DEVELOPMENT OF HAT-RAPD MARKER FOR DETECTION OF *STELLANTCHASMUS FALCATUS* INFECTION

Chalobol Wongsawad

Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

Abstract. The trematode, *Stellantchasmus falcatus*, is one of the members in the Family Heterophyidae, which is reported to be an endemic fluke in northern region of Thailand. A sensitive and specific detection of this parasite is required to determine the epidemiology at larval stages. Specific primers to determine the presence of *S. falcatus* were investigated using high annealing temperature random amplified polymorphic DNA (HAT-RAPD) PCR, with 10 arbitrary primers to generate different polymorphic DNA profiles. Eleven parasite species were used for comparison. A 380 bp HAT-RAPD *S. falcatus*-specific marker was found, and was cloned and sequenced, allowing a pair of primers (St-F 5′-GGCCAACG CAATCGTCATCC-3′ and St-R 5′-GCGTCGGGTTTCAGACATGG- 3′) to be designed to produce a 320 bp amplicon specific for *S. falcatus*. It revealed no cross-reaction with any of the other tested parasite species. The *S. falcatus*-specific primers can be used for epidemiological monitoring and for detection in snail intermediate hosts, which serve as usefulness tools in management and epidemiological control programs.

Keywords: *Stellantchasmus falcatus*, HAT-RAPD, detection, trematode

INTRODUCTION

Stellantchasmus falcatus is a minute intestinal fluke in the Family Heterophyidae. Several kinds of mammals (human, rat, cat, dog and chicken) can serve as definitive hosts (Pearson, 1964; Klick and Tamtachumrun, 1974; Pearson and Ow-Yang, 1978), causing clinical problems in most oriental countries. The larval stages, miracidium, sporocyst, redia, and cercaria, are found in freshwater snails, *Melanoides tuberculata*, *Stenomelania newcombi* and *Thiara granifera* (Martin, 1958; Noda, 1959), which represent the first intermediate hosts, whereas metacercaria (infective stage) is mainly found in brackish water or marine fish, which serve as the 2nd intermediate hosts especially in the group of mullet, such as *Mugil* spp (Vazquez-Colet and Africa, 1940; Noda, 1959; Chai and Sohn, 1988) and *Liza* spp (Komiya and Suzuki, 1966; Pubua and Wongsawad, 2007).

Several reports showed that *S. falcatus* metacercariae can also harbor fresh water fish, such as half-beak (*Dermogenys pusillus*) and climbing perch (*Anabas testudineus*) (Sripalwit *et al*, 2003; Wongsawad *et al*, 2004; Pubua and Wongsawad, 2007), which have high infection rates in

Correspondence: Chalobol Wongsawad, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
Tel: +66 (0) 53 943346 ext 1105
E-mail: cwongsawd@yahoo.com

northern Thailand. People acquire this parasite by consuming undercooked food prepared from fish containing metacercariae or through accidental ingestion from water. The symptomatic feature of *S. falcatus* infection is not clearly determined, but the first clinical report revealed that *S. falcatus* was found by autopsy with many embryonated eggs remaining in blood vessels of cardiac muscles (Klick and Tantachumrun, 1974).

Molecular approaches provide effective and accurate tools for the detection and identification of organism and are suitable for screening of genetic variations among living populations. PCR and filter-hybridization have been introduced to detect bird schistosomes cercariae in lakes (Hertel *et al*, 2002), in snail host, and in fecal and water samples (Hamburger *et al*, 1998a,b, 2001), and in plankton samples (Hertel *et al*, 2004). PCR-RFLP has been developed to differentiate Brazilian *Biomphalaria* snails (Vidigal *et al*, 2002), to identify *Schistosoma haematobium* and *S. bovis* in Kenya (Barber *et al*, 2000), to detect liver fluke, *Fasciola hepatica*, cercariae infecting *Lymnaea columella* snails (Magalhaes *et al*, 2004) and for the identification of transmission sites of schistosomiasis (Melo *et al*, 2006). Due to its rapidity, accuracy and specificity, PCR-based methods have also been used in copro-diagnosis for the detection of several parasites, such as *Echinococcus multilocularis* in definitive host (Dinkel *et al*, 1998) and *Opisthorchis viverrini* in humans (Wongratanacheewin *et al*, 2002; Stensvold *et al*, 2006). High annealing temperature random amplify polymorphic DNA (HAT-RAPD) has high resolution and reproducible results (Anuntalabhochai *et al*, 2000). It had been adopted for use in a trematode, *S. falcatus* identification (Sripalwit *et al*, 2003), for the study of DNA quality and quantities of some trematodes (Wongsawad *et al*, 2006), for investigation of intra-specific variations of tree paramphistome flukes in Thailand (Sripalwit *et al*, 2007) and for construction of specific primers for *H. taichui* detection (Wongsawad *et al*, 2009).

This study screened and developed molecular markers for the specific detection of *S. falcatus* among marine and freshwater populations using HAT-RAPD method.

MATERIALS AND METHODS

Parasite preparation

Eight trematode species used in this study were 4 intestinal flukes (*S. falcatus* (both in mullet and half-beaked), *H. taichui*, *Haplorchoides* sp, and *Centrocestus caninus*), 1 liver fluke (*O. viverrini*) and 3 rumen flukes (*Fischoederius elongatus*, *Orthocoelium streptocoelium, Paramphistomum epiclitum*).

HAT-RAPD PCR

Genomic DNA of all parasites was extracted and purified from adult worms using the GF-1 Tissue DNA Extraction Kit (Vivantis, Selangor, Malaysia) according to manufacturer's instruction. Genomic DNA was diluted to a working concentration of 30 ng/µl and stored at -20ºC until use. Ten arbitrary 10-mer primers (Operon technology, Alameda, CA) were used individually in HAT-RAPD PCR and the reaction was carried out in a final volume of 20 µl containing conventional PCR compositions. PCR was conducted in MyCycler™ Thermocycler (Bio-RAD, Hercules, CA) with a thermal cycling protocol of 1 cycle at 95ºC for 5 minutes; 30 cycles at 95ºC for 45 seconds, 48ºC for 45 seconds, 72ºC for 1 minute; and 1 final cycle at 72ºC for 7 minutes. HAT-RAPD PCR products were separated by 1.4% aga-

rose gel-electrophoresis, stained with ethidium bromide and photographed by Kodak digital camera Gel Logic 100.

Cloning and sequencing of HAT-RAPD fragment

S. falcatus-specific fragment was cloned into a pGEM-T Easy Vector (Promega, Madison, WI) and transfected into *Escherichia coli* (DH5α) by electroporation (Bio-RAD, Hercules, CA) and subjected to sequencing through the bio-information service of Genome Institute, Thailand National Science Park, Klong Luang District, Pathum Thani Province.

Design and evaluation of *S. falcatus*-specific PCR primers

S. falcatus-specific primers were designed based on sequence data of a serotype fragment selected from HAT-RAPD marker using Genetyx-MAC ver 11. The pair of designed primers was tested for specificity using all 8 trematode DNA. The optimum PCR condition for *S. falcatus*-specific detection was determined by varying the total amount of $MgCl_2$ and annealing temperature until only the specific PCR amplicon was clearly generated.

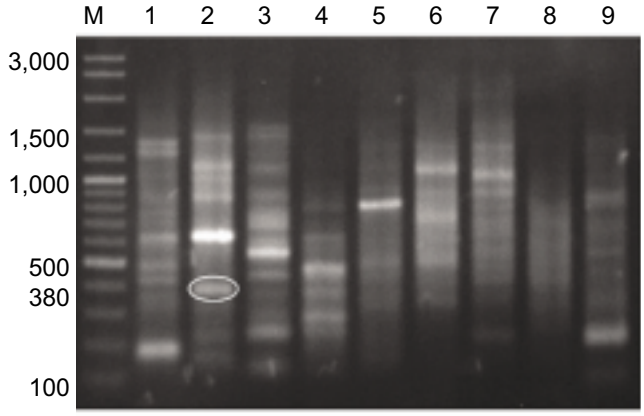

Fig 1–HAT-RAPD–profiles generated by OPA-04 primer. Lane M, DNA markers; 1, *S. falcatus* (in Mullet); 2, *S. falcatus* (in Half-beak); 3, *H. taichui*; 4, *Haplorchoides* sp; 5, *C. caninus*; 6, *O. viverrini*; 7, *O. streptocoelium*; 8, *P. epiclitum*; 9, *F. elongatus.*

AATCGGGCTGACCTTAAAACAGCAATACAAAAA
TTAATGGCCAACGCAATCGTCATCCAGATACATC
GAGCAGCAAGCGATGCAAATATTTTTTCCAGTAT
TTTAAATTTTCAGTTATTCAAGCCGCGTCTGTCAA
TGGTGAAAAAATAGGTAAAGAAATGATCAAAAC
AATTCATTTGAACGTCAGATTTTAATCTACATCAC
CCTGGAAAACTTAAAAACTCTCATGAGCCTGCA
GGTTTCTGCTGAAGCAGCTTTATGCGAACAGGA
TCCTCCCAAATTTAATGTGATTTCTTTGGAATCAT
TCCAAACCAACACATAAGGTTTAGCACCGCGTTC
CCATGTCTGAAACCCGACGCCGCGCTACCTGGG
CTACAGCCCGATTA

Fig 2–Sequence data of 385 bp band generated by OPA-04 primer (underlined) in HAT-RAPD profiles of *S. falcatus*. Sequence and position of *S. falcatus*-specific primer (forward/reverse) are indicated by bold letters.

RESULTS

HAT-RAPD profiles were generated using 10 arbitrary primers showed that a 380 bp amplicon generated from OPA-04 was *S. falcatus*-specific (Fig 1). This fragment was excised, purified and cloned into a pGEM-T Easy Vector for transfection into *E. coli* (DH5α) by electroporation. The insert was subjected to sequencing and sequence of this 380 bp fragment is shown in Fig 2.

A pair of primers designed from this *S. falcatus*-specific-sequence resulted in-(forward) St-F 5′-GGCCAACGCAAT CGTCATCC -3′ and (reverse) St-R 5′-GCG TCGGGTTTCAGACATGG -3′, yielding a product size of 320 bp. The optimal PCR conditions for the specific amplifica-

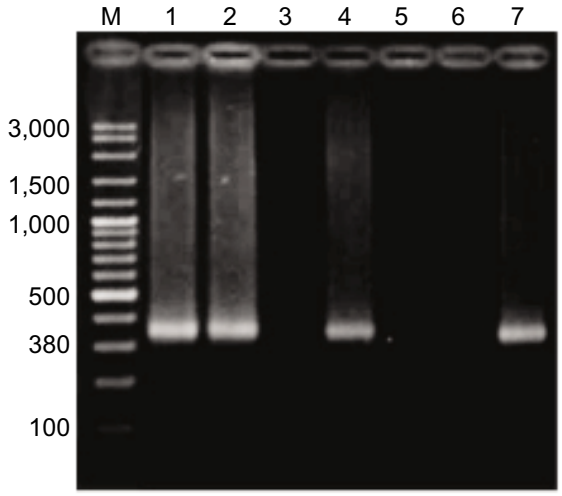

Fig 3–Specific amplification of *S. falcatus*-specific DNA. Primer pair was constructed from the 380 bp fragment obtained from HAT-RAPD profile. Lane M, DNA markers; 1, *S. falcatus* (positive control); 2, *S. falcatus* (metacercariae in half-beaked); 3, *S. falcatus* (metacercariae in mullet); 4, pleurolophocercous cercariae from *T. granifera* snail (Doi Saket District, Chiang Mai); 5, distome cercariae from *M. tuberculata*; 6, monostome cercariae from *Bithynia* sp; 7, pleurolophocercous cercariae from *T. granifera* snail (Saraphi District, Chiang Mai).

tion of *S. falcatus* was 1.0 mM $MgCl_2$ and annealing temperature of 68ºC. All other parameters were the same as for HAT-RAPD PCR.

For St-F and St-R primers, only the 320 bp fragment specific to *S. falcatus* was generated and there was no cross-reaction with any of the other tested parasites (Fig 3). The specificity of St-F and St-R primers was also confirmed by applying them to individual *S. falcatus* adult, cercariae and metacercariae stages. It is worth nothing that pleurolophocercous cercariae from *T. granifera* snail (Doi Saket and Saraphi District, Chiang Mai) were PCR positive for *S. falcatus* (Fig 3).

DISCUSSION

In this study, we report the development of specific primers derived from HAT-RAPD profile specifically targeted to *S. falcatus* (freshwater species). These pecific primers were constructed based on the sequence of 380 bp fragment obtained from HAT-RAPD. These results highlight the advantages of sequence characterized amplified region (SCAR-marker) derived from HAT-RAPD markers. Previous reports designed specific primers based on known sequence data to carry out detection in different locations of such parasite genomes as *Schistosoma mansoni* (Hamburger *et al*, 1998b; Pontes *et al*, 2002), or used of tandem repeated DNA sequences to detect *Trichobilhazia ocellata* (Hertel *et al*, 2002), or mitochondrial 12S rRNA gene for the detection of *E. multilocularis* (Dinkel *et al*, 1998), or complete mitochondrial sequence for *F. hepatica* (Magalhaes *et al*, 2004), *Clonorchis sinensis* and *O. viverrini* identification and discrimination (Le *et al*, 2006), whereas prior DNA sequence data are not require for SCAR-markers.

As HAT-RAPD profiles can give high resolution and reproducible data (Anuntalabhochai *et al*, 2000; Sripalwit *et al*, 2003, 2007), it is frequently employed in several research studies. However, SCAR marker technique has the advantages of lower cost and less time consumption. Although HAT-RAPD for *S. falcatus* detection had already been described by Sripalwit *et al* (2003) but the closely related samples of *S. falcatus* marine species were not included in the report, whereas in this study HAT-RAPD profiles were generated and compared using both freshwater and marine specimens confirming the different DNAs profiles between these two strains

(Fig 1, lanes 1 and 2). In addition, this study differed from the previous report in terms of annealing temperature, which by this study was 68ºC yielding a higher specificity as it could amplify even at low stringent condition whereas the study of Sripalwit *et al* (2003) reported an annealing temperature of 64ºC. Because of the higher annealing temperature, it allows only exactly compatible sequences for binding of DNA templates and primers. Incompatible sequence even at a few positions can not form any bond, which allows more specificity (Magalhaes *et al*, 2004). Overall, the protocol developed by this study represents a highly specific and sensitive method for the specific amplification of *S. falcatus* even under less stringent conditions.

The successful development of *S. falcatus*-specific primers allows their beneficial application in epidemiology and for detection of larval stages in intermediate host and egg stage in fecal specimens of definitive host, which is useful in prevention and management of effective epidemiological control programs.

ACKNOWLEDGEMENTS

The author wishes to thank the Applied Technology in Biodiversity Research Unit, Institute for Science and Technology Research, Chiang Mai University. Grateful thanks are extended to the Parasitology Research Laboratory and the Economic Plant Genomes Research and Service Center, Department of Biology, Faculty of Science, Chiang Mai University for available instruments. Special thanks are also extended to Mr Suksan Chuboon for laboratory assistances.

REFERENCES

Anuntalabhochai S, Chiangda J, Chandet R, Apawat P. Genetic diversity within Lychee (*Litchi chinensis* Soonn.) based on RAPD analysis [Abstract]. Cairhs: International Symposium on Tropical and Subtropical Fruit. 26th November-1st December 2000: 45.

Barber KE, Mkoji GM, Loker ES. PCR-RFLP analysis of the ITS2 region to identify *Schistosoma haematobium* and *S. bovis* from Kenya. *Am J Trop Med Hyg* 2000; 62: 434-40.

Chai JY, Sohn WM. Identification of *Stellantchasmus falcatus* metacercariae encysted in mullet in Korean. *Korean J Parasitol* 1988; 26: 65-8.

Dinkel A, Nickisch-Rosenegk M, Bilger B, Merli M, Lucius R, Romig T. Detection of *Echinococcus multilocullaris* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. *J Clin Microbiol* 1998; 36: 1871-6.

Hamburger J, He N, Xin XY, Ramzy RM, Jourdane J, Ruppel A. A polymerase chain reaction assay for detecting snails infected with Bilharzia parasites (*Schistosoma mansoni*) from very early prepatency. *Am J Trop Med Hyg* 1998a; 59: 872-6.

Hamburger J, Xin XY, Ramzy RM, Jourdane J, Ruppel A. Development and labolatory evaluation of a polymerase chain reaction for mornitoring *Schistosoma mansoni* infestation of water. *Am J Trop Med Hyg* 1998b; 59: 468-73.

Hamburger J, He-Na, Abbasi I, Ramzy RM, Jourdane J, Ruppel A. Polymerase chain reaction assay based on a highly repeated sequence of *Schistosoma haematobium*: A potential tool for monitoring schistosome-infested water. *Am J Trop Med Hyg* 2001; 65: 907-11.

Hertel J, Hamburger J, Harberl B, Hass W. Detection of bird schistosomes in lakes by PCR and filter-hybridization. *Exp Parasitol* 2002; 101: 57-63.

Hertel J, Kedves K, Hassan HM, Haberl B, Haas W. Detection of *Schistosoma mansoni* cercariae in plankton samples by PCR.

Acta Trop 2004; 91: 43-6.

Kliks M, Tantachumrun T. Heterophyid (trematoda) parasites of cats in Northern Thailand, with notes on a human case found at necropsy. *Southeast Asian J Trop Med Public Health* 1974; 5: 547-55.

Komiya Y, Suzuki N. The metacercariae of trematodes belonging to the family Heterophyidae from Japan and adjacent countries. *Jpn J Parasitol* 1966; 15: 208-14.

Le TH, De NV, Blair D, Sithithaworn P, McManus D P. *Clonorchis sinensis* and *Opisthorchis viverrini*: Development of a mitochondrial-based PCR for their identification and discrimination. *Exp Parasitol* 2006; 112: 109-14.

Magalhaes KG, Jannotti-passos LK, Cavalho OS. Detection of *Lymnaea columella* infection by *Fasciola hepatica* through multiplex PCR. *Mem Inst Oswaldo Cruz* 2004; 99: 421-4.

Martin WE. The life histories of some Hawaiian heterophyid trematodes. *J Parasitol* 1958; 44: 305-23.

Melo FL, Gomes ALV, Barbosa CS, Werkhauser RP, Abath FGC. Development of molecular approaches for the identification of transmission sites of schistosomiasis. *Trans R Soc Trop Med Hyg* 2006; 100: 1049-55.

Noda K. The larval development of *Stellantchasmus falcatus* (Trematoda: Heterophyidae) in the first intermediate host. *J Parasitol* 1959; 45: 635-42.

Pearson JC. A revision of the subfamily Haplorchinae Looss, 1988 (Trematoda: Heterophyidae). *Parasitology* 1964; 54: 601-76.

Pearson JC, OW-Yang CK. New species of *Haplorchis* from Southeast Asia, together with keys to the Haplorchis-group of Heterophyid trematodes of the region. *Southeast Asian J Trop Med Public Health* 1982; 13: 30-5.

Pontes LA, Dias-Neto E, Rabello A. Detection by polymerase chain reaction of *Schistosoma mansoni* DNA in human serum and feces. *Am J Trop Med Hyg* 2002; 66: 157-62.

Pubua J, Wongsawad C. Redescription of the Trematode metacercariae from the Mullet (*Liza subviridis*) and Half-beak (*Dermogenus pusillus*). *Southeast Asian J Trop Med Public Health* 2007; 38 (suppl 1): 106-9.

Sripalwit P, Wongsawad C, Chai JY, Rojanapaibul A, Anantalabhochai S. Development of Hat-RADP technique for the identification of *Stellanchasmus falcatus*. [Abstract]. Bangkok: 4th Seminar on Food and Water-borne Parasitic Zoonoses, 2nd International Meeting on Gnathostomiasis and Joint International Tropical Medicine Meeting, 2003: 289.

Sripalwit P, Wongsawad C, Wongsawad P, Anuntalabhochai S. High annealing temperature – random amplified polymorphic DNA (HAT-RAPD) analysis of three paramhhistome flukes from Thailand. *Exp Parasitol* 2007; 115: 98-102.

Wongratanacheewin S, Phumidonming W, Sermsawan RW, Maleewong W. Development of a PCR- based method for the detection of *Opisthorchis viverrini* in experimentally infected hamster. *J Parasitol* 2001; 122: 175-80.

Wongratanacheewin S, Phumidonming W, Sermsawan R W, Pipitgool V, Maleewong W. Detection of *Opisthorchis viverrini* in human stool specimens by PCR. *J Clin Microbiol* 2002; 40: 3879-80.

Wongsawad C, Rojtinnakorn J, Wongsawad P, Rojanapaibul A. Helminths of vertebrates from Maesa stream. *Southeast Asian J Trop Med Public Health*. 2004; 35 (suppl 1): 140-6.

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: 158-61.

Wongsawad C, Wongsawad P, Chai JY, Paratasilpin T, Anantalabhochai S. DNA quantities and qualities from various stages of some trematodes using optical and HAT-RAPD methods. *Southeast Asian J Trop Med Public Health* 2006; 37 (suppl 3): 62-8.

Stensvold C R, Saijuntha W, Sithithaworn P, *et al*. Evaluation of PCR based coprodiagnosis of human opisthorchiasis. *Acta Trop* 2006; 97: 26-30.

Vazquez-Colet A, Africa CM. Morphological studies on various Philippine heterophyid metacercariae with note on the incidence, site and degree of metacercarial infection on three species of marine fish. *Phil J Sci* 1940; 72: 395-419.

Vidigal THDA, Caldeira RL, Simpson AJG, Carvalho OS. Further studies on the molecular systematics of *Biomphalaria* snails from Brazil. *Mem Inst Oswaldo Cruz* 2002; 95: 57-66.