



Mitochondrial and nuclear ribosomal DNA dataset suggests that *Hepatiarius sudarikovi* Feizullaev, 1961 is a member of the genus *Opisthorchis* Blanchard, 1895 (Digenea: Opisthorchiidae)

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Abstract

The taxonomy and classification of the family Opisthorchiidae have been revised by several authors with the exclusion or synonymization of some genera. The genus *Hepatiarius* Feizullaev, 1961 accommodated two species: *Hepatiarius sudarikovi* Feizullaev, 1961 and *H. longissimus* Linstow, 1883. Recently, some experts have suppressed *Hepatiarius* as a junior synonym of *Opisthorchis* Blanchard, 1895 based on morphological features alone. Prior to the present study, no molecular data either from nuclear or from mitochondrial DNA was available for any species of this genus. In the present study, four specimens of *H. sudarikovi* Feizullaev, 1961 were recovered from the bile ducts of the little egret, *Egretta garzetta*. The complete sequences of the internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) and the nearly complete mitochondrial genome sequences were determined and the phylogenetic relationship of *H. sudarikovi* with related taxa was assessed based on the mitochondrial (mt) DNA sequences. The sequence similarity in the ITS rDNA between *H. sudarikovi* and *Opisthorchis felineus* was higher (97.62% in ITS-1 and 96.22% in ITS-2) than with other opisthorchiids. Phylogenetic analysis using Bayesian inference (BI) based on the concatenated amino acid sequences of 12 protein-coding genes (PCGs) clustered *H. sudarikovi* into the clade of opisthorchiids, with *O. felineus* being the closest related species, which supports the affinity of *H. sudarikovi* with trematodes in the genus *Opisthorchis*. This is the first avian liver fluke whose nearly complete mitochondrial genome was sequenced. The mtDNA sequences of *H. sudarikovi*, in combination with its rDNA sequences, provide novel resources of genetic markers for the identification, species differentiation, and systematic studies of *H. sudarikovi* with other avian opisthorchiid flukes.

Keywords *Hepatiarius sudarikovi* · Mitochondrial genome · Nuclear ribosomal DNA · Phylogenetic analysis

Introduction

Opisthorchiidae Looss, 1899 is one of the three well-known families of digenetic trematodes in the superfamily

Opisthorchioidea Looss, 1899 (Bray et al. 2008). Trematodes of the genus *Opisthorchis* Blanchard, 1895 are etiological agents of opisthorchiasis (Hung et al. 2013). These opisthorchiid parasites are cosmopolitan with freshwater snails and fish as the first and second intermediate hosts, respectively (Pakharukova and Mordvinov 2016) and parasitize many vertebrate animals, with birds being the most common definitive hosts (King and Scholz 2001). The hosts, including humans and birds, get infected through eating fish carrying opisthorchiid metacercariae (Doanh and Nawa 2016), causing fish-borne zoonotic trematodiasis (FZT) (Hung et al. 2013; Lun et al. 2005). Humans with opisthorchiasis may suffer from hepatomegaly, pancreatitis, cholangitis, and cholangiocarcinoma, causing severe public health problem (Sripa et al. 2012; Hughes et al. 2017).

In 1961, Feizullaev established a new genus *Hepatiarius* which comprised two species: *Hepatiarius longissimus* (syn. *Distomum longissimum* Linstow, 1883), the type species, and

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Hepatiarius sudarikovi Feizullaev, 1961 recovered from the liver of the little egret *Egretta garzetta* in Azerbaidzhan. The genus *Hepatiarius* Feizullaev, 1961 is different from *Opisthorchis* by having long spirally winding body, anterior body portion broad, and muscular with the ability of strong attachment and contraction. The species *H. sudarikovi* is differentiated from *H. longissimus* by having some peculiar morphological features. It has very small ventral sucker which is not visible or rudimentary, and has almost lost its function, and the testes of *H. sudarikovi* have smooth margins. However, in *H. longissimus*, the ventral sucker is prominent, being larger than oral sucker, and the testes are distinctly two or four-lobed occupying the more posterior position in the body of the worm.

The systematic relationships of opisthorchiid species have been an issue of controversy, and been revised by several authors (Yamaguti 1971; Filimonova 2000; King and Scholz 2001; Scholz 2008). In 2000, Filimonova transferred the type species *H. longissimus* to *Opisthorchis* and proposed *H. sudarikovi* as a single and new type species of the genus *Hepatiarius*. Later, Scholz (2008) in his revision of the family Opisthorchiidae criticized the transferring of *H. longissimus* (type species) to *Opisthorchis* and documented this taxonomic action as the deviation from the rules of International Code of Zoological Nomenclature (ICZN), and thus automatically invalidated the genus *Hepatiarius* and suppressed it as a junior synonym of *Opisthorchis*. All of the hitherto proposed taxonomic positions of *Hepatiarius* spp. were based on morphological features alone, as molecular phylogenetic analysis of *Hepatiarius* spp. has not been conducted, which might provide an alternative approach for assessing the taxonomic position of *Hepatiarius* species.

Nowadays, the nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) sequences offer rich sources of genetic markers which have proven effective for species identification, discrimination, and phylogenetic analysis of trematodes including those of the family Opisthorchiidae (Shekhovtsov et al. 2010; Cai et al. 2012; Choudhary and Agrawal 2017; Sanpool et al. 2018). Therefore, the objectives of the present study were to determine the sequences of the complete internal transcribed spacers (ITS-1 and ITS-2) of rDNA and the nearly complete mt genome sequences of *H. sudarikovi*, and to compare with corresponding sequences of other opisthorchiid trematodes. Phylogenetic analysis was performed in order to assess the systematic relationship of *H. sudarikovi* with other opisthorchiids using the amino acid sequences of 12 mt protein-coding genes (PCGs).

Materials and methods

Parasites and DNA extraction

Four individuals of *H. sudarikovi* were recovered from the liver (bile ducts) of the little egrets (*Egretta garzetta*) collected

from district Swabi (latitude 34°07'07.23" N and longitude 72°36'32.38" E), KP, Pakistan. The live specimens were heat-killed by pipetting with hot water. Two specimens were stained with alum carmine and permanently mounted according to the existing protocol (Lutz et al. 2017). Illustrations of the specimens were done by drawing tube. The two specimens for molecular work were stored in 80% ethanol at −20 °C prior to DNA extraction. The total genomic DNA was extracted from ethanol-preserved specimens following the published protocol (Gasser et al. 2007) using mini-column purification system (Wizard® SV Genomic DNA Purification System, Promega, Madison, USA) according to the manufacturer's instructions. The extracted DNA was stored at −20 °C until further use.

The acquisition of *H. sudarikovi* ITS rDNA

The rDNA region spanning partial 18S rDNA, the complete ITS1-5.8S-ITS2, and partial 28S rDNA were amplified using universal primers BD1 (5'-GTC GTA ACA AGG TTT CCG TA-3') and BD2 (5'-TAT GCT TAA ATT CAG CGG GT-3') (Morgan and Blair 1995). Conventional PCR amplification reactions were conducted in a thermocycler (Bio-Rad, USA) with the following conditions: 94 °C for 5 min (initiation), followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 1 min (extension), with a final extension step of 10 min at 72 °C to complete the amplification. The PCR products were checked on 2% agarose gel using ethidium-bromide staining and the positive amplicons were sequenced by Genewiz sequencing company (Beijing, China). The obtained ITS-1 and ITS-2 rDNA sequences of *H. sudarikovi* were aligned and compared with corresponding sequences of other opisthorchiids available in NCBI (<http://www.ncbi.gov/blast>), and the sequence similarity (%) was determined for each marker (ITS-1 and ITS-2) using BioEdit 7.0.9.0 (Hall 1999).

PCR-based sequencing of *H. sudarikovi* mt genome

Six short fragments of the *H. sudarikovi* mt genome were amplified using universal primers and specific primers designed based on conserved regions of the mt genomes of *Opisthorchis felineus* (Shekhovtsov et al. 2010) and *Opisthorchis viverrini* (Cai et al. 2012) (Table 1). To amplify the remaining sequences in long-PCR reactions, seven pairs of primers were designed based on the acquired sequences from the short fragments of the *H. sudarikovi* mtDNA. Long PCRs were initiated with 2 min denaturation at 98 °C followed by 10 cycles each including denaturation of 10 s at 92 °C, 30 s at 50–55 °C (depending on the primer), 1–3 min at 66 °C (depending on the fragment length), followed by 2 min denaturation at 92 °C. Then, 20 more cycles were performed, each including 10 s at 92 °C (denaturation), 30 s at 50–55 °C, 1–

Table 1 Primers used to amplify the nearly complete mitochondrial genome of *Hepatiarius sudarikovi*

Primer	Sequence (5' → 3')	Location/region amplified	Size (bp/kb)	Reference
OFcox3F	CTGATATTGGCATTGTTGGATTA	Partial <i>cox3</i> -partial <i>cytb</i>	587 bp	This study
OFcytBR	CCGTAGCAGCCCAATAAGACAT			
XcND4F	GADTCBCDATTCDGARCG	Partial <i>nad4</i>	434 bp	Yang et al. (2015)
XcND4R	GCHARCCADCGCTTVCCNTC			
JB11	AGATTCGTAAGGGGCCTAATA	Partial <i>nad1</i>	530 bp	Bowles and McManus (1993)
JB12	ACCACTAACTAATCACTTTC			
JB3	TTTTTTGGGCATAATGAGGTTTAT	Partial <i>cox1</i>	443 bp	Bowles et al. (1992)
JB4.5	TAAAGAAAGAACATAATGAAAATG			
rmLF	GGGATAAGTTACCTCGGGGATAA	Partial <i>rrnL</i> -partial <i>rrnS</i>	1 kb	This study
rmSR	CCAACGTTACCATGTTACGACTT			
XcND5F	ATGCGNGCYCCNACNCCNGTDAG	Partial <i>nad5</i>	469 bp	Yang et al. (2015)
XcND5R1	TGCTTVSWAAAAAANACHCC			
OFtmGF	TGTTGAGTATGCTGTCTTTCCA	Partial tRNA-G-partial <i>cytb</i>	928 bp	This study
HScytbR	CAACCACATTAGCTCGAACC			
F1-F	CTTCCTTGACACCAGATGTCTTAT	Partial <i>cytb</i> -partial <i>nad4</i>	1.8 kb	This study
F1-R	GCAAGCCAACGCTTACCATC			
F2-F	ACGAAGATTCCGTTGTTTCC	Partial <i>nad4</i> -partial <i>nad1</i>	2.8 kb	This study
F2-R	ACCTTATTAGGCCCTTACG			
F3-F	TTGAGAGTGGGTTGAGGTTCTT	Partial <i>nad1</i> -partial <i>cox1</i>	2.2 kb	This study
F3-R	CATATGATGAGCCCAAACAACAC			
F4-F	TGCCATAGTTTGTGTTGGGTAGTG	Partial <i>cox1</i> -partial <i>rrnL</i>	1.7 kb	This study
F4-R	TCAATAGGACCTCTCCTTGCTTC			
F5-F	TTGATTTAGTCGGGTACACAC	Partial <i>rrnS</i> -partial <i>nad5</i>	2.3 kb	This study
F5-R	TAACCAAGGTCGAGGAATGAAC			
HSnad5F	TCCCTCGTTATTCTGGCTCT	Partial <i>nad5</i> -partial tRNA-E	707 bp	This study
OFtmER	CTCCAACACGAAAAATTGGAATGC			

3 min at 68 °C, and 10 min final extension at 68 °C. The amplified fragments were either sequenced directly (size up to 2 kb) or cloned (size above 2 kb) in pMD18-T vector before sequencing.

Genome assembly, annotation, and bioinformatics analysis

The obtained mtDNA sequences of *H. sudarikovi* were assembled manually, and aligned against the complete mt genome sequences of *O. felineus* (EU921260) and *O. viverrini* (JF739555) using the program Clustal X 1.83 (Thompson et al. 1997) in order to identify the gene boundaries. The tRNA genes were inferred using ARWEN (<http://130.235.46.10/ARWEN/>) and by visual inspection with the ability to form their putative secondary structure after aligning mtDNA sequences of the present species against that of *O. felineus* and *O. viverrini*. Nucleotide sequences of the 12 PCGs were translated into their corresponding amino acid sequences using MEGA7 (Kumar et al. 2016). Based on pairwise alignments of

the nucleotide and amino acid sequences of the 12 PCGs and two rRNA genes, sequence identity between *H. sudarikovi* and other opisthorchiid flukes at both nucleotide and amino acid levels was calculated using BioEdit (Hall 1999).

Phylogenetic analysis

To assess the phylogenetic position of *H. sudarikovi*, the concatenated amino acid sequences of the 12 mt PCGs were aligned with those of other selected trematode species, including *O. felineus* (EU921260), *O. viverrini* (JF739555), *Clonorchis sinensis* Russia isolate (FJ381664), *C. sinensis* Korea isolate (JF729304), and *C. sinensis* China isolate (JF729303) (Opisthorchiidae); *Haplorchis taichui* (KF214770) and *Metagonimus yokogawai* (KC330755) (Heterophyidae); *Brachycladium goliath* (KR703278) (Brachycladiidae); *Paragonimus westermani* Japanese isolate (AF219379) (Paragonimidae); *Dicrocoelium chinensis* (KF318786), *D. dendriticum* (KF318787) and *Eurytrema pancreaticum* (KP241855) (Dicrocoeliidae); and

Trichobilharzia regenti (DQ859919). The avian schistosome *T. regenti* (Schistosomatidae: Strigeida) was used as an outgroup. The alignment of amino acid sequences was performed using MAFFT 7.130. The ambiguously aligned regions were excluded using online software Gblocks v. 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) (Talavera and Castresana 2007). The result of Gblocks was subjected to MrBayes (version 3.1.1) for Bayesian Inference (BI) phylogenetic analysis (Ronquist and Huelsenbeck 2003) using a mixed model (Castoe and Parkinson 2006) as the best-fitting amino acid evolution model. BI analysis was conducted with four independent Monte Carlo Markov Chain (MCMC) run for 10,000,000 generations, sampling a tree every 1000th generations. The first 25% generations were discarded as “burn-in.” The analysis was regarded as completed upon finding the average standard deviation of split frequencies lower than 0.01. The phylograms were viewed and drawn using FigTree v. 1.42 (Chen et al. 2014).

Results and discussion

Morphological identification

This avian liver fluke was identified as *Hepatiarius* sp. based on the presence of the broad and muscular anterior body portion and their strong contraction and relaxation (live specimens), especially in its forebody, placed this fluke in the genus *Hepatiarius* within Opisthorchiidae. The specimens were further identified as *H. sudarikovi* Feizullaev, 1961 based on the very similar morphology and morphometrics to the original description of *H. sudarikovi*, with a minor variation in size (Fig. 1, Table 2). Interestingly, the present study reports *H. sudarikovi* from the same definitive host, more than half a century after its discovery and description. The specific characters of the examined specimens, original description of *H. sudarikovi*, *H. longissimus*, and the morphological and morphometric features of the type species of the genus *Opisthorchis* are given in Table 2.

ITS sequence comparison with other flukes within Opisthorchiidae

The ITS rDNA region was 1,143 bp in length (631 bp for ITS-1, 160 bp for 5.8S, and 288 bp for ITS-2 rDNA) (GenBank accession No. MK227161). The gene boundaries were confirmed through comparison with corresponding rDNA sequences of other opisthorchiids available in GenBank. The sequence similarity (%) between *H. sudarikovi* and *O. felineus* (97.62% in ITS-1 and 96.22% in ITS-2) is higher than those with other opisthorchiid flukes (83.12–96.20% in ITS-1, 87.85–94.10% in ITS-2) (Table 3), indicating that *H.*

sudarikovi and *O. felineus* were very closely related within the genus *Opisthorchis*. The inter-specific genetic divergence in ITS-2 region was higher than in ITS-1, which is consistent with previous studies including those of *Opisthorchis* species (Choudhary and Agrawal 2017).

Characterization of the *H. sudarikovi* mt genome

The nearly complete mt genome (excluding tRNA-Glu, LNCr, and tRNA-Gly) of *H. sudarikovi* (GenBank accession No. MK033132) was 13,641 bp in length, containing 12 PCGs (*cox1–3*, *nad1–6*, *nad4L*, *atp6*, and *cytb*), 20 transfer RNA genes (excluding *trnE* and *trnG*), and two ribosomal RNA genes (Table 4). The mt genome arrangement of *H.*

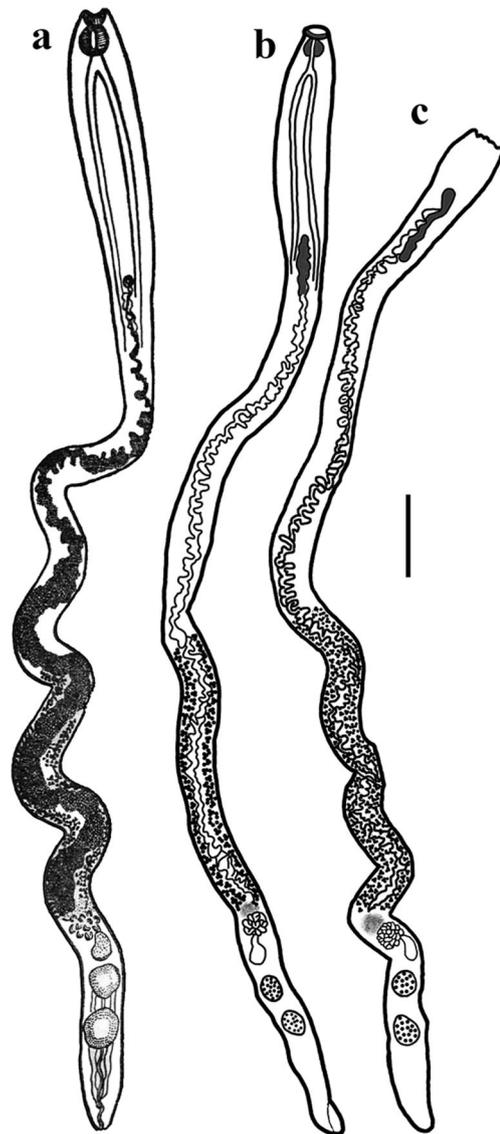


Fig. 1 Line drawing of *Hepatiarius sudarikovi*. **a** Original diagram after Feizullaev, 1961. **b** and **c** Diagrams of the specimens obtained in the present study (anterior body portion is missing for one specimen), scale bar 1 mm

Table 2 Comparison of the specific characters of *Hepatiarius sudarikovi*, *Hepatiarius longissimus*, and *Opisthorchis felineus*

Species	<i>H. longissimus</i>	<i>H. sudarikovi</i> (original)	<i>H. sudarikovi</i> (present study)	<i>O. felineus</i>
Body shape	Very elongate	Elongate	Elongate	Flat, lanceolate
Body length	20 mm	10–13 mm	14.5 mm	7–13 mm
Body breadth	1 mm	0.77–1.02 mm	0.62–0.71 mm	1–3.5 mm
Anterior end	Broad, cylindrical	Broad, cylindrical	Broad, cylindrical	Narrow, conical
Posterior end	Rounded	Rounded	Rounded	Rounded
Spines	Absent	Absent	Absent	Absent
Oral sucker position	Terminal	Terminal	Terminal	Terminal
Oral sucker size	? × 0.28 mm	0.12–0.18 × 0.24–0.3 mm	0.188 × 0.33 mm	0.263–0.280 × 0.228 mm
Pharynx	As large as oral sucker	0.20–0.27 × 0.2–0.39 mm	0.22 × 0.26 mm	0.17–0.20 × 0.16–0.17 mm
Esophagus	As long as pharynx	0.06–0.168 mm	0.166 mm	0.20–0.21 mm
Ventral sucker	Larger than oral, ? × 0.34 mm	Very small, 0.07–0.14 × 0.10–0.16 mm	Very small, not visible	0.25–0.23 × 0.2–0.23 mm
Ovary shape	Follicular, very lobate	Follicular, very lobate	Follicular, very lobate	Smooth-edged, weakly lobed
Seminal receptacle	Large, posterolateral to ovary	Large, posterolateral to ovary	Large, posterolateral to ovary	Large, posterolateral to ovary
Testes shape	Two-four lobed	Smooth-edged	Smooth-edged	Four-five lobed
Testes position	Diagonal, in a more posterior end	Diagonal, in posterior end	Diagonal, in posterior end	Diagonal, in a more posterior end
Cirrus and pouch	Absent	Absent	Absent	Absent
Vitelline fields	In 3/4th of body to ovary	In 3/4th of body to ovary	In 3/4th of body to ovary	Ventral sucker to ovary
Eggs	0.026 × 0.015 mm	0.024 × 0.012 mm	0.023 × 0.01 mm	0.026–0.030 × 0.011–0.015 mm
Host	Hérons and ducks	Little egret	Little egret	Cat, dog
Region	Turkestan	Azerbaijan	Pakistan	Europe
References	von Linstow (1883); Barker (1911)	Feizullaev (1961)	Present study	Barker (1911); Mordvinov et al. (2012)

mm millimeters

sudarikovi is the same as those of other opisthorchiid liver flukes. Genes are located either without intergenic spacers or separated by short intergenic spacers of 1–42 nucleotides, accounting for a total of only 321 bp. The nucleotide contents in the sequenced *H. sudarikovi* mt genome (13,641 bp) have

16.25% A (2,217 bp), 28.87% G (3,938 bp), 43.52% T (5,936 bp), and 11.36% C (1,550 bp), with overall A+T and C+G contents of 59.77% (8153 bp) and 40.23% (5488 bp), respectively. The overall A+T content was similar to that of the mt genomes of *O. felineus*, *O. viverrini*, and *C. sinensis* (Cai et al. 2012).

In the PCGs of *H. sudarikovi* mt genome, ATG or GTG were used as translation initiation codons and TAG or TAA were used as translation termination codons. The 12 PCGs were 10,197 bp in length, encoding 3387 amino acids. Incomplete codons were not used in *H. sudarikovi* mtDNA. The most frequently used codons in the PCGs were codons UUU (Phenylalanine, 328 instances), UUG (Leucine, 290 instances), and GUU (Valine, 220 instances), while the least frequently used codon was UAA (stop codon, 1 instance). A total of 3399 codons, including stop codons, were used to construct 12 PCGs. Codons ending with T were the most frequently used (12.83%, 1741 in number), followed by codons ending with G (8.34%, 1131 in number) and codons ending with A (2.6%, 350 in number), while only 177 codons (1.3%) had C in the third codon position. This bias toward T in the mt genome was

Table 3 Sequence similarity (%) in internal transcribed spacers (ITS-1 and ITS-2) between *Hepatiarius sudarikovi* and members of the family Opisthorchiidae

Similarity in ITS-1		Similarity in ITS-2	
<i>O. felineus</i> EU038134	97.62	<i>O. felineus</i> JN646503	96.22
<i>M. orientalis</i> KX832894	96.20	<i>M. orientalis</i> HM347223	94.10
<i>M. xanthosomus</i> JQ716400	96.20	–	–
<i>M. bilis</i> EU038154	95.72	<i>M. bilis</i> KT740982	93.75
<i>O. viverrini</i> EU038152	95.43	<i>O. viverrini</i> AY584735	92.18
<i>C. sinensis</i> KT020830	94.75	<i>C. sinensis</i> JQ048600	92.33
–	–	<i>O. noverca</i> KC109193	89.76
–	–	<i>Amphimerus</i> sp. AB678442	88.54
<i>O. pedicellata</i> KU716043	83.12	<i>O. pedicellata</i> KU688153	87.85

Table 4 The organization of genes/regions in the mitochondrial genome of *Hepatiarius sudarikovi*

Gene/region	Position 5' to 3'	Length		Ini/Ter codons	tRNA anti-codon	Int. Seq. length (bp)
		bp	aa			
SNCR	1–82	82				
<i>cox3</i>	83–727	645	214	ATG/ TAG		+ 42
tRNA-His (H)	770–837	68			GTG	+ 7
<i>cytb</i>	845–1957	1113	370	ATG/ TAA		+ 10
<i>nad4L</i>	1968–2231	264	87	ATG/ TAG		– 40
<i>nad4</i>	2192–3469	1278	425	GTG/ TAG		+ 13
tRNA-Gln (Q)	3483–3547	65			TTG	+ 38
tRNA-Phe (F)	3586–3656	71			GAA	– 4
tRNA-Met (M)	3653–3721	69			CAT	– 1
<i>atp6</i>	3721–4236	516	171	ATG/ TAG		+ 29
<i>nad2</i>	4266–5135	870	289	ATG/ TAG		+ 8
tRNA-Val (V)	5144–5208	65			TAC	+ 27
tRNA-Ala (A)	5236–5300	65			TGC	+ 4
tRNA-Asp (D)	5305–5371	67			GTC	+ 3
<i>nad1</i>	5375–6277	903	300	GTG/ TAG		+ 4
tRNA-Asn (N)	6282–6353	72			GTT	+ 11
tRNA-Pro (P)	6365–6428	64			TGG	– 1
tRNA-Ile (I)	6428–6491	64			GAT	+ 16
tRNA-Lys (K)	6508–6576	69			CTT	+ 1
<i>nad3</i>	6578–6934	357	118	GTG/ TAG		+ 18
tRNA-SerAGN (S1)	6953–7014	62			GCT	+ 18
tRNA-Trp (W)	7033–7099	67			TCA	+ 1
<i>cox1</i>	7101–8645	1545	514	GTG/ TAG		+ 28
tRNA-Thr (T)	8674–8740	67			TGT	– 10
<i>rrnL</i>	8731–9731	1001				+ 2
tRNA-Cys (C)	9734–9793	60			GCA	0
<i>rrnS</i>	9794–10,565	772				0
<i>cox2</i>	10,566–11,204	639	212	ATG/ TAG		+ 13
<i>nad6</i>	11,218–11,679	462	153	ATG/ TAG		+ 5
tRNA-Tyr (Y)	11,685–11,746	62			GTA	+ 1
tRNA-LeuCUN (L1)	11,748–11,812	65			TAG	– 2
tRNA-SerUCN (S2)	11,811–11,881	71			TGA	+ 5
tRNA-LeuUUR (L2)	11,887–11,952	66			TAA	+ 15
tRNA-Arg (R)	11,968–12,034	67			TCG	+ 2
<i>nad5</i>	12,037–13,641	1605	534	ATG/ TAG		

SNCR short non-coding region, bp base pair, aa amino acid, Ini/Ter initial/Terminal codons, Int. seq. intergenic sequences

also observed for other trematodes including those of Opisthorchiidae (Mordvinov et al. 2009; Shekhovtsov et al. 2010; Cai et al. 2012).

The comparison of PCGs sequences between *H. sudarikovi* and other opisthorchiids showed high sequence similarity with *O. felineus* (83.46% in nt and 85.98% in aa) followed by *C. sinensis* (China isolate) (80.19% in nt and 82.79% in aa) and *O. viverrini* (78.86% in nt and 80.10% in aa). These comparative results suggested a closer

relationship between *H. sudarikovi* and *O. felineus* than with other opisthorchiid trematodes.

Twenty tRNA genes were identified from the nearly complete mt genome sequences (excluding tRNA-Glu, LNCR, and tRNA-Gly) of *H. sudarikovi*, ranging from 60 to 72 bp in length. Eighteen out of the 20 tRNA genes can be folded into a typical “cloverleaf” secondary structure while the tRNA-Cys and tRNA-Ser(AGN) lack DHU arm and show unorthodox structures (data not shown). Their D-arms are missing and

Table 5 Identity (%) of nucleotides and predicted amino acids sequences between *Hepatiarius sudarikovi* and opisthorchiid flukes

Gene/region	Nucleotides identity (%)			Amino acids identity (%)		
	HS/OF	HS/CSC	HS/OV	HS/OF	HS/CSC	HS/OV
<i>cox3</i>	87.28	81.40	81.40	90.65	86.92	84.58
<i>cytb</i>	85.21	84.91	81.42	91.37	89.73	85.68
<i>nad4L</i>	84.85	83.33	82.58	88.51	82.76	89.66
<i>nad4</i>	81.09	78.25	76.76	82.12	79.29	76.94
<i>atp6</i>	82.56	80.81	79.26	84.21	80.70	76.02
<i>nad2</i>	81.38	77.21	75.52	80.62	75.62	71.63
<i>nad1</i>	84.85	82.63	80.64	88.33	86.0	81.67
<i>nad3</i>	84.31	76.75	76.47	86.44	76.27	75.42
<i>cox1</i>	85.16	83.59	83.49	89.23	89.02	88.37
<i>rrnL</i>	89.14	83.09	78.39			
<i>rrnS</i>	83.40	80.15	78.49			
<i>cox2</i>	84.66	78.09	78.14	90.09	82.55	81.78
<i>nad6</i>	83.55	77.49	76.84	84.31	81.05	76.47
<i>nad5</i>	80.37	76.01	74.08	80.71	77.90	74.34
PCGs	83.46	80.19	78.86	85.98	82.79	80.10
Overall	83.20	80.01	78.15			

PCGs protein-coding regions, HS *Hepatiarius sudarikovi*, OF *Opisthorchis felineus*, OV *Opisthorchis viverrini*, CSC *Clonorchis sinensis* (China isolate)

replaced by the loops of different range of base pairs as in some other trematodes including opisthorchiid liver flukes (Le et al. 2002; Shekhovtsov et al. 2010; Cai et al. 2012).

The two mt rRNA genes (*rrnL* and *rrnS*) of *H. sudarikovi* abut directly neighboring genes, located between tRNA-Thr and *cox2* and are separated by tRNA-Cys. The size of the *rrnL* and *rrnS* were 1001 and 772 bp, respectively. The A+T contents of the *rrnL* and *rrnS* of *H. sudarikovi* were 58.04% and 57.38%, similar to other *Opisthorchis* species. Sequence similarities in the *rrnL* and *rrnS* genes between *H. sudarikovi* and other opisthorchiid trematodes were also compared (Table 5). The high sequence similarity (%) in both rRNA genes also suggested that *H. sudarikovi* and *O. felineus* were more closely related opisthorchiid flukes. The nucleotide similarities between the two species were higher in *rrnL* (89.14%) than in

rrnS and PCGs. The short non-coding region (SNCR) is 82 bp in length without any tandem repeats, situated between the tRNA-Gly and *cox3*.

Phylogenetic analysis

The phylogenetic tree (Fig. 2) of 14 trematode species/isolates inferred from the amino acid sequences of 12 mt PCGs showed that members of the family Opisthorchiidae including *H. sudarikovi* clustered together. Within the Opisthorchiidae, *H. sudarikovi*, and *O. felineus* appeared as 100% supported clade nested between other opisthorchiids. The topology indicated that *H. sudarikovi* has closer genetic relationship with *O. felineus* than with other opisthorchiids.

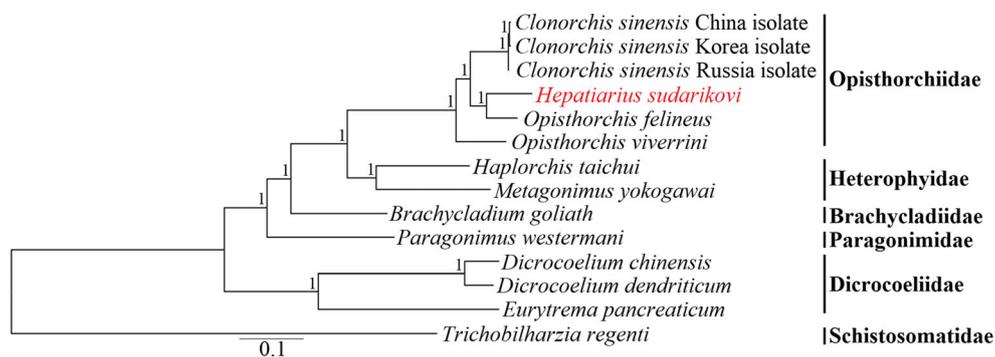


Fig. 2 Phylogenetic relationships of *Hepatiarius sudarikovi* and other trematodes. Tree inferred from the concatenated amino acid sequences of the 12 protein-coding genes (PCGs) from selected trematodes were

constructed using Bayesian inference analysis (BI). *Trichobilharzia regenti* (DQ859919.1) (Schistosomatidae: Strigeida) served as the outgroup

In conclusion, the present study determined the complete ITS rDNA sequences and the nearly complete mt genome sequences of *H. sudarikovi*. This is the first avian opisthorchiid trematode whose nearly complete mt genome was determined and characterized, which provides additional novel genetic markers for identification and specific discrimination of Opisthorchiidae. Phylogenetic analysis based on mt protein sequences suggested that *H. sudarikovi* is genetically closer to *O. felineus*. Further studies are needed to assess the validity of the genus *Hepatiarius* by determining the rDNA and mtDNA sequences of *H. longissimus*, the type species of this genus, when specimens representing *H. longissimus* become available.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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