Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60072-9

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

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ARTICLE INFO

*Article history:*
Received 10 September 2013
Received in revised form 15 December 2013
Accepted 15 March 2014
Available online 20 June 2014

*Keywords:*
Phylogenetic relationship
mCOI
Heterophyidae

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 × 2.0 program. A phylogenetic tree was constructed by maximum likeinghood (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 mtCOI 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]. Heterophydiasis 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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Foundation project: This study was supported by the Graduate School, Chiang Mai University.

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 (Henicorhynchus 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′ TTTTTTGGGCATCCTGACGTTTAT 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 $MgCl_2$, 200 μM of each dNTPs, and 0.5 unit of *Taq* DNA polymerase. The amplification procedure involved an initial denaturation step at 95 ℃ for 3 min, then 40 cycles including denaturation at 95 ℃ for 1 min, primer annealing at 50 ℃ for 1 min, extension at 72 ℃ for 1 minute, and final extension at 7 ℃ 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 likeinghood (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 |
|---|---|---|
| *H. taichui* | Chiang Mai, Thailand | This study |
| *H. taichui* | Thanh Hoa, Viet Nam | JN809909 |
| *H. taichui* | Quang Tri, Viet Nam | JN809893 |
| *H. taichui* | Ha Giang, Viet Nam | JN809875 |
| *H. taichui* | Chumporn, Thailand | EF055885 |
| *H. pumilio* | Bangkok, Thailand | This study |
| *C. caninus* | Chiang Mai, Thailand | This study |
| *S. falcatus* | Chiang Mai, Thailand | This study |
| *H.* sp. | Chiang Mai, Thailand | This study |
| *M.* sp. | Korea | AF096232 |
| *M. yokogawai* | Korea | AB470519 |
| *M. takahashii* | Korea | AF096231 |
| *P. summa* | Korea | AF181884 |
| *F. hepatica* | 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 *Pygidiopsis*). 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.

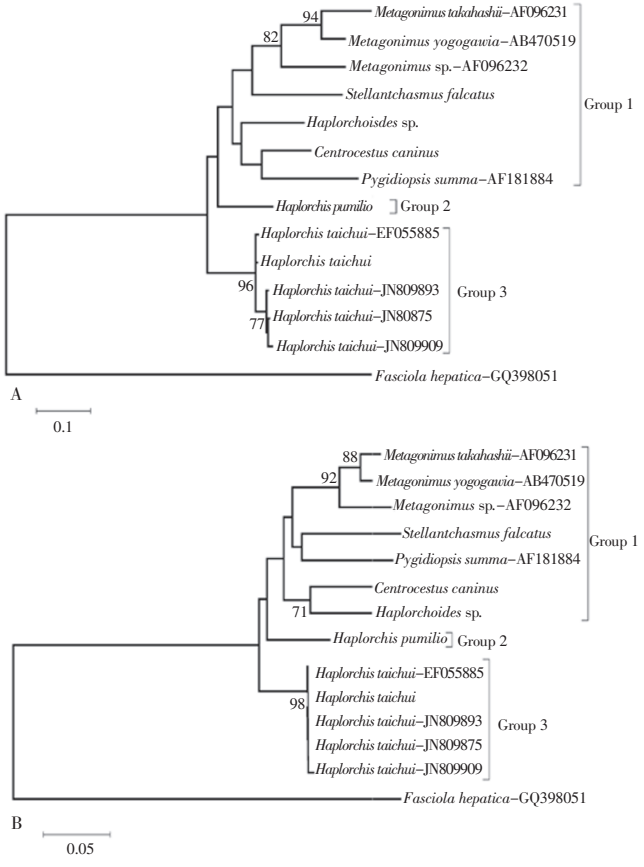


**Figure 1.** The rooted phylogeny form partial mCO Ⅰ sequences of heterophyid trematodes base on the Kimura two-parameter model. Bootstrap values were computed independently for 1 000 resembling. (A) tree form ML method (B) tree form NJ method.

## 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[25,26–28]. 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 *Pygidopsis*) 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 *Pygidopsis* 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.

## Acknowledgements

This study was supported by the Graduate School, Chiang Mai University. We greatly appreciate the Applied Parasitology Research Laboratory, Institute for Science and Technology Research and Economic Plants Genome Research and Service Center, Chiang Mai University, Thailand for providing facilities. Special thanks are given to Dr. Chatmongkon Suwannapoom and Mr. Kittisak Buddhachat for their helpful and suggestions. Finally, we would like to thank Dr. Peter Freeouf and Dr. J.F. Maxwell for editing our manuscript.

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