*4	2059–3342 (1284)	2066–3349 (1284)	ATG/TAG	ATG/TAG		− 40/− 40
tRNA-Gln (Q)	3346–3411 (66)	3355–3424 (70)			TTG	+ 3/+ 5
tRNA-Phe (F)	3419–3484 (66)	3425–3487 (63)			TTG	+ 7/0
tRNA-Met (M)	3518–3580 (63)	3521–3586 (66)			CAT	+ 33/+ 33
*atp*6	3587–4105 (519)	3590–4108 (519)	ATG/TAG	ATG/TAG		+ 6/+ 3
*nad*2	4113–4982 (870)	4116–4985 (870)	ATG/TAG	ATG/TAG		+ 7/+ 7
tRNA-Val (V)	4987–5050 (64)	4990–5053 (64)			TAC	+ 4/+ 4
tRNA-Ala (A)	5075–5142 (68)	5078–5145 (68)			TGC	+ 24/+ 24
tRNA-Asp (D)	5147–5212 (66)	5150–5215 (66)			GTC	+ 4/+ 4
*nad*1	5213–6115 (903)	5216–6118 (903)	GTG/TAG	GTG/TAG		0/0
tRNA-Asn (N)	6122–6188 (67)	6125–6190 (66)			GTT	+ 6/+ 6
tRNA-Pro (P)	6193–6261 (69)	6195–6263 (69)			AGG	+ 4/+ 4
tRNA-Ile (I)	6262–6325 (64)	6264–6327 (64)			GAT	0/0
tRNA-Lys (K)	6333–6401 (69)	6336–6404 (69)			TTT	+ 7/+ 8
*nad*3	6406–6762 (357)	6409–6765 (357)	ATG/TAG	ATG/TAG		+ 4/+ 4
tRNA-Ser (S_1)	6766–6825 (60)	6770–6829 (60)			TCT	+ 3/+ 4
tRNA-Trp (W)	6830–6895 (66)	6834–6899 (66)			TCA	+ 4/+ 4
*cox*1	6899–8437 (1539)	6903–8441 (1539)	GTG/TAA	GTG/TAA		+ 3/+ 3
tRNA-Thr (T)	8473–8542 (70)	8445–8512 (68)			TGT	+ 35/+ 3
*rrn*L	8543–9517 (975)	8513–9521 (1009)				0/0
tRNA-Cys (C)	9518–9584 (67)	9522–9584 (63)			GCA	0/0
*rrn*S	9585–10,333 (749)	9585–10,338 (754)				0/0
*cox*2	10,334–10,942 (609)	10,339–10,947 (609)	ATG/TAG	ATG/TAG		0/0
*nad*6	10,954–11,406 (453)	10,959–11,411 (453)	ATG/TAG	ATG/TAG		+ 11/+ 11
tRNA-Tyr (Y)	11,407–11,473 (67)	11,412–11,477 (66)			GTA	0/0
tRNA-Leu (L_1)	11,475–11,537 (63)	11,478–11,542 (65)			TAG	+ 1/0
tRNA-Ser (S_2)	11,536–11,600 (61)	11,545–11,603 (59)			TGA	− 2/+ 2
tRNA-Leu (L_2)	11,628–11,690 (63)	11,635–11,697 (63)			TAA	+ 27/+ 31
tRNA-Arg (R)	11,691–11,754 (64)	11,700–11,759 (60)			ACG	0/+ 2
*nad*5	11,755–13,320 (1566)	11,762–13,327 (1566)	GTG/TAG	GTG/TAG		0/+ 2
tRNA-Gly (G)	13,340–13,405 (66)	13,347–13,412 (66)			TCC	+ 19/+ 19
tRNA-Glu (E)	13,412–13,478 (67)	13,419–13,485 (67)			TTC	+ 6/+ 6
Non-coding region	13,479–14,468 (990)	13,486–14,416 (931)				0/0

anorexia (Toledo and Esteban 2016). Despite its significance as pathogens, limited information is available about the molecular biology and phylogenetics of echinostomes.

In the present study, the characterization of the mt genome of the echinostome *E. miyagawai* provides a molecular foundation that can be used to improve the diagnosis of human echinostomiasis. Because diagnosis is only based on morphological features, species-specific identification of the adult and larval stages of *Echinostoma* spp. sometimes is not accurate (Gasser 2006). The ITS region of nuclear rDNA and *cox*1 region of mt DNA have been used as genetic markers to molecularly identify *Echinostoma* spp. (Kostadinova et al. 2003; Detwiler et al. 2010; Saijuntha et al. 2011). The increased availability of sequenced mt genomes of echinostomes will allow the development of DNA-based analytical and diagnostic tools for *Echinostoma* spp.

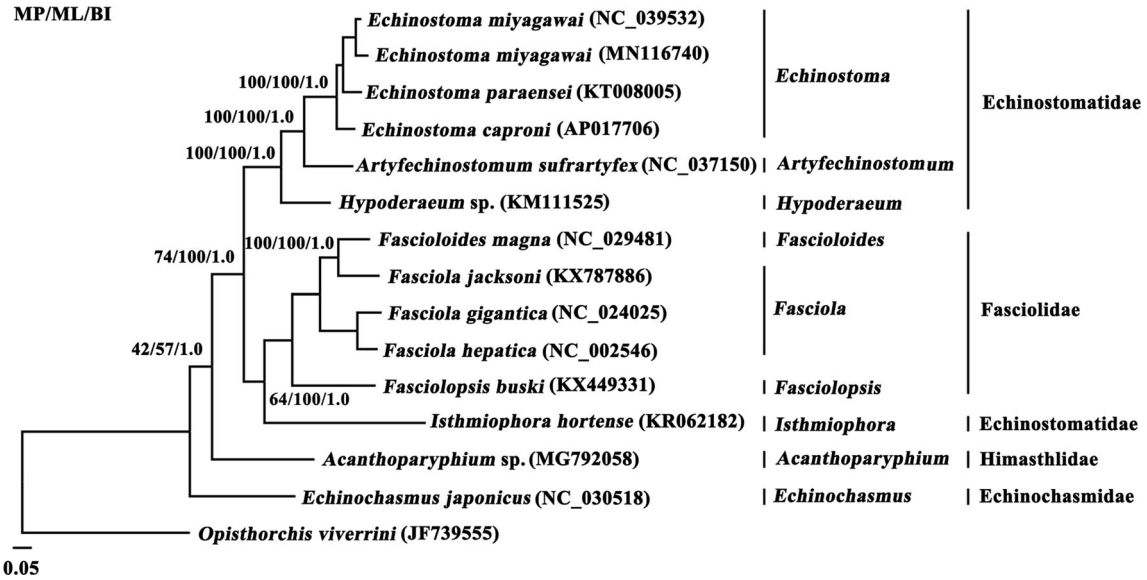

Fig. 2 Inferred phylogenetic relationship among representative suborder Echinostomata trematodes based on concatenated amino acid sequences of 12 protein-coding genes utilizing maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) using *Opisthorchis viverrini* as an outgroup

The taxonomy of *Echinostoma* species is controversial. *Echinostoma* is a complex species; therefore, accurate identification and differentiation of echinostome species can sometimes be challenging (Graczyk and Fried 1998; Toledo and Esteban 2016). In the present study, we characterized the mt genome of *E. miyagawai* to reassess the phylogenetic relationships between the *Echinostoma* species in the suborder Echinostomata using mt genomic/proteomic datasets. Mt genomic sequences are useful molecular markers to study the molecular biology, population genetics, and phylogenetics of many trematodes (Liu et al. 2014; Ma et al., 2016; Yang et al. 2016; Le et al. 2016). In the present study, the analyses of mt genomic sequences provided insight into the phylogenetic relationships among Echinostomatidae or *Echinostoma*; however, many Echinostomatidae species are either not represented or underrepresented. Therefore, the mt genomic sequences of additional Echinostomatidae species should be sequenced and employed in further studies to resolve the taxonomic classification of echinostomes.

Conclusion

The present study determined the entire mt genome sequences of *E. miyagawai* (Hunan isolate) and revealed patterns in genomic organization and gene annotation. Phylogenetic analyses strongly supported monophyly of the genus *Echinostoma*; however, they rejected monophyly of the family Echinostomatidae and the genus *Fasciola*. The *Echinostoma* mt genomic sequences were useful genetic markers to study the population genetics, molecular biology, and phylogenetics of these echinostomes.

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Compliance with ethical standards

All procedures involving animals in the present study were approved, and this study was approved by the Animal Ethics Committee of Hunan Agricultural University (No. 43321503).

Competing interests The authors declared that they have no competing interests.

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