

## Research Article

# Identification of *Fasciola* Species Isolates from Nghe An Province, Vietnam, Based on ITS1 Sequence of Ribosomal DNA Using a Simple PCR-RFLP Method

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Fascioliasis—a disease caused by *Fasciola* spp. (Platyhelminthes: Trematoda: Digenea)—is considered as the most important helminthic infection of bovine, sheep, and buffalo in Vietnam. The aim of this study is to detect the genotype of *Fasciola* spp. isolated from bovine and buffalo in the Nghe An province, central Vietnam, using PCR-RFLP and sequence analysis of the first nuclear ribosomal internal transcribed spacer (ITS1). Adult *Fasciola* spp. were isolated from bile ducts of bovine and buffalo in Nghe An province, Vietnam. Overall, 96 adult flukes from livers of slaughtered animals were collected from abattoirs of different areas. They included 7 samples from infected bovine and 89 samples from infected buffalo. 96/96 samples were identified as *Fasciola* species by ITS1 of rDNA. In this study, a PCR-RFLP method was used to distinguish between *F. hepatica* and *F. gigantica* in ITS1 of rDNA (680 bp) with *RsaI* restriction enzyme. RFLP pattern with *RsaI* produced a consistent pattern of 360, 100, and 60 bp fragments in *F. hepatica*, whereas *F. gigantica* worms had a profile of 360, 170, and 60 bp in size, respectively. The results showed that using PCR-RFLP based on the first internal transcribed spacers (ITS1) of the ribosomal RNA revealed that 93 out of 96 isolates were of *Fasciola gigantica* type, whereas three isolates presented an intermediate *Fasciola*. In the present study, *F. gigantica* and intermediate form were coexisting in bovine and buffalo in the Nghe An province of central Vietnam, whereas *F. hepatica* was not detected.

## 1. Introduction

Fascioliasis, a disease caused by the liver flukes of the genus *Fasciola*, is one of the most important food- and water-borne parasitic zoonoses. *F. hepatica* and *F. gigantica* are two main species which infect humans and animals. *F. hepatica* has a worldwide distribution and both species exist in the tropical and subtropical regions of Africa and Asia [1, 2]. Differentiation of these two species, based on morphological characteristics such as the ratio of body length to width, is difficult due to the variation in their size, particularly with respect to the age of flukes, involved host species, and

fixation techniques used [3]. Because of the limitations of morphological methods, several molecular approaches, using different molecular targets, have been developed for the differentiation of *F. hepatica* and *F. gigantica* [4].

Molecular approaches can be properly distinguished by DNA sequencing of first internal transcribed spacers (ITS1), ITS2, and 28S ribosomal ribonucleic acid genes [5–7]. Several studies using ITS1 of rDNA showed that *F. hepatica*, *F. gigantica*, and their intermediate forms exist in different countries including Vietnam [8–10]. Nevertheless, no reports in bovine and buffalo from Nghe An province exist. This province is located in the centre of Vietnam. Previously, the



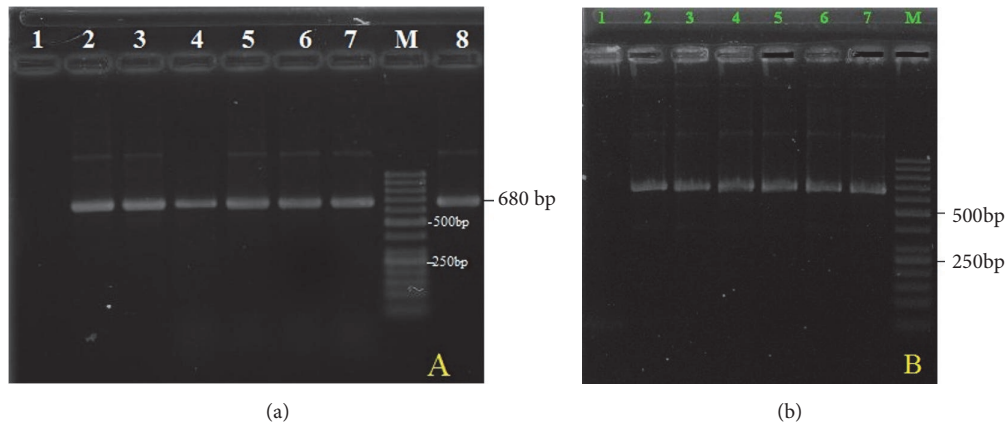


FIGURE 2: PCR patterns of amplified ITS1 ribosomal region. Lane M: 50 bp ladder molecular weight marker; lane 1 (a, b): negative control; lanes 2-8 (a) and 2-7 (b) denoted to different fluke samples amplified as a single band of 680 bp of bovine and buffalo in Nghe An province.

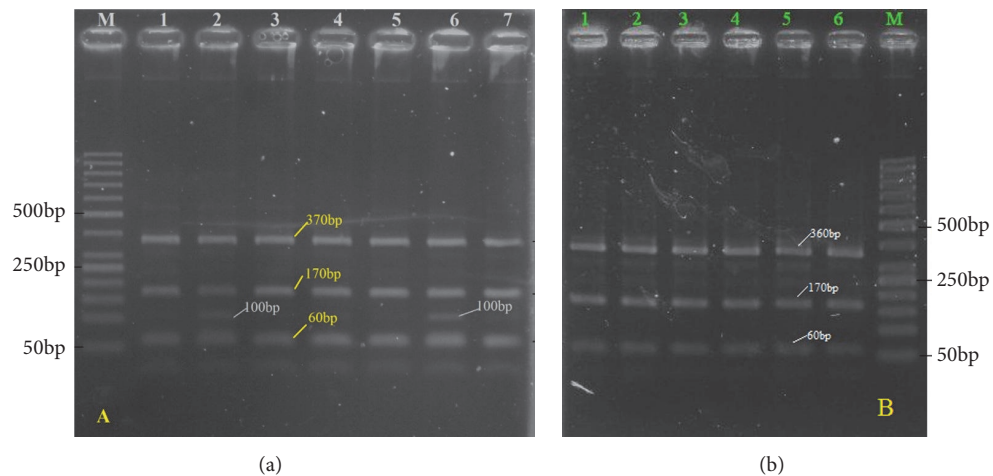


FIGURE 3: RFLP pattern of PCR products of liver flukes collected from bovine (a) and buffalo (b) in Nghe An province, Vietnam, after digestion with *RsaI* enzyme. Lane M: 50 bp ladder molecular weight marker; lanes 1, 3, 4, 5, and 7 (a) denoted to those of *F. gigantica*; lanes 2 and 6 (b) denoted to those of *Fasciola* sp. (intermediate form); lanes 1-6 (b) denoted to those of *F. gigantica*.

*hepatica*, whereas *F. gigantica* worms had a profile of 360, 170, and 60 bp in size, respectively.

**2.5. DNA Sequencing.** PCR products of ITS1 from two isolates, 10B-NA1.2 isolated from bovine and 15Tr-NA5 isolated from buffalo, were sent to First BASE Laboratories Sdn Bhd-service (Kembangan 43300, Selangor, Malaysia) for purification and automatic sequencing in both directions, using the same primers which were used in the PCR. Sequences were read on ABI 3130 Genetic Analyzer software (SeqScape 2.1). The accuracy of data was confirmed by two-directional sequencing. Representative sequences were deposited in the GenBank under accession numbers MH790325 and MH790326.

**2.6. Sequence and Phylogenetic Analyses.** The obtained sequences were analyzed independently by being compared with related sequences available in the GenBank database using BLAST guidelines (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Neighbor joining (NJ) and maximum likelihood (ML)

based on Tamura-Nei model phylogenetic tree of ITS1 sequences were constructed using Mega version 7.09 software. Bootstrap analyses (1,000 replications) were carried out to determine the robustness of the finding. rDNA sequences of *Paragonimus westermani* (AF040935.1) were used as an outgroup.

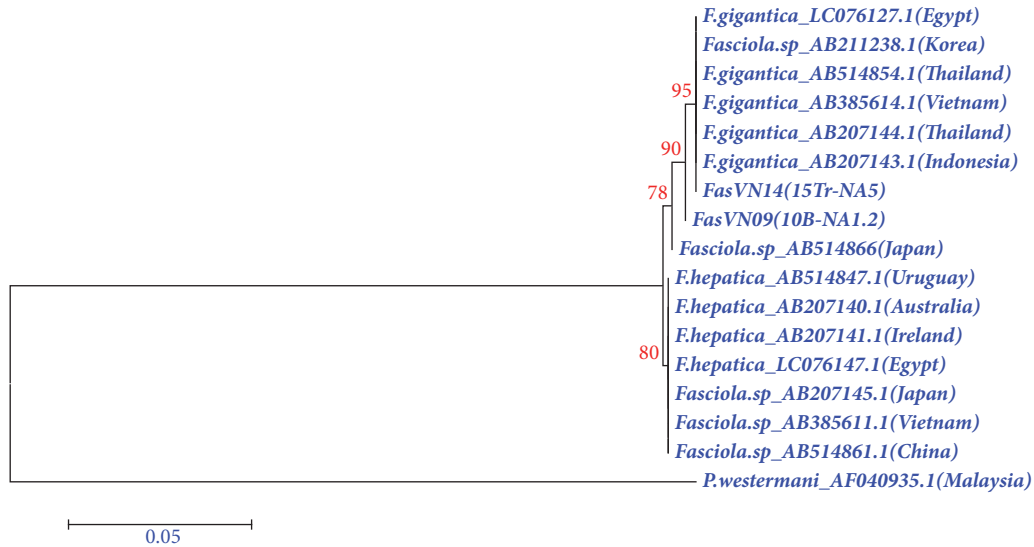
### 3. Results

**3.1. PCR and PCR-RFLP.** A region of approximately 680bp of the ITS1 of rDNA in 96 samples was successfully amplified (Figure 2) as predicted. Negative control did not produce any band on the gels. PCR-RFLP bands profile of *Fasciola* with restriction enzymes *RsaI* was performed. The results of PCR products digestion with *RsaI* were approximately 60, 100bp, and 360bp fragments for *F. hepatica*; 60, 170 bp, and 360 bp for *F. gigantica*; and 60, 100, 170, and 370 bp for intermediate form (Figure 3).

Table 1 illustrates the identified liver flukes in relation to their origins and their definitive hosts. Accordingly, 96

TABLE 1: Frequency of *F. gigantica* and *F. hepatica* identified by PCR-RFLP in different animal hosts in Vietnam.

Host	Bovine	Buffalo	Total number of adult flukes
<i>F. gigantica</i>	5	88	93
<i>Fasciola</i> sp.	2	1	3
<i>F. hepatica</i>	0	0	0
Total number	7	89	96

FIGURE 4: Phylogenetic relationship of ITS1 sequences of *Fasciola* from Vietnam using Neighbor-Joining Tree method. *Paragonimus westermani* (AF040935.1) was used as the outgroup.

*Fasciola* isolates were studied from bovine and buffalo of Nghe An province, Vietnam. Out of 96 analyzed specimens from Nghe An province, 93 (96.88%) flukes were identified as *F. gigantica* and 3 (3.12%) as intermediate *Fasciola* (Table 1).

**3.2. Complete ITS1 Sequencing of rDNA.** DNA sequencing of two ITS1 was complete. BLAST results showed that *F. gigantica* from Thailand (AB207144.1) possessed the sequence most similar to those in these 2 worms with 99.31% identity for FasVN09 (10B-NA1.2) isolate (MH790325) and 100% identity for FasVN14 (15Tr-NA5) isolate (MH790326).

Phylogenetic tree constructed by using ITS1 sequences of the *Fasciola* species is shown in Figure 4. All the isolates of *Fasciola* in different countries clustered together, supported by high bootstrap value (>78%). The phylogenetic tree constructed by Neighbor-Joining Tree method supports the blast results by showing similarity between ITS1 sequences (Nghe An province, Vietnam) and ITS1 in Egypt (LC076127.1), Thailand (AB514854.1), Vietnam (AB385614.1), and Indonesia (AB207143.1). The nucleotide sequence data obtained in current study have been deposited in the GenBank under accession numbers MH790326.1 (15Tr-NA5 isolate) and MH790325.1 (10B-NA1.2 isolate).

#### 4. Discussion

DNA-based molecular methods are accurate and reliable for understanding the different species of *Fasciola* and

considered control procedures in endemic areas [13, 14]. PCR-RFLP assay is a powerful method to distinguish between *F. hepatica* and *F. gigantica*. In this study, PCR-RFLP based on the partial rDNA of ITS1 and restriction *RsaI* enzyme was used for differentiation and identification of *Fasciola* species in Nghe An province. These techniques have been utilized for differentiation among *Fasciola* species based on the profiles generated by the effects of endonucleases on ITS genes of these parasites [15]. Ichikawa et al. (2011) used *RsaI* enzyme based on ITS1 region to specifically distinguish *F. hepatica* and *F. gigantica* in Myanmar and did not report *F. hepatica* in their study. In a study in Iran, Aryaeipour et al. (2014) showed that *RsaI* restriction enzyme may be utilized for the differentiation of two species [16]. Saki et al. (2011) used *AvaII* and *DraII* to differentiate between *F. hepatica* and *F. gigantica* in 28S DNA [17]. Ghavami et al. (2009) showed that a digested pattern of 230, 340, and 341bp is specific to *F. hepatica* species and has no effect on *F. gigantica* [18]. Ashrafi et al. (2004) used ITS2 nucleotide sequencing to identify *Fasciola* spp. In their study, two species of *F. hepatica* and *F. gigantica* were seen in Guilan, northern Iran [19].

Our study did detect *F. gigantica* and mixed patterns in Nghe An province but not *F. hepatica*. The results of our study were similar to those of some previous researches in Vietnam [20, 21]. The majority of *Fasciola* species in this study were molecularly identified as *F. gigantica* which was concordant with the knowledge that *F. gigantica* mainly infected buffalo and bovine in Vietnam. In addition the



hybrid form of *Fasciola* had been confirmed based on analysis of ITS1 sequence. The intermediate form from a human case and animals had been reported in Vietnam [10, 22]. The absence of *F. hepatica* in the current study was supported by recent studies of a large number of *Fasciola* from animals in the country [10] and some neighboring countries such as Myanmar and Thailand [3, 23]. Intermediate *Fasciola* form has been reported to coexist in areas sympatric for both *F. hepatica* and *F. gigantica* such as some Asian countries (Japan, Korea, China, and Iran) [12]. In this study, the coexistence of intermediate *Fasciola* and *F. gigantica* in the absence of *F. hepatica* was detected in Vietnam as well as in Thailand and Myanmar [3, 23].

## 5. Conclusion

Using ITS1 marker has identified both *F. gigantica* and intermediate form of *Fasciola* coexisting in the Nghe An province of central Vietnam.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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## References

- [1] S. Mas-Coma, M. D. Bargues, and M. A. Valero, "Fascioliasis and other plant-borne trematode zoonoses," *International Journal for Parasitology*, vol. 35, no. 11-12, pp. 1255–1278, 2005.
- [2] S. Mas-Coma, M. A. Valero, and M. D. Bargues, "Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control," *Advances in Parasitology*, vol. 69, pp. 41–146, 2009.
- [3] M. Ichikawa and T. Itagaki, "Molecular analysis of aspermic Fasciola flukes from Korea on the basis of the nuclear ITS1 region and Mitochondrial DNA markers and comparison with Japanese aspermic Fasciola flukes," *Journal of Veterinary Medical Science*, vol. 74, no. 7, pp. 899–904, 2012.
- [4] W. Y. Huang, B. He, C. R. Wang, and X. Q. Zhu, "Characterisation of Fasciola species from Mainland China by ITS-2 ribosomal DNA sequence," *Veterinary Parasitology*, vol. 120, no. 1-2, pp. 75–83, 2004.
- [5] L. Ai, M.-X. Chen, S. Alasaad et al., "Genetic characterization, species differentiation and detection of Fasciola spp. by molecular approaches," *Parasites & Vectors*, vol. 4, no. 1, article no. 101, 2011.
- [6] T. Itagaki, M. Kikawa, K. Terasaki, T. Shibahara, and K. Fukuda, "Molecular characterization of parthenogenic Fasciola sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene," *Journal of Veterinary Medical Science*, vol. 67, no. 11, pp. 1115–1118, 2005.
- [7] A. Marcilla, M. D. Bargues, and S. Mas-Coma, "A PCR-RFLP assay for the distinction between Fasciola hepatica and Fasciola gigantica," *Molecular and Cellular Probes*, vol. 16, no. 5, pp. 327–333, 2002.
- [8] M. Ichikawa and T. Itagaki, "Discrimination of the ITS1 types of Fasciola spp. based on a PCR-RFLP method," *Parasitology Research*, vol. 106, no. 3, pp. 757–761, 2010.
- [9] T. Itagaki, K. Sakaguchi, K. Terasaki, O. Sasaki, S. Yoshihara, and T. Van Dung, "Occurrence of spermic diploid and aspermic triploid forms of Fasciola in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA," *Parasitology International*, vol. 58, no. 1, pp. 81–85, 2009.
- [10] S. Nguyen, S. Amer, M. Ichikawa, T. Itagaki, Y. Fukuda, and Y. Nakai, "Molecular identification of Fasciola spp. (Digenea: Platyhelminthes) in cattle from Vietnam," *Parasite*, vol. 19, no. 1, pp. 85–89, 2012.
- [11] T. D. Bui, P. N. Doanh, C. Saegerman, and B. Losson, "Current status of fasciolosis in Vietnam: An update and perspectives," *Journal of Helminthology*, vol. 90, no. 5, pp. 511–522, 2016.
- [12] T. Itagaki, M. Kikawa, K. Sakaguchi et al., "Genetic characterization of parthenogenic Fasciola sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA," *Parasitology*, vol. 131, no. 5, pp. 679–685, 2005.
- [13] M. Peng, M. Ichinomiya, M. Ohtori, M. Ichikawa, T. Shibahara, and T. Itagaki, "Molecular characterization of Fasciola hepatica, Fasciola gigantica, and aspermic Fasciola sp. in China based on nuclear and mitochondrial DNA," *Parasitology Research*, vol. 105, no. 3, pp. 809–815, 2009.
- [14] R. Shafiei, B. Sarkari, and A. Moshfe, "A consistent PCR-RFLP assay based on ITS-2 ribosomal DNA for differentiation of Fasciola species," *Iranian Journal of Basic Medical Sciences*, vol. 16, no. 12, pp. 1266–1269, 2013.
- [15] Y. Dar, S. Amer, A. Mercier, B. Courtioux, and G. Dreyfuss, "Molecular identification of Fasciola spp. (Digenea: Fasciolidae) in Egypt," *Parasite*, vol. 19, no. 2, pp. 177–182, 2012.
- [16] M. Aryaeipour, S. Rouhani, M. Bandehpour, H. Mirahmadi, B. Kazemi, and M. B. Rokni, "Genotyping and phylogenetic analysis of fasciola spp. Isolated from sheep and cattle using PCR-RFLP in Ardabil Province, Northwestern Iran," *Iranian Journal of Public Health*, vol. 43, no. 10, pp. 1364–1371, 2014.
- [17] J. Saki, S. Khademvatan, and E. Yousefi, "Molecular identification of animal Fasciola isolates in southwest of Iran," *Australian Journal of Basic and Applied Sciences*, vol. 5, no. 11, pp. 1878–1883, 2011.
- [18] M. B. Ghavami, P. Rahimi, A. Haniloo, and S. N. Mosavinasab, "Genotypic and phenotypic analysis of fasciola isolates," *Iranian Journal of Parasitology*, vol. 4, no. 3, pp. 61–70, 2009.
- [19] K. Ashrafi, *A Survey on Human and Animal Fascioliasis And Genotypic and Phenotypic Characteristics of Fasciolids and their Relationship with Lymnaeid Snails in Gilan Province [Ph.D. thesis]*, PhD, Tehran University of Medical Sciences, northern Iran, 2004.
- [20] T. H. Le, N. Van De, T. Agatsuma et al., "Molecular confirmation that Fasciola gigantica can undertake aberrant migrations in human hosts," *Journal of Clinical Microbiology*, vol. 45, no. 2, pp. 648–650, 2007.

- [21] T. G. T. Nguyen, N. van de, J. Vercruyssen, P. Dorny, and T. H. Le, "Genotypic characterization and species identification of *Fasciola* spp. with implications regarding the isolates infecting goats in Vietnam," *Experimental Parasitology emphasizes*, vol. 123, no. 4, pp. 354–361, 2009.
- [22] T. H. Le, N. V. De, T. Agatsuma et al., "Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam," *International Journal for Parasitology*, vol. 38, no. 6, pp. 725–730, 2008.
- [23] A. Wannasan, P. Khositharattanakool, P. Chaiwong, S. Piangjai, P. Uparanukraw, and N. Morakote, "Identification of *Fasciola* species based on mitochondrial and nuclear DNA reveals the co-existence of intermediate *Fasciola* and *Fasciola gigantica* in Thailand," *Experimental Parasitology emphasizes*, vol. 146, pp. 64–70, 2014.