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Research paper

Development of *Cytochrome B*, *a* new candidate gene for a high accuracy detection of *Fasciola* eggs in fecal specimens

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ABSTRACT

Fasciolosis among domestic ruminants has resulted in a decrease in the production of milk products and has occasionally led to the deaths of young ruminants due to of acute infections. This study aimed to discriminate between the eggs of *Fasciola gigantica* and other trematode eggs in samples collected from ruminant feces specimens using PCR-based methods with the new candidate gene *Cytochrome B* (*CYTB*). A species-specific primer was developed with a high degree of sensitivity (3.285 pg). The primer was able to amplify the *F. gigantica* genomic DNA and there were no positive results with the other related trematodes (*Paramphistomum* sp., *Orthocoelium* sp., *Fischoederius* sp., *Calicophoron* sp., *Echinostoma revolutum*, *E. cinetorchis*, *E. ilocanum* and *Isthmiophora hortensis*), freshwater snails (*Lymnaea auricularia*, *Bithynia siamensis*, *Indoplanorbis exustus*, *Melanoides tuberculata*, *Tarebia granifera*) or definitive hosts (*Bos primigenius* and *Bubalus bubalis*). The minimum concentration of DNA from eggs that could be give a positive result was 3.285 pg. Moreover, the results of the study confirmed the existence of *F. gigantica* in Nakhon Pathom Province with a high prevalence (28.57%) and revealed the area of infection through epidemiological mapping. Thus, the species-specific primer and epidemiological data in this study may be helpful for use in epidemiological studies, phylogenetic studies and veterinary studies in the future.

1. Introduction

Domestic and free-living ruminants are frequently infected by various intestinal, stomach or liver trematodes including digenetic species of the *Paramphistomum*, *Orthocoelium*, *Fischoederius, Calicophoron* and *Fasciola* genera (Dorchies, 2006; Eduardo, 1980; Sanabria and Romero, 2008; Swarnakar et al., 2014; Zhao et al., 2017). Most of these trematode species have been induced similar clinical signs (Bazsalovicsová et al., 2010). The trematode belonging to the genus *Fasciola* is a particularly important zoonosis trematode that is infectious and causes fasciolosis in both ruminants and humans (Bargues et al., 2016). This disease effects almost half of the ruminants in the world, has caused losses amounting to billions of US dollars in the livestock industry, and has resulted in the deaths of animals in the tropical regions of Africa and Asia (Demerdash et al., 2011; Ichikawa-Seki et al., 2017a; Rast et al., 2017). Fasciolosis commonly occurs in the gallbladder and the biliary canals of various livestock species such as cattle (*Bos primigenius),* water buffalos (*Bubalus bubalis*), goats (*Capra aegagrus*) and other herbivorous ruminants (Ashrafi et al., 2014). The fasciolosis of domestic ruminants are often accompanied by various clinical signs including diarrhea, weight loss, decreased milk production and occasionally deaths in young ruminants due to acute and heavy infections (Elkhatam and Khalafalla, 2016; Umur et al., 2018). The *F. gigantica* is the dominant species of *Fasciola* genus in Southeast Asia (Wannasan et al., 2014). In order to diagnose *F. gigantica* infections in both humans and ruminants, fecal examination is the classical method that is most commonly used to identify eggs in the fecal specimens. However, it is difficult to distinguish between the eggs of *F. gigantica* and those of other trematodes, such as *Paramphistomum* (Bazsalovicsová et al., 2010) and *Echinostoma* (Bless et al., 2015). These eggs display a mophological apperance that is similar to *F. gigantica* egg (referred to as *F. gigantica*-like eggs in this study), as these eggs possess very similar sizes, shapes and internal structures (Bless et al., 2015).

The validation of the current epidemic area of *F. gigantica* is important for the surveillance or prevention program for the the livestock. Several studies have focused on classifying the *F. gigantica* species by molecular data and phylogenetic analysis based on various genes or regions, including *Cytochrome c oxidase* subunit I (*CO*1) (Ichikawa-Seki et al., 2017b), *Nicotinamide Adenine Dinucleotide Hydrogen* (*NADH*) *dehydrogenase* subunit I (*ND*1) (Hayashi et al., 2016; Ichikawa-Seki et al.,

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2016), *28S ribosomal DNA* (Raina et al., 2015) and the internal-transcribed spacers 2 (Ai et al., 2010; Chaudhry et al., 2016; Raina et al., 2015). However, to identify the eggs in fecal samples, it has various inhibitors that may affect PCR reactions and led to decrease the detection limit of PCR reaction (Monteiro et al., 1997). The most suitable genes were selected as a target for a specifically designed and species-specific primer that was determined to be acceptable for species discrimination among degraded samples. Therefore, a new candidate gene for the identification of *F. gigantica* eggs was selected. The *Cytochrome B* gene (*CYTB*) holds various informative sites in the nucleotide sequences that are conserved at the genus or species level (Blasco-Costa et al., 2016; Valadas et al., 2016). Various reports have used the conserved sequences in this gene for the purposes of taxonomic identification of several animal species (Ali et al., 2015; Stevanovic et al., 2016; Xu et al., 2015). Moreover, this gene is suitable for the identification of difficult or degraded specimens, such as those found in processed foods (Ali et al., 2014, 2015) and with degraded DNA, such as that which occurs in forensic samples (Staats et al., 2016). Additionally, it has been recommended for use as a marker gene because has species specific regions that can be separated among trematode species (Blasco-Costa et al., 2016). Thus, this gene has features that could potentially be used to construct species-specific primers to amplify the DNA that is present in egg specimens.

Therefore, this study aimed to develop new high-performance species-specific primers for *F. gigantica* based on the *CYTB* gene in order to discriminate between this trematode egg and other *F. gigantica*-like eggs in the fecal specimens with high specificity. Moreover, this study aimed to investigate the epidemiological situation of *F. gigantica* in Nakhon Pathom Province by identifying *F. gigantica* infected areas in Thailand by using both classical and molecular methods based on PCR techniques. This study will then present any differences that are found in the infection area using both methods. The epidemiological data and the specific primers obtained from this study could be applied to medical and veterinary studies in order to develop a prevention program of fasciolosis in future studies.

2. Materials and methods

2.1. Animal ethics

All experimental procedures involving animals in this study were conducted in accordance with the National Research Council of Thailand and were approved by the Committee for Biological Experimentation on Animals from the Faculty of Science at Srinakharinwirot University, Thailand (NO. 037/2561).

2.2. Specimen collection

Between June 2017 and October 2017, about 30 g of fecal specimens were obtained from 35 cattle from different livestock farms that were randomly selected from 16 local farms in Nakhon Pathom Province, Central Thailand. Each specimen was collected in a plastic cup, which was then capped and labeled with the relevant information (i.e. sample number, date of sampling and Global Positioning System (GPS) data). All specimens were then transferred to the Applied Parasitology Research Laboratory (Srinakharinwirot University, Thailand) for fecal examination.

Adult trematodes, including *F. gigantica, Paramphistomum epicitum*, *Orthocoelium streptocoelium*, *Fischoederius elongatus, Calicophoron calicophorum*, were harvested from a local slaughterhouse. The intestinal trematode, *Echinostoma revolutum*, was developed from the metacercarial stage in an experimental host (*Phodopus roborovskii*). Regarding other echinostome species, *E. cinetorchis*, *E. ilocanum* and *Isthmiophora hortensis*, were obtained from the Department of Parasitology and Tropical Medicine at the Seoul National University College of Medicine. All the adult trematodes were identified using taxonomic keys (Yamaguti, 1958).

2.3. Fecal examination

The *F. gigantica*-like eggs in fecal specimens were collected using the modified formalin-ether technique (Allen and Ridley, 1970). This technique is a concentration method that was designed to separate the eggs from fecal debris by centrifugation (Allen and Ridley, 1970) and has typically been used for trematode egg detection in feces (Kaewpitoon et al., 2015). First, two grams of the fecal specimens were ground into 10 mL of normal saline solution (NSS). Then, the samples were placed in the collection tube (15 mL tube; Thermo Fisher Scientific, USA) of the centrifuge and spun at a speed of $2500 \times g$ for 1 min. The supernatant was then removed, and all sediment was retained. Subsequently, 10 mL of a 10% formalin was added to the tube. The specimens were vigorously shaken and left in the tube for at least 5 min at room temperature. Next, 3 mL of diethyl ether was added, and the samples were vigorously shaken for one minute. Finally, the specimens were put in the collection tube of the centrifuge and spun at a speed of $2000 \times g$ for 3 min to separate the solution into 4 layers based on their specific gravity (Allen and Ridley, 1970). The layers comprised of ether at the top, debris, formalin, and a sediment mixed with parasite eggs at the bottom. The sediments were used to examine the presence of the eggs under a microscope. All egg specimens were then kept at a temperature of -20 °C.

2.4. Epidemiological map

All of *F. gigantica*-like eggs were separated from other parasitic eggs based on the morphological characteristics that were observed through a high magnification compound light microscope (100x). Each of the sites infected with *F. gigantica*-like eggs in Nakhon Pathom Province were recorded in order to construct an epidemiological map using the Geographic Information Systems Software (GIS). The infected area was indicated by positive results of *F. gigantica*-like eggs in the feces.

2.5. Molecular studies and species-specific primer designs

The adult stage of *F. gigantica* and other related trematodes were washed with distilled water to remove any traces of host tissue or any debris before the DNA extraction process. Each adult specimen was homogenized by sterile pistil in the distilled water (one specimen for each species). For the host specimens, 25 mg of the tissues from each species of host was collected for DNA extraction. The worms and hosts DNA were then extracted using the DNeasy® blood and tissue kit (Qiagen) following the manufacturer's instruction. The *F. gigantica*-like eggs, they were washed with distilled water before the extraction process using the QIAamp® DNA stool mini-kit (Qiagen) following the manufacturer's instruction.

All PCR reactions in this study used distilled water as a negative control. For the initial amplification of the *CYTB* gene, the primer set was developed in this study that was comprised of the following: forward primer; Tre_CYTB-F (5′- AAT GTT GTG GAT TTG CCT AC - 3′) and reverse primer; Tre_CYTB-R (5′- GGA TAC TCA GGA TGA CAA GC-3′). These primers are novel and were specifically designed based on conserved region in *CYTB* sequences of *F. gigantica* (KF543342.1) and other related species, including the following: *F. hepatica* (AP017707.1), *Calicophoron microbothrioides* (KR337555.1), *Explanatum explanatum* (KT198989.1), *Fischoederius cobboldi* (KX169164.1), *Fischoederius elongatus* (KM397348.1), *Gastrothylax crumenifer* (KM400624.1), *Orthocoelium streptocoelium* (KM659177.1) and *Paramphistomum cervi* (KT198987.1) (Fig. 1). The PCR conditions were began with a pre-denaturation at 94 °C for 5 min, followed by a round of 30 cycles of DNA amplification reaction with 45 s for denaturation at 94 °C, 45 s for primer annealing at 51 °C, and for 50 s at 72 °C for primer extension. A final extension was then performed at 72 °C for 7 min. All amplicons

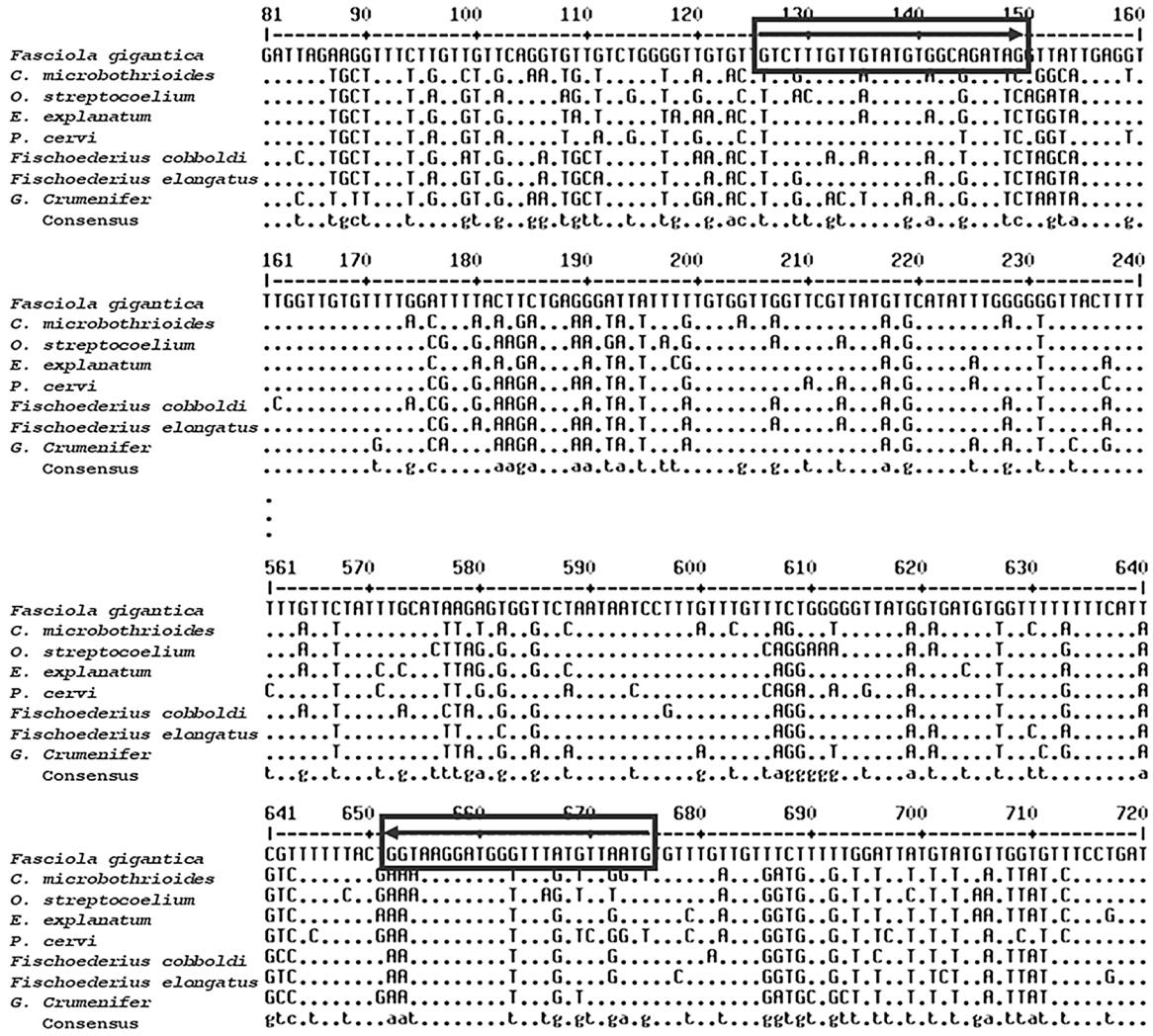

Fig. 1. The novel primers were designed based on *CYTB* sequences of *Fasciola gigantica* (KF543342.1) and other related species, including *Calicophoron microbothrioides* (KR337555.1), *Orthocoelium streptocoelium* (KM659177.1), *Explanatum explanatum* (KT198989.1), *Paramphistomum cervi* (KT198987.1), *Fischoederius cobboldi* (KX169164.1), *Fischoederius elongatus* (KM397348.1) and *Gastrothylax crumenifer* (KM400624.1).

were sent to be purified and sequenced by 1st BASE DNA Sequencing Services (Axil Scientific Pty. Ltd). After that, all the sequencing results were aligned with the sequences that were acquired from the GENBANK database. The ClustalW program was used for aligning the sequences of each trematode species to design the species-specific primers for *F. gigantica* (FG primer set). The specificity of the species-specific primers were verified using the *in-silico* program (Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the results revealed no positivity for any trematode sequences or their definitive hosts (data not shown). This primer set consisted of forward primer; FG_F (5′- GTC TTT GTT GTA TGT GGC AGA TAG-3′) and reverse primer; FG_R (5′- CAT TAA CAT AAA CCC ATC CTT ACC-3′), and the expected length of a PCR product was found to consist of 549 base pairs.

2.6. Validation of specificity and sensitivity test of primers

The optimal annealing temperatures for the species-specific primer were optimized by the gradient PCR process. The PCR reaction was performed in a volume of 25 µl (The reaction contained 1.6 mM of $MgCl_2$, 0.2 µM of each primer, 50 µM of each dNTP, 0.2 Unit of *Taq* polymerase (Vivantis), 2.5 µl of 1x PCR buffer (Vivantis) and 82.63 ng of *F. gigantica* DNA), under similar conditions employed for the Tre_*CYTB* primer, but the annealing temperatures in each reaction were changed to 55.0, 55.7, 56.9, 58.8, 61.2, 63.1, 64.3 and 65.0 °C, respectively. The performance of the species-specific primer set was validated by the specificity and sensitivity test.

The PCR reaction was performed in 25 µl using the same conditions as the gradient-PCR, but the annealing temperature was fixed at the optimal temperature. The specificity of the primers was validated using PCR amplification on the DNA of other related trematodes displaying a morphological appearance that was similar to *F. gigantica*-like eggs, comprised of *F. gigantica, P. epicitum*, *O. streptocoelium*, *Fischoederius elongatus, C. calicophorum*, *E. revolutum*, *E. cinetorchis*, *E. ilocanum* and *I. hortensis*, respectively. All DNA concentration were validated by a NanoDrop™ machine (Thermo Scientific NanoDrop 2000, USA) and diluted to 5 ng/µl). To verify the cross-reactions between the primers and the hosts, specificity was tested on freshwater snails (first intermediate host) that have been identified as the host of gymnocephalous cercaria (cercarial type of Fasciolidae) infections in Thailand. These samples consisted of *Lymnaea auricularia* (43.27 ng/µl) and *Bithynia siamensis* (34.13 ng/µl), *Indoplanorbis exustus* (21.14 ng/µl), *Melanoides tuberculata* (13.22 ng/µl), *Tarebia granifera* (47.38 ng/µl). For the tissues of the definitive hosts, *Bos primigenius* and *Bubalus bubalis,* DNA concentration that used for specificity test was 65.21 ng/µl and 43.90 ng/µl, respectively.

For the sensitivity test, two steps were performed involving PCR reactions with a ten-fold serial dilution DNA. First, the sensitivity of the

Table 1
The positive result of *Fasciola gigantica*-like eggs in each collecting site of Nakhon Pathom Province based on classical and molecular methods.

Farms	Districts	No. of specimens	No. of infected *Fasciola gigantica*-like eggs and codes	
			Classical method	Molecular method
1	Mueang Nakhon Pathom	2	0	0
2	Mueang Nakhon Pathom	3	1 (A1)	1 (A1)
3	Mueang Nakhon Pathom	1	0	0
4	Mueang Nakhon Pathom	2	1 (A2)	0
5	Mueang Nakhon Pathom	2	0	0
6	Don Tum	1	0	0
7	Don Tum	1	0	0
8	Don Tum	2	2 (A3, A4)	1 (A3)
9	Don Tum	3	0	0
10	Nakhon Chaisi	2	1 (A5)	1 (A5)
11	Nakhon Chaisi	2	2 (A6, A7)	2 (A6, A7)
12	Kamphaeng Saen	5	5 (A8, B1 – B4)	2 (A8, B3)
13	Kamphaeng Saen	1	0	0
14	Kamphaeng Saen	3	3 (B5 – B 7)	2 (B6, B7)
15	Kamphaeng Saen	3	1 (B8)	1 (B8)
16	Kamphaeng Saen	2	0	0
Totals		35	16	10

eggs was amplified with an initial DNA value of 500 *F. gigantica* eggs (concentration of 32.85 ng/μl) and the next step involved the mixed DNA of adult *F. gigantica* and the definitive hosts that were comprised of *Bos primigenius* and *Bubalus bubalis*. The DNA concentrations of adult *F. gigantica*, *Bos primigenius* and *Bubalus bubalis* were recorded at 82.63, 65.21 and 43.90 ng/μl, respectively, while the mixed DNA concentration (with an equal volume of 10 μl of each DNA specimen) was recorded as 47.30 ng/μl. Both of these experiments used amplified DNA under the same PCR conditions as described above to find the minimum concentration of DNA from pure eggs and DNA with host DNA interference that can be detectable.

2.7. Phylogenetic tree construction and infection mapping analysis

After a complete validation of species-specific primers, these primers were used to investigate the DNA of *F. gigantica*-like eggs in the study area and classify the species of these eggs based on the PCR method. The positive amplicons were purified and sequenced using 1st BASE DNA Sequencing Services (Axil Scientific Pty. Ltd). All of the sequences of the positive results were used to construct Maximum-Likelihood (ML) trees with the Kimura-2-parameter model with 10,000 bootstraps for re-sampling using the MEGA7® program (Proprietary freeware; Pennsylvania State University, USA) for the validation of amplification results. The epidemiological data acquired from the classical and molecular methods at each local farm in this study were imported to a GIS program to construct an epidemiological map to compare the differences between the results of the infection rates from each method.

3. Results

3.1. Fecal examination

The examinations revealed that 16 of 35 specimens (45.71%) were positive for *F. gigantica*-like eggs (Table 1). The highest number of *F. gigantica*-like eggs were found in Kamphaeng Saen District (9 specimens), followed by Nakhon Chai Si District (3 specimens), Mueang Nakhon Pathom District (2 specimens) and Don Tum District (2 specimens). The morphological appearance of *F. gigantica*-like eggs was recorded as large operculated eggs and appeared a little yellowish, with a thin shell and an oval body. The size of the egg was found to be 130–170 × 70–90 μm (Fig. 2).

3.2. Validation of specificity and sensitivity of the primers

The gradient-PCR results revealed that the optimal annealing temperature (T_a) of the FG primer set was 61.2 °C, and the PCR products were comprised of a single band with 549 base pairs (Fig. 3). The results of the specificity test revealed that the primers could amplify the *CYTB* gene of *F. gigantica* with negative results with other trematodes (Fig. 4A), freshwater snails and the definitive hosts (Fig. 4B). Regarding the sensitivity results, the high performance of the FG primer set was demonstrated by amplified *F. gigantica* DNA, which was diluted up to

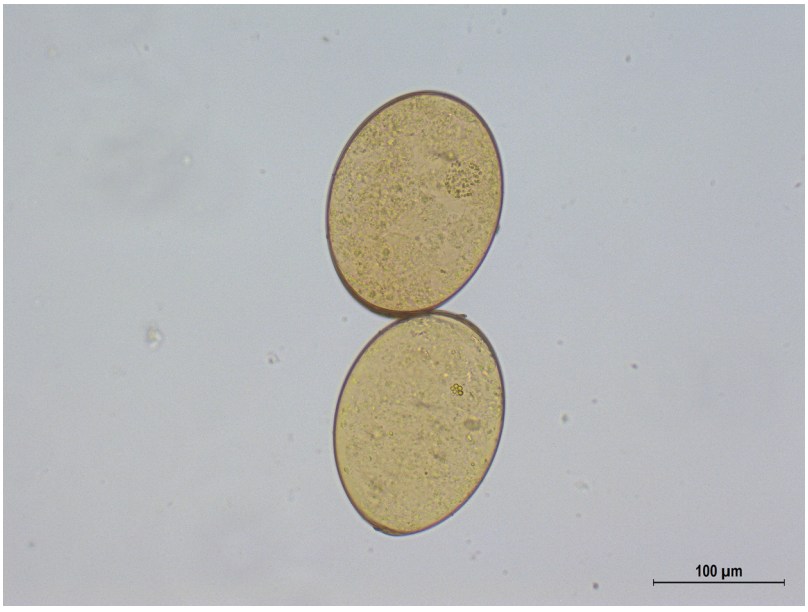

Fig. 2. *F. gigantica*- like eggs found in ruminant feces after examination by the formalin-ether technique.

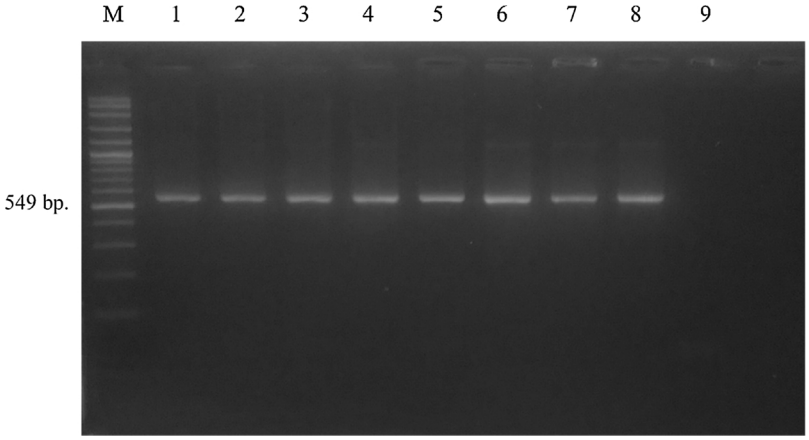

Fig. 3. Gradient-PCR revealing each result of the annealing temperature (T_a) of the FG primer set. Lane M was the DNA ladder; the results of the PCR reaction that used *F. gigantica* as the DNA template. The annealing temperatures were varied from 55.0, 55.7, 56.9, 58.8, 61.2, 63.1, 64.3 and 65.0 °C, in lanes 1 to 8, respectively. Lane 9 was the negative control (distilled water). The PCR product was a single band at 549 base pairs.

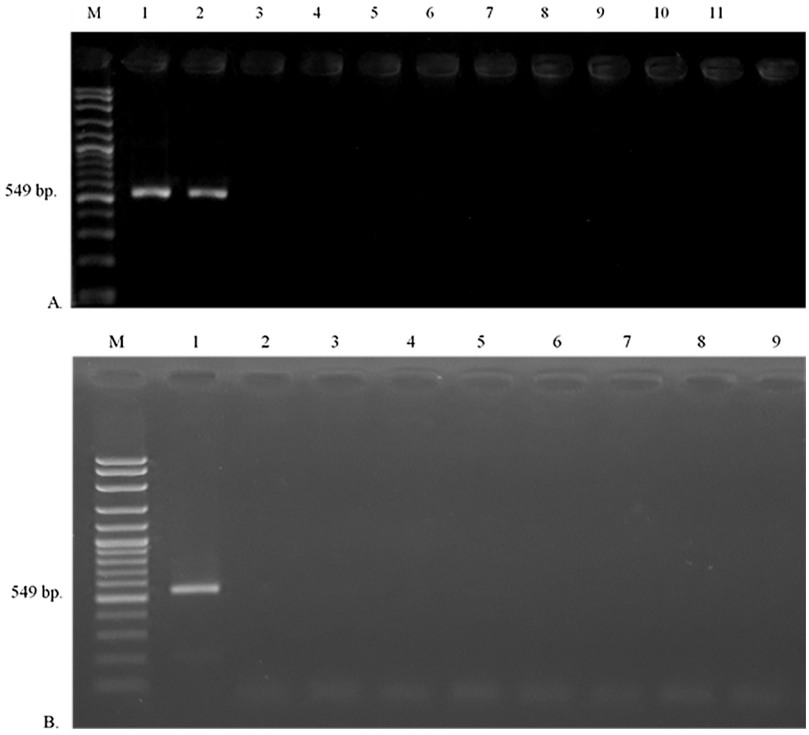

Fig. 4. Specificity tests of the primer set revealed that it could amplify the *CYTB* gene of *F. gigantica* with no cross-reactions with other specimens and the PCR product was 549 base pairs.
A. Lane M was the DNA ladder; the trematode in Lane 1 was DNA from *F. gigantica* eggs, lane 2 referred to the DNA of adult *F. gigantica*, Lanes 3 to 11 referred to *Paramphistomum epicitum*, *Orthocoelium streptocoelium*, *Fischoederius elongatus*, *Calicophoron calicophorum*, *Echinostoma revolutum*, *E. cinetorchis*, *E. ilocanum*, *Isthmiophora hortensis* and negative control, respectively.
B. Lane M was the DNA ladder; Lane 1 referred to the DNA of adult Fasciola gigantica, Lanes 2–6 referred to the freshwater snails, *Lymnaea auricularia*, *Bithynia siamensis*, *Indoplanorbis exustus*, *Melanoides tuberculata*, *Tarebia granifera*, respectively. Lane 7 referred to *Bos primigenius* DNA, lane 8 referred to *Bubalus bubalis* and lane 9 was the negative control.

10^{-4} fold in both eggs and the adult DNA specimens (Fig. 5A and B). The minimum DNA concentration that could be detected was 3.285 pg for the egg specimens and 8.263 pg for the mixed DNA of the adult *F. gigantica* and the definitive hosts.

3.3. Phylogenetic tree construction and infection mapping analysis

All *F. gigantica*-like egg specimens collected from the infection sites were amplified with a species-specific primer. Six of the sixteen *F. gigantica*-like egg specimens were collected from the following infection sites: (1) site 4 in Mueang Nakhon Pathom District; (2) site 8 in Don Tum District; (3) site 12 in Kamphaeng Saen District, and (4) site 14 in Kamphaeng Saen District. These specimens were not amplified using the FG primer set (Fig. 6A and B). From these results, the infection mapping of *F. gigantica* was reconstructed to differentiate the *F. gigantica*-like eggs and the true *F. gigantica* eggs using a separate color code (Fig. 7). All the positive FG primer results were sequenced and an ML phylogenetic tree was constructed with 10,000 bootstraps for re-sampling. The results found that the *F. gigantica* and the positive eggs were clustered in the same clade, which was distinct from *F. hepatica* and other related species (Fig. 8).

4. Discussion

The general diagnostic protocol that was used to confirm *Fasciola* species infection was conducted by examining the eggs in the fecal samples. This is usually performed by finding the eggs in fecal specimens examined under a high magnification light microscope. However, more time and personal experience are required for accurate identification through this method and the misidentification of *F. gigantica*-like eggs has been known to occur (Bazsalovicsová et al., 2010; Bless et al., 2015). In order to overcome these problem, this study applied the PCR method to develop a high accuracy detection tools in order to discriminate between *F. gigantica* eggs and other *F. gigantica*-like eggs in the feces of livestock using DNA extracted from the fecal samples. The FG primer set has been designed based on the *CYTB* gene and was identified as one of the best genes for use as a target for degraded sample amplification (Ali et al., 2014, 2015; Staats et al., 2016). This property is important for species identification of egg DNA in feces, as formalin and ether were used in the method that separated the eggs

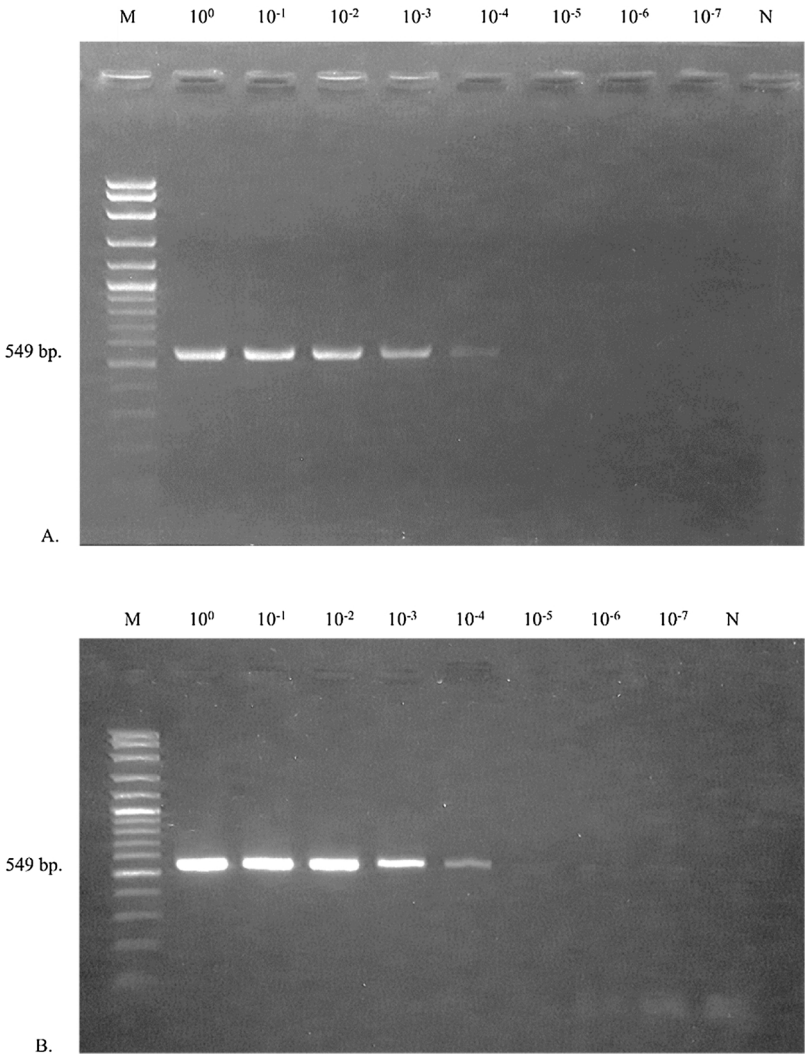

Fig. 5. Sensitivity results of FG primer set. The initial DNA concentration was started with the DNA of 500 eggs of *Fasciola gigantica* (Lane 1), then in Lanes 2–8 DNA was diluted using a ten-fold dilution method; Lane N was used for the negative control.
A. Ten-fold dilution of *F. gigantica* DNA from 500 eggs.
B. Ten-fold dilution of mixed DNA of adult *F. gigantica*, *Bos primigenius* and *Bubalus bubalis*.

from the feces and it can degrade the DNA of eggs. Moreover, the feces also have various inhibitor that decrease the detection limit of PCR reaction (Monteiro et al., 1997). The primer set present in our study can amplify the egg DNA of *F. gigantica* with a high specificity and dose not display cross-reaction with their hosts or other related trematode species. Moreover, the lowest concentration of DNA that could be amplified was 3.285 pg for pure egg DNA specimens and 8.263 pg for the mixed hosts and *F. gigantica* DNA. Thus, the FG specific primer was found to be able to amplify DNA at lower concentrations than other primers described in most of the previous reports that had used DNA for PCR amplification at a nanogram scales (Ai et al., 2010; Hayashi et al., 2016; Ichikawa-Seki et al., 2016, 2017a; Ichikawa-Seki et al., 2017b; Shoriki et al., 2016). Moreover, the degree of sensitivity of the species-specific primer of *F. gigantica* was found to be higher than that which was based on the design of the ITS-2 primer (DSJf/DSJ4) with a sensitivity of 0.35 ng (Ai et al., 2010). Thus, this primer revealed a very high level of sensitivity and could hopefully be used in epidemiological, phylogenetic and veterinary studies in the future.

The epidemiological map of *Fasciola gigantica* egg was constructed using specific amplification. Notably, this method was found to be more accurate than the classical methods. It may be challenging to identify these eggs at the species level because of similarities in the morphological appearance of the eggs (Bazsalovicsová et al., 2010). Therefore, this study used the FG primer to discriminate between other *F. gigantica*-like eggs and revealed the precise areas of *F. gigantica* infection that were then employed to improve epidemiological mapping with greater accuracy.

Regarding the epidemiological situation of *F. gigantica* present in Nakhon Pathom Province, Thailand, Chaichanasak and colleagues reported finding *F. gigantica* in Mueang Nakhon Pathom six years ago (Chaichanasak et al., 2012). The results revealed that more than 45% of the livestock farms were infected with *F. gigantica.* This confirms that *F. gigantica* was still in existence at that time in the same district. Moreover, a heavy infection rate was found in nearby districts namely, Kamphaeng Saen District. This can be very dangerous for people residing in the area of study. Residents of the area could be infected by ingesting raw vegetables which may contain the metacercarial stage of *F. gigantica*, which is known to be the infective stage. Additionally, for over two decades, the *Fasciola* genus has been established as one of drug resistant trematodes in the world (Gaasenbeek et al., 2001; Hanna, 2015; Hanna et al., 2015; Overend and Bowen, 1995). Therefore, it is possible that drug resistance in *F. gigantica* may enhance the distribution of this trematode from district to district. Importantly, this presupposition needs to be further researched in the future before an

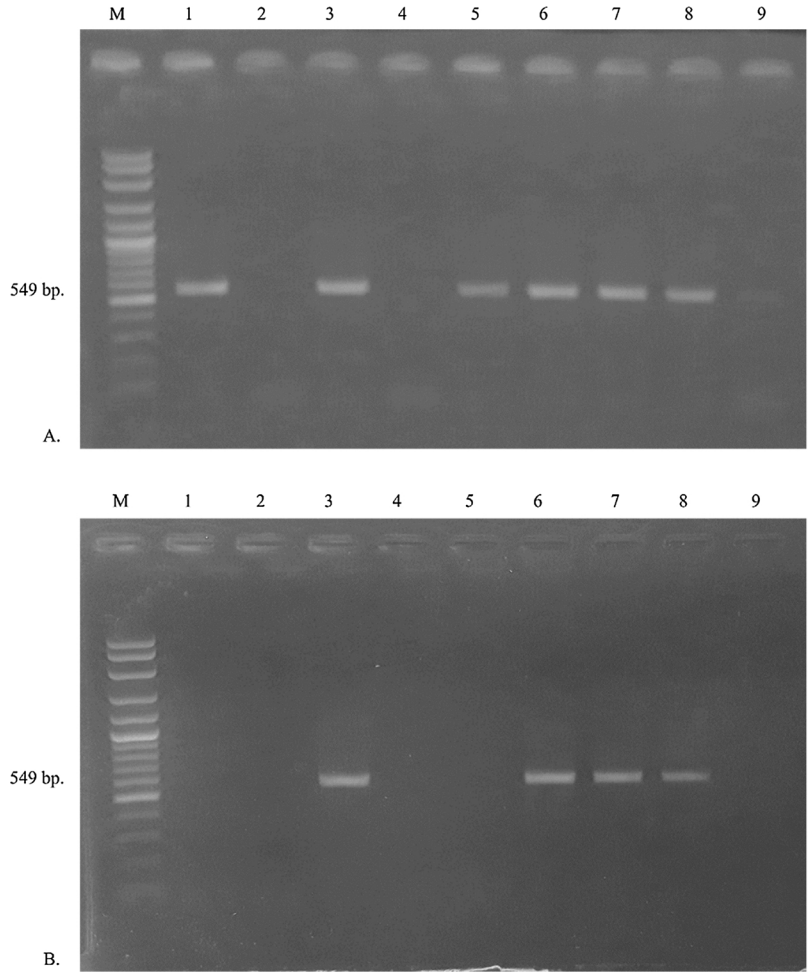

Fig. 6. Six of the sixteen *Fasciola gigantica*-like egg specimens collected from four infection sites. The numbers A1 – A8 and B1 – B8 indicate the positive result of FG-like eggs in each collecting site of Nakhon Pathom Province and the numbers A9 and B9 are the negative controls (distilled water). The specimens from site 4 in Mueang Nakhon Pathom District (A2); site 8 in Don Tum District (A4); site 12 in Kamphaeng Saen District (B1, B2, B4) and site 14 in Kamphaeng Saen District (B5) were not amplified with the FG primer set.

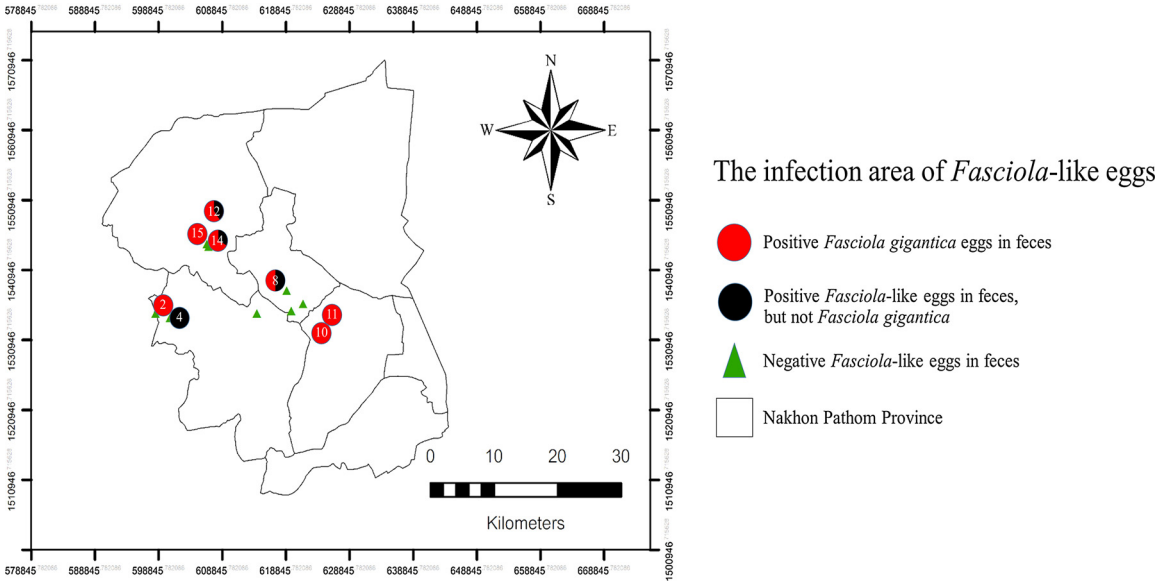

Fig. 7. The comparative epidemiological map among classical and molecular methods. In some areas shown, the results of the classical method are faulty when verified with the species-specific primers. The numbers infected with *Fasciola gigantica*-like eggs in the collection sites are shown.

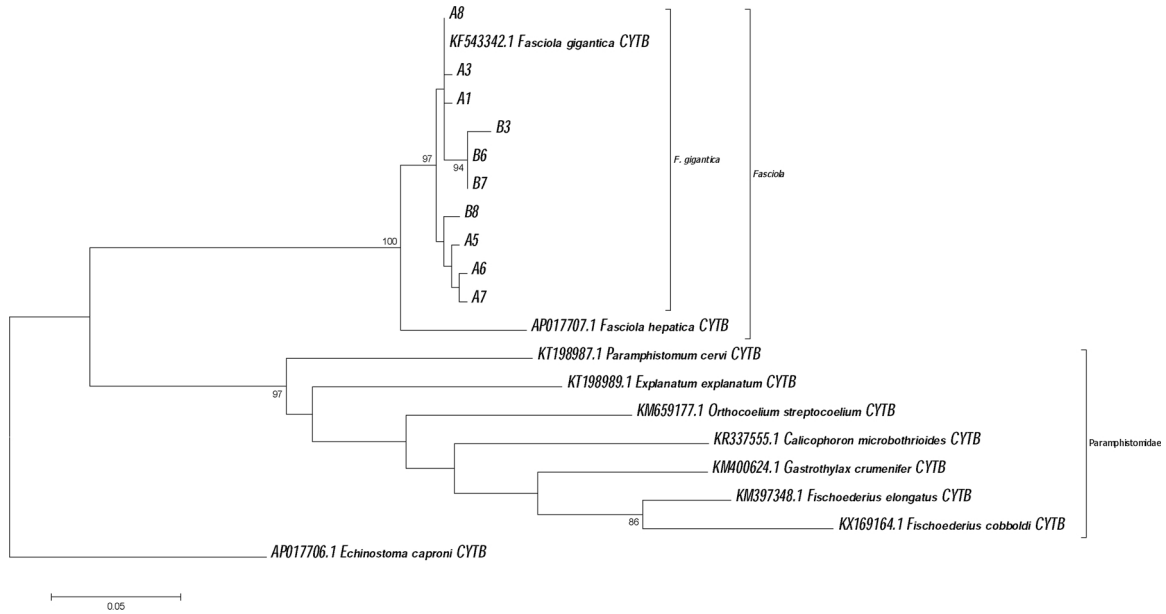

Fig. 8. The sequences of positive fecal specimens were employed to construct the maximum likelihood phylogenetic tree, with 10,000 bootstraps for resampling. The results found that *F. gigantica* and the positive eggs were clustered in the same clade, which were distinct from other related species usually found in the fecal specimens. The details of each code are shown in Table 1.

outbreak of *F. gigantica* occurs.

For phylogenetic tree analysis, all the nucleotide sequence data on positive egg specimens were clustered in the same clade of *F. gigantica.* The efficiency of the primer that separated genuine *F. gigantica* eggs from the *F. gigantica*-like eggs had improved an accuracy and convenience as compared to classical method. Additionally, the variations within *F. gigantica* sequences that separated them into sub-groups will be of interest for future studies on the phylogenetic relationship of this species.

5. Conclusion

This study aimed to successfully develop a highly accurate species-specific detection method to discriminate between the eggs of *Fasciola gigantica,* and other *F. gigantica*-like eggs found in fecal specimens. The limitation of the concentration for detection was recorded at 3.285 pg for the pure egg DNA specimens and 8.263 pg for the mixed hosts and the *F. gigantica* DNA. No cross-reaction among the hosts or related species was found in any amplification. Additionally, the present study confirmed the existence of *F. gigantica*, which was found to be present in the same district six years ago, as well as in nearby districts. This can be dangerous for the human residing in the infection area as this trematode can easily be transmitted to humans and other livestock when ingested along with raw vegetables that contain metacercaria at the infective stage. The presupposition of the high prevalence of *F. gigantica* in this study may cause by drug resistance in this species similar to other regions. This important point needs to be further researched in the future.

Declaration of Competing Interest

We declare that all of the authors hold no conflict of interest.

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