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ORIGINAL PAPER

Genetic analysis of *Fasciola* isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA

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Abstract Nuclear ribosomal DNA sequence of the second internal transcribed spacer (ITS-2) has been used efficiently to identify the liver fluke species collected from different hosts and various geographic regions. ITS-2 sequences of 19 *Fasciola* samples collected from Korean native cattle were determined and compared. Sequence comparison including ITS-2 sequences of isolates from this study and reference sequences from *Fasciola hepatica* and *Fasciola gigantica* and intermediate *Fasciola* in Genbank revealed seven identical variable sites of investigated isolates. Among 19 samples, 12 individuals had ITS-2 sequences completely identical to that of pure *F. hepatica*, five possessed the sequences identical to *F. gigantica* type, whereas two shared the sequence of both *F. hepatica* and *F. gigantica*. No variations in length and nucleotide composition of ITS-2 sequence were observed within isolates that belonged to *F. hepatica* or *F. gigantica*. At the position of 218, five *Fasciola* containing a single-base substitution (C>T) formed a distinct branch inside the *F. gigantica*-type group which was similar to those of Asian-origin isolates. The phylogenetic tree of the *Fasciola* spp. based on complete ITS-2 sequences from this study and other representative isolates in different locations clearly showed that pure *F. hepatica*, *F. gigantica* type and intermediate *Fasciola* were observed. The result also provided additional genetic evidence for the existence of three forms of *Fasciola* isolated from native cattle in Korea by genetic approach using ITS-2 sequence.

Introduction

Fasciola hepatica and *Fasciola gigantica* are considered as the most common trematodes which infected and caused fasciolosis in both domestic ruminants and humans (Mas-Coma et al. 2005). Classically, these two species have been discriminated based on their morphological properties, but in some areas, the inter-breed of two species giving hybrid/intermediate *Fasciola* can lead to aberrant morphology (Lotfy and Hillyer 2003). For genetic characterization and identification of *Fasciola*, internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) has been used widely because they contain many variable regions (Hillis and Dixon 1991; Morgan and Blair 1995). ITS-2 sequence is located between the 5.8S and 28S coding regions of rDNA and has proved useful for genetic polymorphic analysis of liver flukes worldwide because it showed high repetition and variable

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regions flanked by more conserved sequences (Hillis and Dixon 1991). Nucleotide sequence analysis of ITS-2 of several isolates of *F. hepatica* and *F. gigantica* from different countries showed that their sequences were different at six nucleotide sites which were 2.8% variation in a total of 213 nucleotides compared (Adlard et al. 1993). This result revealed that the ITS-2 region offered inter-specific variability in nucleotide sequence and clearly allowed discrimination at the species level of *F. hepatica* and *F. gigantica* (Morgan and Blair 1995; Leon-Regagnon et al. 1999; Tkach et al. 2000; Scholz et al. 2004; Semyenova et al. 2005). Several studies on genetic characterization and species identification of *Fasciola* sampled from various hosts in different parts of the world based on ITS-2 sequence as molecular marker have been conducted (Itagaki and Tsutsumi 1998; Agatsuma et al. 2000; Huang et al. 2004; Itagaki et al. 2005a, b; Semyenova et al. 2005; Alasaad et al. 2007; Ali et al. 2008; Le et al. 2008; Prasad et al. 2008; Erensoy et al. 2009; Nguyen et al. 2009; Peng et al. 2009).

Cattle are heavily infected with *Fasciola* in Korea before 2000 with the prevalence of up to 50% (unpublished data). After that time, the liver flukes were rarely detected in cattle because the improvement in cattle breeding system was applied in the whole country. Taxonomical studies on Korean liver fluke species were carried out based on their morphology by Giemsa and C-band staining methods (Rhee et al. 1987), isoelectric focusing electrophoresis (Lee and Zimmerman 1993), and DNA sequences of mitochondria and nuclear DNAs (Agatsuma et al. 2000). Using two mitochondrial DNAs (cytochrome c-oxidase subunit 1–cox 1 and NADH dehydrogenase I–NDI) and two nuclear DNAs (ITS-2 and D2 regions), the taxonomic status of five Korean *Fasciola* worms from an unknown host was analyzed. Molecular genetics of Korean *Fasciola* isolates from a cattle slaughterhouse in Seoul, Korea, were characterized by DNA sequence of ITS-1 and NDI (Itagaki et al. 2005a). The results demonstrated that three forms of the Korean liver flukes, which were *F. hepatica*, *F. gigantica*, and intermediate *Fasciola*, were co-existent in Korea, but it is yet to be known whether the occurrence of those forms of *Fasciola* in cattle collected from other locations in Korea could be confirmed by using ITS-2 data as a genetic approach.

In the present study, we determined the nucleotide sequence of the ITS-2 in liver fluke isolates collected from cattle in Gangwon province, northeastern Korea, in 2009 and compared them with the published sequences of *F. hepatica*, *F. gigantica* and intermediate *Fasciola* isolates from Korea and Japan, and representative isolates from other regions to characterize and evaluate the genetic differences between them.

Materials and methods

Fasciola samples

A total of 19 liver flukes were sampled from the liver of different animals (all of them were native cattle) collected in three slaughterhouses in Gangwon province, northeastern Korea, during 2009. All were kept in 70% ethanol for DNA extraction.

DNA isolation and amplification

Genomic DNA was extracted from small pieces of adult worms using QIAamp DNA extraction kit (QIAGEN, Hilden, Germany) according to manufacturer's guidance. Total DNA was eluted in 50 μl and stored at −20°C for further use. The ITS-2 sequence was amplified by PCR using the specific primer pair which spans to 5.8S and 28S sequences. The following forward primer, 5′-CGGTGGAT CACTCGGCTCGT-3′, and reverse primer, 5′-CCTGG TTAGTTTCTTTTCCTCCGC-3′, were used to amplify the complete ITS-2 sequence. Amplification reaction was carried out in a final volume of 20 μl of AccuPower PCR PreMix (BiONEER, Korea) using 100 ng of genomic DNA as template. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 50 s, annealing at 55°C for 50 s, extension at 72°C for 40 s, and a final synthesis at 72°C for 10 min, in the C1000 Thermal Cycler (Bio-Rad, USA). The PCR products were purified by QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and directly used for sequencing (Macrogene, Seoul, Korea).

Sequence analysis and phylogenetic tree

Complete ITS-2 sequences were edited by GenDoc version 2.6.002 (Nicholas and Nicholas 1997). Multiple alignments were performed for sequences obtained from 19 isolates from this study using BioEdit 7.0.9.0 (Hall 1999). ITS-2 sequences representative for *F. hepatica*, *F. gigantica*, and intermediate *Fasciola* available in Genbank were included in the sequence analysis. The phylogenetic relationship was analyzed based on complete ITS-2 sequences of 19 *Fasciola* spp., and other reference sequences isolated from different hosts and localities, in which ITS-2 sequences of three Korean *Fasciola* spp. from an unknown host were included (sequence data from the study of Agatsuma et al. 2000). Phylogeny was constructed by Mega 4.1 package (Tamura et al. 2007) using the neighbor-joining (NJ) method. The reliable tree was tested by bootstrap value using 1,000 times of re-sampling.

Results

Sequence comparison

ITS-2 nucleotide sequences obtained from 19 liver flukes collected from Korean native cattle in 2009 were submitted to Genbank and assigned with the accession numbers listed in Table 1. Comparison of the total 45 ITS-2 sequences at variable sites was also presented in Table 1. All ITS-2 sequences of the Korean *Fasciola* spp. were 361 and 362 bp in length. Among 19 samples, the ITS-2 sequence of 12 *Fasciola* was identical to that of pure *F. hepatica*, five had the ITS-2 sequence identical to *F. gigantica* type, whereas two shared the ITS-2 sequence of both *F. hepatica* and *F. gigantica*. ITS-2 sequence comparison showed that no variation in length and nucleotide composition were found within 12 sequences that belonged to *F. hepatica* and five sequences of *F. gigantica*.

Data shown in Table 1 revealed seven identical variable sites in which nucleotides at the positions of 207, 218, 231, 270, 276, and 334 are single-base substitutions, and nucleotide at 327 is a single-base deletion (T nucleotide). In detail, the main differences between *F. gigantica* and *F. hepatica* in ITS-2 sequence were as follows: the single-base substitution C>T at nucleotide sites of 207 and 231, T>C at the sites of 270 and 276, A>G at the site of 334, and T deletion at the site of 327. Nucleotide change (C>T) at the position of 218 was observed in five isolates, which created the distinct subgroup in the *F. gigantica* type. Alignment of complete ITS-2 sequences was carried out using the total 39 sequences, in which 19 were from the present study, and 20 were from representative *Fasciola* available in Genbank. The sequence comparison result was then used for phylogenetic study.

Phylogenetic tree

The phylogenetic tree resulting from ITS-2 data is shown in Fig. 1. It clearly shows that the Korean isolates are *F. hepatica* or *F. gigantica*, and a number of isolates belong to the group of intermediate *Fasciola*. Among 19 *Fasciola* isolates, twelve and five are claded to pure *F. hepatica* and *F. gigantica*, respectively, while two are grouped into the intermediate group. Out of two intermediate *Fasciola*, Fsp (GW5)-KR contained the typical sequence of *F. gigantica* with a single-base T deletion at the position of 327, resulting in an ITS-2 length of 361 bp, but it also possessed two single-base substitutions C>T at both sites 207 and 231, which is identical to *F. hepatica*. The other was 362 bp in length and identical to *F. hepatica*, but it had two single-base substitutions C>T at both sites 270 and 276, which is found in all pure *F. gigantica* (Table 1). With the values higher than 80% shown in Fig. 1, the bootstrapping replicates of the tree showed a precise topology among analyzed samples.

Discussion

For comparison purpose, ITS-2 sequences of *F. hepatica* and *F. gigantica* were defined that they differ at six nucleotide sites (Adlard et al. 1993; Itagaki and Tsutsumi 1998). The ITS-2 sequences of *Fasciola* species from naturally infected cattle and sheep collected from several countries showed the number of nucleotide differences between *F. hepatica* and *F. gigantica*, and they were grouped into identical clades (Semyenova et al. 2005). ITS-2 sequences from our study were aligned and compared with published sequences of *F. hepatica*, *F. gigantica*, and intermediate *Fasciola* from different geographic distributions. A number of variable sites in ITS-2 sequence within *Fasciola* isolates originated from cattle occurred among Korean worms. Nucleotide differences at seven variable sites in the ITS-2 sequence of *Fasciola* species obtained from the present study and from various hosts and geographical regions published previously were clearly listed in Table 1. Twelve sequences are identical to that of pure *F. hepatica*; five are identical to that of *F. gigantic* type, and two share both sequences of *F. hepatica* and *F. gigantica* to form intermediate *Fasciola*. *F. hepatica* and *F. gigantica* differed by six nucleotides in their ITS-2 sequence, and a typical difference by deletion of the T nucleotide at site 327 leads to variations in length of 362 and 361 bp, respectively. Previously, *Fasciola* worms in Korean and Japan have been confirmed by morphologically resembling *F. hepatica*, *F. gigantica*, and their intermediate type (Itagaki and Akane 1959; Oshima et al. 1968); cytological showing parthenogenic diploid, triploid, and mixoploid (Morriyama et al. 1979; Rhee et al. 1987). Genetic characterization of *Fasciola* isolated from cattle in Seoul, Korea, using the ITS-1 sequence and mitochondrial NDI gene (Itagaki et al. 2005a), in which the occurrence of intermediate genotypes of *Fasciola* was demonstrated. Together, variation in ITS-2 and D2 region sequences of Korean *Fasciola* isolates from an unknown host possessed sequences of both types of *F. hepatica* and *F. gigantica* implying cross-hybridization between two species coexisting in Korea (Agatsuma et al. 2000). The present study also provided additional genetic evidence of ITS-2 data for the occurrence of intermediate *Fasciola* in Korean native cattle. Unfortunately, ITS-2 sequence data from the study by Nguyen et al. (2009) were not assigned in Genbank, and there was insufficiency of ITS-2 data of isolates Fsp-Korea1 and Fsp-Kore3 (appearing as Fsp(Kor1/3) in Table 1) from the study by Agatsuma et al. (2000). Therefore, those could not be included in our sequence alignment and phylogenetic study.

Table 1 Comparison of ITS-2 variable sites in different *Fasciola* isolates

No	Sample	Host	Location	Sites of variation							Size of ITS-2 (bp)	Accession number	Assessment for
				207	218	231	270	276	327	334			
1	Fg-IN	Cattle	Indonesia	C	T	C	T	T	-	A	361	EU260080	*F. gigantica*
2	FspI-JP	N/A	Japan	C	T	C	T	T	-	A	361	AB010979	*F. gigantica*
3	FgGx-CH	Buffalo	China	C	T	C	T	T	-	A	361	EU260079	*F. gigantica*
4	FgBDB-VN	Cattle	Vietnam	C	T	C	T	T	-	A	361	EU260057	*F. gigantica*
5	FspTH-VN	Cattle	Vietnam	C	C	C	T	T	-	A	361	EU260076	*F. gigantica*-type
6	FspB3-VN	Cattle	Vietnam	C	C	C	T	T			361	EU260061	*F. gigantica*-type
7	Fsp(Koi)-JP	Cattle	Japan	C	C	C	T	T	-	A	361	AB207152	*F. gigantica*-type
8	Fsp(Sai)-JP	Cattle	Japan	C	C	C	T	T	-	A	361	AB207151	*F. gigantica*-type
9	Fsp(Ko4)-KR	N/A	Korea	C	C	C	T	T	-	A	361	**)	*F. gigantica*-type
10	Fsp(Ko5)-KR	N/A	Korea	C	C	C	T	T	-	A	361	**)	*F. gigantica*-type
11	**Fsp(GW3)-KR**	**Cattle**	**Korea**	**C**	**C**	**C**	**T**	**T**	**-**	**A**	**361**	HQ821452	***F. gigantica*-type**
12	**Fsp(GW4)-KR**	**Cattle**	**Korea**	**C**	**C**	**C**	**T**	**T**	**-**	**A**	**361**	HQ821451	***F. gigantica*-type**
13	**Fsp(GW8)-KR**	**Cattle**	**Korea**	**C**	**C**	**C**	**T**	**T**	**-**	**A**	**361**	HQ821454	***F. gigantica*-type**
14	**Fsp(GW9)-KR**	**Cattle**	**Korea**	**C**	**C**	**C**	**T**	**T**	**-**	**A**	**361**	HQ821455	***F. gigantica*-type**
15	**Fsp(GW15)-KR**	**Cattle**	**Korea**	**C**	**C**	**C**	**T**	**T**	**-**	**A**	**361**	HQ821453	***F. gigantica*-type**
16	**Fsp(GW5)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**T**	**T**	**-**	**A**	**361**	HQ821457	***F. gigantica*-type**
17	Fsp(He2)-CN	Sheep	China	T	T	C	T	T	-	A	361	AJ557571	*F. gigantica*-type
18	Fg-ZA	Cattle	Zambia	T	T	C	T	T	-	A	361	AB010976	*F. gigantica*-type
19	Fg(Bobo)-BF	Cattle	Burkina Faso	T	T	C	T	T	-	A	361	AJ853848	*F. gigantica*-type
20	Fsp(Ko1/3)-KR	N/A	Korea	C/T	C/T	C/T	C/T	C	-/T	A/G	361	**)	*F. gigantica*-type/ *F. hepatica*-type
21	FspYB11-VN	Goat	Vietnam	T	T	C	T	T	T	G	362	N/A *)	*F. hepatica*-type
22	FspYB-VN	Goat	Vietnam	T	T	T	T	T	T	G	362	N/A *)	*F. hepatica*-type
23	FspYB1-VN	Goat	Vietnam	T	T	T	T	T	T	G	362	N/A *)	*F. hepatica*-type
24	FspT-VN	Cattle	Vietnam	C	T	T	C	C	T	G	362	N/A *)	*F. hepatica*-type
25	**Fsp(GW2)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**T**	**T**	**T**	**G**	**362**	HQ821456	***F. hepatica*-type**
26	**Fsp(GW1)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821448	***F. hepatica***
27	**Fsp(GW6)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821441	***F. hepatica***
28	**Fsp(GW7)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821445	***F. hepatica***
29	**Fsp(GW10)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821450	***F. hepatica***
30	**Fsp(GW11)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821440	***F. hepatica***
31	**Fsp(GW12)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821447	***F. hepatica***
32	**Fsp(GW13)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821439	***F. hepatica***
33	**Fsp(GW14)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821443	***F. hepatica***
34	**Fsp(GW16)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821446	***F. hepatica***
35	**Fsp(GW17)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821444	***F. hepatica***
36	**Fsp(GW18)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821442	***F. hepatica***
37	**Fsp(GW19)-KR**	**Cattle**	**Korea**	**T**	**T**	**T**	**C**	**C**	**T**	**G**	**362**	HQ821449	***F. hepatica***
38	Fsp(Ko2)-KR	N/A	Korea	T	T	T	C	C	T	G	362	**)	*F. hepatica*
39	Fsp(Hok)-JP	Cattle	Japan	T	T	T	C	C	T	G	362	AB207175	*F. hepatica*
40	FspII-JP	N/A	Japan	T	T	T	C	C	T	G	362	AB010978	*F. hepatica*
41	Fsp(He1)-CH	Sheep	China	T	T	T	C	C	T	G	362	AJ557570	*F. hepatica*
42	FspCB2-VN	Buffalo	Vietnam	T	T	T	C	C	T	G	362	EU260065	*F. hepatica*
43	FspN-VN	Human	Vietnam	T	T	T	C	C	T	G	362	EU260073	*F. hepatica*
44	Fh-UR	Cattle	Urugoay	T	T	T	C	C	T	G	362	AB010974	*F. hepatica*
45	Fh-AU	Cattle	Australia	T	T	T	C	C	T	G	362	EU260058	*F. hepatica*

*): Indicates data from Nguyen et al., (2009)
**): Indicates data from Agatsuma et al., (2000)
N/A not available

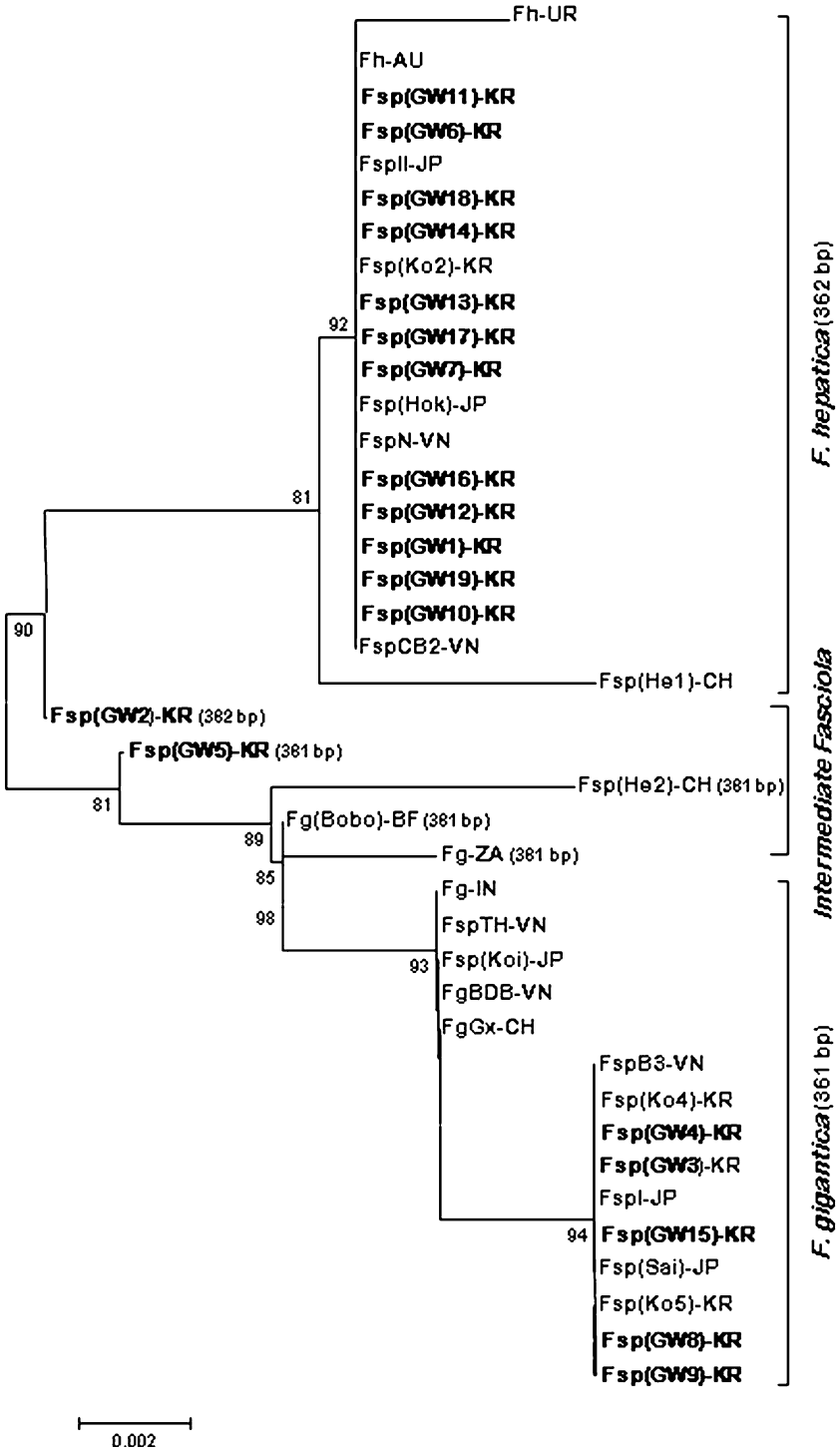

Fig. 1 The phylogenetic tree based on ITS-2 sequence of *Fasciola* isolates collected from native cattle in Korea and other representative isolates collected from different hosts and locations. Topology was constructed by MEGA 4.1 (Tamura et al. 2007) using the neighbor-joining method. Isolates from this study are shown in *bold*. Codes for individuals are the same as in Table 1. The length of the ITS-2 sequence is indicated in *brackets*. *Numerals* indicate bootstrap values (percent) from 1,000 replicates

In terms of geographical distribution, it can be assumed that *F. hepatica* was of European origin and now has cosmopolitan distribution, while *F. gigantica* was reported prevalent in the tropical and subtropical regions of Africa and Asia (Bargues et al. 2001; Mas-Coma et al. 1999). The existence of intermediate *Fasciola* was confirmed in different hosts in mainland China (Huang et al. 2004; Lin et al. 2007; Peng et al. 2009). The presence of hybrid/introgressed forms of *F. hepatica* and *F. gigantica* collected from different hosts (human, goat, cattle) in the regions of Vietnam had also been confirmed by ITS-2 sequence (Le et al. 2008; Nguyen et al. 2009). Taken together, intermediate *Fasciola* has a broader geographical distribution, especially in the Asian countries of China, Vietnam, Japan, and Korea. This suggests that the intermediate *Fasciola* in those countries originated from the same ancestors and has

recently spread out because of the migration of animals and the close geographical location between these countries.

Our phylogenetic tree clearly showed the two main clades of *F. hepatica* and *F. gigantica*, in which 12 and five Korean *Fasciola* isolates were grouped, respectively. The new cluster in the middle of those branches was formed "intermediate" contained Fg from Burkina Faso, Zambia, Fsp from China and two Korean isolates from this study. Phylogenetic study conducted by Nguyen et al. (2009) also showed that four Vietnamese *Fasciola* isolates collected from indigenous goat were distributed in the cluster between *F. hepatica* and *F. gigantica*, and two of them shared the same variable sites in ITS-2 sequence with Korean isolates (Table 1). The intermediate group that included *F. hepatica*-type isolates shared the ITS-2 sequence of *F. gigantica*, and *F. gigantica*-type isolates contained the ITS-2 sequence of *F. hepatica*. Together with two Korean isolates Fsp(Ko4)-KR and Fsp(Ko5)-KR, five *F. gigantica* types from this study contained single-base change (T=>C) at the position of 218 in ITS-2 sequence which is the same variable site found in previously published sequences of the Vietnamese and Japanese isolates. Constructed topology apparently revealed a separate cluster inside the *F. gigantica* type. A similar pattern was seen in Vietnamese *Fasciola* isolates with the same single-base change (Nguyen et al. 2009).

No variations in length and nucleotide composition of ITS-2 were detected within 12 *F. hepatica* isolates from our study. Supporting our result, no nucleotide change was observed among 43 liver flukes of *F. hepatica* collected from different locations such as Russia, Belarus, Ukraine, Armenia, and Turkmenistan (Semyenova et al. 2005). Among 12 *Fasciola* ITS-2 sequences from a goat collected in Vietnam, none was pure *F. hepatica* (Nguyen et al. 2009), whereas Erensoy et al. (2009) found that all Turkish isolates sampled from sheep belonged to pure *F. hepatica*. Similar to that, 25 liver flukes collected from nine host species and 19 geographical locations in Spain represented the single species of *F. hepatica* (Alasaad et al. 2007). This finding implied that ITS-2 sequence variation seems not to be related to particular host species and/or geographical origins of *Fasciola*.

Our study confirmed that nucleotide overlapping between ITS-2 sequences of *F. hepatica* and *F. gigantica* also occurred in *Fasciola* liver flukes collected from a Korean native cattle host in northeastern Korea. The nucleotide change (T>C at position 218) found in five Korean worms from this study showed the formation of a distinct subgroup inside the *F. gigantica*-type group which was similar to those of Asian-origin isolates.

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