

**Molecular Identification of Trematode,
Haplorchis taichui Cercariae
(Trematoda: Heterophyidae) in *Tarebia granifera*
Snail Using ITS2 Sequences**

Suksan Chuboon* Chalobol Wongsawad* and Pheravut Wongsawad*

Abstract

Minute intestinal fluke, *Haplorchis taichui*, remain clinical importance, especially in the north-eastern and northern regions of Thailand. For obtaining an effective epidemiological control program, a sensitive, accurate, specific detection are required. Sequences of Internal transcribed Spacer subunit 2 were performed to identify cercarial trematodes in snail intermediate hosts. The results showed that phylogram depicting phylogenetic relationships was constructed based on combined sequence data of ITS2 and showed that, 2 distinct clusters were formed by first containing with the group of middle-larger sizes trematode while the remaining was minute size with all of them were belonged to family Heterophyidae. Within the minute size cluster, *H.taichui* and *Pleurolophocercous cercaria* were placed in the same branch which can confirm the identities of PC found in this study that they will be developed and identified to *H. taichui*. Additionally, results obtained in this study were effective to determine the presence of parasites in snail intermediate hosts that can be use for epidemiological monitoring, preventing management and control program.

Keywords : Molecular identification Trematode Cercariae *Tarebia granifera*
ITS2 Sequence

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การระบุชนิดเชิงโมเลกุลของพยาธิใบไม้ *Haplorchis taichui*
ระยะเชอร์คาเรีย (Trematoda: Heterophyidae)
ในหอย *Tarebia granifera*
โดยใช้ลำดับนิวคลีโอไทด์บริเวณ ITS2

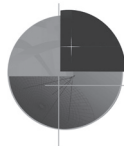
สุขสรรค์ ชูบุญ* ชโลบล วงศ์สวัสดิ์* และ พิระวุฒิ วงศ์สวัสดิ์*

บทคัดย่อ

พยาธิใบไม้ลำไส้ขนาดเล็ก *Haplorchis taichui* ยังคงมีความสำคัญทางการแพทย์ โดยเฉพาะในภาคตะวันออกเฉียงเหนือ และภาคเหนือของไทย การควบคุมการระบาดที่มีประสิทธิภาพต้องการวิธีการตรวจสอบที่มีความจำเพาะเจาะจงและแม่นยำสูง ลำดับนิวคลีโอไทด์บริเวณ Internal transcribed Spacer subunit 2 (ITS2) จึงถูกนำมาใช้ในการจำแนกตัวอ่อนพยาธิระยะเชอร์คาเรียในหอยที่เป็นโฮสต์กึ่งกลาง ผลการศึกษาพบว่า phylogram ที่แสดงความสัมพันธ์เชิงวิวัฒนาการจากข้อมูลลำดับนิวคลีโอไทด์ของดีเอ็นเอบริเวณ ITS2 สามารถแบ่งกลุ่มของพยาธิออกเป็น 2 กลุ่ม ประกอบด้วยพยาธิใบไม้ขนาดกลางถึงขนาดใหญ่ และพยาธิใบไม้ขนาดเล็ก ซึ่งพยาธิทั้งหมดในกลุ่มนี้จัดอยู่ในกลุ่มพยาธิใบไม้ลำไส้ขนาดเล็กในวงศ์ Heterophyidae และภายในกลุ่มนี้พบว่า *H. taichui* และ *Pleurolophocercous cercariae* จัดอยู่ในแขนงเดียวกัน ซึ่งเป็นการยืนยันว่า *Pleurolophocercous cercariae* จากหอย *T. granifera* ที่พบจากการศึกษาครั้งนี้ จะพัฒนาไปเป็นพยาธิ *H. taichui* ผลการศึกษานี้มีประสิทธิภาพในการจำแนกตัวอ่อนพยาธิใบไม้ระยะเชอร์คาเรียในหอย ซึ่งเป็นประโยชน์ในการติดตามตรวจสอบและการควบคุมระบาดวิทยาของพยาธิใบไม้ต่อไปได้

คำสำคัญ : การระบุเชิงโมเลกุล พยาธิใบไม้ เชอร์คาเรีย หอยเจดีย์ ลำดับนิวคลีโอไทด์

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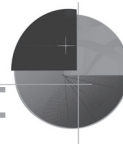
Introduction

In Thailand, in addition to Opisthorchiasis that seems to be the most common food-borne trematode infection in the northeastern part (1-2). Heterophyiasis caused by the infection of heterophyid flukes is widely distributed and also occupied over all parts of Thailand including neighboring countries in Southeast Asia. *Haplorchis taichui*, *Stellantchasmus falcatus* and *Centrocestus caninus*, has been reported as common flukes found in the northern region (3-4). High prevalence of *H. taichui* metacercaria in cyprinid fish has been documented by it can infect to a wide ranges of fish species and also resulting a high intensity of infection in Siamese Mud Carp, *Henicorhynchus siamensis* (4-5). While some of other heterophyid flukes, *H. pumilio*, *H. yokogawai* and *Procerrovum varium* are preferably found in the central part than in the north. Humans acquire these flukes by consuming improperly cooked fish containing infective metacercaria (4, 6). Encapsulated eggs of *Haplorchis* species can be deposited in spinal cord and brain tissues of humans (7). Host immune response to *H. taichui* infection in rat with significantly increasing of mucosal mast cells (MMC)s has been described (8). In this context, heterophyid trematodes still remain their clinical importance and have been suggested to be causative agent of public health concern problems worldwide.

A number of PCR-based molecular approaches have been developed to identify several trematode species in every life stages forms. Specific DNA probes/primers have been

developed to detect *O. viverrini* in hamsters and human stool (9-10), and in fish (11), to detect *Fasciola gigantica* infection in snails (12) and *F. hepatica* in field-collected *L. columella* and *L. viatrix* snails (13). Multiplex-PCR has been successfully developed for detection of *F. hepatica* in *Lymnaea columella* snail (14) and for discriminate *C. sinensis* and *O. viverrini* (15). PCR diagnosis targeting rDNA has been investigated to discriminate *O. viverrini*, *Clonorchis sinensis*, *H. taichui*, and *H. pumilio* (16), mitochondrial COI sequence marker has been introduced to separate *O. viverrini* and *H. taichui* (17) and ITS2 sequences have attempted to determine life cycle stages of heterophyid trematode in Vietnam (18). Random amplified polymorphic DNA (RAPD) analysis is an approach for DNA fingerprinting and for designing specific primers as well as Sequence Characterized Amplifying Region (SCAR-marker) which no prior DNA sequence information is needed. Increasing the annealing temperature to 46–48°C results in greater polymorphism, higher reproducibility and higher resolution (19) reported that. This method has been used in numerous studies such as genetic variation of *O. viverrini* from north-east Thailand and Laos PDR (20), molecular identification of larval trematodes in Chiang Mai, Thailand (21), a phylogenetic study of *Stellantchasmus falcatus* (22), molecular detection of *H. taichui* (23) and to identify human pathogenic heterophyid trematode metacercariae by PCR-RFLP (24).

Our study aimed to investigate habitat/ ecological preference and to design specific



primers using SCAR-marker based on fragments generated by the HAT– RAPD PCR which are potentially useful for *H. taichui* detection and identification and for long-term monitoring of transmission foci in epidemic areas.

Methods

Four trematode species were used in this study where 3 metacercarial species; *H. taichui*, *S. falcatus* and *O. viverrini* were collected from the Siamese's Mud Carp (*Henicorhynchus siamensis*), the Half-beak (*Dermogynus pusillus*) and the White-eyed Barb (*Cyclocheilichthys armatus*), respectively by using a digestion technique with 1% pepsin solution and then were force-fed to an experimental host 1 day-old chicks (*Gallus gallus domesticus*), except for the liver fluke, which was force-fed to hamsters (*Mesocricetus auratus*), to obtain the adult stage.

Adults of *Ganeo tigrinus* were gathered from frogs (*Limnonectes limnocharis*). ITS2 sequences of 5 trematode species acquired from Genbank were integrated for determining phylogram as same as for identify trematode cercariae by comparing with their adult stage.

Total genomic DNA extraction

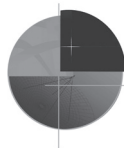
All adult trematodes and cercariae collected were subjected for Genomic DNA extraction using the GF-1 Tissue DNA extraction Kit (Vivantis, Malaysia), according to the manufacturer's instructions. The *H. taichui* - specific fragments obtained from HAT–RAPD PCR were purified from agarose gel, using the GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia).

All extracted genomic DNA was diluted to a working concentration of 40 ng/μl and stored at -20°C until used.

Amplification of ITS2 region

The reaction was carried out in a final volume of 20 μl by using primer pair of 3SF (5'-GGTACCGGTGGATCACTCGGCTCGTG-3') and BD2R (5'TATGCTTAAATTCAGCGGGT-3'). Reaction mixture containing 2 mM of each ribonucleotide triphosphate, 2 μl of 10X reaction buffer, 2 μM of primer 20 ng of DNA template and 1 U of Taq DNA polymerase (Vivantis, Malaysia). The reactions were performed in a MyCycler™ Thermocycler (Bio-RAD) and the PCR conditions used were as follows: 1 cycle of 95°C for 2 min, 40 cycles of 95°C for 45 sec, 50°C for 45 sec, 72°C for 1 min and 1 cycle of final extension at 72°C for 7 min. PCR products were separated on 1.4% TBE agarose gel electrophoresis, stained with ethidium bromide, and photographed by a Kodak digital camera (Gel Logic 100).

All ITS2 fragments obtained were then purified and therefore subjected for sequencing directly by without cloned and transformed. Based on the sequence data, they were trimmed to provide an equivalent sequences among each trematode species and then were aligned using multiple sequence alignment method in available online ClustalW software (www.align.genomes.jp). They were then aligned and compared to ITS2 databases available in Genbank using Basic Local Alignment Search Tools (BLASTN).



Results

The results showed that, the 495 bp PCR product of ITS2 region was generated in all 4 trematode species including *Pleurolophocercous cercariae* with a few over hanging in fragment sizes between species as showed in Figure 1.

Based on ITS2 sequence data and the BLASTN results obtained in this study, the result showed that, it could be confirmed that, ITS2 sequences found in this study were identically satisfied by revealing high alignment scores (>200), *Pleurolophocercous cercariae* showed maximum identities to *H. taichui* adult worm with high alignment scores by revealing 265 scores, identities 94% (309/329 bp) with expected values of 3e-135 compared with database available in Genbank, (Figure 2).

Sequences of ITS2 region obtained in this study were consequently assembled to phylogenetic analyses using an unweighted pair group method using arithmetic averages (UPGMA) analysis. Phylogram depicting

phylogenetic relationships was constructed based on combined data set for all primers used and showed that 2 distinct clusters were formed by first containing with the group of middle-larger sizes trematode while the remaining was minute size with all of them were belonged to family Heterophyidae (Figure 3). Within the minute size cluster, *H.taichui* and *Pleurolophocercous cercariae* were placed in the same branch which can confirm the identities of *Pleurolophocercous cercariae* collected from *T. granifera* snail will developed and identified to be *H. taichui* (Figure 3).

Discussion

Currently, Heterophyiasis are the most common flukes found in Thailand and neighboring countries. Although, our result can validate the valuable supplemented data that required for understanding of the epidemiological promotion factors which can providing an effective control program.

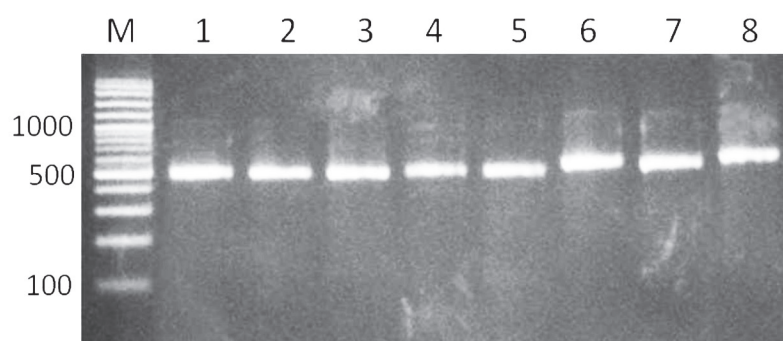
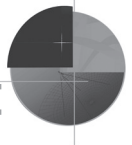


Figure 1 Agarose gel electrophoresis determine amplification of Internal Transcribed Spacer subunit 2 (ITS2) region Lane M: 100 bp ladder, 1: *Pleurolophocercous cercariae* (from *T. granifera* snail), 2-3: *H. taichui* adult, 4-5: *S. falcatus* adult, 6-7: *O. viverrini* adult, 8: *G. tigrinus* adult

**Pleurolophocercous cercariae**

Score = 490 bits (265), Expect = 3e-135
 Identities = 309/329 (94%), Gaps = 0/329 (0%)
 Strand=Plus/Plus

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Query 2   AAATAGTCCNGGCTTGGGTCTTGCCAGCTGGCGTGAATTCCTTGTGCAATGCATAGGGT 61
          |||
Sbjct 22  AAATAGTCCNGGCTTGGGTCTTGCCAGCTGGCGTGAATTCCTTGTGCTTTTGCATAGGGT 81

Query 62  GCCAGATCTATGGCATTACCTAATGTGCGGACGCAACCATGTCTCGGCTGACGGCCTC 121
          |||
Sbjct 82  GCCAGATCTATGGCTTTTCCCTAATGTGCGGACGCAACCATGTCTGGGCTGACGGCCTG 141

Query 122 GATGAGGAAGTGGCGGAGTCGTGCTCAATGTAAATGTGCGCGCTCCTAAGCTTAA 181
          |||
Sbjct 142 GATGAGGAAGTGGCGGAGTCGTGCTCAATGTAAATGTGCGCGCTCCTAAGCTTAA 201

Query 182 CGTCTGTCTTGCTGACGGCTTGGATGAGGAAGCGGCGGCGGAGTCACGCTCAATGATTA 241
          |||
Sbjct 202 CCTCTGTCTTGCTGACGGCTTGGATGAGGAAGCGGCGGCGGAGTCACGCTCAATGAAA 261

Query 242 TTGTGCGCGCTCCAAAGCTTAACCTCTGTCTGAGCTGACGGCTAGGACATGGCANTGCAT 301
          |||
Sbjct 262 TTGTGCGCGCTCCAAAGCTTAACCTCTGTCTGAGCTGACGGCTAGGACATGGCANTGCAT 321

Query 302 CCGATCGAATTCAATGTGCACITGAATGT 330
          |||
Sbjct 322 CCGATGCAATTCTTTGTGCACITGAATGT 350
  
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Figure 2 Alignment of Internal Transcribed Spacer subunit2 (ITS2) gene sequences of *Pleurolophocercous cercariae* compared with subjects (*H. taichui* adult worm Accession Number GQ176379.1) available in Genbank

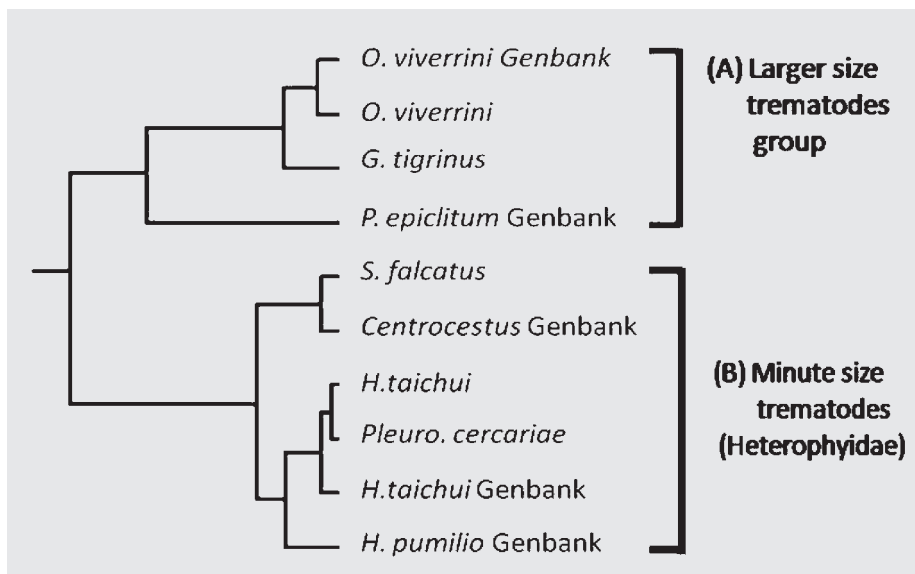
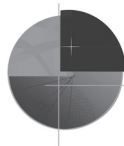


Figure 3 Phylogram derived from an UPGMA analysis depicting phylogenetic relationships of trematodes used in this study by basing on ITS 2 sequences



Discrimination and identification of cryptic fluke species have always been problematic due to the morphological similar and resemble particularly in egg and larval stages in their transmission sites. Molecular approaches can validate the executive diagnostic tools and overcome these limitations. Our results correspond to several molecular approaches that has been developed for these confusion, viz PCR targeting rDNA has been investigated to discriminate *O. viverrini*, *Clonorchis sinensis*, *H. taichui* and *H. pumilio* (16), COI sequence marker has been introduced to separate *O. viverrini* and *H. taichui* (17) and ITS2 sequences to determine life cycle stages of heterophyid trematodes in Vietnam (18). Although, these methods conducted on gene sequencing which required more facilities, budget and much time consumption, but they can yield maximum identities at nucleotide level. This phenomenon confirms a high magnitude ITS2 sequences for identification aspect of such larval trematodes in their transmission site atmosphere. Contrasting to the HAT-RAPD profiles that has been introduced frequently in a number of reports described previously (6), that attempted to identify *Pleurolophocercous cercariae*, the major cercarial type infected in *T. granifera* snail to *H. taichui* by providing similar DNA patterns as same as they were clustered in the same branch in UPGMA dendrogram. This mentioned method seems to appropriate for investigate genetic variation of such parasites as described previously (19-20,

22) including specific detection as well as SCAR marker derived from HAT-RAPD method (23). Randomly annealed between primer and DNA template may affect stabilities of reaction and profiling results.

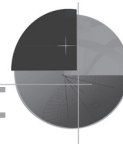
Our results provide beneficial applications for demonstrating the epidemiology and detection of cercariae in snail intermediate hosts as biological control and monitoring of parasitic disease, particularly at transmission site level. Thus it is useful for prevention, management and epidemiological control programs.

Acknowledgments

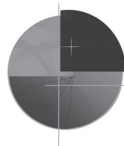
Special thanks are given to the Applied Technology in Biodiversity Research Unit, Institute for Science and Technology Research, Chiang Mai University. Greatful thanks are extended to The Applied Parasitology Research Laboratory and The Economic Plant Genomes Research and Service Center, Department of Biology, Faculty of Science, Chiang Mai University for instrumental available.

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