ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 51, No. 6: 719-726, December 2013
http://dx.doi.org/**10.3347/kjp.2013.51.6.719**

▣ ORIGINAL ARTICLE

Complete Mitochondrial Genome of *Haplorchis taichui* and Comparative Analysis with Other Trematodes

Dongmin Lee[1], Seongjun Choe[1], Hansol Park[1], Hyeong-Kyu Jeon[1], Jong-Yil Chai[2], Woon-Mok Sohn[3], Tai-Soon Yong[4], Duk-Young Min[5], Han-Jong Rim[6] and Keeseon S. Eom[1,*]

[1]*Department of Parasitology, Medical Research Institute and Parasite Resource Bank, Chungbuk National University School of Medicine, Cheongju 361-763, Korea;* [2]*Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, Seoul 110-799, Korea;* [3]*Department of Parasitology and Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju 660-70-51, Korea;* [4]*Department of Environmental Medical Biology, Institute of Tropical Medicine and Arthropods of Medical Importance Resource Bank, Yonsei University College of Medicine, Seoul 120-752, Korea;* [5]*Department of Immunology and Microbiology, Eulji University School of Medicine, Daejeon 301-746, Korea;* [6]*Department of Parasitology, Korea University College of Medicine, Seoul 136-705, Korea*

Abstract: Mitochondrial genomes have been extensively studied for phylogenetic purposes and to investigate intra- and interspecific genetic variations. In recent years, numerous groups have undertaken sequencing of platyhelminth mitochondrial genomes. *Haplorchis taichui* (family Heterophyidae) is a trematode that infects humans and animals mainly in Asia, including the Mekong River basin. We sequenced and determined the organization of the complete mitochondrial genome of *H. taichui*. The mitochondrial genome is 15,130 bp long, containing 12 protein-coding genes, 2 ribosomal RNAs (rRNAs, a small and a large subunit), and 22 transfer RNAs (tRNAs). Like other trematodes, it does not encode the *atp8* gene. All genes are transcribed from the same strand. The ATG initiation codon is used for 9 protein-coding genes, and GTG for the remaining 3 (*nad1*, *nad4*, and *nad5*). The mitochondrial genome of *H. taichui* has a single long non-coding region between *trnE* and *trnG*. *H. taichui* has evolved as being more closely related to Opisthorchiidae than other trematode groups with maximal support in the phylogenetic analysis. Our results could provide a resource for the comparative mitochondrial genome analysis of trematodes, and may yield genetic markers for molecular epidemiological investigations into intestinal flukes.

Key words: *Haplorchis taichui*, trematode, mitochondrial genome, molecular phylogeny

INTRODUCTION

The intestinal trematode, *Haplorchis taichui*, is a medically important parasite infecting humans and livestock. Haplorchiasis is a major public health threat in Asia and in parts of Africa and the Americas [1-3]. *H. taichui* is the most frequently reported species among the minute intestinal flukes from Southeast Asia, including Thailand, Lao PDR, China, and Vietnam [3,4-7]. Mitochondrial (mt) genomes exhibit a relatively conserved suite of protein-coding sequences, but also relatively rapid rates of evolutionary change [8,9]. In recent years, complete mitochondrial DNA (mtDNA) sequences have been extensively used to infer higher level phylogenies [10,11] and also for taxonomy and population genetics at lower taxonomic levels [12-14]. To date, quite a number of complete mt genomes of metazoan species, including helminths, have been deposited in GenBank and published [15]. Information from flatworm mitochondrial genomes is strongly biased toward parasitic species of medical importance. For this reason, recent mitochondrial genome scale phylogenetic surveys have emphasized the need to collect data for the major groups of flatworms that have not been sampled [16,17]. However, most of them still remain poorly understood at the molecular level, in particular, the complete mt genomes of the species in the family Heterophyidae. Parasitic flatworm mt genomes, ranging in size usually from 13 to 14 kb but far bigger up to 24 kb sometimes, are typically circular and usually encode 36 genes, including 12 protein-coding genes, and without introns and with short intergenic regions [18]. The Digenea currently contains about 18,000 nominal species parasitizing vertebrates, and sometimes humans as the definitive host [19]. The purpose of the present study was to sequence the mt genome of *H. taichui*

• Received 21 June 2013, revised 2 October 2013, accepted 11 October 2013.
* Corresponding author (kseom@chungbuk.ac.kr)

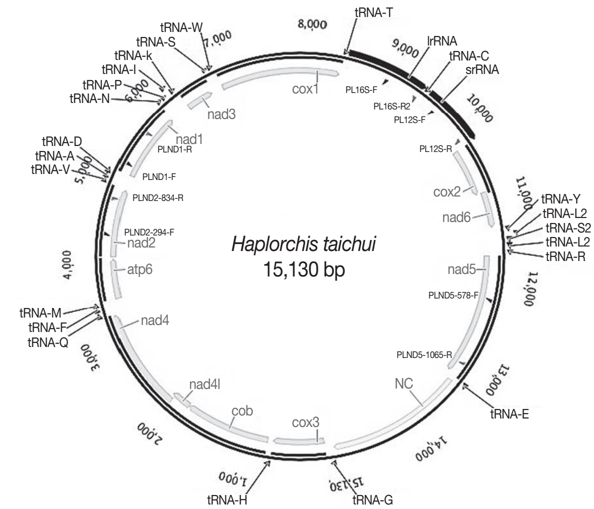
for comparison with the organization and sequence of the mt genomes of other trematodes. In addition, we wished to reconstruct the phylogenetic relationships of the family Heterophyidae within the class Trematoda using mtDNA sequences.

MATERIALS AND METHODS

Long PCR amplification and sequencing of the *H. taichui* mtDNA molecule

Adult *H. taichui* worms were obtained from naturally infected Laotian people during the activity of “Korea-Lao PDR Collaborative Project for Control of Foodborne-Trematode Infections (esp. Opisthorchiasis) in Lao PDR (2007-2011)”. The specimens were washed in normal physiological saline and identified based on morphological characters (gonotyl bears 12-16 spines in *H. taichui*). The worms were stored in 70% ethanol prior to DNA extraction. Total genomic DNA was extracted from 200 specimens using a QIAamp tissue kit (Qiagen Inc., Valencia, California, USA), according to the manufacturer's instruction and used as the template DNA. The complete mt genome was PCR-amplified in overlapping fragments. The nucleotide sequences and relative positions of the PCR primers are shown in Fig. 1. PCR reactions were performed in a 50 μl reaction volume consisting of 10 units of EF-Taq polymerase (Solgent Co., Daejeon, Korea), 2.5 mM dNTP mixture, 2.5 mM $MgCl_2$, 20 *p*mole of each primer, and 10 μg of genomic DNA in a thermocycler (Biometra Co., Goettingen, Germany) under the following conditions: 92˚C for 2 min (initial denaturation), then 92˚C for 10 sec (denaturation), 50˚C for 30 sec (annealing), and 68˚C for 2 min to 10 min (extension) for 10 cycles, followed by 92˚C for 2 min, then 92˚C for 10 sec, 48˚C for 30 sec, and 68˚C for 2 min to 10 min for 20 cycles, and a final extension at 68˚C for 8 min.

A negative control (no template) was also included for every PCR reaction to detect contamination. Each amplicon (3 μl) was examined by agarose (1%) gel electrophoresis, stained with ethidium bromide and photographed using a gel documentation system (UVItec, Cambridge, UK), excised under long-wavelength UV light, extracted using a Doc-do purification kit (Elpis Co., Daejeon, Korea), and then used as a template for sequencing reactions. The primer walking method was employed to obtain overlapping sequences for each of the amplified fragments. Cyclic sequencing from both ends of the fragments was performed with a Big-Dye, and the amplified products were subjected to electrophoresis on an ABI 3100 automated DNA sequencer.



Primers	Primer sequence (5'-3')	Direction	Lacation
PLND 2-294-F	TDAAGTTTGGWKTDTTYCCDTTT	Forward	*nad2*
PLND 2-834-R	ARAWAAARYTGYTCWRAAAANCTATANA	Reverse	*nad2*
PLND 1-F	KCGTAAGGGBCCWAAHAAGGTTGG	Forward	*nad1*
PLND 1-R	AATCATAACGAAYACGHGGA	Reverse	*nad1*
PL 16S-F	WYYGTGCDAAGGTAGCATAAT	Forward	*rrnl*
PL 16S-R2	AWAGATAAGAACCRACCTGGCT	Reverse	*rrnL*
PL 12S-F	CAGTGCCAGCAKCYGCGGTTADWCTG	Forward	*rrnS*
PL 12S-R	AYCSWGRKTGWCGGGCGRTRTGTAC	Reverse	*rrns*
PLND 5-578-F	ATGCGKGCYCCNACNCCNGTWAGTTC	Forward	*nad5*
PLND 5-1065-R	ARARMATGCTTMSTAAAAWABAM	Reverse	*nad5*

Fig. 1. A map of the complete mitochondrial genome of *Haplorchis taichui*. The primer sequences used for amplification of respective mitochondrial genes are indicated in the map.

Sequence analysis and characterization of the *Haplorchis taichui* mt genome

Gene annotation, genome organization, translation initiation, translation termination codons, and the boundaries between protein-coding genes of mt genomes were identified based on comparison with mt genomes of other trematodes reported previously [17,18]. Sequences were assembled manually and aligned against the complete mt genome sequences of our own *Metagonimus yokogawai* sequence (will be published elsewhere) and trematode parasites available in the GenBank database (http://www.ncbi.nlm.nih.gov WebGenBank) using BLAST searches. Open reading frames and gene boundaries were confirmed by comparing with *M. yokogawai* nucleotide sequences. The codon usage profiles of 12 protein-coding genes and their nucleotide composition were calculated using Geneious 6.1.5 (Biomatters Co., Auckland, New Zealand) program [20]. Putative secondary structures of 22 tRNA genes were identified manually by recognizing potential secondary structures and anticodon sequences.

Phylogenetic analysis

To assess the phylogenetic position of *H. taichui* and the utility of mt genomes in resolving the interrelationships of trematode orders, complete mitochondrial genome sequences of 15 flatworms were analyzed. The mtDNA sequences were as follows: *H. taichui* (KF214770), *Trichobilharzia regenti* (NC_009680), *Schistosoma spindale* (DQ157223), *S. haematobium* (DQ157222), *S. mansoni* (NC_002545), *S. japonicum* (NC_002544), *S. mekongi* (NC_002529), *Fasciola hepatica* (NC_002546), *Paragonimus westermani* (NC_002354), *Opisthorchis felineus* (EU921260), *O. viverrini* (JF739555), *Clonorchis sinensis* (FJ381664/ JF729303/ JF729304), and 1 Monogenea species (*Gyrodactylus thymalli*, NC_009682) as the outgroup. Each gene was translated into an amino acid sequence using the trematode mt genetic code by translation table 21 in Geneious 6.1.5, and was aligned based on its amino acid sequence using default settings. A conserved block of concatenated alignment was selected using the Gblocks program [21], (http://molevol.cmima.csic.es/castresana/Gblocks_server) for 12 protein-coding genes examined. Phylogenetic relationships among trematodes were inferred using different tree construction algorithms, i.e., maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML), Bayesian phylogeny (BP) [22], PAUP* [23], PhyML 3.0 [24], and MrBayes [25]. Bootstrap analysis was done using 1,000 random replications. Models of amino acid substitution were determined for each data partition independently using ModelTest supported in Geneious 6.1.5. The MP analysis was performed using the exhaustive search option. ML analysis was performed using the substitution model Le Gascuel (LG). In BP analysis, the following settings were applied: the number of cycles = 1,100,000; sampling frequency = 200; heated chains = 4; burn-in = 100,000.

RESULTS

General features of the mt genome of *H. taichui*

The complete mt genome of *H. taichui* Lao PDR isolate (GenBank accession no. KF214770) is 15,130 bp in length (Fig. 1). It encodes 36 genes; 12 protein-coding genes (*cox1*-3, *nad1*-6, *nad4L*, *atp6*, *cob*, and lacking *atp8*), 22 transfer RNA genes, and 2 ribosomal RNA genes.The relative positions and lengths of each gene are given in Table 1. An AT-rich region (1,710 bp) is located between *tRNA-Glu* and *tRNA-Gly*. All genes are transcribed in the same direction. The 2 adjacent genes, *nad4L* and *nad4*, overlap each other by 40 nt in different reading frames.

Table 1. Position and characteristics of protein-coding and non-coding sequences in the mt genome of *Haplorchis taichui*

Gene/Region	No. of		Codons		Positions
	Nucleo-tides	Amino acids	Initiation	Termina-tion	(5'-3')
cox3	657	218	ATG	TAG	1-657
tRNA-H	66				660-725
cob	1,110	369	ATG	TAG	732-1841
nad4L	264	87	ATG	TAG	1843-2106
nad4	1,281	426	GTG	TAA	2067-3347
tRNA-Q	66				3357-3422
tRNA-F	62				3427-3488
tRNA-M	64				3489-3552
atp6	516	171	ATG	TAG	3553-4068
nad2	870	289	ATG	TAA	4094-4963
tRNA-V	60				4968-5027
tRNA-A	63				5029-5091
tRNA-D	67				5094-5160
nad1	906	301	GTG	TAG	5161-6066
tRNA-N	66				6069-6134
tRNA-P	64				6139-6202
tRNA-I	65				6199-6263
tRNA-K	65				6267-6331
nad3	360	119	ATG	TAG	6332-6691
tRNA-S	62				6705-6766
tRNA-W	64				6774-6837
cox1	1,542	513	ATG	TAG	6842-8383
tRNA-T	61				8385-8445
rrnL	979				8446-9424
tRNA-C	64				9426-9489
rrnS	747				9490-10236
cox2	624	207	ATG	TAG	10237-10860
nad6	459	152	ATG	TAG	10847-11305
tRNA-Y	67				11306-11372
tRNA-L1	66				11370-11435
tRNA-S2	64				11434-11497
tRNA-L2	70				11504-11573
tRNA-R	64				11585-11648
nad5	1,587	528	GTG	TAA	11650-13236
tRNA-E	73				13248-13320
NR	1,710				13323-15032
tRNA-G	59				15035-15093

Codon usage and protein-coding genes

The mt genome of *H. taichui* encodes 12 protein-coding genes, identical with the situation in other trematodes. The start and termination codons of these were identified by sequence comparison with homologs in other trematodes. The ATG codon was used in 9 protein-coding genes, and the GTG codon in 3 genes (*nad1*, *nad4*, and *nad5*). The TAG stop codon was used in 9 genes (*cox3*, *cob*, *nad4L*, *atp6*, *nad2*, *nad1*, *nad3*, *cox1*, and *nad6*) and the TAA termination codon in the remain-

Table 2. Properties of trematode mtDNA protein-coding genes and similarity comparison between *Haplorchis taichui* and other trematodes

Gene	Species											
	H.t	S.s	S.h	S.m	S.j	S.ml	S.mk	F.h	P.w	O.f	O.v	C.s
No. of amino acids												
cox3	218	221	221	218	215	217	217	214	215	214	214	213
cob	369	364	367	365	372	373	373	371	373	371	369	370
nad4L	87	84	86	87	88	88	88	91	86	87	87	87
nad4	426	420	421	420	425	424	424	424	402	425	425	425
atp6	171	173	174	174	173	174	174	173	171	171	171	171
nad2	289	279	279	280	285	284	284	289	289	289	289	290
nad1	301	291	298	297	297	298	296	301	297	300	300	300
nad3	119	122	122	121	120	121	121	119	119	118	118	118
cox1	513	587	601	511	509	>433	511	511	498	520	516	519
cox2	207	200	198	198	203	u	233	201	200	212	214	211
nad6	152	155	157	150	153	u	154	151	151	153	153	153
nad5	528	528	527	528	529	>333	531	523	528	534	534	534
Amino acid similarity (%)												
cox3	100	28.7	28.2	27.3	24.2	30.0	28.2	49.5	54.0	49.5	50.0	49.3
cob	100	48.5	51.1	52.9	54.9	53.0	53.8	75.6	75.9	82.4	77.8	80.5
nad4L	100	32.9	322.6	32.6	32.2	31.0	33.3	64.4	67.1	72.4	73.6	71.3
nad4	100	34.0	30.9	32.8	29.4	30.4	31.3	50.7	52.7	59.6	59.1	58.9
atp6	100	38.5	34.5	33.9	39.9	41.4	38.7	62.8	58.5	64.9	66.1	67.8
nad2	100	32.3	30.6	33.3	32.3	33.3	34.4	47.1	46.4	51.9	53.3	52.2
nad1	100	41.1	40.2	43.1	46.8	42.1	43.1	69.9	69.6	72.6	70.9	70.2
nad3	100	32.8	37.0	37.3	37.0	41.7	37.0	63.0	60.5	67.2	62.2	63.9
cox1	100	68.0	68.0	68.2	67.9	nc	66.5	74.7	78.5	76.8	78.9	78.0
cox2	100	44.6	42.6	45.8	45.4	nc	41.6	48.8	48.5	59.5	58.5	61.0
nad6	100	34.0	29.	34.2	35.7	nc	36.4	51.0	54.0	57.2	60.5	57.2
nad5	100	34.0	32.8	31.2	32.5	nc	32.4	52.0	50.3	46.5	44.6	46.0
Inferred initiation/termination codon[a]												
cox3	A/G	A/A	A/G	G/G	A/G	A/A	A/G	A/G	A/G	A/G	A/G	A/G
cob	A/G	A/G	A/A	G/G	A/G	A/A	A/A	A/G	A/G	A/G	A/G	A/G
nad4L	A/G	A/A	A/A	A/A	A/A	A/A	A/A	G/G	G/G	A/G	A/G	A/G
nad4	G/A	A/A	A/G	A/A	A/G	A/A	A/A	G/A	A/G	A/G	A/G	G/G
atp6	A/G	A/A	A/G	A/G	A/A	A/G	A/A	A/G	A/G	A/G	A/G	A/G
nad2	A/A	A/A	A/A	G/A	A/G	A/G	A/A	A/G	A/A	A/G	A/G	G/G
nad1	G/G	A/A	A/G	G/G	A/G	A/A	A/A	G/G	A/G	G/G	G/G	G/G
nad3	A/G	A/A	A/A	A/G	A/G	A/G	A/G	A/G	A/G	G/G	G/G	G/G
cox1	A/G	A/A	A/G	A/G	G/G	A/nc	A/A	A/G	A/G	G/G	G/G	G/A
cox2	A/G	A/A	A/G	A/A	A/A	nc/nc	A/A	A/G	A/G	A/G	A/G	A/G
nad6	A/G	A/G	A/A	A/A	A/G	nc/nc	G/A	A/G	A/G	A/G	A/G	G/A
nad5	G/A	A/G	A/G	A/G	A/G	nc/G	G/A	G/G	G/G	A/G	A/A	G/A

Inferred initiation codons have not been determined for some genes and species (u, undetermined), and other genes for *S. malayensis* have yet to be characterized (nc, not characterized), giving rise to partial lengths for *cox1* and *nad5*. H.t, *Haplorchis taichui*; S.s, *Schistosoma spindale*; S.h, *S. haematobium*; S.m, *S. mansoni*; S.j, *S. japonicum*; S.ml, *S. malayensis*; S.mk, *S. mekongi*; F.h, *F. hepatica*; P.w, *Paragonimus westermani*; O.f, *Opisthorchis felineus*; O.v, *O. viverrini*; C.s, *Clonorchis sinensis*. [a]A or G (TG)/(TA) A or G.

ing 3 genes (*nad4*, *cox2*, and *nad5*). Pairwise comparisons were made among the amino acid sequences inferred from individual protein-coding genes in the *H. taichui* genome with those representing 12 other trematodes (Table 2). The amino acid sequence similarities in individual inferred proteins ranged from 76.8% (*cox1*) to 82.4% (*cob*) between *H. taichui* and *O. felineus*; and from 78.0% (*cox1*) to 80.5% (*cob*) between *H. taichui* and *C. sinensis*. The amino acid sequence similarities between *H. taichui* and *S. japonicum* ranged from 24.2% (*cox3*) to 67.9% (*cox1*); and from 47.1% (*nad2*) to 75.6% (*cob*) with *F. hepatica* (Table 2). The 12 protein-coding genes were 10,176 bp in length and composed of 43% T, 17.1% A, 28% G, and,

Table 3. Nucleotide content of protein-coding genes from complete or almost complete mitochondrial genomes of flatworms

Species	GenBank accession no.	Base composition (%)					Total bp usage	Total no. of codons
		T	C	A	G	A+T		
Schistosoma spindale	DQ157223	45.2	7.0	28.1	19.7	73.3	10,308	3,424
Schistosoma haematobium	DQ157222	44.9	7.6	27.8	19.5	72.7	10,389	3,451
Schistosoma mansoni	NC_002545	45.6	8.2	23.3	23.0	68.9	10,083	3,349
Schistosoma japonicum	NC_002544	48.3	8.0	23.0	20.7	71.3	10,143	3,369
Schistosoma malayensis[a]	AAG60031	48.8	6.6	23.6	20.9	72.4	8,271	2,745
Schistosoma mekongi	NC_002529	48.4	6.7	24.3	20.6	72.7	10,254	3,406
Fasciola hepatica	NC_002546	49.4	9.6	14.2	26.8	63.6	10,140	3,368
Paragonimus westermani	NC_002354	38.3	17.9	13.2	30.6	51.5	10,023	3,329
Opisthorchis felineus	EU921260	45.3	12.1	15.3	27.2	60.6	10,217	3,394
Opisthorchis viverrini	JF739555	44.9	12.3	15.5	27.3	60.4	10,206	3,390
Clonorchis sinensis	FJ381664	45.1	11.9	15.7	27.3	60.8	10,209	3,391
Haplorchis taichui	KF214770	43.0	11.9	17.1	28.0	60.1	10,176	3,380

[a]*S. malayensis* is an incomplete mt genome.

Table 4. Nucleotide codon usage for 12 protein-encoding genes of the mitochondrial genome of *Haplorchis taichui*

AA	Codon	No.	%	AA	Codon	No.	%	AA	Codon	No.	%	AA	Codon	No.	%	AA	Ab	No.	%
Phe	UUU(F)	308	9.19	Ser	UCU(S)	96	2.87	Tyr	UAU(Y)	136	4.06	Cys	UGU(C)	98	3.93	Ala	A	146	4.3
Phe	UUC(F)	48	1.43	Ser	UCC(S)	20	0.60	Tyr	UAC(Y)	35	1.04	Cys	UGC(C)	22	0.66	Cys	C	101	3.0
Leu	UUA(L)	145	4.33	Ser	UCA(S)	22	0.66	***	UAA(*)	3	0.09	Trp	UGA(W)	49	1.46	Asp	D	71	2.1
Leu	UUG(L)	204	6.09	Ser	UCG(S)	34	1.01	***	UAG(*)	9	0.27	Trp	UGG(W)	76	2.27	Glu	E	78	2.3
																Phe	F	336	9.9
Leu	CUU(L)	93	2.78	Pro	CCU(P)	35	1.04	His	CAU(H)	36	1.07	Arg	CGU(R)	34	1.01	Gly	G	303	9.0
Leu	CUC(L)	23	0.69	Pro	CCC(P)	16	0.48	His	CAC(H)	8	0.24	Arg	CGC(R)	7	0.21	His	H	54	1.6
Leu	CUA(L)	22	0.66	Pro	CCA(P)	13	0.39	Gln	CAA(Q)	8	0.24	Arg	CGA(R)	8	0.24	Ile	I	107	3.2
Leu	CUG(L)	52	1.55	Pro	CCG(P)	15	0.45	Gln	CAG(Q)	17	0.51	Arg	CGG(R)	31	0.93	Lys	K	44	1.3
																Leu	L	565	16.7
Ile	AUU(I)	98	2.93	Thr	ACU(T)	47	1.40	Asn	AAU(N)	33	0.99	Ser	AGU(S)	63	1.88	Met	M	186	5.5
Ile	AUC(I)	14	0.42	Thr	ACC(T)	11	0.33	Asn	AAC(N)	6	0.18	Ser	AGC(S)	14	0.42	Asn	N	72	2.1
Met	AUA(M)	65	1.94	Thr	ACA(T)	9	0.27	Asn	AAA(N)	33	0.99	Ser	AGA(S)	31	0.93	Pro	P	92	2.7
Met	AUG(M)	108	3.22	Thr	ACG(T)	16	0.48	Lys	AAG(K)	41	1.22	Ser	AGG(S)	62	1.85	Gln	Q	24	0.7
																Arg	R	67	2.0
Val	GUU(V)	171	5.10	Ala	GCU(A)	70	2.09	Asp	GAU(D)	51	1.52	Gly	GGU(G)	100	2.99	Ser	S	350	10.4
Val	GUC(V)	32	0.96	Ala	GCC(A)	18	0.54	Asp	GAC(D)	15	0.45	Gly	GGC(G)	40	1.19	Thr	T	88	2.6
Val	GUA(V)	58	1.73	Ala	GCA(A)	10	0.30	Glu	GAA(E)	15	0.45	Gly	GGA(G)	49	1.46	Val	V	402	11.9
Val	GUG(V)	122	3.64	Ala	GCG(A)	29	0.87	Glu	GAG(E)	55	1.64	Gly	GGG(G)	141	4.21	Trp	W	118	3.5
																Tyr	Y	175	5.2

AA, Amino acid; Ab, Abbreviation; No., Number of codons.

11.9% C, accounting for 67.3% of the full length of the genome (Table 3). All 64 codons were used (Table 4). However, some codons, such as CGC, CGA for arginine, ACA for threonine, or GCA for alanine, were very uncommon, reflecting the nucleotide composition. Several amino acids, histidine (1.6%), lysine (1.3%), and glutamine (0.7%), were rarely used. Five of the most frequently used amino acids were leucine (16.7%), valine (11.9%), serine (10.4%), phenylalanine (9.9%), and glycine (9.0%). These collectively constituted 57.9% of the total number of amino acids. Amino acids encoded by T-rich codons (≥ 2 Ts in a triplet) were the most abundant and accounted for 42.7% of the total amino acid composition, whereas C-rich codons (≥ 2 Cs in a triplet) were the least used (they accounted for merely 5.0% of the total amino acid composition). As shown in Table 4, unequal usage of synonymous codons avoiding C at the third codon position was prominent in most cases; for instance, the relative frequency of using TTT for Phe was 9.2%, but the frequency of using TTC was only 1.4%.

Transfer RNA genes, ribosomal RNA genes, and non-coding regions

The sizes of 22 tRNA genes identified in mt genomes of *H. taichui* ranged from 60 to 73 nucleotides (nt) in length. Of the 22 tRNA genes, 20 could be folded into the conventional cloverleaf structure, including a 7 bp amino-acyl stem, a 2-4 bp DHU arm with a 3-10 nt loop, a 5 bp anticodon stem with a loop of 7 nt, and a 2-8 bp of the TψC arm with a 3-10 nt loop. The 2 tRNAs specifying serine were exceptions (Fig. 2). The *rrnL* was located between *tRNA-Thr* and *tRNA-Cys*, and *rrnS* was located between *tRNA-Cys* and *cox2*. The *rrnS* and *rrnL* of *H. taichui* were 747 nt and 979 nt in length. Each ribosomal gene was assumed to directly abut neighboring genes. The A+T contents of the *rrnL* was 58.5%, respectively, and the A+T content of the *rrnS* was 55.9%. Just 1 long non-coding region (LNR) was identified, located between the *tRNA-Gly* and *tRNA-Glu*, and lacked any tandem repeats. The size was 1,710 bp, comprising 11.3% of the genome, and the A+T content was 58.3%.

Phylogenetic analysis

A concatenated alignment set of 3,380 homologous amino acid positions from conserved blocks was used. Phylogenetic relationships among the 15 flatworms using different analytical approaches (MP, ML, NJ, and BP methods) were the same in their topology (Fig. 3). Phylogenetic relationships among species were well resolved with very high nodal support throughout. In this tree, Schistosomatidae and (Fasciolidae+Paragonimidae+Opisthorchiidae+Heterophyidae) formed monophyletic groups. *H. taichui* was resolved as sister to Opisthorchiidae with a very high support in the phylogenetic analysis.

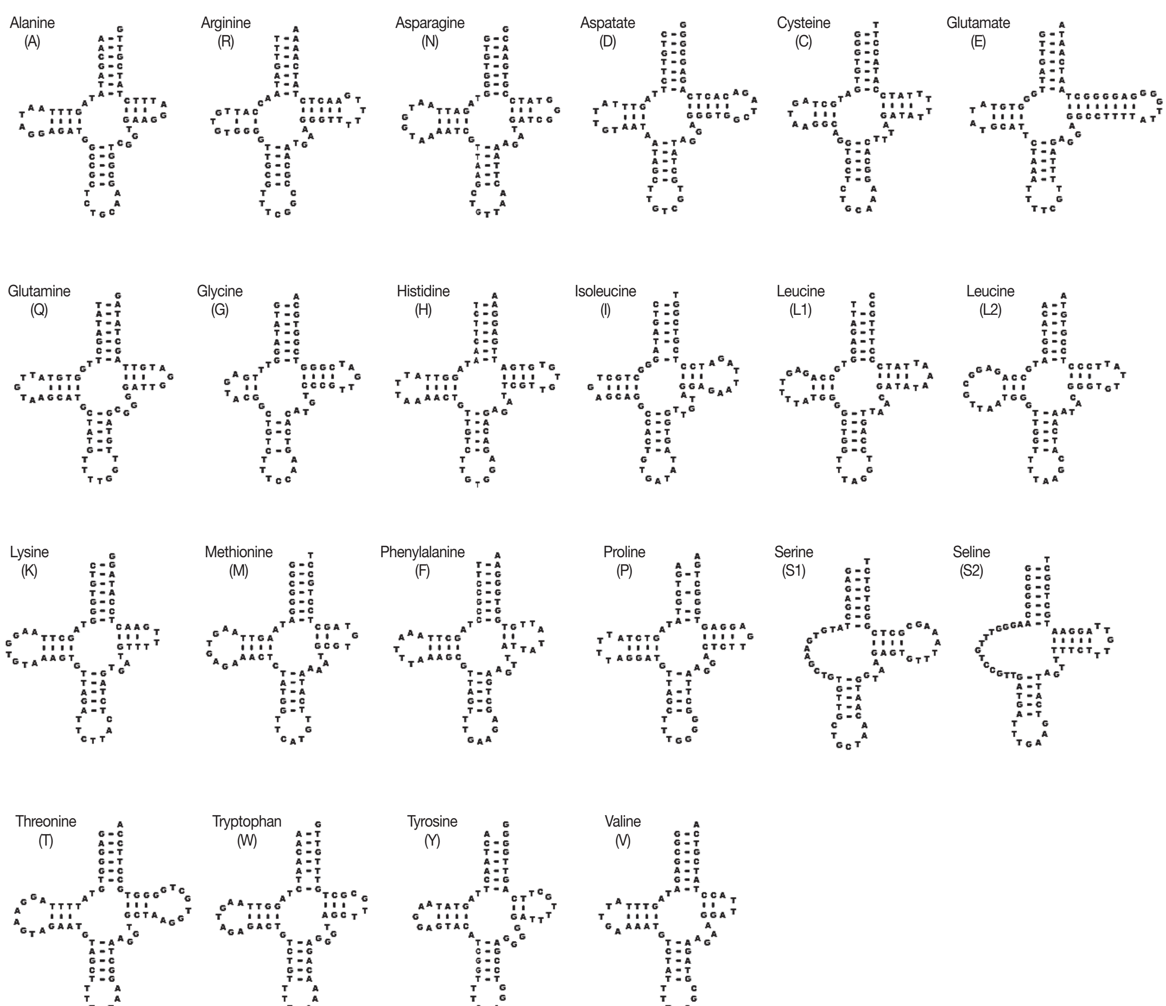


Fig. 2. DNA sequences for 22 tRNA genes of *H. taichui* mtDNA folded into inferred secondary structures.

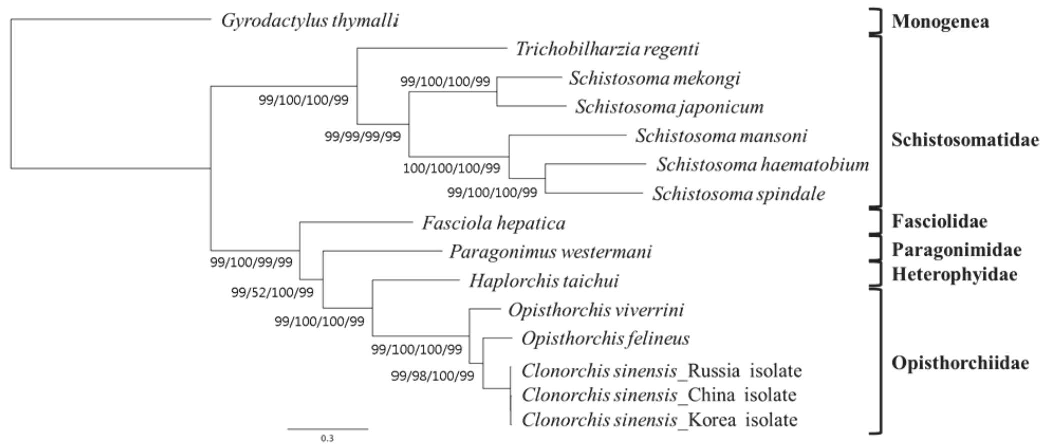


Fig. 3. Phylogenetic relationships among trematode parasites based on inferred amino acid sequences of 12 mitochondrial protein-coding gene loci for 11 species 14 individual using the outgroup, *G. thymalli* (GenBank accession no. NC_009682) by MP/ML/NJ/BP, respectively (branch length for ML).

DISCUSSION

The mt genome arrangement of *H. taichui* was the same as that of *Fasciola hepatica* (NC_002546), *Paragonimus westermani* (NC_002354) and *Opisthorchis* spp., but distinct from the arrangement seen in some *Schistosoma* spp. [9,18]. All genes were transcribed in the same direction, as in other flatworms for which data are available [26]. *H. taichui* lacks *atp8*, a gene that is not seen in any flatworm species [18]. The majority of metazoan mtDNA sequences contain 2 non-coding regions of significant size difference, a long non-coding region (LNR) and a short non-coding region (SNR). In the case of *H. taichui*, a single long non-coding region was present. The nucleotide composition of the entire mtDNA of *H. taichui* was biased toward T and G, with T being the most common nucleotide and C the least favored, in accordance with mt genomes of other trematodes except for *P. westermani* (Table 3). Two genes, *nad4L* and *nad4*, overlapped by 40 nucleotides (Table 1), similar to the situation in other digeneans. The tRNA genes generally resemble those of other digeneans. A standard cloverleaf structure can be inferred for most tRNAs. Exceptions are tRNA-S in which the paired dihydrouridine (DHU) arm is missing in all parasitic flatworm species [18], although secondary structures including this arm are feasible for some species [15] including *H. taichui*. The *rrnS* was 747 nt in length, shorter than that of the homologs from other trematodes except for *S. mansoni* (744 nt) and *P. westermani* (744 nt, not registered on GenBank). The *rrnS* of *S. mekongi* was noted as being 709 nt (GenBank accession no. NC_002529), but it could be 39 nt longer if it directly abuts *cox2* in that species. The *rrnL* of *H. taichui*, at 979 nt, was the shortest among trematodes recorded yet. Morphological data have traditionally been used for taxonomic studies on flatworms. Such data are now being supplemented by data from ultra-structural and biochemical studies [27,28] and, increasingly, from molecular sequences. Published phylogenies using nuclear ribosomal genes [19,29] indicate that the Heterophyidae is paraphyletic with respect to the Opisthorchiidae. Our mitochondrial sequences have yielded a tree consistent with this finding, with *H. taichui* seen as a sister to *Clonorchis*+*Opisthorchis* (family Opisthorchiidae). Sequences from additional heterophyid and opisthorchiid mt genomes will be required to confirm the findings from nuclear genes.

In conclusion, the present study reported the complete mtDNA sequence and genome organization of *H. taichui* for the first time. Its constituent genes were compared with homologs from other trematodes. Our phylogenetic analysis of concatenated protein-coding genes supports a sister group relationship between families Opisthorchiidae and Heterophyidae. These data will provide tools for the molecular diagnosis of haplorchiasis and for studies on the biology of the species.

ACKNOWLEDGMENTS

The Parasite Resource Bank of Korea National Research Resource Center, Republic of Korea, provided the materials for morphological identification. The National Research Foundation of Korea (NRF-2010-0024745) financially supported this work.

REFERENCES

1. Chai JY, Murrell KD, Lymbery AJ. Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 2005; 35: 1233-1254.
2. Dung DT, De NV, Waikagul J, Dalsgaard A, Chai JY, Sohn WM, Murrell KD. Fishborne zoonotic intestinal trematodes, Vietnam. Emerg Infect Dis 2007; 13: 1828-1833.
3. Chai JY, Shin EH, Lee SH, Rim HJ. Foodborne intestinal flukes in Southeast Asia. Korean J Parasitol 2009; 47(suppl): S69-S102.
4. Thaenkham U, Visetssuk K, Dung DT, Waikagul J. Discrimination of *Opisthorchis viverrini* from *Haplorchis taichui* using COI sequence marker. Acta Trop 2007; 103: 26-32.
5. Jeon HK, Lee DM, Park HS, Min DY, Rim HJ, Zhang H, Yang Y, Li X, Eom KS. Human infections with liver and minute intestinal flukes in Guangxi, China: analysis by DNA sequencing, ultrasonography, and immunoaffinity chromatography. Korean J Parasitol 2012; 50: 391-394.
6. Chai JY, De NV, Sohn WM. Foodborne trematode metacercariae in fish from northern Vietnam and their adults recovered from experimental hamsters. Korean J Parasitol 2012; 50: 317-325.
7. Wongsawad C, Wongsawad P, Chuboon S, Anuntalabhochai S. Coprodiagnosis of *Haplorchis taichui* infection using sedimentation and PCR-based methods. Southeast Asian J Trop Med Public Health 2009; 40: 924-928.
8. Jex AR, Waeschenbach A, Littlewood DTJ, Hu M, Gasser RB. The mitochondrial genome of *Toxocara canis*. PLos Negl Trop Dis 2008; 2: e273.
9. Lawton SP, Hirai H, Elronside JE, Johnston DA, Rollinson D. Genomes and geography: genomic insights into the evolution and phylogeography of the genus *Schistosoma*. Parasit Vectors 2011; 4: 131.
10. Cavalier-Smith T. Protist phylogeny and the high-level classification of Protozoa. Eur J Protistol 2003; 39: 338-348.
11. Gissi C, Iannelli F, Pesole G. Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. Heredity 2008; 101: 301-320.
12. Jeon HK, Kim KH, Eom KS. Complete sequence of the mitochondrial genome of *Taenia saginata*: comparison with *T. solium* and *T. asiatica*. Parasitol Int 2007; 56: 243-246.
13. Park JK, Kim KH, Kang S, Jeon HK, Kim JH, Littlewood DTJ, Eom KS. Characterization of the mitochondrial genome of *Diphyllobothrium latum* (Cestoda: Pseudophyllidea)-implications for the phylogeny of eucestodes. Parasitology 2007; 134: 749-762.
14. Liu GH, Wu CY, Song HQ, Wei SJ, Xu MJ, Lin RQ, Zhao GH, Huang SY, Zhu XZ. Comparative analyses of the complete mitochondrial genomes of *Ascaris lumbricoides and Ascaris suum* from humans and pigs. Gene 2012; 492: 110-116.
15. Shekhovsov SV, Katokhin AV, Kolchanov NA, Mordvinov VA. The complete mitochondrial genomes of the liver flukes *Opisthorchis felineus* and *Clonorchis sinensis* (Trematoda). Parasitol Int 2010; 59: 100-103.
16. Kim KH, Jeon HK, Kang S, Sultana T, Kim GJ, Eom KS, Park JK. Charaterization of the complete mitochondrial genome of *Diphyllobothrium nihonkaiense* (Diphyllobothriidae: Cestoda), and Development of molecular markers for differentiating fish tapeworms. Mol Cells 2007; 23: 379-390.
17. Cai XQ, Liu GH, Song HQ, Wu CY, Zou FC, Yan HK, Yuan ZG, Lin RQ, Zhu XQ. Sequences and gene organization of the mithochondrial genomes of the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* (Trematoda). Parasitol Res 2012; 110: 235-243.
18. Le TH, Blair D, McManus DP. Mitochondrial genomes of parasitic flatworms. Trends Parasitol 2002; 18: 206-213.
19. Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ. Phylogeny and classification of Digenea (Platyhelminthes: Trematoda). Int J Parasitol 2003; 33: 733-755.
20. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meinthes P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinfomatics 2012; 28: 1647-1649.
21. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 2007; 56: 564-577.
22. Masters BC, Fan V, Ross HA. Species delimitation-a geneious plugin for the species boundaries. Mol Ecol Resour 2010; 11: 154-157.
23. Swofford DL. Paup*: phylogenetic analysis using parsimony (* and other methods). Version 4. Sunderland, Massachusetts, USA. Sinauer Associates. 2003.
24. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 2003; 52: 696-704.
25. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 2001; 17: 754-755.
26. Johnston DA. Genomes and genomics of parasitic flatworms. In Maule AG, Marks NJ eds, Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology. Wallingford, Oxford, UK. CAB International. 2006, p 37-80.
27. Justine JL. Phylogeny of parasitic Platyhelminthes: a critical study of synapomorphies proposed on the basis of the ultrastructure of spermiogenesis and spermatozoa. Can J Zool 1991; 69: 1421-1440.
28. Jeon HK, Eom KS. Immunoblot patterns of *Taenia asiatica* taeniasis. Korean J Parasitol 2009; 47: 73-77.
29. Thaenkham U, Blair D, Nawa Y, Waikagul J. Families Opisthorchiidae and Heterophyidae: are they distinct? Parasitol Int 2012; 61: 90-93.