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Molecular phylogeny of trematodes in Family Heterophyidae based on mitochondrial cytochrome c oxidase subunit I (mCOI)

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ABSTRACT

Objective: To analyze a phylogenetic tree for understanding the molecular systematic of trematode in Family Heterophyidae, which are highly distributed in Thailand. **Methods:** Based on thirteen sequences of mitochondrial cytochrome c oxidase subunit I (mCOI) gene from six genera of heterophyid trematodes, viz. *Haplorchis*, *Stellantchasmus*, *Centrocestus*, *Metagonimus*, *Pygidopsis*, and *Haplorchoides* were aligned automatically using the Clustal X 2.0 program. A phylogenetic tree was constructed by maximum likelihood (ML) and neighbor-joining (NJ) methods, with 1 000 bootstrap using the 5.0 program. **Results:** The phylogenetic relationship from both methods was similar and separated into three groups consisting of *Haplorchoides pumilio* group, *Haplorchoides taichui* group and another heterophyid genera. **Conclusions:** The sequence data of mCOI can be used to investigate the phylogenetic relationships of trematodes at the genus level. Each clade of different genera of heterophyid trematodes can be separated into sister groups that correlated with the morphological characteristic, kind of secondary intermediate host and geographic distribution.

1. Introduction

Fish-borne trematodes in Family Heterophyidae are minute intestinal parasites of various definitive hosts such as birds, cats, rodents, dogs, and humans[1]. Heterophyidiasis has now a wide distribution in South-east Asia, especially in Thailand, Laos, and Vietnam[2–4], and is contracted by humans by eating raw or improperly cooked fish[5–7]. In Thailand, there are in five genera of trematodes in the family Heterophyidae viz. *Haplorchis*, *Centrocestus*, *Stellantchasmus*, *Procerovum*, and *Haplorchoides*[8–10].

Several epidemiological investigations of trematode infections in Thailand have shown a high prevalence of heterophyid trematodes infections[11–13]. Freshwater fish from the Mae Sa village stream in Chiang Mai Province have been investigated for trematode infection. Four species of heterophyid trematodes were found, viz. *Haplorchis* sp., *Haplorchoides* sp., *Centrocestus caninus* (*C. caninus*) and *Stellantchasmus falcatus* (*S. falcatus*)[14]. In the metacercarial stage of *Haplorchis taichui* (*H. taichui*), there was a high prevalence of infection in cyprinoid fish collected from Chiang Mai Province[15]. Nithikathkul and Wongsawad have reported a high infection rate of *H. taichui* and *Haplorchoides* sp. (83.90%) in the same area[16].

Various conventional PCR-based methods have been developed to study the evolution, phylogeny, biogeography, and population genetics[17]. Mitochondrial-based PCR has been extensively used to study the phylogenetic relationship because mitochondrial genomes are apparent in the maternal mode of inheritance and with high

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mutation rates[18–19]. Such sequences have proven useful for investigating species variation in trematodes such as *Clonorchis sinensis* (*C. sinensis*) and *Opisthorchis viverrini* (*O. viverrini*)[20], *Schistosoma nasale* (*S. nasale*)[21] and *H. taichui*[22]. Three species of heterophyid trematodes, viz. *Metagonimus yokogawai* (*M. yokogawai*), *Metagonimus takahashii* (*M. takahashii*), and *Metagonimus miyatai* (*M. miyatai*) have been compared in a phylogenetic relationship, using 28S rDNA and mitochondrial cytochrome c oxidase subunit I (mCOI) gene. The results showed that the phylogenetic tree from 28S ribosomal DNA was similar with mCOI sequence data[23]. The PCR–RFLP utilizes the mCOI gene sequence marker to distinguish *O. viverrini* from *H. taichui*[24]. There are few reports concerning the phylogenetic relationship of heterophyid trematodes based on the mCOI gene.

We investigated the phylogenetic relationship of heterophyid trematodes in Thailand using mCO I sequence data to investigate species which have not been previously investigated. This study provides a new more systematic data on heterophyid trematodes in Thailand.

2. Materials and methods

2.1. Parasite specimens

Five species of heterophyid trematodes, viz. *H. taichui*, *Haplorchis pumilio* (*H. pumilio*), *C. caninus*, *S. falcatus*, and *Haplorchoides* sp. were collected in northern Thailand. Four species of heterophyid trematodes at the metacercarial stage were collected from various species of fish, viz. *H. taichui* from Siamese Mud Carp (*Hemicorhynchus siamensis*), *H. pumilio* from Moonlight Gourami (*Trichogaster microlepis*), *C. caninus* from Golden Little Barb (*Puntius brevis*), and *S. falcatus* from Half-Beak (*Dermogenys pusillus*). Metacercariae were collected from various fish species using the digestion technique with 1% pepsin solution[25] and then 200 metacercariae of each species of heterophyid trematodes were inoculated into one-day chicks (*Gallus gallus domesticus*). The adult stage of *Haplorchoides* sp. were collected from Bagrid catfish (*Hemibagrus filamentus*).

The genomic DNA of trematodes was extracted from 40 adult specimens of each heterophyid species using a commercial GF–1 tissue DNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions. All genomic DNA of each specimen were diluted to a concentration at 50 ng/μL with elution buffer and kept at –20 oC until used.

2.2. mCO I amplification protocol

The PCR amplification of partial mCO I fragment uses by

a pair of primers is described by Yu *et al*[26]. It consists of (JB3) 5' TTTTTGGGCATCCTGACGTTTAT 3/ as a forward primer and (JB 4,5) 5' TAAAGAAAGAACATAATGAAAATG 3' as a reverse primer. The PCR amplifications were carried out in a final volume of 20 mL, including 50 ng of DNA template, 50 pM of each primer (JB3 and JB4,5), 1.5 mM of MgCl₂, 200 μM of each dNTPs, and 0.5 unit of *Taq* DNA polymerase. The amplification procedure involved an initial denaturation step at 95 °C for 3 min, then 40 cycles including denaturation at 95 °C for 1 min, primer annealing at 50 °C for 1 min, extension at 72 °C for 1 minute, and final extension at 7 °C for 7 minutes, respectively. PCR products were analyzed after electrophoresis separation at 100 volt for 30 minutes on 1.8% agarose gels stained with ethidium bromide in TBE buffer. Gels were visualized by a Kodak digital camera (GelLogic 100). The sequence was carried out for checking by using the BLAST program in the National Center for Biotechnology Information database, to confirm the PCR target. The eletropherograms of each sequence were examined for sequence accuracy using a Sequence Scanner version 1.0 and Bioedit version 7.1. All sequences were aligned automatically using Clustal X version 2.0.

2.3. Phylogenetic construction

All phylogenetic trees were constructed using Mega version 5.0. All molecular data were analyzed by both maximum likelihood (ML) and neighbor-joining (NJ) methods. The reliability of internal branches in both trees was assessed using the bootstrap method, 1 000 replicates. Both methods used the Kimura two – parameter model. The sequences of *Fasciola hepatica* (*F. hepatica*) were used as an out-group for phylogenetic analysis. The five isolates of *H. taichui*, three of *Metagonimus* spp., and of *Pygidopsis summa* (*P. summa*), *S. falcatus*, *C. caninus*, *Haplorchoides* sp. were used for constructing the phylogenetic tree (Table 1).

Table 1

List of material and sequences of mCO I used for constructed phylogenetic analysis.

Species of trematodes	Locations	References
<i>H. taichui</i>	Chiang Mai, Thailand	This study
<i>H. taichui</i>	Thanh Hoa, Viet Nam	JN809909
<i>H. taichui</i>	Quang Tri, Viet Nam	JN809893
<i>H. taichui</i>	Ha Giang, Viet Nam	JN809875
<i>H. taichui</i>	Chumporn, Thailand	EF055885
<i>H. pumilio</i>	Bangkok, Thailand	This study
<i>C. caninus</i>	Chiang Mai, Thailand	This study
<i>S. falcatus</i>	Chiang Mai, Thailand	This study
<i>H. sp.</i>	Chiang Mai, Thailand	This study
<i>M. sp.</i>	Korea	AF096232
<i>M. yokogawai</i>	Korea	AB470519
<i>M. takahashii</i>	Korea	AF096231
<i>P. summa</i>	Korea	AF181884
<i>F. hepatica</i>	Chiang Mai, Thailand	GQ398051

3. Results

The partial mCOI nucleotide sequences of heterophyid trematode samples were used to understand their phylogenetic relationships. The length of partial mtCO I nucleotide sequence data was 370–390 bp. Heterophyid trematodes appear to be monophyletic using *F. hepatica* as an out-group.

Phylogenetic trees were constructed for the mCO I (Figure 1) with sequence data from the character method (ML) and the distance method (NJ). Both methods show that the topology is similar among the trees. It is clear that the ML and NJ methods can be separated into three groups, viz. *H. pumilio* group, *H. taichui* group, and the other heterophyid genera group (*Metagonimus*, *Stellantchasmus*, *Haplorchoides*, *Centrocestus*, and *Pygidioopsis*). The *Metagonimus* species are in the same group with *S. falcatus*, *Haplorchoides* sp., *C. caninus*, and *P. summa*. The five data sequences of *H. taichui* were grouped together as closely related species. *Metagonimus* spp. is in other heterophyid genera groups are grouped with some closely related species. The *H. taichui* group and *H. pumilio* group are more closely related than to the heterophyid species group.

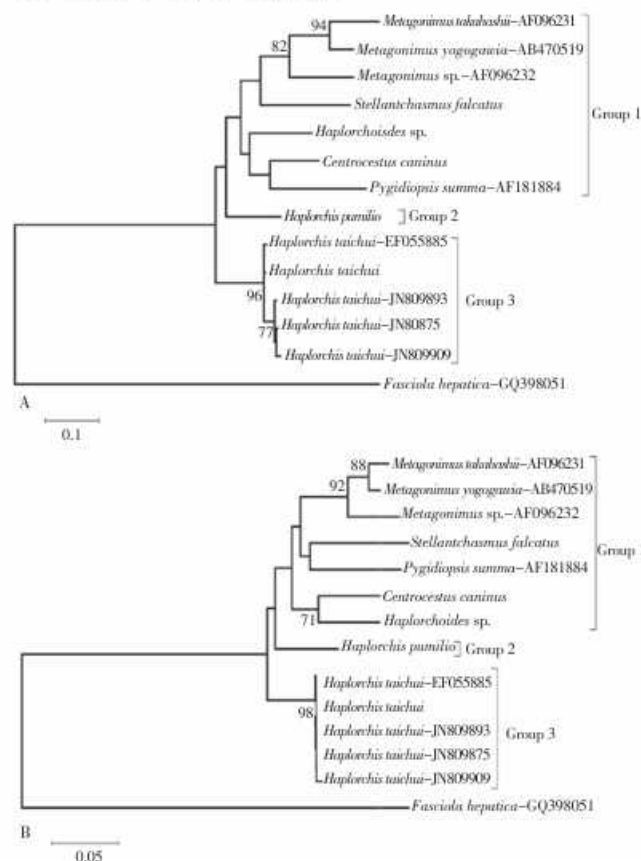


Figure 1. The rooted phylogeny from partial mCO I sequences of heterophyid trematodes base on the Kimura two-parameter model. Bootstrap values were computed independently for 1000 resampling. (A) tree from ML method (B) tree from NJ method.

4. Discussion

Only a few studies have demonstrated the relationship of mCOI of heterophyid species in Thailand [8, 24]. The molecular approach and DNA sequencing technologies have been successfully developed for studying their phylogenetic relationships.

Our analysis revealed invariably a monophyletic tree of trematodes in the Family Heterophyidae. This agrees with reports on PCR-RFLP and RAPD patterns and the phylogenetic relationship of heterophyid trematodes [23, 26–28]. Moreover, in this study we have used the species of heterophyid trematodes that have not been previously analyzed for phylogenetic relationship using mCOI sequences in Thailand.

The mCO I has been used to study the systematic biology of some heterophyid trematodes viz., *Haplorchis* [22], *Metagonimus* [23]. In our opinion this work did not come from *Heterophyidae* species. Three species of *Trematodes*; *Stellantchasmus*, *C. caninus*, and *Haplorchoides* commonly occur with high prevalence in Thailand, but the systematic of these parasites has not been studied previously.

The phylogenetic tree was derived using sequence data of mCO I and shows similar relationships. Each clade of different genera of heterophyid trematodes was separated into groups that correlated with the kind of secondary intermediate host in terms of morphological characteristics and geographic distribution. Our hypothesis is that trematodes whose cycle ends in fish are able to switch to definitive hosts. In the three groups of heterophyid trematodes, the first group (*H. pumilio*) uses *Trichogaster microlepis* (*T. microlepis*) and *Rivulus harti* (*R. harti*) as their second intermediate hosts [29], the second group (*H. taichui*) uses cyprinoid fish in the genera *Henicorhynchus* and *Puntioplites* [30], and the third group (*Metagonimus*, *Stellantchasmus*, *Centrocestus*, and *Pygidioopsis*) uses diverse fish genera, viz. *Lisa*, *Mulgi*, *Tribolodon*, *Lateolabex*, *Cichlasoma*, *Plecoglossus*, and *Salmo* [30–33]. *Haplorchoides* sp. use fish in the family Bagridae (*Hemibagrus filamentus*) as a definitive host.

Morphological characteristics of the ventrogenital complex and the number of testes can be separated into three groups. *H. taichui* and *H. pumilio* have single testes and a modified ventrogenital sac. *Metagonimus*, *Centrocestus*, *Stellantchasmus*, *Metagonimus*, and *Pygidioopsis* have two testes and a simple ventrogenital sac. *Haplorchoides* has a single testis and a modified ventrogenital sac. The identification of heterophyid trematodes should consider

the intermediate host of parasites, the number of testes, and the modification of the ventrogenital sac. Surprisingly, the geographic distribution of *Metagonimus* spp. includes Korean and Japan, while it has not been previously reported from Thailand. *Metagonimus* and other heterophyid trematodes do not have a similar geographic distribution nor ecological conditions. *H. taichui* and *H. pumilio* are to be separated into related but different groups on the basis that the location of the second intermediate host is different. Hence, our findings agree with Radomyos *et al* who reported that *H. taichui* is widely distributed and occurs with high prevalence in the northern provinces of Thailand while the geographic distribution of *H. pumilio* is restricted in lower part of northern Thailand^[34].

The ML and JN trees show the groupings of multiple species as being closely related to *H. taichui*. The bootstrapping of sequences with both trees indicates significant support for this grouping.

The phylogenetic and systematic biology of heterophyid trematodes can be determined by a molecular approach using mCOI for DNA barcoding. Hence, the mtCOI sequences can demonstrate their relationship. Three groups were defined according to systematic criteria.

Conflict of interest statement

We declare that we do not have of conflict of interest.

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References

[1] Watthanakulpanich D, Waikagul J, Muipanich W, Nuamtanong S, Sanguankiat S, Puhampen S, et al. *Haplorchis taichui* as a possible etiologic agent of irritable bowel syndrome-like symptoms.

Korean J Parasitol 2010; 48(3): 225–229.

[2] Sato M, Sanguankiat S, Puhampen S, Kusolsenk T, Muipanich W, Waikagul J. Egg laying capacity of *Haplorchis taichui* (Digenea: Heterophyidae) in human. *Korean J Parasitol* 2009; 47(3): 315–318.

[3] Chai JY, Yong TS, Eom KS, Min DY, Shin EH, Banoung V, et al. Prevalence of the intestinal flukes *Haplorchis taichui* and *H. yokogawai* in a mountainous area of Phongsaly Province, Lao PDR. *Korean J Parasitol* 2010; 48(4): 339–342.

[4] Skov J, Kania PW, Dalsgaard A, Jørgensen TR, Buchmann K. Life cycle stages of heterophyid trematode in Vietnamese freshwater fishes traced by molecular and morphometric methods. *Vet Parasitol* 2009; 160(1): 66–75.

[5] Dzikowski R, Levy MG, Poore MF, Flowers JR, Paperna I. Use of rDNA polymorphism for identification of *Heterophyidae* infecting freshwater fishes. *Emerg Infect Dis* 2004; 59(1): 35–41.

[6] Clausen JH, Madsen H, Murrell KD, Van PT, Thu HNT, Dung DT, et al. Prevention and control of fish-borne zoonotic trematodes in fish nurseries, Vietnam. *Emerg Infect Dis* 2012;18(9): 1438–1445.

[7] Van KV, Phan VT, Ernbjell AK, Nguyen T, Nguyen KV, Nguyen HT, et al. Freshwater aquaculture nurseries and infection of fish with zoonotic trematode, Vietnam. *Emerg Infect Dis* 2010; 16(12): 1905–1909.

[8] Thuengkham U, Dekumyoy P, Komalamisra C, Sato Megumi, Dung DT, et al. Systematics of the subfamily *Haplorchiinae* (Trematoda: Heterophyidae), based on nuclear ribosomal DNA gene and ITS2 region. *Parasitol Int* 2010; 59(1): 460–465.

[9] Wongsawad P, Wongsawad C. DNA fingerprints of some heterophyid trematodes from adult and metacercarial stages in Thailand. *Southeast Asian J Trop Med Public Health* 2007; 38(suppl 1): 110–114.

[10] Chuboon S, Wongsawad C. Molecular identification of larval trematode in intermediate hosts from Chiang Mai, Thailand. *Southeast Asian J Trop Med Publ Health* 2009; 40(1): 1216–20.

[11] Wongsawad C, Wongsawad P, Chai JY, Anuntalabhochai S. *Haplorchis taichui*, Witenberg, 1930: Development of a HAT-RAPD marker for the detection of minute intestinal fluke infection. *Exp Parasitol* 2009; 123(1): 158–161.

[12] Chontanarath T, Wongsawad C. Prevalence of *Haplorchis taichui* in field-collected snails: A molecular approach. *Korean J Parasitol* 2010; 48(1): 343–346.

[13] Chontanarath T, Wongsawad C. *Haplorchis taichui* infection of the freshwater snails and molecular identification. *Trend Res Sci & Tec* 2010; 2(1): 7–12.

[14] Wongsawad C, Rojtinnakorn J, Wongsawad P, Rojanapaibul A, Marayong T, Suwattanacupt S, et al. Helminthes of vertebrate in Mae Sa stream, Chiang Mai, Thailand. *Southeast Asian J Trop Med Public Health* 2004; 35(1): 140–147.

[15] Kumchoo K, Wongsawad C, Chai JY, Vanittanakom P,

- Rojanapaibul A. High prevalence of *Haplorchis taichui* metacercariae in cyprinoid fish from Chiang Mai province, Thailand. *Southeast Asian J Trop Med Public Health* 2005; 36(2): 451–455.
- [16] Nithikathkul C, Wongsawad C. Prevalence of *Haplorchis taichui* and *Haplorchoides* sp. metacercariae in freshwater fish from water reservoirs, Chiang Mai, Thailand. *Korean J Parasitol* 2008; 46(1): 109–112.
- [17] Anna M, Sean AL, Daniel M, David JM, Teresa JC. Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminthes. *Mol Eco Res* 2009; 9(1): 75–82.
- [18] Travessa D, Costanzo F, Iorio R, Aroch T, Lavy E. Mitochondrial cytochrome c oxidase subunit I (COX1) gene sequence of *Sporocerca lupi* (Nematoda, Spirurida): avenues for implications. *Vet Parasitol* 2007; 146(1): 263–270.
- [19] Saijunha W, Sithithaworn P, Wongkham S, Laha T, Chilton NB, Petney TN, et. al. Mitochondrial DNA sequence variation among geographical isolates of *Opisthorchis viverrini* in Thailand and Lao PDR, and phylogenetic relationships with other trematodes. *Parasitol* 2008; 135(1): 1479–1486.
- [20] Park GM. Genetic comparison of liver fluke, *Clonorchis sinensis* and *Opisthorchis viverrini*, based on rDNA and mtDNA gene sequence. *Parasitol Res* 2007; 100(1): 351–357.
- [21] Sato Y, Le TH, Hiraike R, Yukawa M, Sakai TC, Rajapakse RPV, et al. Mitochondrial DNA sequence and gene order of the Sri Lankan *Schistosoma nasale* is affiliated to the African/Indian group. *Int Parasitol* 2008; 57(1): 450–464.
- [22] Dung DT, Hop NT, Theankham U, Waikagul J. Genetic difference among Vietnamese *Haplorchis taichui* populations using The COI genetic marker. *J Helmintho* 2012; 6(1): 1–5.
- [23] Lee SU, Huh S, Sohn W, Chai JY. Sequence comparisons of 28S ribosomal DNA and mitochondrial cytochrome c oxidase subunit I of *Metagonimus yokogawai*, *M. takahashi* and *M. miyatai*. *Korean J Parasitol* 2004; 42(3): 129–135.
- [24] Theankham U, Visetsak K, Dung DT, Waikagul J. Discrimination of *Opisthorchis viverrini* form *Haplorchis taichui* using COI sequence marker. *Acta Trop* 2007; 103(1): 26–32.
- [25] Srisawangwong T, Sithithaworn P, Tesana S. Metacercariae isolated from cyprinoid fishes in Khon Kaen district by digestion technique. *Southeast Asian J Trop Med Public Health* 1997; 28(1): 224–226.
- [26] Yu JR, Chung JS, Huh S, Lee SH, Chai JY. PCR-RFLP patterns of three kinds of *Metagonimus* in Korea. *Korean J Parasitol* 1997; 35(4): 271–276.
- [27] Van KV, Dalsgaard A, Blair D, Le TH. *Haplorchis pumilio* and *H. taichui* in Vietnam discriminated using ITS-2 DNA sequence data from adult and larvae. *Exp Parasitol* 2009; 123(1): 146–151.
- [28] Wongsawad C, Wongsawad P, Chuboon S, Anuntalabhochai S. Copro-diagnosis of *Haplorchis taichui* infection using sedimentation and PCR-based methods. *Southeast Asian J Trop Med Public Health* 2009; 40: 924–928.
- [29] Diaz MT, Hernandez LE, Bashirullah AK. Studies on the life cycle of *Haplorchis pumilio* (Looss, 1896) (Trematode: Heterophyidae) in Venezuela. *Rev Cient* 2008; 17(1): 35–42.
- [30] Rim HJ, Sohn WM, Yong TS, Keeseon SE, Chai JY, Min DY, et.al. Fishborne trematode metacercariae detected in freshwater fish from Vientiane municipality and Savannakhet Province, Lao PDR. *Korean J Parasitol* 2008; 46(4): 253–260.
- [31] Tomas S, Guillermo SM. The introduction and dispersal of *Centrocestus formosanus* (Nishigiri 1924) (Digenea: Heterophyidae) in Mexico: A review. *Ame Mid Nat* 2000; 143(1): 185–200.
- [32] Shin EH, Guk SM, Kim HJ, Lee HY, Chai JY. Trends in parasitic diseases in the Republic of Korea. *Trans Parasitol* 2008; 24(3): 143–150.
- [33] Chai JY, Murrell KD, Lamberty AJ. Fish-borne parasitic zoonoses: status and issues. *Int J Parasitology* 2005; 35(1): 1233–1254.
- [34] Radomyos B, Wongsaroj T, Wilaratana P, Radomyos P, Praevanich R, Meesomboon V, et al. Opisthorchiasis and intestinal fluke infections in Northern, Thailand. *Southeast Asian J Trop Med Public Health* 1998; 29(1): 123–127.