Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60022-5

Prevalence of infection and molecular confirmation by using ITS-2 region of *Fasciola gigantica* found in domestic cattle from Chiang Mai province, Thailand

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ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 25 November 2013
Accepted 15 December 2013
Available online 20 March 2014

Keywords:
Fasciola gigantica
Domestic cattle
Chiang Mai
Molecular confirmation
ITS-2 region

ABSTRACT

Objective: To investigate the infection of *Fasciola gigantica* (*F. gigantica*) in domestic cattle from Chiang Mai province and molecular confirmation using ITS-2 region. **Methods:** The liver and gall bladder of *Bubalus bubalis* (*B. bubalis*) and *Bos taurus* (*B. taurus*) from slaughterhouses were examined adult worms and prevalence investigation. The species confirmation with phylogenetic analysis using ITS-2 sequences was performed by maximum likelihood and UPGMA methods. **Results:** The total prevalences of infection in *B. bubalis* and *Bubalus taurus* (*B. taurus*) were 67.27% and 52.94% respectively. The respective prevalence in both *B. bubalis* and *B. taurus* were acquired from Doi-Saket, Muang, and Sanpatong districts, with 81.25%, 62.50% and 60.00% for *B. bubalis* and 62.50%, 50.00% and 47.06% for *Bos taurus* respectively. The species confirmation of *F. gigantica* and some related species by basing on maximum likelihood and UPGMA methods used, 4 groups of trematodes were generated, first *F. gigantica* group including specimen of Chiang Mai, second 2 samples of *F. hepatica*, third group of 3 rumen flukes; *Orthocoelium streptocoelium*, *F. elongatus* and *Paramphistomum epliclitum* and fourth group of 3 minute intestinal flukes; *Haplorchis taichui*, *Stellantchasmu falcatus*, *Haplorchoides* sp. and liver fluke; *Opisthorchis viverrini* respectively. **Conclusions:** These results can be confirmed the Giant liver fluke which mainly caused fascioliasis in Chiang Mai was identified as *F. gigantica* and specimens were the same as those of *F. gigantica* recorded in other different countries. Nucleotide sequence of ITS-2 region has been proven as effective diagnostic tool for the identification of *F. gigantica*.

1. Introduction

Giant liver flukes, genus Fasciola have been recognized as importantly plant-borne trematode infection. These parasites are reported as mainly caused of fascioliasis in domestic ruminants and humans[1] and including they are an economically important-helminthic infection disease which having a significant impact on growth rate, development and productivity in domestic ruminants[2]. Humans are accidental hosts, with a few cases of human fascioliasis have been reported. *Fasciola hepatica(F. hepatica)* has a cosmopolitan distribution, mainly in temperate zones, while *Fasciola gigantica* (*F. gigantica*) is foundin tropical regions of Africa and Asia[3]. Furthermore, in some areas, inter-breed of *F. gigantica* and *F. hepatica* may be occurred which consequently giving intermediate or hybrid Fasciola that can launch an aberrant morphology[4]. Sibling morphology or cryptic trematode species can cause problematic case of identification using morphological characteristics alone. Various molecular approaches have been approved as effective identification tools such as sequences of nuclear ribosomal; ITS-1 and ITS-2[5] and mitochondrial; NDI and COI[6] were used to identify Fasciola species isolated from northern Iran. Amor *et al*[7] reported molecular characterization of *F. gigantica* from Mauritunia based on mitochondrial and nuclear ribosomal DNA sequences. Additionally, the ITS-2 sequences were used for the genetic identification of *F. hepatica* which it is related species of *F. gigantica* in Turkey[8] and designed the species-specific ribosomal ITS-2 markers for isolating of *F. hepatica* is included[9].

Internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) has been widely used, due to its highly repeated

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and contains variable regions flanked by more conserved regions[10,11]. Characterize of *Fasciola* samples from different host animals and geographical localities by sequences of the first and second internal transcribed spacer of ribosomal DNA was study in Niger, Spain, Tunisia, and Northern Iran[7,12-14]. Remarkably, according to the *Fasciola* samples, most studies and researches frequently conducted on *F. hepatica*, *F. gigantica* and intermediate *Fasciola* in different aspects. ITS-2 sequences of nuclear ribosomal DNA have been famously used to determine the genetic variation and identification of *F. hepatica* from different geographical region[15-17]. Moreover, molecular identification of liver fluke on the basis of ITS sequences has also been report in India, Vietnam, and China[18-22]. From these, ITS-2 sequences have been approved to be effective diagnostic region for specific identification of *Fasciola* species.

Although, present study is aimed to investigate the infection of *F. gigantica* in domestic cattle from Chiang Mai province, Thailand and to describe the molecular confirmation by using ITS-2 sequences comparing with *Fasciola* species and other related samples acquired in GenBank databases. The phylogenic tools can be applies to discriminate species of our samples and other samples.

2. Materials and methods

2.1. Prevalence study

Fifty five of *Bubalus bubalis* (*B. bubalis*) and 51 of *Bos taurus* (*B. taurus*) were examined in three districts of Chiang Mai province, during October 2010 to September 2012. Adult worms of *F. gigantica* were collected in liver and gall bladder of *B. taurus* and *B. bubalis* from slaughterhouses. The flukes were removed and counted for the prevalence calculation.

2.2. Molecular study

Genomic DNAs of all parasites were extracted and purified from adult worms using the GF-1 Tissue extraction kit (Vivantis, Malaysia), according to the manufacturer's instructions. All extracted genomic DNA were diluted to a working concentration of 50 ng/μL and stored at -20℃ until used.

Amplification of Internal Transcribed Spacer Subunit 2 (ITS-2) region. Primers to amplify the entire nuclear second internal transcribed spacer region (ITS-2) were as described by Bowles *et al*[23]. The pair of primers were 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′ (3S-F) as a forward primer, and 5′-TATGCTTAAATTCAGCGGGT-3′ (BD2-R) as a reverse primer. The final volume of each amplification reaction was 20 μL, with common PCR composition. The PCR was carried out in a My cyclerTM Thermal Cycler (Bio-Rad, USA) with PCR conditions are as follows; 1 cycle of 94℃for 4 min, 30 cycle of 94℃ for 1 min, 50℃ for 30 s, 72℃ for 45 s and 1 cycle of final extension at 72℃ for 7 min. PCR products were separated on 1.4% agarose gel electrophoresis, stained with ethidium bromide, visualized on UV trans-illuminator and photographs digitally using Kodak Gel logic. The PCR products were purified and subjected for sequencing directly by without cloned and transformed.

2.3. Data analysis

ITS-2 sequence data obtained in this study were compared with ITS-2 sequences of *F. gigantica* and related-species currently available in NCBI-GenBank. All ITS-2 sequence data were aligned and trimmed to provide an equivalent sequences among each trematode species using BioEdit 5.0.6 software. Phylogenetic tree of each trematodes were performed by maximum likelihood and UPGMA method using MEGA 5.05 software. The nucleotides diversity was calculated by DNAsp[24] and confirmed the distinct species according to "4x rule" as following Birky *et al*[25].

3. Results

3.1. Prevalence study

The flukes were collected in liver and gallbladder of *B. bubalis* and *B. taurus* in 3 slaughterhouses from Muang, Doi Saket, and Sanpatong districts of Chiang Mai province, during October 2010 to September 2012. Fifty five of *B. bubalis* and 51 of *B. taurus* were examined. The infection of *F. gigantica* in *B. bubalis* were higher than *B. taurus*, total prevalence of infection were 67.27% and 52.94%, respectively. The respective prevalences (highest to lowest) of *F. gigantica* infection in both *B. bubalis* and *B. taurus* were acquired from Doi-Saket, Muang, and Sanpatong districts, with 81.25% (13/16), 62.50% (15/24) and 60.00% (9/15) for *B. bubalis* and 62.50% (10/16), 50.00% (9/18) and 47.06% (8/17) for *B. taurus* respectively (Figure 1).

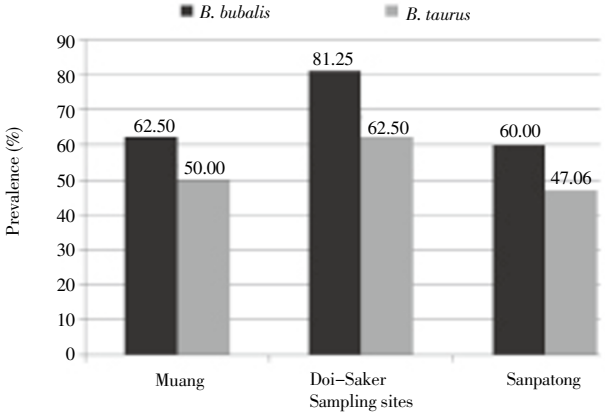

Figure 1. The prevalences of adult worm infection were found in *B. bubalis* and *B. taurus* of Muang, Doi-Saket, and Sanpatong districts of Chiang Mai province.

3.2. Sequence data of ITS-2 region

ITS-2 sequences of *F. gigantica* were comparative analyzed with related trematodes species that available in

Genbank. Supplemented fluke samples such as; *Fishoederius elongatus(F. elongatus), Paramphistomum epiclitum(P. epiclitum), Orthocoelium streptocoelium(Or. streptocoelium), Haplorchis taichui(H. taichui), Stellantchasmus (S. falcatus), Haplorchoides* sp., *Opisthorchis viverrini(O. viverrini)* were also included. PCR products of ITS-2 region generated by primer 3S-F (forward primer) and BD2-R (reverse primer) were found approximately 500 base pairs (bps.). A few over hanging in fragment sizes between species were also found (Figure 2). Then, all PCR products were purified and subjected for sequencing directly by without cloned and transformed. Sequences alignment of *F. gigantica* found in this study demonstrated that, maximum length obtained was 533 base pairs (Figure 3).

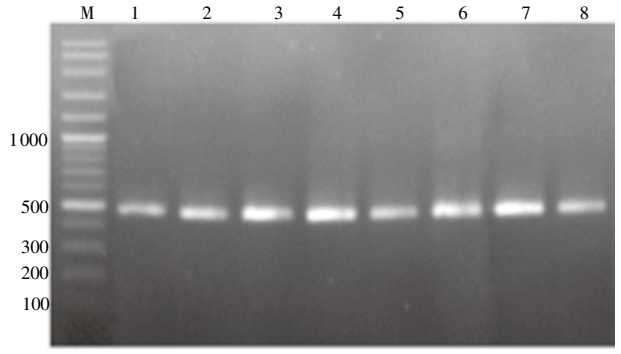

Figure 2. DNA profiles of ITS-2 region derived from BD2-R and 3S-F primer.
Lane M: DNA marker (VC 100 bp DNA Ladder); lane 1: *F. gigantica*; lane 2: *F. elongatus*; lane 3: *P. epiclitum*; lane 4: *Or. streptocoelium*; lane 5: *H. taichui*; lane 6: *S. falcatus*; lane 7: *Haplorchoides* sp. ; lane 8: *O. viverrini*.

Figure 3. Illustration demonstrated 533 ITS-2 nucleotide sequences of *F. gigantica* found in this study.

Based on the ITS-2 sequence data, they were then aligned and compared with 12 accession numbers of *F. gigantica* ITS-2 sequences including *F. hepatica* that available in NCBI-GenBank and some supplemented fluke species (this study). The result showed that *F. gigantica* found in this study revealed 98%-99% maximum identities as demonstrated in Table 1.

Table 1
Demonstrate the maximum identities and query coverage of *F. gigantica* found in this study compared with closely related-trematodes available in NCBI-GenBank.

Accession	Geographical location	Query coverage (%)	Maximum identity (%)
HQ700438.1	China	95	99
AB536921.1	Viet Nam	95	99
AB553695.1	Egypt	95	99
JF930346.1	India	95	99
AB010977.1	Indonesia	93	99
AB010979.1	Japan	93	99
AB010976.1	Zambia	93	99
JN828958.1	Iran	85	99
AM850108.1	Niger	87	99
AB207149.1	Thailand	93	99
JF432078.1	Iran	86	98
JF708029.1	USA	87	98

The genetic diversity of *F. gigantica* in this study compared with *F. gigantica* and *F. hepatica* that available in GenBank. The results shown that *F. gigantica* in this study are the same species with other countries ($K<4\theta$) while, *F. gigantica* difference from *F. hepatica* ($K>4\theta$) (Table 2).

3.3. Analysis of phylogenetic relationships

Based on comparative study of ITS2 sequences among *F. gigantica* obtained in this study with closely related samples in Genbank, phylogenetic relationships was analyzed, while 7 supplemented trematodes; *H. taichui*, *S. falcatus*, *Haplorchoides* sp., *O. viverrini*, *Or. streptocoelium*, *F. elongatus* and *P. epliclitum* were used as an out group. Maximum likelihood (ML) and UPGMA methods with bootstrap values of 1 000 replicates set were performed. The results showed that, 4 groups of trematodes were generated, first was *F. gigantica* group including the specimen of Chiang Mai, second was 2 samples of *F. hepatica* (GenBank), third was group of 3 rumen flukes; *O. streptocoelium*, *F. elongatus* and *P. epliclitum* (this study) and fourth was group of 3 minute intestinal flukes; *H. taichui*, *S. falcatus*, *Haplorchoides* sp. and liver fluke; *O. viverrini* respectively (Figure 4&5). These results can be confirmed that the Giant liver fluke which mainly caused fascioliasis in Chiang Mai, Thailand was identified as *F. gigantica*.

Table 2.
The variables speciation of genetic diversity of *F. gigantica* and *F. hepatica*.

Population	Nucleotide diversity (π)	θ	$\theta \times 4$	Sequence divergence between clades (K)	If $K \geqslant 4\theta$
F. gigantica	0.00242	0.00251	0.01004	0.00142	No
F. hepatica	0.00236	0.00236	0.00944	0.01071	Yes

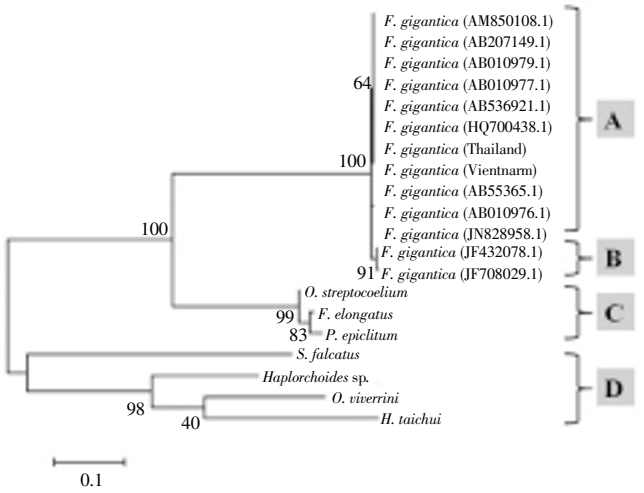

Figure 4. Phylogram derived from maximum likelihood analysis depicting phylogenetic relationships of trematodes used in this study by basing on ITS-2 sequences: (A): group of *F. gigantica* obtained from both Chiang Mai and GenBank; (B): group of *F. hepatica* (GenBank); (C): group of rumen flukes (*O. streptocoelium*, *F. elongatus* and P. epliclitum); (D): group of minute intestinal fluke (*H. taichui*, *S. falcatus*, *Haplorchoides* sp.) and liver flukes (*O. viverrini*).

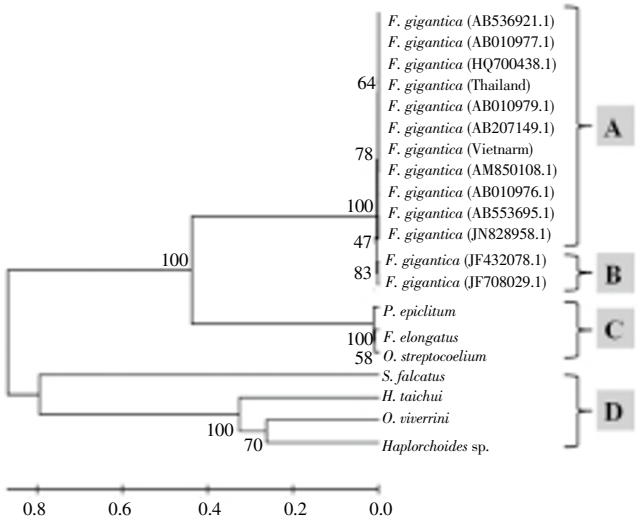

Figure 5. Phylogram derived from an UPGMA analysis depicting phylogenetic relationships of trematodes used in this study by basing on ITS2 sequence data: (A): group of *F. gigantica* obtained from both Chiang Mai and GenBank; (B): group of *F. hepatica* (GenBank); (C): group of rumen flukes (*O. streptocoelium*, *F. elongatus* and *P. epliclitum*); (D): group of minute intestinal fluke (*H. taichui*, *S. falcatus*, *Haplorchoides* sp.) and liver flukes (*O. viverrini*).

4. Discussion

In Chiang Mai province, infection of fasciolasis in domestic cattle is still high. Based on our result, it can be confirmed that, the giant liver flukes found in Chiang Mai province were identified as only *F. gigantica*. There was no any evidence that provides information concerning the infection of *F. hepatica* in northern Thailand. This result corresponds with the study of Sukhapesna *et al*[26] that revealed the main cause of fascioliasis in domestic ruminants from Thailand is *F. gigantica*. The prevalence of *F. gigantica* infecting domestic cattle in Chiang Mai province was already determined. Prevalence of infection in *B. bubalis* was higher than *B. taurus*, with total prevalence of 65.71% and 46.67%, respectively. However, our findings are contradicted with the study of Sobhon *et al*[27] that demonstrate the prevalence of *F. gigantica* infection in cattle were only 4%-24%, with highest incidences in the north and northeastern, and the lowest in the south. A few different of *F. gigantica* infection among *B. bubalis* and *B. taurus* was observed and it may depend on the preference of feeding behavior. *B. bubalis* can be feed a variety of grazing and aquatic plants and its can be feed food in both terrestrial and aquatic whereas *B. taurus* was mostly cultured in farmland system that it has been greatly management and frequently treated by anthelmintics. Moreover, the incidence of *F. gigantica* found in Chiang Mai was higher than the other regions of Thailand which may due to the *B. bubalis* and *B. taurus* from slaughterhouses of Chiang Mai province was mostly imported from neighboring countries and tended to increase which may be the point of higher fluke diversity in this region.

By basing on the comparative study of nucleotide sequences of *F. gigantica* ITS-2 sequences, it was found that *F. gigantica* collected in Chiang Mai, Thailand are grouped with *F. gigantica* in GenBank while, *F. hepatica* and including other related-species are separated individually. The molecular analysis of the ITS-1 and ITS-2 genes of *Fasciola* spp. were commonly reported in Asia countries[5]. In accordance with the constructed phylogenetic trees, *F. gigantica* in this study showed highly relationship with the groups of *F. gigantica* that acquired from GenBank. The K>4 θ criterion proposed by Birky[28] are presented in Table 2 show that the *F. gigantica* from Thailand (group A) are genetically closely with *F. gigantica* from other countries. Moreover the K>4 θ criterion are indicated *F. gigantica* in this study diverge with *F. hepatica*. The degree of separation between these groups was sufficient for diagnosed as distinct species using four times rule for speciation[29]. Our finding is in agreement with Amor *et al*[30] who reported confirmation Fasciola spp. using ITS-1 and ITS-2 sequences showed closely relationship of the Mauritanian samples with isolates of *F. gigantica* from different localities of Africa and Asia. Moreover NCBI databases can be use for compared with other species of trematodes in the family Fasciolidae[19].

The results can be confirmed that the Giant liver fluke which mainly caused fascioliasis in Chiang Mai, Thailand was identified as *F. gigantica* as same as found in other different countries with the maximum identities of 98%-99%. The infection of *F. gigantica* in *B. bubalis* was higher than *B. taurus*. The molecular identification and phylogenetic analysis by using nucleotide sequence of ITS-2 region is approved to be effective diagnostic tool to confirmed species of *F. gigantica* which useful for specific identification particularly in developmental stages. The four times rule for speciation is useful to separate between these groups.

Conflict of interest statement

The authors have no any financial or personal interests and

final article have been approved by all authors.

Acknowledgements

The authors wish to thank Miss Warapron Noikong and Mr. Suksan Chuboon for their assistances. Special thanks are extended to the Parasitology Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University for lab facilities and the grateful thanks the Graduated School, Chiang Mai University for some financial support.

References

[1] Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol* 2005; **35**(11-12): 1255-1278.

[2] Dargie JD. The impact on production and mechanisms of pathogens of trematodes infections in cattle and sheep. *Int J Parasitol* 1987; **17**: 453-463.

[3] Mas-Coma S, Bargues MD. Human liver flukes: a review. *Res Rev Parasitol* 1997; **57**: 145-218.

[4] Lotfy WM, Hillyer GV. *Fasciola* species in Egypt. *Exp Pathol Parasitol* 2003; **6**: 9-22.

[5] Amer S, Dar Y, Ichikawa M, Fukuda Y, Tada C, Itagaki T, et al. Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS-1 and ITS-2) and mitochondrial (NDI and COI) gene markers. *Parasitol Int* 2011; **60**(1): 5-12.

[6] Sharifiyazdi H, Moazeni M, Rabbani F. Molecular characterization of human *Fasciola* samples in Gilan Province, Northern Iran on the basis of DNA sequences of ribosomal and mitochondrial DNA genes. *Comp Clin Path* 2012; **21**(5): 889-894.

[7] Amor N, Halajian A, Merella P, Farjallah S, Said K, Slimane BB. Genetic characterization of *Fasciola* spp. from Tonekabon City (Northern Iran) based on the ribosomal internal transcribed spacer regions. *Pak J Zool* 2011; **43**(6): 1061-1067.

[8] Erensoy A, Kuk S, Ozden M. Genetic identification of *Fasciola hepatica* by ITS-2 sequence of nuclear ribosomal DNA in Turkey. *Parasitol Res* 2009; **105**: 407-412

[9] Bazsalovicsova E, Kralova-Hromadova I, Spakulova M, Reblanova M, Oberhauserova K. Determination of ribosomal internal transcribed spacer 2 (ITS-2) interspecific markers in *Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum* and *Paramphistomum cervi* (Trematoda), parasites of wild and domestic ruminants. *Helminthologia* 2010; **47**: 76-82.

[10] Hillis DM, Dixon MT. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 1991; **66**(4): 411-453.

[11] Morgan JAT, Blair D. Nuclear rDNA ITS sequence variation in the trematode genus Echinostoma: An aid to establishing relationships within the 37-collar spine group. *Parasitology* 1995; **111**: 609-615.

[12] Ali H, Ai L, Song HQ, Ali S, Lin RQ, Seyni B, et al. Genetic characterisation of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. *Parasitol Res* 2008; **102**: 1021-1024.

[13] Alasaad S, Huang CQ, Li QY, Granados JE, García-Romero C, Pérez JM, et al. Characterization of *Fasciola* samples from different host species and geographical localities in Spain by sequences of internal transcribed spacers of rDNA. *Parasitol Res* 2007; **101**: 1245-1250.

[14] Farjallah S, Sanna D, Amor N, Mehel BB, Piras MC, Merella P, et al. Genetic characterization of *Fasciola hepatica* from Tunisia and Algeria based on mitochondrial and nuclear DNA sequences. *Parasitol Res* 2009; **105**: 1617-1621.

[15] Adlard RD, Barker SC, Blair D, Cribb TH. Comparison of the second internal transcribed spacer (Ribosomal DNA) from populations and species of fasciolidae (Digenea). *Int J Parasitol* 1993; **23**(3): 423-425.

[16] Huang WY, He B, Wang CR, Zhu XQ. Characterisation of *Fasciola* species from mainland China by ITS-2 ribosomal DNA sequence. *Vet Parasitol* 2004; **120**: 75-83.

[17] Erensoy A, Kuk S, Ozden M. Genetic identification of *Fasciola hepatica* by ITS-2 sequence of nuclear ribosomal DNA in Turkey. *Parasitol Res* 2009; **105**: 407-412.

[18] Prasad PK, Tandon V, Biswal DK, Goswami LM, Chatterjee A. Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions. *Parasitol Res* 2008; **103**: 1247-1255.

[19] Le TH, De NV, Agatsuma T, Blair D, Vercruysse J, Dorny P, et al. Molecular confirmation that *Fasciola gigantica* can undertake aberrant migrations in human hosts. *J Clin Microbiol* 2007; **45**(2): 648-650.

[20] Nguyen TGT, Van DN, Vercruysse J, Dorny P, Le TH. Genotypic characterization and species identification of *Fasciola* spp. with implications regarding the isolates infecting goats in Vietnam. *Exp Parasitol* 2009; **123**(4): 354-361.

[21] Ashrafi K, Massoud J, Holakouie Naieni K, Jo-Afshani MA, Mahmoodi M, Ebadati N, et al. Nuclear ribosomal DNA ITS-2 sequence characterization of *Fasciola hepatica* and Galba truncatula. *Iran J Public Health* 2007; **36**(4): 42-49.

[22] Lin RQ, Dong SJ, Nie K, Wang CR, Song HQ, Li AX, et al. Sequence analysis of the first internal transcribed spacer of rDNA supports the existence of the intermediate *Fasciola* between *F. hepatica* and *F. gigantica* in mainland China. *Parasitol Res* 2007; **101**: 813-817.

[23] Bowles J, Blair D, Mcmanus DP. A molecular phylogeny of the human schistosomes. *Mol Phylogenet Evol* 1995; **4**: 103-109.

[24] Rozas J, Sanchez-DeBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 2003; **19**: 2496-2497.

[25] Birky CW, Adams J, Gemmel M, Perry J. Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. *PLoS ONE* 2010; **5**(5): e10609.

[26] Sukhapesna V, Tuntasuvan D, Sarataphan N, Imsab K. Economic impact of fascioliasis in buffalo production. *J Thai Vet Med Assoc* 1994; **45**: 45-52.

[27] Sobhon P, Anantavara S, Dangprasert T, Viyanant V, Krailas D, Upatham ES, et al. *Fasciola gigantica*: studies of the tegument as a basis for the developments of immune diagnosis and vaccine. *Southeast Asian J Trop Med Public Health* 1998; **29**: 387-400.

[28] Birky CW. Species detection and identification in sexual organisms using population genetic theory and DNA sequences. *PLoS ONE* 2013; **8**(1): e52544.

[29] Walker SM, Prodohl PA, Hoey EM, Fairweather I, Hanna REB, Brennan G, et al. Substantial genetic divergence between morphologically indistinguishable populations of *Fasciola* suggests the possibility of cryptic speciation. *Int J Parasitol* 2012; **42**: 1193-1199.

[30] Amor N, Farjallah S, Salem M, Lamine DM, Merella P, Said K, et al. Molecular characterization of *Fasciola gigantica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. *Exp Parasitol* 2011; **129**(2): 127-136.