

A molecular phylogeny of Asian species of the genus *Metagonimus* (Digenea)—small intestinal flukes—based on representative Japanese populations

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Abstract *Metagonimus* Katsurada, 1912 is a genus of small intestinal parasites. The genus comprises eight species, primarily from far-eastern Asia, with two exceptions reported from Europe. *Metagonimus yokogawai*, the most widespread species, is the main agent responsible for the intestinal disease, metagonimiasis, in Japan and some other East Asian countries. On the basis of the ratio of the size of the ventral and oral suckers, *Metagonimus* has traditionally been morphologically divided into two groups; however, the genus has not been extensively studied using molecular data. To reveal phylogenetic relationships within *Metagonimus* based on molecular data, we analyzed six of the seven species present in Asia using samples collected in central Japan. Maximum likelihood and Bayesian analyses of a combined 28S ribosomal DNA (rDNA), internal transcribed

spacer 2 (ITS2), and mitochondrial *cox1* gene sequence dataset separated the six species into two well-supported clades. One clade comprised *M. yokogawai*, *M. takahashii*, *M. miyatai*, and *M. hakubaensis*, whereas the other consisted of *M. otsurui* and *M. katsuradai*. Genetic distances calculated from 28S rDNA and ITS2 nucleotide sequences and a comparison of the predicted amino acid sequences of *cox1* gene suggested that *M. otsurui* and *M. katsuradai* may have diverged recently. None of the four main morphological characters used to delimit species of *Metagonimus* (i.e., sucker ratio, positions of the uterus and testes, and distribution of vitelline follicles) was consistent with the distribution of species in the molecular tree.

Keywords *Metagonimus* · 28S rDNA · *cox1* · ITS2 · Interrelations · Morphology · Incongruence

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Introduction

Trematodes of the genus *Metagonimus* Katsurada, 1912 (Digenea: Heterophyidae) are small intestinal parasites of fish-eating mammals and birds. Some of these species have been reported to be potential human parasites (Chai et al. 2005). Infection in the definitive hosts (humans and various fish-eating mammals and birds) occurs through consumption of raw flesh of freshwater and brackish-water fish infected with metacercariae (the infective stage). Infection in humans is generally asymptomatic, but heavy infections may cause symptoms of metagonimiasis: severe diarrhea, abdominal pain, and weight loss (Chai et al. 1991).

The *Metagonimus* life cycle includes two intermediate hosts, with the metacercariae encysted in the musculature and other organs of teleost fishes (Sohn 2009; Yu and Chai 2012). Many fish, including members of the genera *Plecoglossus*, *Tribolodon*,

and *Carassius*, have been reported as second intermediate hosts (Kino et al. 2006; Sohn 2009; Yu and Chai 2012). In some cases, metacercariae of more than one *Metagonimus* species can be found in a single fish. For example, metacercariae of *M. yokogawai* Katsurada, 1912, *M. takahashii* Suzuki, 1930, and *M. miyatai* Saito et al., 1997 have been found in *Plecoglossus altivelis* from Korea and Japan (Kino et al. 2006; Yu and Chai 2012).

Based on an adult worm of *M. yokogawai*, the genus was originally described from Taiwan and Japan (Katsurada 1912; Kobayashi 1912). Seven additional species have been subsequently described. Two of these species, *M. romanicus* Ciurea, 1915 and *M. minutus* Katsuta, 1932, have been recorded in Europe and Taiwan, respectively; these species are of uncertain validity (Shimazu and Urabe 2002; Yu and Chai 2012). The most widely distributed species, *M. yokogawai*, has been found in far eastern Asia (Korea, China, Taiwan, and Japan), the Russian Far East, southeastern Europe (the Balkan Peninsula and Romania), and southwestern Europe (Spain) (Ito 1964; Yu and Mott 1994; Chai et al. 2009). *M. minutus* is known only from Taiwan (Katsuta 1932), whereas *M. takahashii* and *M. miyatai* have been found in Japan and Korea. The remaining three species, *M. hakubaensis* Shimazu, 1999, *M. katsuradai* Izumi, 1935, and *M. otsurui* Saito & Shimizu, 1968, have been recorded only from Japan (Shimazu 1999, 2003; Shimazu and Urabe 2002; Kino et al. 2006). Three of the six species known to occur in Japan, *M. yokogawai*, *M. takahashii*, and *M. miyatai*, have been reported as human pathogens causing metagonimiasis, with *M. yokogawai* being the main species causing the disease in Japan and other Asian countries (Chai et al. 2000; Uppal and Wadhwa 2005; Yamada et al. 2008).

Species of *Metagonimus* are differentiated from one another by the oral and ventral sucker size ratio, position of testes and uterus, and distribution of vitelline follicles (Saito et al. 1997; Shimazu 1999, 2003; Kim et al. 2006). Among these morphological characters, the sucker size ratio is the major diagnostic character dividing the genus into two major groups: *M. yokogawai*, *M. miyatai*, *M. takahashii*, and *M. minutus*, having an oral sucker smaller than the ventral one, and *M. katsuradai* and *M. otsurui*, of which the oral sucker is larger (Saito et al. 1997; Shimazu and Urabe 2002; Shimazu 2003; Shimazu and Kino 2015). In *M. hakubaensis*, this ratio is variable, but the oral sucker is typically larger than the ventral one (Shimazu 1999; Shimazu and Kino 2015). A preliminary molecular study on species of *Metagonimus* was performed by Lee et al. (2004), who used partial 28S ribosomal DNA (rDNA) and mitochondrial cytochrome *c* oxidase subunit I (*cox1*) sequences to analyze relationships among *M. yokogawai*, *M. takahashii*, and *M. miyatai* from Korea. Their results appear to support the monophyly of *Metagonimus* and to confirm the close relationship among the three species inferred by morphology. To further clarify relationships within *Metagonimus*, we analyzed six of the

seven nominal species of *Metagonimus* distributed in the Manchurian subregion. For this purpose, samples were collected from central Japan, and phylogenetic relationships were reconstructed based on three DNA markers likely to have different levels of resolution: 28S rDNA (D1–D3 regions), the rDNA internal transcribed spacer 2 (ITS2) region, and the mitochondrial *cox1* gene. We then compared these molecular phylogenetic relationships with the original published morphological descriptions of the species.

Materials and methods

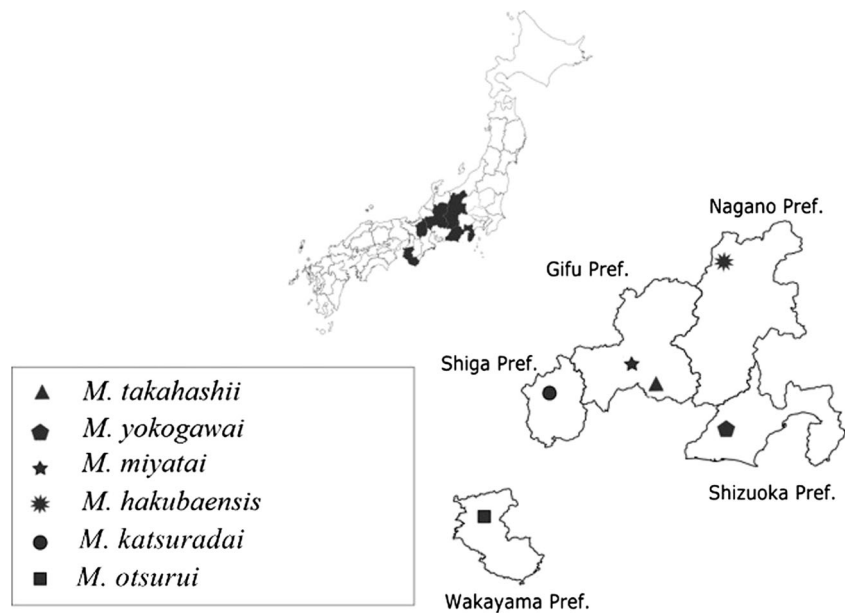
Sample collection

Metacercariae of *Metagonimus* were collected from fishes from different localities in Japan (Fig. 1) by digestion with artificial gastric juice (Table 1). Doses of 36–100 metacercariae from each locality were orally administered to Syrian golden hamsters (*Mesocricetus auratus* Waterhouse, 1839). Adult worms were collected from the small intestines of hamsters 14–15 days post-infection. The hamsters were handled and maintained according to the guidelines provided in the Guide for the Care and Use of Laboratory Animals of Hamamatsu University School of Medicine, Japan (approval nos. 2007085 and 2012012). The worms were identified under a light microscope according to described morphological characteristics (Hong and Seo 1969; Chai et al. 1991; Saito et al. 1997; Shimazu 1999; Shimazu and Urabe 2002; Shimazu and Kino 2015). Adult worms of four species, *M. yokogawai*, *M. takahashii*, *M. miyatai*, and *M. hakubaensis*, were collected. Metacercariae of *M. otsurui* and *M. katsuradai* were collected and identified by one of the authors (Takeshi Shimazu) according to published morphological criteria and host specificity of these two parasites (Shimazu and Urabe 2002; Shimazu 2003; Shimazu and Kino 2015). These two species were not obtained from an infected experimental host. All samples were preserved in absolute alcohol and stored at -20°C until use.

DNA extraction, PCR, and DNA sequencing

Genomic DNA from three individuals of each species was extracted using a Genomic DNA mini kit (Tissue) (Geneaid, Taipei, Taiwan) according to the manufacturer's protocol. A portion of the 28S rRNA gene (D1–D3 regions), the ITS2 region of rDNA, and the mitochondrial *cox1* gene were amplified by polymerase chain reaction (PCR) using the primers listed in Table 2. Amplification conditions for the 28S rDNA, modified from Olson et al. (2003), consisted of one cycle of denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min. PCR amplification for the ITS2 region followed Skov et al. (2009), whereas the PCR conditions used for the

Fig. 1 Collection localities of six *Metagonimus* spp. in central Japan



cox1 gene were according to Bowles and McManus (1994). PCR amplicons were size-checked by 1.0 % agarose gel electrophoresis and visualized under a UV transilluminator. All amplicons were sequenced in both directions with the same primers used for the PCRs on an ABI Prism 3730XL DNA sequencer (Biobasic, Toronto, Canada).

DNA analysis

The generated DNA sequences were checked using BioEdit version 7.1.3.0 (Hall 2011) and aligned using CLUSTAL X2 (Larkin et al. 2007). For inclusion in the phylogenetic analyses, out-groups were selected on the basis of known relationships among heterophyid intestinal flukes (Thaenkham et al. 2012); their corresponding sequences were downloaded from GenBank and incorporated along with two additional published *Metagonimus* ITS2 sequences into the aligned sequence datasets (Table 1). Genetic distances among 28S rDNA, ITS2, and *cox1* sequences were estimated by MEGA 6.06 (Tamura et al. 2013). To examine genetic differentiation within *Metagonimus*, the *cox1* sequences were translated into amino acid sequences using MEGA.

Phylogenetic analysis

Before conducting the phylogenetic analyses, nucleotide substitution saturation was measured to evaluate the degree of saturation of each marker using the DAMBE (data analysis in molecular biology and evolution) program (Xia 2013). Phylogenetic analyses were conducted on individual 28S rDNA, ITS2, and *cox1* sequence datasets. Phylogenetic trees were reconstructed using maximum likelihood (ML) as implemented in MEGA 6.06 and MrBayes v.3.2 (Ronquist et al.

2012). For the ML analyses, best-fit models of nucleotide substitution were selected based on the Akaike information criterion as determined in MEGA. The ML trees were constructed with and without out-groups, and support for each node in the resulting trees was estimated using 1000 bootstrap replicates. *Haplorchis taichui* Nishigori, 1924 (Trematoda: Heterophyidae) was used as a potential out-group because of indicating close phylogenetic position to genus *Metagonimus* (Thaenkham et al. 2012).

Phylogenetic analyses were also conducted on a combined dataset consisting of concatenated sequences of all three DNA regions. Before generating the combined dataset, partition homogeneity testing in PAUP* 4.0b10 (Swofford 1998) was carried out to confirm congruence between the three partitions at a significance level of $P < 0.02$ (Cunningham 1997). Maximum likelihood and Bayesian inference were used to generate trees from the combined dataset using *H. taichui* as an out-group. The ML tree was constructed following the method described above. For the Bayesian analysis, Bayesian posterior probabilities were calculated from the combined sequence dataset under the same best-fit model of evolution used in the ML analysis (Ronquist et al. 2012) using the default priors in MrBayes. Two simultaneous Markov chain Monte Carlo (MCMC) runs, each comprising four chains (one cold and three heated), were conducted. Each MCMC run was performed for 1,000,000 generations, with sampling every 100 generations. The convergence between runs was visually estimated by plotting the number of generations vs. log-likelihood values and using the potential scale reduction factor provided in the “sump” output of MrBayes (Ronquist et al. 2012). After discarding results from the first 25 % of generations as “burn in,” majority-rule consensus trees were generated from the remaining trees. Node support was assessed using the Bayesian posterior probabilities.

Table 1 The six *Metagonimus* spp. used in this study. Developmental stages of worms, sources of metacercariae, and localities of collected samples are given

Species	Stage	Fish	Locality in Japan	GenBank accession nos.		
				28S rDNA	ITS2	cox1
<i>M. yokogawai</i>	Adult	<i>Plecoglossus altivelis</i>	Tenryu River, Shizuoka	HQ832639	<i>HQ832621</i>	KM061412
				HQ832640	HQ832622	KM061413
				HQ832641	HQ832623	KM061414
<i>M. takahashii</i>	Adult	<i>Carassius auratus</i>	Kiso River, Gifu	HQ832636	<i>HQ832618</i>	KM061406
				HQ832637	HQ832619	KM061407
				HQ382638	HQ832620	KM061408
<i>M. miyatai</i>	Adult	<i>Plecoglossus altivelis</i>	Nagara River, Gifu	HQ832633	HQ832615	KM061409
				HQ832634	HQ832616	KM061410
				HQ832635	HQ832617	KM061411
<i>M. hakubaensis</i>	Adult	<i>Lethenteron reissneri</i>	Hime River, Nagano	KM061388	KM061397	KM061415
				KM061389	KM061398	KM061416
				KM061390	KM061399	KM061417
<i>M. katuradai</i>	Metacercariae	<i>Tanakia limbata</i>	Irrigation canal, Miyake, Shiga	KM061391	KM061400	KM061418
				KM061392	KM061401	KM061419
				KM061393	KM061402	KM061420
<i>M. otsurui</i>	Metacercaria	<i>Rhinogobius flumineus</i>	Nyukawa River, Wakayama	KM061394	KM061403	KM061421
				KM061395	KM061404	KM061422
				KM061396	KM061405	KM061423
<i>Haplorchis taichui</i>	Adult			<i>HM004181</i>	<i>AB517574</i>	<i>JN809909</i>

Accession numbers that were set in italic refer to previously published sequences; all other sequences were generated in this study

Results

Genetic distances among species of *Metagonimus*

Pairwise genetic distances were estimated from the 28S rDNA (1140 bp), ITS2 (406 bp), and *cox1* (355 bp) aligned sequences (Table 3). The average genetic distance among six *Metagonimus* species based on the 28S rDNA sequences was approximately 0.012, although the genetic distance between *M. katuradai* and *M. otsurui* was 0.002. Using the ITS2 sequence dataset, the average distance among six *Metagonimus* was approximately 0.03, with a distance of 0.008 calculated between *M. katuradai* and *M. otsurui*. Based on the *cox1* sequences, the genetic distance among six *Metagonimus* species was about 0.15; the genetic distance between *M. katuradai* and *M. otsurui* was 0.115, close to the average.

Amino acid differences based on the *cox1* gene

Because of the relatively large genetic distances between *cox1* sequences compared with distances estimated from 28S rDNA and ITS2 sequences, especially those between

M. katuradai and *M. otsurui*, we examined differences in amino acid sequences predicted from *Metagonimus cox1* nucleotide sequences. A comparison of 115 predicted amino acids within this region uncovered no differences between *M. katuradai* and *M. otsurui* (Table 4). In contrast, amino acid sequence differences were found among the other four *Metagonimus* species, i.e., *M. takahashii*, *M. yokogawai*, *M. miyatai*, and *M. hakubaensis*.

Phylogenetic relationships

To show phylogenetic relationships among six *Metagonimus* species studied, trees were constructed from 28S rDNA, ITS2, and *cox1* sequences using *H. taichui* as an out-group. None of these genetic markers showed evidence of nucleotide substitution saturation; consequently, they could all be used for revealing phylogenetic relationships. As shown in the Supplement Data 1, the topologies recovered using the three different markers were incongruent with one another. The same was true for the phylogenetic trees constructed without an out-group. As indicated by the high ML bootstrap proportions and Bayesian posterior probabilities, most nodes in each tree were well supported.

Table 2 Primers used for PCR amplification and sequencing of each DNA marker region

Marker	Primer name	Primer sequence (5'-3')	Size	Species used	Reference
LSU (28S)	LSU_F	TAG-GTC-GAC-CCG-CTG-AAY-TTA-AGC-A	~1140	<i>M. yokogawai</i> , <i>M. takahashii</i> , <i>M. miyatai</i>	Olson et al. 2003
	LSU_R	GCT-ATC-CTG-AGG-GAA-ACT-TCG			
	128S1_F	AGT-AAC-GGC-GAG-TGA-ACA-GG	296	<i>M. hakubaensis</i> , <i>M. katsuradai</i> , <i>M. otsurui</i>	Our study
	2.28S1_R	CCT-CAC-GGT-ACT-TGT-TCG-CT	377		
	3.28S2-1_F	TAG-ACC-TTG-GAG-TCG-GGT-TG	509		
	4.28S2-1_R	CAA-TGC-CGG-TCG-TGG-TGA			
	5.28S2-2_F	AGC-ATG-GTT-TGA-GCT-TGG-TC	475		
	6.28S2-2_R	TCA-GGT-GGA-AAG-TCT-ACC-GC			
7.28S3_F	GTC-CTC-GGG-TGT-AAT-CAG				
8.28S3_R	AGT-TCA-CCA-TAT-TTA-GGG				
ITS2 region	OPHET_F	CTC-GGC-TCG-TGT-GTC-GAT-GA	406	6 species	Skov et al. 2009
	OPHET_R	GCA-TGC-ART-TCA-GCG-GGT-A			
<i>cox1</i> gene	JB3	TTT-TTT-GGG-CAT-CCT-GAG-GTT-TAT	354	<i>M. yokogawai</i> , <i>M. takahashii</i> , <i>M. miyatai</i>	Bowles and McManus 1994
	JB4.5	TAA-AGA-AAG-AAC-ATA-ATG-AAA-ATG			
	COI_F	TCC-TGA-GGT-TTA-TGT-GYT-GA	355	<i>M. hakubaensis</i> , <i>M. katsuradai</i> , <i>M. otsurui</i>	Our study
	COI_R	GCA-ATM-ACR-AAC-CAA-GTM-TCM-TG			

To more fully resolve phylogenetic relationships using all available evolutionary evidence, the three sequence datasets were combined and analyzed using *H. taichui* as the out-group. Although the trees generated from the individual datasets were incongruent, a partition homogeneity test indicated that the 28S rDNA, ITS2, and *cox1* sequence partitions could be combined (nonsignificant for the homogeneity test; $P=0.5$). ML and Bayesian analyses were run on the combined dataset under the HKY+G+I model of nucleotide substitution. As shown in Fig. 2, the topologies recovered by these analyses were congruent, with the inferred phylogenetic relationships among *Metagonimus* species generally well supported—as indicated by ML bootstrap proportions and most Bayesian posterior probabilities. In the tree in Fig. 2, species of *Metagonimus* are separated into two clades. Within clade I, *M. yokogawai* and *M. takahashii* constitute a sister clade to *M. hakubaensis*, with these three species in turn forming a sister clade to *M. miyatai*. Clade II comprises the remaining two species, *M. katsuradai* and *M. otsurui*.

We compared the inferred molecular phylogenetic relationships of the six *Metagonimus* species with their previously described morphological characteristics (Hong and Seo 1969; Chai et al. 1991; Saito et al. 1997; Shimazu 1999, 2002, 2003; Shimazu and Urabe 2002; Shimazu and Kino 2015). Mapping of morphological characteristics used to

distinguish individual species of *Metagonimus*, namely position of testes and uterus position and distribution of vitelline follicle (see Materials and Methods for references), onto the phylogenetic tree has revealed incongruence of molecular and morphological data (Fig. 2). No morphological character was consistent with respect to the two main lineages of *Metagonimus*. Another taxonomically important characteristic, sucker ratio, was also inconsistent because of the variable size of the suckers of *M. hakubaensis*.

Discussion

Pairwise comparison of genetic distances based on three different DNA markers—28S rDNA, ITS2, and *cox1*—revealed the evolutionary divergence of the six *Metagonimus* species collected from central Japan. According to genetic distances estimated from 28S rDNA and ITS2 sequences, *M. yokogawai*, *M. takahashii*, *M. miyatai*, and *M. hakubaensis* were clearly highly genetically differentiated from one another at the species level, while *M. katsuradai* and *M. otsurui* were closely related to each other. Interestingly, the divergence between *cox1* sequences of the latter two species was similar to the average genetic distance among all six *Metagonimus* species. No changes

Table 3 Pairwise genetic distances among six *Metagonimus* species based on 28S rDNA, ITS2, and *cox1* sequences

	<i>Mt</i>	<i>My</i>	<i>Mm</i>	<i>Mh</i>	<i>Mo</i>	<i>Mk</i>	<i>Ht</i>
A) 28S rDNA sequences							
<i>Mt</i>							
<i>My</i>	0.010						
<i>Mm</i>	0.012	0.010					
<i>Mh</i>	0.014	0.011	0.011				
<i>Mo</i>	0.017	0.014	0.013	0.011			
<i>Mk</i>	0.017	0.014	0.013	0.010	0.002		
<i>Ht</i>	0.085	0.079	0.079	0.080	0.080	0.080	
B) ITS2 sequences							
<i>Mt</i>							
<i>My</i>	0.038						
<i>Mm</i>	0.034	0.042					
<i>Mh</i>	0.027	0.034	0.023				
<i>Mo</i>	0.046	0.046	0.027	0.034			
<i>Mk</i>	0.053	0.053	0.034	0.042	0.008		
<i>Ht</i>	0.218	0.221	0.214	0.218	0.191	0.183	
C) <i>cox1</i> sequences							
<i>Mt</i>							
<i>My</i>	0.136						
<i>Mm</i>	0.161	0.118					
<i>Mh</i>	0.150	0.142	0.162				
<i>Mo</i>	0.180	0.158	0.123	0.157			
<i>Mk</i>	0.169	0.142	0.147	0.162	0.115		
<i>Ht</i>	0.235	0.218	0.206	0.215	0.204	0.229	

Mt *Metagonimus takahashii*, *My* *M. yokogawai*, *Mm* *miyatai*, *Mh* *M. hakubaensis*, *Mo* *M. otsurui*, *Mk* *M. katsuradai*, *Ht* *Haplorchis taichui*

in amino acids were evident between *M. otsurui* and *M. katsuradai*, whereas the predicted amino acid sequences of the other four *Metagonimus* species were different from one another (Table 4). This similarity in amino acids supports the evidence obtained from 28S rDNA and ITS2

Table 4 Variable amino acids within the translated *cox1* gene region of six *Metagonimus* spp.

Species	Amino acid position					
	28	41	53	80	89	105
<i>M. takahashii</i>	V	V	I	V	V	I
<i>M. yokogawai</i>	–	L	V	–	–	–
<i>M. miyatai</i>	–	L	–	–	–	–
<i>M. hakubaensis</i>	–	L	V	–	I	V
<i>M. otsurui</i>	I	L	V	I	–	–
<i>M. katsuradai</i>	I	L	V	I	–	–

V valine, I isoleucine, L leucine

sequences that *M. otsurui* and *M. katsuradai* are closely related.

To confirm the genetic relationships suggested above, we reconstructed rooted and unrooted phylogenies of the six *Metagonimus* species using the three genetic markers (28S rDNA, ITS2, and *cox1*). Most nodes in the resulting trees had high statistical support, but the topologies were largely incongruent (Supplement Data 1). Such high levels of incongruence have not typically been observed in analyses of trematode genera such as *Paragonimus* Braun, 1899 and *Schistosoma* Weinland, 1858, where trees constructed from different genetic markers were generally congruent (Lockyer et al. 2003; Thaenkham and Waikagul 2008; Doanh et al. 2007). In the genus *Haplorchis*, some inconsistencies were uncovered between rDNA and ITS2 trees; in that case, however, the tree constructed from the ITS2 region was characterized by low bootstrap support (Thaenkham et al. 2010). After performing a partition homogeneity test (Cunningham 1997), which supported the merging of the three data partitions, we analyzed the combined dataset to better resolve phylogenetic relationships among the six *Metagonimus* species. The phylogenetic tree obtained from analysis of the combined DNA sequences (Fig. 2) contained two distinct *Metagonimus* clades. Clade I comprised *M. yokogawai*, *M. takahashii*, *M. miyatai*, and *M. hakubaensis*. *M. yokogawai* and *M. takahashii*, which were most closely related to one another, were in a sister relationship with *M. hakubaensis*. Within this clade, *M. miyatai* was distinct from the other three species. Clade II consisted of *M. katsuradai* and *M. otsurui*. The high similarity of their 28S rDNA and ITS2 sequences and their identical *cox1* translated amino acid sequences suggest that these two species may have recently diverged from one another.

To look for correlations between the molecular phylogeny and the morphological characters used to delimit the six *Metagonimus* species (described in the legend in Fig. 2), we labeled the species in the tree in Fig. 2 according to their morphological characteristics. No clear correspondence was found between the two phylogenetic clades and sucker size ratio, which is considered to be the major defining morphological character within *Metagonimus*. In *M. hakubaensis*, this ratio has been reported to range from 0.94 to 1.33 (Shimazu 1999). The minor morphological diagnostic characters, including position of testes and uterus, and distribution of vitelline follicles, were also incongruent with phylogenetic relationships. No congruence between morphological characters and phylogenetic clades was thus observed in the six representatives of *Metagonimus* from Japan. This result contrasts with the situation observed in members of the subfamily Haplorchiinae (Heterophyidae), where the monophyletic clade of the genus *Haplorchis* is characterized by a single testis and is distinct from other heterophyid genera (Thaenkham et al. 2010).

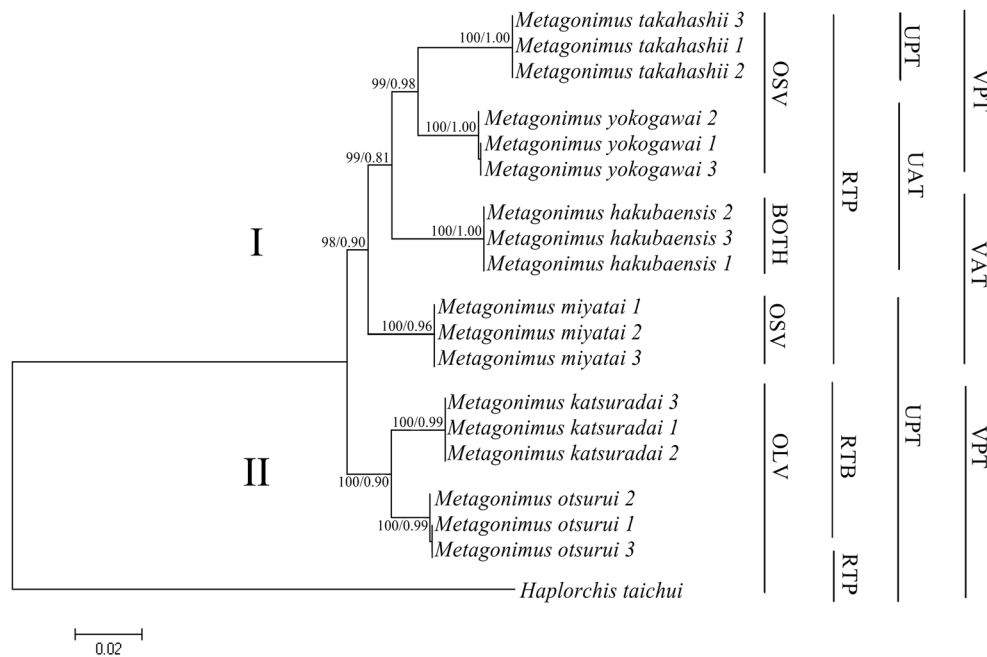


Fig. 2 Phylogenetic tree of *Metagonimus* spp. based on a combined 28S rDNA, ITS2, and *cox1* sequence dataset. *H. taichui* was used as an out-group. Maximum likelihood bootstrap support values/Bayesian posterior probabilities are shown above branches. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions. Morphological characters traditionally used to delimit *Metagonimus* spp. are indicated to the right of the tree as follows: OSV, oral sucker smaller than ventral sucker; OLV, oral sucker larger than ventral sucker; RTP, right testis located at the posterior end of the body;

RTB, right testis located at a distance from the posterior extremity, in the intercecal field of the body; UAT, uterus occupies the space from the ventral sucker to the anterior border of the testes; UPT, uterus extends between the testes near the posterior end of the body; VAT, vitelline follicles distributed along the lateral side of the body and mostly terminating at the front of the anterior two-thirds of the right testis; and VPT, vitelline follicles distributed along the lateral side of the body and extending beyond the posterior end of the right testis

Morphological characters of the Japanese representatives investigated in this study seem to be homoplastic, but a more robust phylogenetic hypothesis should be available. Morphological adaptations among species of *Metagonimus*, which may have diverged within a small region of central Japan, should be clarified by future studies on morphological polymorphisms and genetic variation of *Metagonimus* populations from other geographical localities, especially the mainland region, but also using other molecular markers such as microsatellites.

In conclusion, this study was the first to reveal phylogenetic relationships of six of eight species of *Metagonimus*, which have obviously diverged into two separate clades. The results of our phylogenetic analyses also suggest that the major morphological diagnostic character, sucker ratio, may have no correspondence with evolutionary divergence within *Metagonimus*. Because our sampling was limited to a small number of Japanese populations, more samples from other regions should be analyzed to provide more robust data on the evolutionary history of these sympatrically living parasites. Additionally, another Asian species, *M. minutus* endemic to Taiwan, should be collected to assess its relationships with the Japanese taxa. For future analyses, samples should be collected more broadly from various geographical localities to assess genetic variation within and among species. For phylogenetic analysis of *Metagonimus*, 28S rDNA, ITS2,

and *cox1* markers can be used in combination to reconstruct a reliable phylogenetic tree.

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