DNA QUANTITIES AND QUALITIES FROM VARIOUS STAGES OF SOME TREMATODES USING OPTICAL AND HAT-RAPD METHODS

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Abstract. The aim of this experiment was to minimize DNA quantity and quality for detection by optical (spectrophotometer at 260 nm and 280 nm) and HAT-RAPD methods. Total DNA from different stages, adult, metacercaria and eggs of 6 trematode species were isolated for analysis. In this experiment, the adult trematodes were classified into 3 groups by size: small, *Haplorchis taichui* and *Stellantchasmus falcatus*; medium, *Opisthorchis viverrini* and *Ganeo tigrinus*; and large, *Paramphistomum epiclitum* and *Fischoederius elongatus*. The adult minimal DNA quantities and qualities of all specimen samples detected by optical method were 97.22, 72.28, 3,167.00, 1,490.62, 21,382.66, and 27,321.77 ng; eggs were 3.92, 3.57, 3.72, 6.23, 17.53, and 14.01 ng, respectively; and metacercarial stages 50.70 and 40.98 ng in *H. taichui* and *S. falcatus*. In addition, the HAT-RAPD technique was chosen to amplify the minimal DNA qualities and quantities of all trematode specimens. Total DNA was 1-1x10^{-12}ng; DNA templates in each dilution were used for amplification by primer OPA-09. DNA concentrations ranging between 1x10^{-8} and 1x10^{-11}ng were amplified with high polymorphism. Our experiment concluded that only a single specimen of each egg, metacercaria, or adult stage could be amplified with distinct bands.

INTRODUCTION

The PCR method is commonly used to identify parasitic infection and species diversity for helminths. The applications of this method were reported to investigate the genetic variations of the global cestode, *Taenia solium* (Okamoto *et al*, 2001), to detect the liver fluke, *Opisthorchis viverrini* in bithynid snails and cyprinoid fishes (Maleewong *et al*, 2003), and to identify the minute intestinal trematode, *Stellantchasmus falcatus*, infecting half-beaked fish (*Dermogenus pusillus*) (Sripalwit *et al*, 2003). Optimal quantities and qualities of parasite genomic DNA are essential for 100% sensitivity and specificity of results. Shiff *et al* (2000) estimated the quantity of genomic DNA of the cercarial-stage blood fluke, *Schistosoma haematobium*, infecting snails and found that 2.5 ng DNA was optimal for the RAPD method. For DNA fingerprinting of *O. viverrini* detected by specific primer (OV-61 and OV-6R), the lowest DNA quantity was 2X10^{-17} ng extracted from only 1 egg, which was taken directly from the fluke or from a patient's feces (Wongratanachewin *et al*, 2001) while in former reports, DNA quantities from 5 eggs were employed (Sermsawan *et al*, 1991; Sirisinha *et al*, 1991). However, 100% result sensitivity could also be obtained by using genomic DNA of the liver fluke from 1 cercaria or 1 metacercaria in snail and fish intermediate hosts, respectively (Maleewong *et al*, 2003). For DNA fingerprinting of the lung fluke, *Paragonimus heterotremus*, genomic DNA from 5 eggs taken from the feces of an infected cat was used with pPH-13 specific DNA probe and produced 100% sensitivity (Intapan *et al*, 2005).

DNA quantity and purification by optical method using a spectrophotometer were investigated with the ratio OD_{260} : OD_{280} in the range 1.65-1.85. Absorbance of nucleic acid and protein was highest in wavelengths of 260 and 280 nm, respectively (Thai Society for Biotechnology, 1993; Piyachochanakul, 2003). The differences in species, size and stage of parasite may affect the quantities of genomic DNA used in the PCR method; the HAT-RAPD method (Anuntalabhochai *et al*, 2000) was used effectively to identify *Stellantchasmus falcatus* in half-beaked fish by Sripalwit *et al*. (2003).

This study aims to investigate the DNA quantity and quality of 6 species of trematodes of 3 different sizes by spectrophotometric method and the optimal quantity and quality by PCR and HAT-RAPD methods, which could be used with other helminths.

MATERIALS AND METHODS

Parasite materials

Six species of trematodes were divided into 3

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sizes; small, *Haplorchis taichui* and *Stellantchasmus falcatus*; medium, *Opisthorchis viverrini* and *Ganeo tigrinus*; and large, *Paramphistomum epiclitum* and *Fischoederius elongatus*. The metacercarial stage was used only for the small size, while the adult and egg stages were used for all species.

The metacercariae of *H. taichui* were collected from Jullien's mud carp (*Henicorhynchus siamensis*) and *S. falcatus* from half-beaked fish (*Dermogenus pusillus*), in Chiang Mai Province, using 1% pepsin solution in a shaking waterbath at 37˚C for 2 hours. Adults of each trematode were prepared by metacercariae being orally force-fed to 3-day-old chicks (*Gallus gallus domesticus*); the adults were collected 7 days post-infection from the intestines with Bearman's apparatus and kept in 0.85% NaCl at -20˚C.

The adult *Opisthorchis viverrini* was prepared by being orally force-fed to a hamster with metacercaria of the cyprinoid fish (*Cyclocheilichthys amatus*) from Khon Kaen Province. *Ganeo tigrinus* was collected from a frog (*Rana kuhlii*) in Chiang Mai.

Adults of the large trematodes, *Paramphistomum epiclitum* and *Fischoederius elongatus*, were collected from the rumen of cows in Lamphun Province. The egg stages of all trematodes were collected from the adult stage.

Morphological preparation

All trematodes were confirmed to species using permanent slides, fixed in 4% formalin, stained with hematoxylin, passed through alcohol series, and mounted with permount. A permanent slide was prepared for drawing using a camera lucida. Species identification was examined under a light microscope.

DNA preparation

The adults were grouped as small (100, 50, 20, 10, 5 and 1 specimens in each subgroup); medium (5, 2, and 1), and large (2 and 1). Fresh adult specimens were disrupted in a ceramic tissue grinder on liquid nitrogen. Metacercarial stages were grouped using 100, 50, 20, 10, 5 and 1 specimens in each group. The eggs were divided as follows; 100, 50, 20, 10, 5 and 1 specimens in each group. Each stage of each species was extracted using the DNeasy Tissue Kit (QIAGEN), 25 mg in a 1.5-ml microcentrifuge tube. The DNA was eluted in 5 mM Tris-HCl, pH 8.5.

DNA quality and quantity

Spectrophotometric method. Spectrophotometric optical densities of 260 nm and 280 nm were used to investigate the DNA quantity in a worm. DNA purity was measured using the appropriate ratio of $OD_{260:}$ OD_{280} (1.65-1.85).

Agarose gel electrophoresis examination. To confirm the existence of sufficient DNA to produce banding in every specimen tested, agarose gel electrophoresis was employed.

PCR-based HAT-RAPD method. Four arbitrary primers (10 nucleotides) from Operon Technology, USA (OPA-02, OPA-04, OPA-08, OPA-09) were used to examine the appearance of DNA banding to screen for only 1 primer for further investigation. The concentration of DNA was ten-fold serially diluted into various concentrations from $1\text{-}1x10^{-12}$ng. One microliter of all concentrations in an Appendoff tube was used to detect DNA quality using PCR-based HAT-RAPD (High Annealing Temperature-RAPD) technique (Anuntalabhochai *et al*, 2000). The PCR products were separated by 1.7% agarose gel electrophoresis at 60 volts for 2 hours 30 minutes, stained in 1 mg/ml of ethidium bromide for 10 minutes and then destained in distilled water for 10 minutes. The DNA patterns were examined under an UV transluminator and photographed by Kodak digital camera Gel Logic 100.

Data analysis

The lowest concentrations of DNA from both spectrophotometric and PCR methods were used to analyze the optimal concentration for DNA quality and quantity for each size of the 6 trematodes species. DNA banding was compared with 100-bp DNA ladder plus (Fermentas) and 100-bp DNA ladder plus (BIO-RAD), and the molecular weight examined by Kodak ID Image.

RESULTS

Morphological investigation

The widths and lengths of the adult and metacercarial stages of the trematodes are shown in Table 1, and the details of the morphological characteristics are demonstrated in Fig 1 (A-F). The mean length of the eggs is shown in Table 2. Data on worm size and DNA quantities were analyzed.

DNA quality and quantity

Spectrophotometric method. DNA quantities and the ratios of OD_{260}:OD_{280} of all trematodes in adult and metacercarial stage were shown in Table 1. DNA quantities increased with sizes. The amount of DNA of metacercarial stage was lower than that of adult stage. The ratios of OD_{260}:OD_{280} were 1.66-1.84, which confirmed DNA purity. DNA quantities in each of the

Table 1
The width and length of the trematode adult and metacercarial stages, quantity of DNA and ratio of OD_{260}:OD_{280}.

Trematode species	Width / Length (mm)	DNA (ng) per 1		OD_{260}:OD_{280}	
		Adult	Meta	Adult	Meta
Small size					
Haplorchis taichui	0.18-0.25 / 0.63-0.70	97.22	50.70	1.79	1.84
Stellantchasmus falcatus	0.11-0.12 / 0.16-0.19	72.28	40.98	1.73	1.74
Medium size					
Opisthorchis viverrini	1.17-1.23 / 4.70-5.50	3,167.00		1.76	
Ganeo tigrinus	0.76-1.37 / 1.32-3.26	1,490.62		1.72	
Large size					
Paramphistomum epiclitum	3.00-4.50 / 11.00-14.00	21,382.66		1.66	
Fischoederius elongatus	2.80-6.16 / 9.00-24.50	27,321.77		1.69	

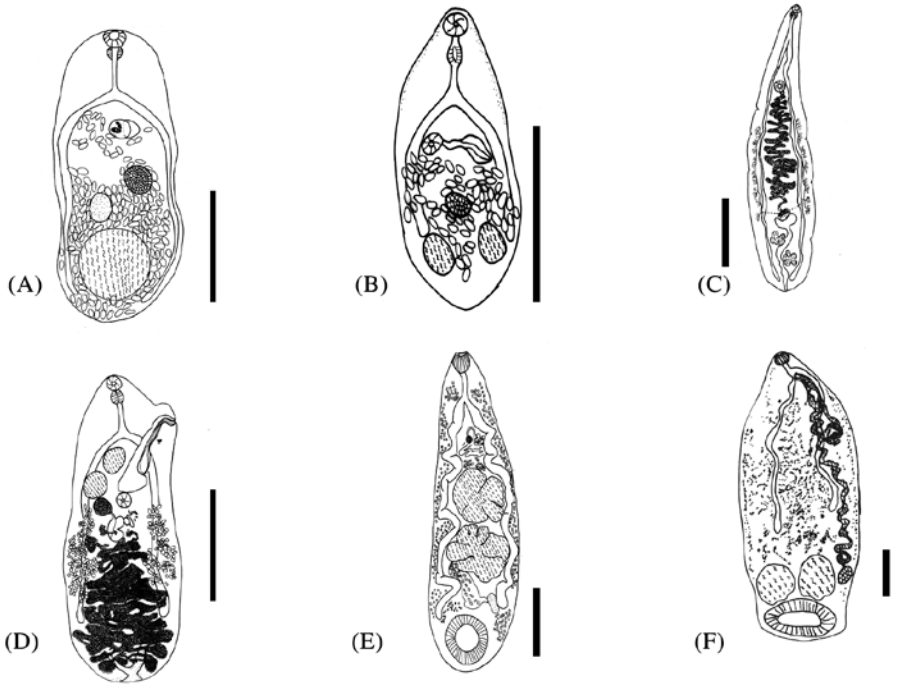

Fig 1- (A-F) Drawings of trematodes. (A) *Haplorchis taichui* (B) *Stellantchasmus falcatus*, C) *Opisthorchis viverrini*, (D) *Ganeo tigrinus*, (E) *Paramphistomum epiclitum*, (F) *Fischoederius elongatus* (scale bar A= 0.2 mm, B = 0.1 mm; C,D = 1 mm; E,F = 2 mm).

small trematode eggs were 3.57-3.92 ng, medium 3.72-6.23 ng, and large 14.01-17.53 ng (Table 2). The ratios of OD_{260}:OD_{280} for all trematode eggs ranged between 1.73-1.85, so their DNA qualities were acceptable.

Agarose gel electrophoresis examination. Agarose gel electrophoresis examination showed genomic DNA bandings in all specimens. The appearance and clearness of the bands depended on the number of specimens used in each subgrouping. Therefore, all trematode groupings could be used for PCR investigation.

PCR-based HAT-RAPD method. The results from PCR-based HAT-RAPD method revealed that, of the 4 primers tested, only OPA-04, OPA-08, and OPA-09 produced DNA bandings in every trematode. However, OPA-09 was selected for further investigation, since

Table 2
Mean lengths of trematode eggs, quantity of DNA and ratio of OD_{260}:OD_{280}.

Trematode species	Mean length (μm)	DNA / 1 egg (ng)	OD_{260}:OD_{280}
Small size			
Haplorchis taichui	20.50-25.00	3.92	1.77
Stellantchasmus falcatus	17.50-22.50	3.57	1.73
Medium			
Opisthorchis viverrini	22.50-27.50	3.72	1.85
Ganeo tigrinus	22.50-30.00	6.23	1.75
Large			
Paramphistomum epiclitum	120.00-150.00	17.53	1.78
Fischoederius elongatus	110.00-140.00	14.01	1.77

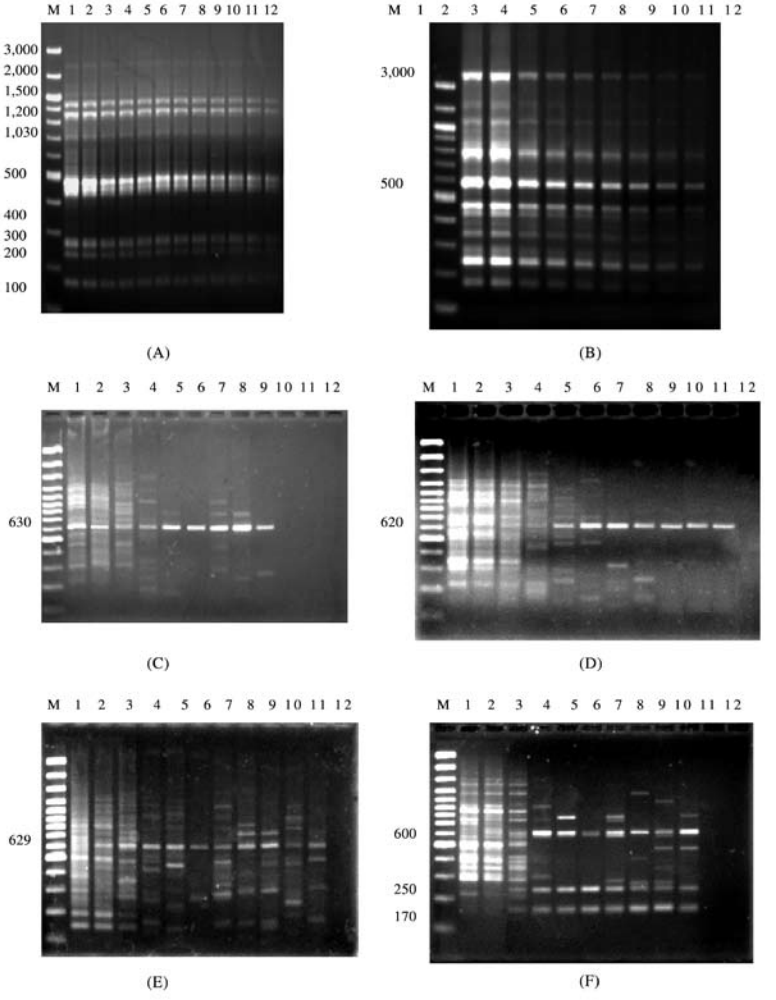

Fig-2 (A-F) Various concentrations of DNA template of adult stage amplified with OPA-09. (A) *H. taichui*, (B) *S. falcatus,* (C) *O. viverrini*, (D) *G. tigrinus*, (E) *P. epiclitum*, (F) *F. elongatus* (lane M, 100 bp ladder; DNA template in lane 1 = 1 ng; lane 2 = $1x10^{-1}$ng; lane 3 = 1 x 10^{-2}ng; lane 4 = 1 x 10^{-3}ng; lane 5 = $1x10^{-4}$; lane 6 = $1x10^{-5}$ng; lane 7 = $1x10^{-6}$ng; lane 8 = $1x10^{-7}$ng; lane 9 = $1x10^{-8}$ng; lane 10 = $1x10^{-9}$ng; lane 11 = $1x10^{-10}$ ng; lane 12 = $1x10^{-11}$ng).

its bandings were most distinct.

Of various concentrations of DNA amplified with OPA-09, the bandings of 6 trematodes are shown in Fig 2 (A-F).

Haplorchis taichui: DNA bandings were observed from concentrations at 1-1x10^{-11}ng. The optimal DNA concentration was observed at the minimal DNA concentration of clearest banding, 10^{-11}ng. The molecular weight of the DNA was 280-1,005 bp (Fig 2-A).

Stellantchasmus falcatus: DNA patterns were shown from concentrations at 1-1x10^{-8}ng. The minimal DNA concentration was 10^{-8}ng. The band of DNA molecular weight 180-1,750 bp and 600 bp appeared in all concentrations (Fig 2-B).

Opisthorchis viverrini: DNA patterns were shown from concentrations at 1-1x10^{-8}ng. The minimal DNA concentration was 10^{-8}ng. The band of DNA molecular weight of 200-1,300 bp and 630 bp appeared in all concentrations (Fig 2-C).

Ganeo tigrinus: DNA patterns were shown from concentrations at 1-1x10^{-10}ng. The minimal DNA concentration was 10^{-10}ng. The band of DNA molecular weight 270-1,470 bp and 620 bp appeared in all concentrations (Fig 2-D).

Paramphistomum epiclitum: DNA patterns were shown from concentrations at 1- 1x10^{-10}ng. The minimal DNA concentration was 10^{-10}ng. The band of DNA molecular weight 150-1,400 bp and 629 bp appeared in all concentrations (Fig 2-E).

Fischoederius elongatus: DNA patterns were shown from concentrations at 1- 1x10^{-10}ng. The minimal DNA concentration was 10^{-10}ng. The band of DNA molecular weight 170-2,000 bp and 170, 250, 600 bp appeared in all concentrations (Fig 2-F).

DNA patterns of adult and metacercarial stages in various subgroups were investigated only in the small worms, *H. taichui* and *S. falcatus* (Fig 3). DNA banding of high-to-low numbers of specimens appeared distinctively as high-to-low intensities. However, the 100 adult specimens of *H. taichui* (lane 1A) were observed to have lower band intensity. In Fig 3B, OPA-09 could not show distinct banding in *S. falcatus* (lane 1). The clear DNA patterns of the egg stage were found in all trematodes from this study of every subgroup (Fig 4).

DISCUSSION

The DNA quantities investigated by optical method from adult, metacercaria and egg stages were high-to-low, respectively. The maturation of the body, especially the reproductive system in adults, was higher than metacercariae and eggs, which affected their DNA quantities. The maturation of eggs began from unicellular, multicellular to miracidium, depending on the age and species of trematode (Sukontason *et al*, 2001). The qualities of the DNA for all specimens in this study were 1.66-1.85, showing high purity double-stranded DNA (Thai Society for Biotechnology, 1993;

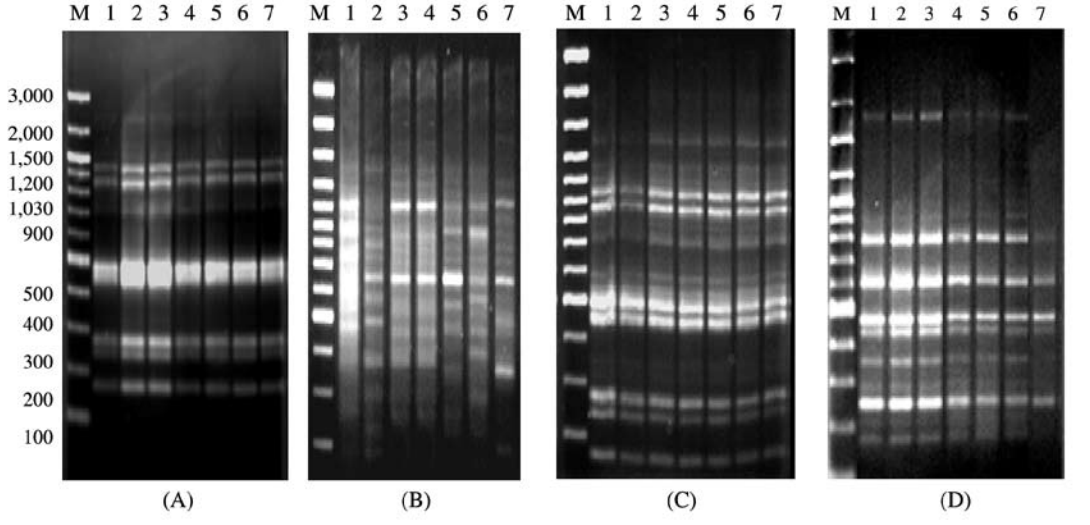

Fig 3- The DNA patterns of adult and metacercarial stage in various numbers amplified with OPA-09. Adult A. *H. taichui* B. *S. falcatus*; metacercaria C. *H. taichui* D. *S. falcatus*, (lane M, 100 bp ladder; DNA-template numbers in lane 1 = 1oo; lane 2 = 50; lane 3 = 20; lane 4 = 10; lane 5 = 5; lane 6 = 2; lane 7 =1).

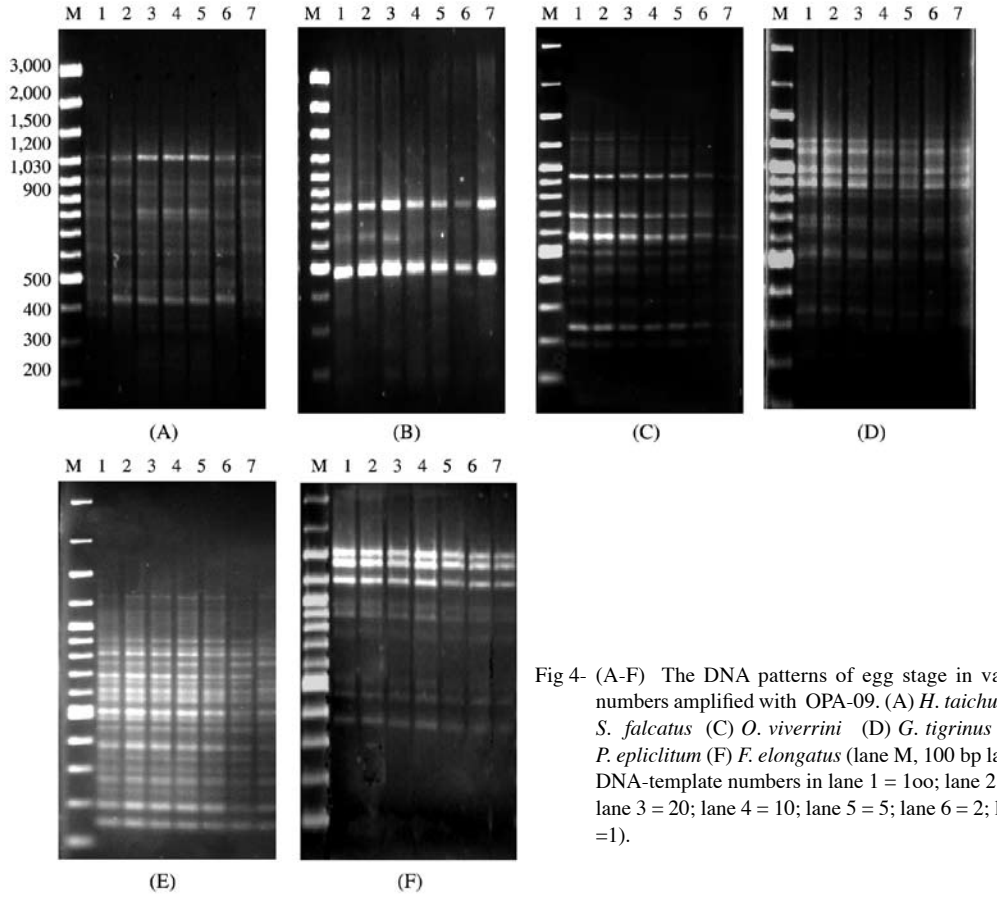

Fig 4- (A-F) The DNA patterns of egg stage in various numbers amplified with OPA-09. (A) *H. taichui* (B) *S. falcatus* (C) *O. viverrini* (D) *G. tigrinus* (E) *P. epliclitum* (F) *F. elongatus* (lane M, 100 bp ladder; DNA-template numbers in lane 1 = 1oo; lane 2 = 50; lane 3 = 20; lane 4 = 10; lane 5 = 5; lane 6 = 2; lane 7 =1).

Piyachochanakul, 2003). Piyachochanakul (2003) mentioned that the limitation of the optical method was the large sample quantity required for DNA detection. The results from this work showed that only one individual egg sample could yield enough DNA for detection by this method.

Genomic DNA investigated by gel electrophoresis method showed distinct banding. However, a large number of samples, *ie*, at least 20 eggs and 10 metacercariae, were needed for enough DNA to be detected by gel electrophoresis method.

The PCR method was used to study the optimal quantity and quality of DNA. The primer OPA-09 out of the 4 tested primers was chosen for further use in this study. The previous reports also used this primer to identify the heterophyid trematodes and *O. viverrini* (Sripalwit *et al*, 2003) and *Metagonimus* spp (Yu *et al*, 1997a, b). The lowest DNA concentrations from this work produced distinct bands for DNA analysis between 1-1x10^{-11}ng.

The lowest DNA quantities investigated in this research ranged between 1x10^{-6}-1x10^{-10}ng. The DNA quantity observed in this study was higher than reported by Wongratanachewin *et al* (2001), but lower than Intapan *et al* (2005), at 2x10^{-17}ng and 1x10^{-4}ng, respectively, which might be due to trematode species differences and personal technique. The annealing between the primer and DNA template produced repeated DNA synthesis, and distinct bands appeared (Piyachochanakul, 2003). The minimal DNA concentration of all stages in this study depended on species and could be used for preparing these trematodes for further study.

The optimal lowest quantity of DNA for PCR method was investigated. The adult *H. taichui* in the subgroup of 100 specimens showed lower distinct banding but the rest did. The high concentration of DNA template might cause annealing between themselves instead of with the primers (Piyachochanakul, 2003). One metacercaria, 1 egg or 1 adult from this study was used in the PCR method, while 1 egg of *Opisthorchis viverrini* (Wongratanachewin *et al*, 2001), 1 larval stage in a bithynid snail or 1 metacercaria in a cyprinoid

fish of *O. viverrini* were used in other studies by specific primer (Maleewong *et al*, 2003). However, higher quantities were also reported using 5 eggs of *Paragonimus westermani* in experimentally infected cats (Intapan *et al*, 2005), and 25-30 cercariae of *Schistosoma haematobium* in snails (Shiff *et al*, 2000). The practical usefulness of this work will be used to identify only single samples of trematodes in mixed-species specimens. Although the minimal quantity of DNA from this research was obtained from only 1 /adult/ metacercaria/egg, the specific DNA primer for each trematode could be detected appropriately.

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