Veterinary Parasitology 273 (2019) 45–51

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Genetic Characterization of Cambodian *Fasciola gigantica* and Dispersal Direction of the Species in Asia

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ARTICLE INFO

Keywords:
Fasciola gigantica
Cattle
Phosphoenolpyruvate carboxykinase gene
NADH dehydrogenase subunit 1 gene
Population diversity
Gene flow

ABSTRACT

Fasciola gigantica and hybrid *Fasciola* are distributed throughout Asia. Herein, we investigated the species of the *Fasciola* fluke distributed in three hotspots of fascioliasis in Cambodia. A total of 92 flukes collected from 21 slaughtered cattle from Kandal (44), Battambang (41), and Kratie (7) Provinces were identified as *F. gigantica* using multiplex PCR for a nuclear phosphoenolpyruvate carboxykinase (*PEPCK*) gene. The overall prevalence of *F. gigantica* infestation was 7.14% (21/294). Phylogenetic as well as population genetics analyses were performed using the mitochondrial NADH dehydrogenase subunit 1 (*ND1*). The 19 *ND1* haplotypes were identified from Cambodian *F. gigantica* (haplotype diversity, 0.83). All of the haplotypes were classified into *F. gigantica* haplogroup C, which includes *ND1* haplotypes detected from Thailand, Vietnam, Indonesia, Myanmar, and China. Among haplogroup C, novel and unique haplotypes of Cambodia were found in the Battambang and Kandal Provinces, and the nucleotide diversity of the Cambodian population (0.00532) was the highest. Pairwise fixation indices among the *F. gigantica* populations from these countries indicated that the Cambodian and Thailand populations were related to each other. The highest genetic diversity in the Cambodian population suggests that *F. gigantica* in Cambodia may be the ancestor of the populations in Southeast Asian countries. Most likely, livestock movement, including *Zebu* cattle, played an important role in the transmission of *F. gigantica*. In this study, the hybrid *Fasciola* flukes that are commonly found in neighboring countries, were not found in Cambodia. Further comprehensive investigations of *Fasciola* prevalence should be conducted by analyzing a wider range of hosts throughout Cambodia to reach a more solid conclusion about the absence of hybrid flukes.

1. Introduction

Fascioliasis is one of the most important parasitic diseases of livestock, reducing the productivity of animal industries and imposing an economic burden of at least 2 billion dollars annually worldwide (Mas-Coma et al., 2009). Furthermore, fascioliasis is included in the World Health Organization's list of neglected zoonotic tropical diseases of humans (WHO, 2012), and at least 90 million people worldwide are at risk of *Fasciola* infection (Keiser and Utzinger, 2009). *Fasciola hepatica* and *Fasciola gigantica* cause fascioliasis in animals and humans. *F. hepatica* resides in temperate zones, whereas *F. gigantica* is restricted to tropical regions, mainly in Africa and Asia (Torgerson and Claxton, 1999).

Classically, the differentiation between *F. hepatica* and *F. gigantica* was based on their morphological criteria such as body size and shape; however, this is generally difficult because of the substantial variations found in their morphological features. In addition, hybrid forms of *F. hepatica* and *F. gigantica* have been identified in Asian countries, which

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https://doi.org/10.1016/j.vetpar.2019.07.013
Received 27 February 2019; Received in revised form 30 July 2019; Accepted 31 July 2019

indicates taxonomic complexities in the genus (Ashrafi et al., 2006). It has been accepted that specific differentiation of liver flukes cannot be achieved solely by morphological examination (Periago et al., 2006). Application of molecular approaches is therefore required to identify *Fasciola* flukes at the species level.

Analysis of the sequences of nuclear ribosomal internal transcribed spacers ITS1 and ITS2 were employed for the differentiation of *Fasciola* spp. (Agatsuma et al., 2000; Le et al., 2008). In addition, the mitochondrial NADH dehydrogenase subunit 1 (*ND1*) gene is commonly used for phylogenetic studies (Itagaki et al., 2005a). However, it has been suggested that PCR analysis of these genes is unable to distinguish the hybrids from *F. hepatica* and *F. gigantica* (Shoriki et al., 2016). A multiplex PCR using a nuclear marker, the phosphoenolpyruvate carboxykinase (*PEPCK*) gene, could distinguish among *F. hepatica, F. gigantica*, and the hybrid fluke in East and Southeast Asian (SEA) countries (Shoriki et al., 2016).

Approximately 28%–56% of free-grazing Cambodian cattle and buffaloes are at risk of *Fasciola* infection in the seasonal flooding areas, mainly in the vicinity of Tonle Sap Lake and along the Mekong and Bassac Rivers (Tum et al., 2004). A study conducted at an abattoir in Kandal Province found that 18.9% of 644 cattle livers were positive for *Fasciola*, and approximately 24.7% of fecal samples were positive (Sothoeun et al., 2006). An increasing number of humans in SEA are being infected with *F. hepatica, F. gigantica,* or their hybrid in countries such as Thailand (Wannasan et al., 2014) and Vietnam (Itagaki et al., 2009; Hayashi et al., 2018). Bless et al. (2015) reported the *Fasciola* seropositivity of children in two schools of Kandal Province in Cambodia.

We hypothesized that the hybrid fluke might arise in Cambodia. However, the molecular characterization of *Fasciola* flukes has never previously been performed in Cambodia. Therefore, the objectives of this study were to investigate the *Fasciola* species distributed in the hot spot districts of Cambodia, using the *PEPCK* gene; and to determine the distribution route of *F. gigantica* by analyzing its phylogenetic relationships with populations in neighboring Asian countries based on the *ND1* gene.

2. Materials and methods

2.1. Collection of Fasciola flukes and morphological analysis

In July 2017, sample collections were conducted in 4 slaughterhouses from three provinces, which are considered fascioliasis hotspots in Cambodia, Kandal (1 abattoir), Kratie (1 abattoir), and Battambang (2 abattoirs) (Tum et al., 2004; Sothoeun et al., 2006). These abattoirs are located in the capital of each province and are the center houses where the slaughtered cattle are submitted daily. We collected samples consecutively over seven nights in each abattoir.

Out of 294 slaughtered cattle, 21 cattle were infected with liver flukes in their bile ducts. Depending on the degree of infection, one to 30 adult flukes were collected from each infected liver. Then, one to ten flukes were randomly selected from each liver for further laboratory examination that resulted in a total of 92 adult flukes. The number of abattoirs, the infected cattle, and the selected adult flukes from each province are presented in Fig. 1 and Table S1. Fresh adult flukes were washed in 0.9% saline solution and laid flat on a glass slide to measure the distance between the ventral sucker to the posterior end (VS-P), body length (BL), and body width (BW). Each worm was gently pressed between two slides to avoid distortion and then preserved in 70% ethanol and stored at −20 °C. A one-way ANOVA test was employed for statistical analyses. *P* values below 0.05 were considered significant.

2.2. DNA extraction

Each fluke was punctured (approximately 0.5 cm diameter) around their posterior ends to harvest tissue for DNA extraction. Total genomic DNA (gDNA) was extracted using the NucleoSpin DNA RapidLyse Kit (Macherey-Nagel GmbH, Germany) according to the manufacturer's instructions. The resulting DNA was eluted with 100 μl of Buffer BE at the final step. All gDNA samples were stored at −20 °C until the next step. A hybrid worm (Wannasan et al., 2014) was included as the positive extraction control.

2.3. DNA analyses

2.3.1. Multiplex PCR (mPCR) analysis

The mPCR primers to amplify the *PEPCK* gene were Fg-pepcK-F, Fh-pepcK-F, and Fcmn-pepcK-R (Shoriki et al., 2016). Reactions were performed in a 20 μl reaction mixture containing 5 ng template gDNA, 0.5 μM of each forward Fh-pepcK-F and Fg-pepcK-F, 1 μM of Fcmn-pepcK-R, 0.5 unit of KOD-Multi & Epi-polymerase (Toyobo, Japan), and the manufacturer's supplied reaction buffer. The amplification cycles were as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles at 98 °C for 10 s, 61 °C for 10 s, and 68 °C for 30 s, using a T100™Thermal Cycler (Bio-Rad, USA). Distilled water (DW) as a negative control and the gDNA of *F. hepatica*, *F. gigantica*, and a hybrid form (Wannasan et al., 2014) as positive controls were included in the reactions. Amplicons were analyzed using agarose (1.5%) gel electrophoresis.

2.3.2. Nucleotide sequence analysis of the mitochondrial ND1 gene

The mitochondrial *ND1* gene was amplified using primers ita10 and ita2 (Itagaki et al., 2005a). PCR amplification of *ND1* was performed in a 40 μl reaction mixture containing 10 ng template gDNA, 0.2 μM of each primer, and the manufacturer's supplied PCR Master Mix (Quick Tag HS DyeMix; Toyobo, Japan). Amplifications were performed as follows: 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 68 °C for 30 s, using a T100™Thermal Cycler (Bio-Rad, USA). DW as a negative control and gDNA of *F. gigantica* as a positive control (Wannasan et al., 2014) were included in the reactions. All PCR products, which were purified using a NucleoSpin PCR Clean-up Kit (Macherey-Nagel GmbH, Germany), were sequenced by Macrogen Inc. (South Korea) in the sense and antisense directions using the PCR primers described above.

2.4. Phylogenetic analysis

ND1 sequences were analyzed using BLAST (NCBI) and aligned using ClustalW as implemented in MEGA version 6 (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2013). We generated a neighbor-joining (NJ) tree using Kimura 2-Parameter distances with bootstrapping (1000 replicates) to assemble phylogenetic trees representing the clustering patterns. The sequence of *Paragonimus westermani* (Accession number. AF219379) was used as an out-group. In addition, the sequences of *F. hepatica* from Australia, China and Japan; *F. gigantica* from Zambia, Egypt, India, Nepal, Bangladesh, China, Thailand, Indonesia, Vietnam and Myanmar; and aspermic *Fasciola* sp. from Bangladesh, Korea, Japan, China, Thailand, Vietnam and Myanmar were used as references for tree construction (Table S3).

We used Network 4.6.1.2 (Bandelt et al., 1999) to generate a median-joining (MJ) network of *ND1* haplotypes and reference haplotypes. The reference haplotypes and their frequencies have been published in previous papers: Japan (Ichikawa et al., 2010; Itagaki et al., 2005b), South Korea (Itagaki et al., 2005b), China (Ichikawa-Seki et al., 2017; Peng et al., 2009), Vietnam (Itagaki et al., 2009), Thailand (Chaichanasak et al., 2012), Myanmar (Ichikawa et al., 2011), India (Hayashi et al., 2015, 2016b), Bangladesh (Mohanta et al., 2014), Nepal (Shoriki et al., 2014), and Indonesia (Hayashi et al., 2016a). In the MJ network, a median vector is the proposed haplotype required to connect haplotypes within the network.

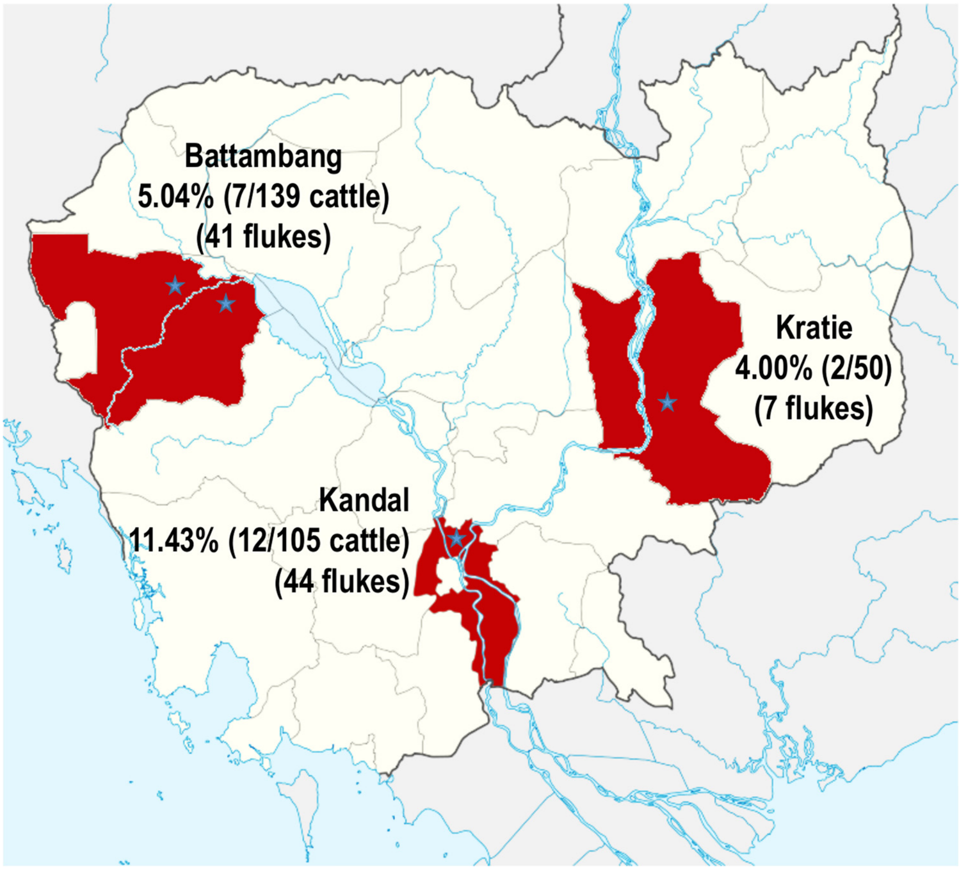

Fig. 1. Map of Cambodia with the red color indicating the three provinces where *Fasciola* specimens were collected. Blue stars indicate the location of slaughterhouses. The prevalence (infected/total cattle) and the actual numbers of fluke specimens are displayed for each province. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.5. Population genetic analysis

For the populations of haplogroup C of *F. gigantica* from Cambodia and other reference countries (Thailand, Vietnam, Indonesia, and China), we used DnaSP 6.11.01 (Rozas et al., 2017) to calculate the number of flukes (*N*), number with variable sites (*S*), number of haplotypes (*h*), and nucleotide diversity (π). The extent of gene flow among populations was estimated using the pairwise fixation index (*F*st), which provides a relative measure of strain differentiation. *Fst* was calculated using Arlequin 3.5 (Excoffier and Lischer, 2010). *F*st values approaching 1 indicated extreme genetic differentiation between populations. We conducted pairwise analyses of five populations of *F. gigantica* from Cambodia (present study), Thailand (Chaichanasak et al., 2012), Vietnam (Itagaki et al., 2009), Indonesia (Hayashi et al., 2016a), and China (Ichikawa-Seki et al., 2017; Peng et al., 2009).

3. Results

3.1. Prevalence of fascioliasis

Out of 294 slaughtered cattle, the overall prevalence of *Fasciola* infection was 7.14% (21/294). The *Fasciola* infection rates in the cattle were 11.43% (12/105), 5.04% (7/139) and 4.00% (2/50) from Kandal, Battambang, and Kratie, respectively (Fig. 1 and Table S1).

3.2. Morphometric analysis

The morphometric indices of 82 intact bodies and appendages of the flukes were measured and grouped according to their occurrence in geographic hotspots. *Fasciola* flukes in the present study had average and SD values (ranges) of BLs = 3.60 ± 0.04 cm (2.90–4.70 cm), BW = 0.92 ± 0.01 cm (0.60–1.2 cm), BL/BW ratio = 3.98 ± 0.64 (2.53–2.90), and VS-P = 3.14 ± 0.05 cm (2.30–4.20 cm) (Table 1).

3.3. mPCR identification of species

The mPCR of the *PEPCK* gene discriminates a long amplicon size of *F. gigantica* (approximately 500 bp) from a shorter amplicon of *F. hepatica* (approximately 240 bp), and two amplicons generated from *F. gigantica* and *F. hepatica* sequences for the hybrid *Fasciola*. In comparison to the positive controls, the 92 Cambodian flukes were accordingly identified as *F. gigantica*. None of the Cambodian specimens displayed the hybrid fragment pattern.

3.4. Mitochondrial ND1 haplotypes

A total of 21 substitutions were detected in the partial nucleotide sequence of the *ND1* (535 bp) gene, and the 19 haplotypes were distinguished. They were designated as Fg-ND1-CM1 through Fg-ND1-CM19 (DDBJ/*EMBL*/*GenBank* Accession No. LC373018-LC373036). The substitutions included six transversions, 16 transitions, and 4 nonsynonymous variations. These *ND1* haplotypes were 98.70%–99.60% identical. The Fg-ND1-CM12 haplotype was detected in all hotspots at the highest frequency (35.87%, 33/92 flukes) (Table 2). The second and third highest frequencies were those of Fg-ND1-CM9 (13.04%, 12/92 flukes) found in Kandal and Kratie, and Fg-ND1-CM5 (10.87%, 10/92 flukes) obtained in Battambang and Kratie, respectively. There were 15 haplotypes of 39 flukes from Kandal, 10 haplotypes of 38 flukes from Battambang, and two haplotypes of five flukes from Kratie (Table S1). The 21 nucleotide variations of the 19 haplotypes are shown in Table S2.

Analysis of the NJ phylogenetic tree revealed that the 19 haplotypes of Cambodian flukes were included in the Asian clade of *F. gigantica*, which was distinct from the African *F. gigantica* clade (bootstrap value = 86%) (Fig. 2).

The MJ network shown in Fig. 3 displays the relationship among the 19 ND1 haplotypes together with the haplotypes detected from the

Table 1
Morphometric measurement of *F. gigantica* flukes from Cambodian cattle.

Measurement (cm)	All flukes		Kandal		Battambang		Kratie	
	Range	X ± SD	Range	X ± SD	Range	X ± SD	Range	X ± SD
	n = 82		n = 39		n = 38		n = 5	
BL	2.90-4.70	3.60 ± 0.04	2.90-4.70	3.80 ± 0.74	2.90-4.50	3.48 ± 0.06	3.30- 3.50	3.44 ± 0.04
BW	0.60-1.20	0.92 ± 0.01	0.60-1.20	0.92 ± 0.02	0.60-1.20	0.93 ± 0.02	0.70-0.90	0.84 ± 0.04
VS-P	2.30-4.20	3.14 ± 0.05	2.40-4.20	3.33 ± 0.07	2.30-4.00	2.98 ± 0.06	2.80-3.10	2.98 ± 0.05
BL/BW	2.53-2.90	3.98 ± 0.64	3.27-5.38	4.18 ± 0.09	2.90-5.43	3.76 ± 0.08	3.78-4.71	4.13 ± 0.17

BL, body length; BW, body width; VS-P, distance between ventral sucker to posterior end. Incomplete worms were not included in the measurements.

Table 2
Number of *Fasciola* flukes, sites, DNA types, and species identification.

ND1-haplotype	Accession no.	Number of fluke	Province
Fg-ND1-CM1	LC373018	3	Battambang, Kandal
Fg-ND1-CM2	LC373019	2	Kandal
Fg-ND1-CM3	LC373020	1	Kandal
Fg-ND1-CM4	LC373021	1	Kandal
Fg-ND1-CM5	LC373022	10	Battambang, Kandal
Fg-ND1-CM6	LC373023	2	Battambang
Fg-ND1-CM7	LC373024	6	Battambang, Kandal
Fg-ND1-CM8	LC373025	2	Kandal
Fg-ND1-CM9	LC373026	12	Kandal,Kratie
Fg-ND1-CM10	LC373027	2	Kandal
Fg-ND1-CM11	LC373028	7	Battambang, Kandal
Fg-ND1-CM12	LC373029	33	Battambang, Kratie, Kandal
Fg-ND1-CM13	LC373030	1	Kandal
Fg-ND1-CM14	LC373031	1	Battambang
Fg-ND1-CM15	LC373032	1	Battambang
Fg-ND1-CM16	LC373033	2	Kandal
Fg-ND1-CM17	LC373034	3	Battambang, Kandal
Fg-ND1-CM18	LC373035	1	Battambang
Fg-ND1-CM19	LC373036	2	Kandal
Total		92	

reference countries. The Cambodian *F. gigantica* haplotypes clustered into haplogroup C could be distinguished from those of haplogroups A and B. The Fg-ND1-CM1 haplotype was uniquely found in Cambodian *F. gigantica*. A median vector connected this haplotype to three clusters of the other 18 haplotypes. One of the predominant haplotypes, Fg-ND1-CM12, had an identical sequence to those of Thailand (T7: AB207160) and of Myanmar (M10: AB604016). The satellite haplotypes, Fg-ND1-CM11, CM13, CM15, and CM16, were derived from CM12 with single nucleotide substitutions (Table S2). The second predominant haplotype of Fg-ND1-CM17 was identical to those of Thailand (T27: AB603736) and Indonesia (ID12: LC127275), with satellite haplotypes Fg-ND1-CM7, CM8, CM9, and CM19. Fg-ND1-CM4, CM5, CM6, CM10, and CM14 were related to the haplotypes from Thailand (T17: AB603726, T19: AB603728), Indonesia (ID1: LC127264), Vietnam (V2: AB385616), and China (C1: AB477364). The Fg-ND1-CM2 and CM3 were derived from single nucleotide substitutions from the haplotypes of Thailand (T18: AB603727), Vietnam (V1: AB385615), and China (C9: AB604933).

3.5. Genetic diversities between F. gantica populations

The *ND1* haplotype and nucleotide diversity indices of the haplogroup C were compared with the flukes from the reference Asian countries (Table 3). The *ND1* haplotype diversity (Hd) value of the 92 Cambodian *F. gigantica* flukes was 0.836, and the highest number (15) of haplotypes represented flukes present in Kandal (Table S1). The highest nucleotide diversity (π) (0.00532) was found in the Cambodian *F. gigantica* population, followed by the populations from Thailand, Indonesia, China and Vietnam (Table 3). Moreover, the differences among these values were statistically significant ($P < 0.05$).

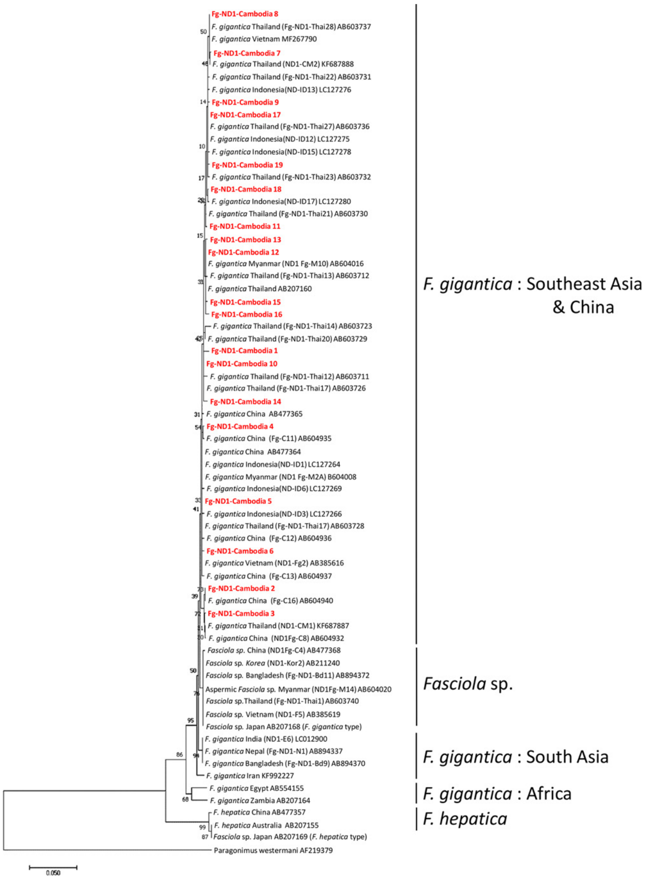

Fig. 2. The neighbor-joining phylogenetic tree based on the mitochondrial *ND1* haplotype of *F. gigantica* from Cambodia and other countries. The 19 haplotypes (Fg-ND1-Camboida1-19) were detected from Cambodia. The scale bar indicates the nucleotide substitution per site.

The highest *F*st value was observed between Cambodia and Vietnam (0.41438), followed by the values with China (0.34294), Indonesia (0.16002) and Thailand (0.02904). The *F*st values were statistically significant, except for that between the Chinese and Vietnamese populations (Table 4).

4. Discussion

The accurate differentiation of *Fasciola* species is useful for understanding the epidemiology, prevention, and control of fascioliasis. Epidemiological studies conducted in SEA, including Cambodia, have shown that *F. gigantica* is the predominant species (Gray et al., 2008). In the present study, our analysis of the *PEPCK* and *ND1* genes revealed that the 92 *Fasciola* flukes collected from naturally infected cattle from different provinces of Cambodia (Kandal, Battambang, and Kratie) were *F. gigantica*. We consider these analyses to be highly convincing, because the findings of the analyses of nuclear *PEPCK* and mitochondrial

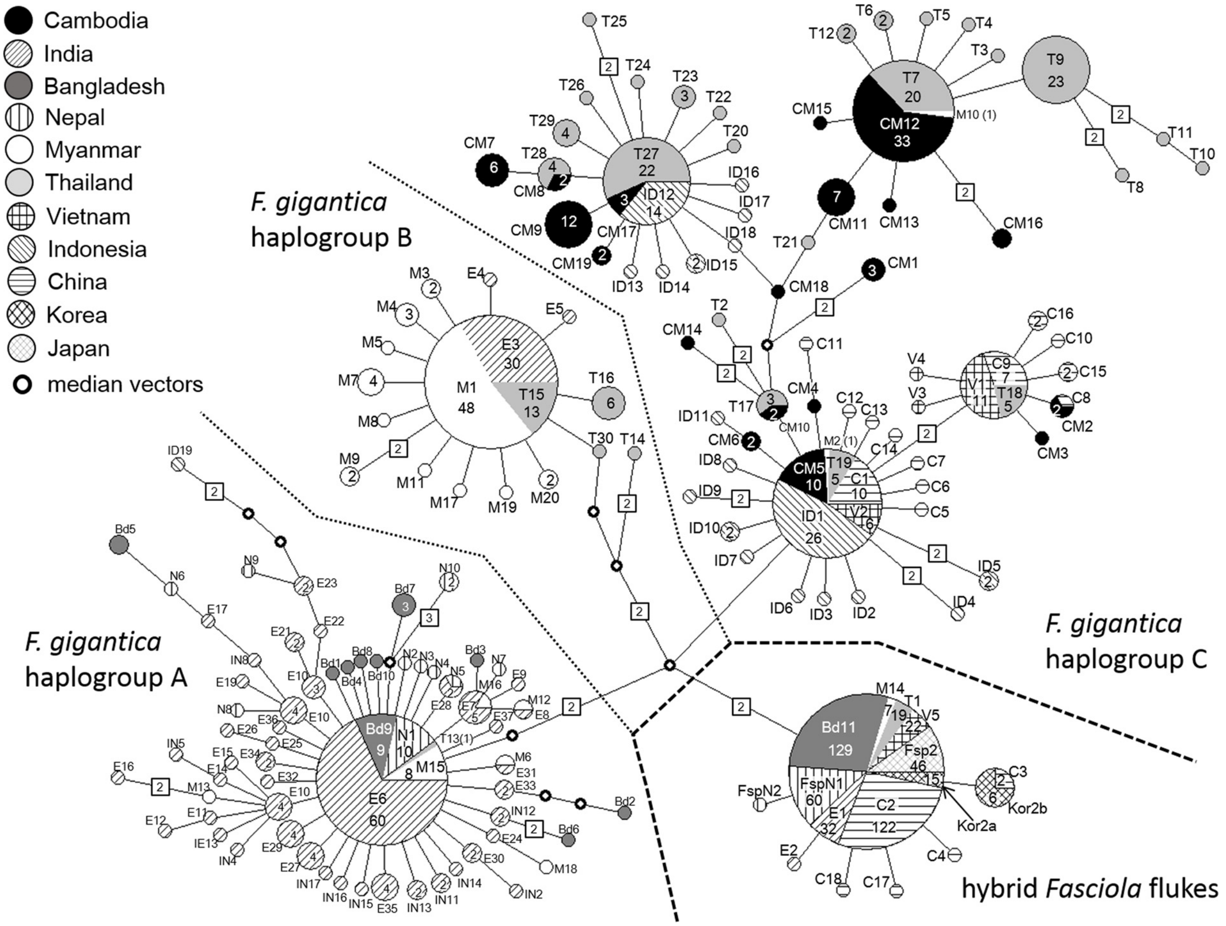

Fig. 3. A median-joining network based on *ND1* haplotypes of *F. gigantica* from Cambodia together with those from reference countries, including India, Bangladesh, Nepal, Myanmar, Thailand, Vietnam, Indonesia, China, Korea, and Japan. *Fasciola* flukes of Cambodia are shown in black. A circle indicates a haplotype. Haplotype codes are labeled within or adjacent to the circles. The prefix of each haplotype code, *ND1*, used in Table S1, was omitted. Numbers on each circle and node indicate the number of flukes and substitution sites, respectively. Circles and nodes without labeled numbers mean only one fluke and one substitution site. Small, dark circles on the node represent median vectors. A thick dashed line separates *F. gigantica* from the hybrid *Fasciola* fluke, and two thin dashed lines divide the three haplogroups of *F. gigantica.*

Table 3
Diversity indices for the populations of *F. gigantica* haplogroup C on the basis of the nucleotide sequences of mitochondrial *ND1* gene.

Populations	N	Diversity			
		S	h	Hd ± SD	π ± SD
Cambodia	92	21	19	0.836 ± 0.030*	0.00532 ± 0.00037
Thailand	106	25	24	0.872 ± 0.017	0.00507 ± 0.00027
Vietnam	19	4	4	0.591 ± 0.088	0.00210 ± 0.00035
China	30	13	13	0.844 ± 0.050*	0.00350 ± 0.00034
Indonesia	59	20	18	0.755 ± 0.048	0.00398 ± 0.00034

N numbers of flukes used, S numbers of variable sites, h numbers of haplotypes, Hd haplotype diversity, SD standard deviation, π nucleotide diversity.

* Statistically non-significant.

ND1 were consistent. In this study, we found an *F. gigantica* prevalence of 7.14% in selected slaughterhouse cattle.

Morphological observations of *F. gigantica* in the present study revealed the average VS-P (3.14 ± 0.05, 2.30–4.20) of Cambodian *F. gigantica* seemed to be larger than ones from Thailand (2.81 ± 0.67, 1.71–3.60). The average value of the BL/BW (3.98 ± 0.64, 2.53–2.90) was comparable to that of *F. gigantica* from China (3.88 ± 0.55, 3.00–4.46) (Peng et al., 2009) and Vietnam (4.01 ± 0.25, 3.57–4.46) (Itagaki et al., 2009), but the value was higher than that of *F. gigantica* from Thailand (3.18 ± 0.94, 1.64–4.69) (Wannasan et al., 2014). These observations indicated that the adult worms were *F. gigantica*, which supported the results of molecular identification. However, there is considerable variation in morphological features and the size of *Fasciola* adult worms depending on such factors as the age of the fluke; the species of the final host and the host immunity; the levels of infection (massive infection tends to result in smaller flukes); the intermediate host; or various genealogy factors (Ghavami et al., 2009; Spithill et al., 1999; Kendall, 1965), and therefore, molecular characterization is required for precise identification.

Table 4
Pairwise fixation index (*F*st) values among *F. gigantica* populations from haplogroup C of mitochondrial *ND1* gene.

Populations	Cambodia	Thailand	Vietnam	China	Indonesia
Cambodia	–				
Thailand	0.02904	–			
Vietnam	0.41438	0.45905	–		
China	0.34294	0.39410	0.04924*	–	
Indonesia	0.16002	0.20309	0.39448	0.25696	–

* Statistically non-significant ($P = 0.09$); all other values were statistically significant ($P < 0.0001$).

The NJ phylogram showed the 19 *ND1* haplotypes were grouped with the *F. gigantica* from the Asian countries and were distinct from those from Africa (Fig. 2), indicating that the distribution route of *F. gigantica* should be considered inside Asia.

The *Fasciola* population exhibits surprising power to expand, which is associated with the parasite's ability to colonize new habitats accompanied by the migration of its intermediate hosts. The rate of response to selection imposed by changes in the environment of the parasite affect its genetic variation. Generally, the ancestral population exhibits higher genetic diversity. Here, the highest genetic variation was observed in the Cambodian *F. gigantica* population compared with those of other SEA countries as well as that of China. That is because unique haplotypes in Cambodia were found in this study (ex. Fg-ND1-CM1) (Fig. 3). According to the diversity values, it can be inferred that the F. gigantica population belonging to haplogroup C was dispersed from Cambodia into other countries. In Cambodia, it is not surprising that the greatest diversity was found in Kandal Province because that is where the highest number of flukes were examined.

Moreover, the comparatively lower *F*st value between the Cambodian and Thai populations of *F. gigantica* suggests a high level of genetic flow was maintained between the two countries. It can be concluded that *F. gigantica* dispersed from Cambodia to Thailand. On the other hand, differences between the Vietnam and China populations were non-significant ($P = 0.09$). The Chinese and Vietnamese populations seemed to be derived from the SEA populations, although fewer haplotypes migrated (Fig. 3). Again, this study indicates that the Indonesian *F. gigantica* population may have expanded from Thailand (Hayashi et al., 2016a) and Cambodia. Most likely, anthropogenic movement of cattle (e.g., for trading and exchange) between SEA and China have allowed the spread of *F. gigantica* (Hayashi et al., 2016b; Mas-Coma et al., 2009). *Zebu* cattle have been exchanged among SEA countries; in recent years, the majority of cattle trade through Cambodia went to Thailand (Pham et al., 2015).

In conclusion, the present study provided the phylogeny as well as the genetic diversity of the *ND1* haplotypes of Cambodian liver flukes. The results are important to understand the epidemiology of these economically important parasites. However, we did not detect any hybrid *Fasciola* flukes, despite numerous reports of its presence in countries such as Thailand, Vietnam, and Myanmar. Further large-scale molecular investigations of *Fasciola* infestation are required.

CRediT authorship contribution statement

Vandara Loeurng: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing - original draft. **Madoka Ichikawa-Seki:** Data curation, Formal analysis, Software, Writing - original draft, Writing - review & editing. **Anchalee Wannasan:** Resources. **Tum Sothyra:** Investigation, Resources. **Warangkhana Chaisowwong:** Investigation. **Saruda Tiwananthagorn:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgements

This research project is supported by Chiang Mai University, in part via the Veterinary Public Health Centre for Asia Pacific, Excellence Center for Veterinary Public Health, and Excellent Center in Veterinary Bioscience, Faculty of Veterinary Medicine, Chiang Mai University. Laboratory support of National Veterinary Research Institution, Cambodia, and veterinarians of Kandal, Battambang, and Kratie Provinces are greatly appreciated.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.07.013.

References

Agatsuma, T., Arakawa, Y., Iwagami, M., Honzako, Y., Cahyaningsih, U., Kang, S.Y., Hong, S.J., 2000. Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. Parasitol. Int. 49, 231–238.

Ashrafi, K., Valero, M., Panova, M., Periago, M., Massoud, J., Mas-Coma, S., 2006. Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan. Iran. Parasitol. Int. 55, 249–260.

Bandelt, H.J., Forster, P., Rohl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Bless, P.J., Schär, F., Khieu, V., Kramme, S., Muth, S., Marti, H., Odermatt, P., 2015. High prevalence of large trematode eggs in schoolchildren in Cambodia. Acta Trop. 141, 295–302.

Chaichanasak, P., Ichikawa, M., Sobhon, P., Itagaki, T., 2012. Identification of *Fasciola* flukes in Thailand based on their spermatogenesis and nuclear ribosomal DNA, and their intraspecific relationships based on mitochondrial DNA. Parasitol. Int. 61, 545–549.

Ghavami, M.B., Rahimi, P., Haniloo, A., Mosavinasab, S.N., 2009. Genotypic and phenotypic analysis of Fasciola isolates. Iran. J Parasitol. 4, 61–70.

Gray, G.D., Copland, R., Copeman, D., 2008. Overcoming liver fluke as a constraint to ruminant production in South-East Asia. ACIAR Monograph No. 133. 155 pp.

Excoffier, I., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetic analyses under Linux and Windows. Mol. Ecol. Res. 10, 564–567.

Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Singh, T.S., Shoriki, T., Sugiyama, H., Itagaki, T., 2015. Molecular phylogenetic analysis of *Fasciola* flukes from eastern India. Parasitol. Int. 64, 334–338.

Hayashi, K., Ichikawa-Seki, M., Allamanda, P., Wibowo, P.E., Mohanta, U.K., Guswanto, A., Nishikawa, Y., 2016a. Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from western Java. Indonesia. Parasitol. Int. 65, 424–427.

Hayashi, K., Mohanta, U.K., Neeraja, T., Itagaki, T., 2016b. Molecular characterization of *Fasciola gigantica* in Delhi, India and its phylogenetic relation to the species from South Asian countries. J. Vet. Med. Sci. 78, 1529–1532.

Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Shoriki, T., Chaichanasak, P., Itagaki, T., 2018. Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, pepck and pold. J. Vet. Med. Sci. 80, 98–102.

Ichikawa, M., Iwata, N., Itagaki, T., 2010. DNA types of aspermic *Fasciola* species in Japan. J. Vet. Med. Sci. 72, 1371–1374.

Ichikawa, M., Bawn, S., Maw, N.N., Htun, L.L., Thein, M., Gyi, A., Sunn, K., Katakura, K., Itagaki, T., 2011. Characterization of *Fasciola* spp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers. Parasitol. Int. 60, 474–479.

Ichikawa-Seki, M., Peng, M., Hayashi, K., Shoriki, T., Mohanta, U.K., Shibahara, T., Itagaki, T., 2017. Nuclear and mitochondrial DNA analysis reveals that hybridization between *Fasciola hepatica* and *Fasciola gigantica* occurred in China. Parasitology 144, 206–213.

Itagaki, T., Kikawa, M., Sakaguchi, K., Shimo, J., Terasaki, K., Shibahara, T., Fukuda, K., 2005a. Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. Parasitology 131, 679–685.

Itagaki, T., Kikawa, M., Terasaki, K., Shibahara, T., Fukuda, K., 2005b. Molecular characterization of parthenogenic *Fasciola* sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. J. Vet. Med. Sci. 67, 1115–1118.

Itagaki, T., Sakaguchi, K., Terasaki, K., Sasaki, O., Yoshihara, S., Van Dung, T., 2009. Occurrence of spermic diploid and aspermic triploid forms of *Fasciola* in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA. Parasitol. Int. 58, 81–85.

Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin. Microbiol. Rev. 22, 466–483.

Kendall, S.B., 1965. Relationships between the Species of *Fasciola* and their Molluscan Hosts. Adv. Parasitol. 3, 59–98.

Le, T.H., De, N.V., Agatsuma, T., Thi Nguyen, T.G., Nguyen, Q.D., McManus, D.P., Blair, D., 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. Int. J. Parasitol. 38, 725–730.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.

Mohanta, U.K., Ichikawa-Seki, M., Shoriki, T., Katakura, K., Itagaki, T., 2014. Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. Parasitol. Res. 113, 2493–2501.

Peng, M., Ichinomiya, M., Ohtori, M., Ichikawa, M., Shibahara, T., Itagaki, T., 2009. Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. Parasitol. Res. 105, 809.

Periago, M., Valero, M., Panova, M., Mas-Coma, S., 2006. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). Parasitol. Res. 99, 368.

Pham, L., Smith, D., Sotheun, S., Vitau, S., 2015. The Cambodian beef cattle industry.

Regional Workshop on Beef Markets and Trade in Southeast Asian and China. Ben Tre, Vietnam, 30th November – 3rd December, 2015. pp. 1–13.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A., 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol. Biol. Evol. 34, 3299–3302.

Shoriki, T., Ichikawa-Seki, M., Devkota, B., Rana, H.B., Devkota, S.P., Humagain, S.K., Itagaki, T., 2014. Molecular phylogenetic identification of *Fasciola* flukes in Nepal. Parasitol. Int. 63, 758–762.

Shoriki, T., Ichikawa-Seki, M., Suganuma, K., Naito, I., Hayashi, K., Nakao, M., Aita, J., Mohanta, U.K., Inoue, N., Murakami, K., 2016. Novel methods for the molecular discrimination of *Fasciola* spp. on the basis of nuclear protein-coding genes. Parasitol. Int. 65, 180–183.

Sothoeun, S., Davun, H., Copeman, B., 2006. Abattoir study on *Fasciola gigantica* in Cambodian cattle. Trop. Anim. Health Prod. 38, 113–115.

Spithill, T., Smooker, P., Copeman, D.B., 1999. *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In: Dalton, J.P. (Ed.), 1999. Fasciolosis. CABI, Oxon, UK, pp. 465–525.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA 6: molecular evolutionary genetics analysis version 6. 0. Mol. Biol. Evol. 30, 2725–2729.

Torgerson, P., Claxton, J., 1999. Epidemiology and control. In: Dalton, J.P. (Ed.), Fasciolosis. CABI, Oxon, UK, pp. 113–149.

Tum, S., Puotinen, M., Copeman, D.B., 2004. A geographic information systems model for mapping risk of fasciolosis in cattle and buffaloes in Cambodia. Vet. Parasitol. 122, 141–149.

Wannasan, A., Khositharattanakool, P., Chaiwong, P., Piangjai, S., Uparanukraw, P., Morakote, N., 2014. Identification of *Fasciola* species based on mitochondrial and nuclear DNA reveals the co-existence of intermediate *Fasciola* and *Fasciola gigantica* in Thailand. Exp. Parasitol. 146, 64–70.

WHO, 2012. Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases: A Roadmap for Implementation: Executive Summary. World Health Organization, Geneva 22 pp.