Genetic Analysis of *Stellantchasmus falcatus* on HAT-RAPD Technique

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ABSTRACT

Twenty random primers were selected to detect the molecular marker in *Stellantchasmus falcatus* with other four heterophyid flukes *(Haplorchis taichui, Centrocestus caninus, Metagonimus yokogawai* and *Haplorchoides* sp.) and liver fluke *(Opisthorchis viverrini*) by using high annealing temperature random amplification of polymorphic DNA (HAT-RAPD) technique. In total, HAT-RAPD had generated 354 genetic markers of which only 0.56 % was shared by the six species studied and separated the six species studied into two major clusters: *S. falcatus, H. taichui, C. caninus* and *M. yokogawai* were grouped together; the second cluster is *Haplorchoides* sp. with *O. viverrini* as outgroup.

Keywords : *Stellantchasmus falcatus* HAT-RAPD molecular marker

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การวิเคราะห์พันธุกรรมของพยาธิใบไม้ *Stellantchasmus falcatus* โดยเทคนิค HAT-RAPD

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บทคัดย่อ

การวิจัยนี้ได้ศึกษา molecular marker ของพยาธิใบไม้ *Stellantchasmus falcatus* และพยาธิใบไม้อีก 4 ชนิด ได้แก่ *Haplorchis taichui*, *Centrocestus caninus*, *Metagonimus yokogawa*i, *Haplorchoides* sp. ซึ่งอยู่ในวงศ์ Heterophyidae และพยาธิใบไม้ตับ *Opisthorchis viverrini* ด้วยเทคนิค HAT-RAPD โดยใช้ไพรเมอร์เดี่ยวแบบสุ่ม 20 ไพรเมอร์ ได้แถบดีเอ็นเอ ทั้งหมด 354 แถบ ซึ่งมีเพียง 0.56 เปอร์เซ็นต์ที่พบจากพยาธิทั้ง 6 ชนิดที่ทำการศึกษา และสามารถจัดกลุ่มออกเป็น สองกลุ่มใหญ่ พยาธิใบไม้กลุ่ม *S. falcatus*, *H. taichui*, *C. caninus*, *M. yokogawai* จัดอยู่ในกลุ่มเดียวกัน และพยาธิใบไม้ *Haplorchoides* sp. อยู่ในกลุ่มที่สอง โดยมีพยาธิใบไม้ *O. viverrini* เป็น outgroup

คำสำคัญ : *Stellantchasmus falcatus* HAT-RAPD molecular marker

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Introduction

In Thailand, the heterophyid flukes and opisthorchiid fluke *(Opisthorchis viverrini)* are the common trematodes infecting humans. The infection rate and density of *O. viverrini* metacercariae in fish decreased markedly during the last ten years due to the National Liver Fluke Control Program (1-3). On the other hand, the prevalence of heterophyid metacercariae in fishes tended to increase, also with increase of the prevalence in humans (4). Recent fish surveys in several areas in the northern Thailand revealed that most trematode metacercariae were heterophyid flukes (3,5 - 8). Human infections with heterophyid flukes in Thailand are confined to the subfamilies Haplorchiinae and Centrocestinae; five species of *Stellantchasmus falcatus*, *Haplorchis pumilio, H. taichui, H. yokogawai, Centrocestus caninus* and have been recorded (9,10). *S. falcatus* is one of the heterophyid flukes that were reported in humans in the northern and northeastern Thailand (4,11,12).

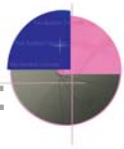
Heterophyid flukes such as *Haplorchis* spp., has been reported as causing intestinal irritation accompanied by colicky pain and mucous diarrhea, with production of excess mucus and superficial necrosis of the mucous coat (13). However, some of heterophyid flukes perhaps wander in the deep layers of the intestinal wall, become imprisoned and die. Their eggs that degenerate are taken up by the lymphatic or blood circulation and are carried to various organs of the body, including the brain, heart and spinal cord. The cardiac, brain and spinal cord lesions were also recorded (14 -16). On the genomic level, many studies have been performed on the opisthorchiid fluke (17,18) while few studies were done on the species of heterophyid flukes. The present study was undertaken to differentiate *S. falcatus* with other four species of heterophyid flukes and the liver fluke and determine the genetic relationships among them based on HAT-RAPD analysis.

Methods

Parasitic materials

Metacercariae of *S. falcatus, Haplorchis taichui, Centrocestus caninus* were collected from half-beaked fish *(Dermogenus pusillus)*, Jullien's mud carp *(Henicorhynchus siamensis)* and goldfish *(Carassius auratus),* respectively, in Chiang Mai and Bangkok provinces. These adult parasites were obtained by orally force fed to mice *(Mus musculus)* or to chick *(Gallus domesticus)* with metacercariae.

Adults of *Metagonimus yokogawai* were obtained from Prof. Jong-Yil Chai, Department of Parasitology and Tropical Medicine, College of Medicine, Seoul Nation University, Korea. Whereas, adults of



Haplorchoides sp. were gathered from yellow mystus *(Hemibagrus filamentus)* from Doi Tao reservoir, Chiang Mai province and adults of *Opisthorchis viverrini* were provided by Assoc. Prof. Paiboon Sithithaworn, Department of Parasitology, Faculty of Medicine, Khon Kaen University.

DNA extraction

Each specimen was placed in a 1.5 ml microcentrifuge tube and followed by extraction using the DNeasy Tissue Kit (QIAGEN). The DNA was eluted in 5 mM Tris-HCl, pH 8.5 and 1 ml was used in the PCR reaction.

HAT-RAPD Reaction

Twenty decamer oligonucleotide primers Kit A (Operon Technologies Inc., California) were screened by polymerase chain reaction (PCR). PCR reactions were performed by using a 20 ml mixture, containing sample DNA (10-25 ng), 1 x QIAGEN PCR buffer, 100 mM of each dNTP, 40 ng of each primer and 0.5 units of Taq DNA polymerase. For DNA amplification, the PCR thermocycler (Perkin Elmer: Gene Amp System 2400) was programmed as follows: incubation at 94^0C for 5 min; 30 cycles at 94^0C for 45 sec, 48^0C for 45 sec, 72^0C for 1 min, followed by one final extension cycle of 7 min at 72^0C. The amplification products were separated by electrophoresis in 1.4% (w/v) agarose gels with 1 x TBE buffer, stained by 0.5 mg/ml of ethidium bromide (EtBr) and photographed under exposure to UV light.

Data analysis

Amplified HAT-RAPD markers were scored as present (1) or absent (0) for each sample. Ambiguous bands that could not be easily distinguished were not scored (19). The similarity of samples was calculated as follow: Similarity = $2\ N_{AB}/N_A+N_B$, N_{AB} is the number of bands shared by individuals A and B, N_A and N_B are the number of bands in individuals A and B, respectively Phylogenetic relationships among 6 species were analyzed using parsimony method and bootstrap analysis in the PAUP program (version 4.0b10).

The total number of amplified bands per primer varied from 0 (OPA-05) to 31 (OPA-04). Most of the differences among the genetic profiles of these species were provided by primers OPA-04, OPA-07, OPA-10, OPA-13 and OPA-18 (Figure 1). In total, HAT-RAPD had generated 354 genetic markers of which only 0.56% was shared by the six species studied. The most polymorphic genetic profiles for *S. falcatus* were generated by the following primers, OPA-01 with 3 of the 22 fragments amplified being polymorphic, OPA-03 with 3 of the19 amplified, OPA-07 with 3 of the 24 amplified and OPA-18 with three out of the 25. For *H. taichui,* the most polymorphic marker was produced by the primer OPA-15 with 5 of the

Results

HAT-RAPD fingerprints

The HAT-RAPD technique produces 6 species with all tested primers except very different genetic profiles among OPA-05 (Table 1).

Table 1 Nucleotide sequence of primers used for generating RAPD markers among six studied species.

Primers	Sequence $5^{/}\rightarrow3^{/}$	Number of total	Number of polymorphic Markers
OPA-01	CAGGCCCTTC	22	12
OPA-02	TGCCGAGCTG	23	17
OPA-03	AGTCAGCCAC	19	12
OPA-04	AATCGG GCTG	31	17
OPA-05	AGGGGTCTTG	0	0
OPA-06	GGTCCCTGAC	1	1
OPA-07	GAAACGGGTG	24	17
OPA-08	GTGACGTAGG	14	13
OPA-09	GGGTAACGCC	19	9
OPA-10	GTGATCGCAG	23	6
OPA-11	CAATCGCCGT	16	10
OPA-12	TCGGCGATAG	8	8
OPA-13	CAGCACCCAC	25	12
OPA-14	TCTGTGCTGG	8	8
OPA-15	TTCCGAACCC	16	10
OPA-16	AGCCAGCGAA	21	9
OPA-17	GACCGCTTGT	19	11
OPA-18	AGGTGACCGT	25	11
OPA-19	CAAACGTCGG	20	9
OPA-20	GTTGCGATCC	20	11
Total		354	203

16 amplified products while the OPA-02 was the most for *C. caninus* with 4 out of 23. The most polymorphic genetic profiles for *M. yokogawai, Haplorchoides* sp. and *O. viverrini* were generated by the primer OPA-14, OPA-07 and OPA-08 with 6 of 8, 5 of 24 and 7 of 14 fragments, respectively.

Data analysis

The results obtained with the HAT-RAPD data were consistent with the separation of species. The similarity between *S. falcatus* and *H. taichui, C. caninus, M. yakogawai, Haplorchoides* sp.and *O. viverrini* were 0.269, 0.240, 0.179, 0.140 and

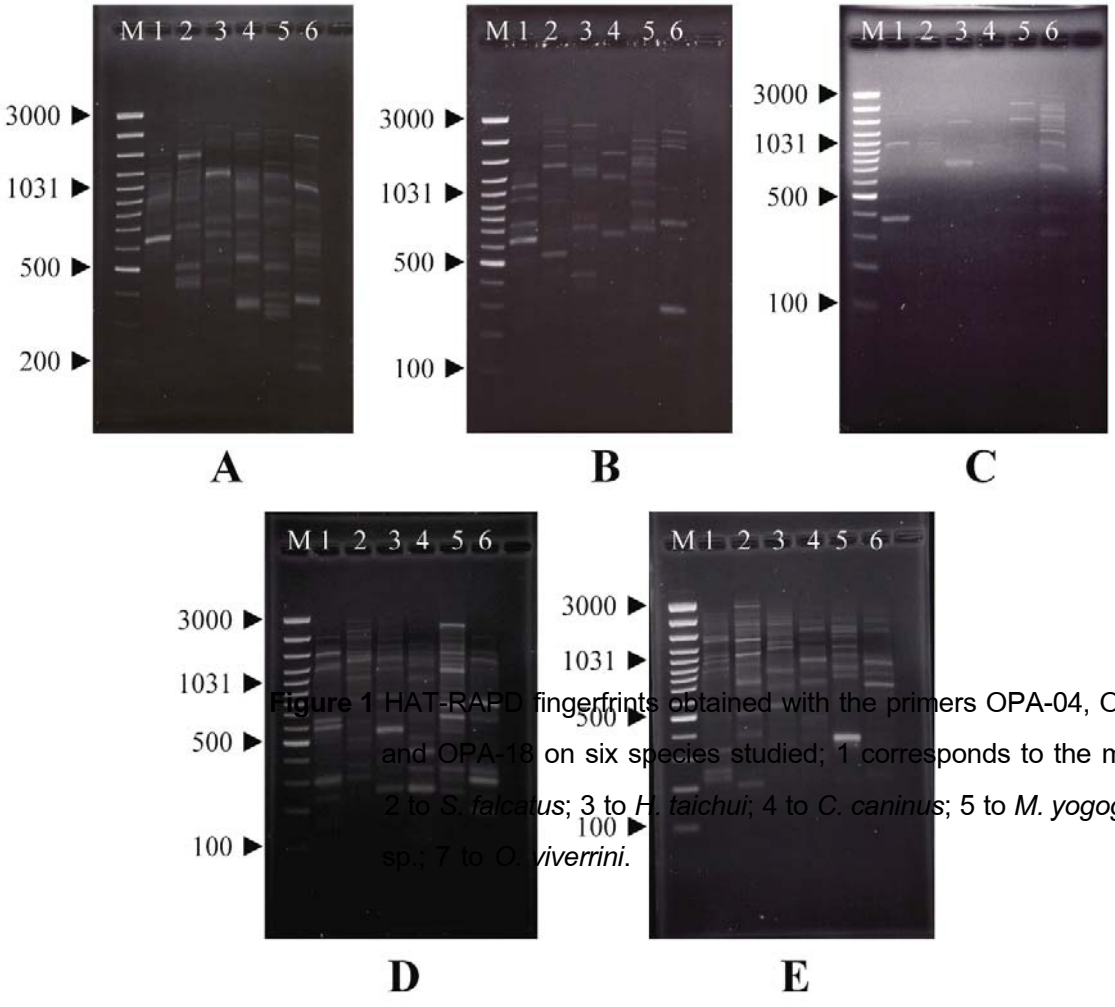


Figure 1 HAT-RAPD fingerfrints obtained with the primers OPA-04, OPA-07, OPA-08, OPA-10 and OPA-18 on six species studied; 1 corresponds to the molecular weight markers; 2 to *S. falcatus*; 3 to *H. taichui*; 4 to *C. caninus*; 5 to *M. yogogawai*; 6 to *Haplorchoides* sp.; 7 to *O. viverrini*.

0.168, respectively. The phylogenetic tree was constructed using the parsimony method with *O. viverrini* as outgroup. The evolutionary relationships obtained with the HAT-RAPD data are shown in Figure 2. The trees grouped the heterophyid fluke together and placed *Haplorchoides* sp. and *O. viverrini* outside the group. This clustering was supported by 80% of bootstrap replicas.

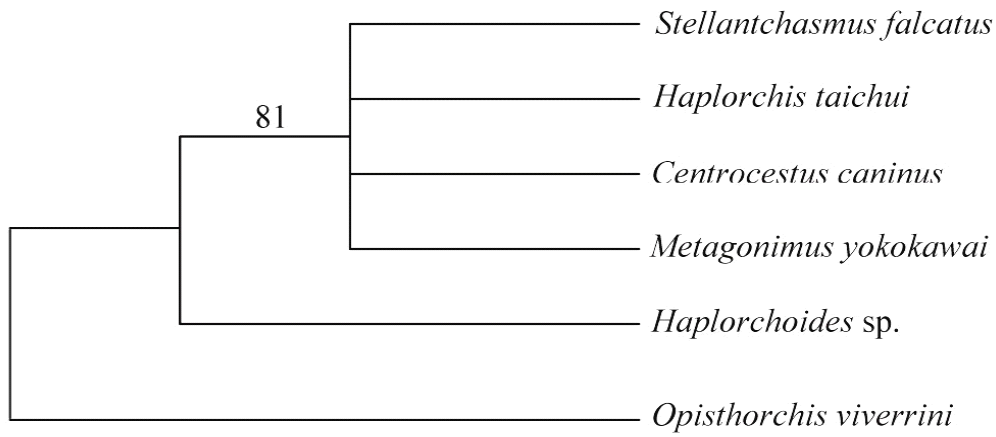
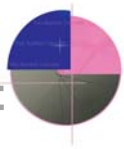


Figure 2 Evolutionary tree (consensus tree) constructed with 354 genetic markers generated with the HAT-RAPD technique using the parsimony method. The number in each bifurcation indicates the number of times the two groups are separated in the 5000 trees constructed with bootstrap analysis with the species *O. viverrini* as an outgroup (CI = 0.704)

Discussion

Among the twenty primers screened, 19 primers produces very different genetic profiles among 6 species studied, whereas OPA-05 was not able to amplify PCR product. Of these primers, the primers OPA-04, OPA-07, OPA-10, OPA-13 and OPA-18 yielded the best product for HAT-RAPD analysis. In total, HAT-RAPD had generated 354 genetic markers varied in size ranging from 185 to 5,200 bp. Among *S. falcatus, H. taichui, C. caninus, M. yakogawai, Haplorchoides* sp. and *O. viverrini,* 77, 109, 98, 79, 95 and 101 HAT-RAPD markers were revealed, with 21, 40, 33, 28, 39 and 42 of specific markers for each species,respectively. The sources of polymorphism in RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification and deletions or insertions that change the size



of a DNA fragment without preventing its amplification (19). The differences in RAPD profiles are determined by a competition between potential priming sites in genomic DNA rather than by the total number of priming sites available (20).

The consensus tree generated with the HAT-RAPD data separated the six species studied into two major clusters with the species *O. viverrini* as outgroup. For the first cluster, the heterophyidflukes were grouped together with supported by 80% of bootstrap replicas. For the second cluster, member was *Haplorchoides* sp. The Within the heterophyid species, *S. falcatus and H. taichui* (0.269) was more similar than (0.240) whereas *S. falcatus* and *M. yokogawai* (0.179) was less similar than *S. falcatus* and *C. caninus.* The similarity between *M. yokogawai* and *C. caninus* (0.237) was smaller than between *H. taichui* and *C. caninus* (0.280). Moreover, it was unexpected the similarity between *S. falcatus* and *O. viverrini* (0.168) and between *C. caninus* and *O. viverrini* (0.181) were higher than between *S. falcatus* and *Haplorchoides* sp. (0.140) and between *C. caninus* and *Haplorchoides* sp. (0.166), respectively. It indicated that *S. falcatus* and *C. caninus* were more similar with *O. viverrini* than *Haplorchoides* sp. The phenomena could not be explained well and might be due to the confusion of the classification of the genus *Haplorchoides* which has still not cleared. Genus *Haplorchoides* was erected and place by in the subfamily Haplorchinae, family Heterophyidae (21). Chatterji took the retrograde step of considering it as a synonym of *Haplorchis* (22), while Yamaguti removed it from the Haplorchinae and placed it in the family Cryptogonimidae (23). Pearson returned it to the family Heterophyidae (24). So the heterophyid flukes should similar with *Haplorchoides* sp. than *O. viverrini* which more different families. However, these heterophyid flukes and *O. viverrini* have reported in human whereas Haplorchoides occurred in fish, rather than birds or mammals (4,10,24,25).

In conclusion, a HAT-RAPD method could be used for distinguishing five species of heterophyid flukes and liver flukes *(Opisthorchis viverrini).* For further study, we will develop the PCR-based technique for the detection *S. falcatus* and other four heterophyid from specific markers of each species from the HAT-RAPD data of this study

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