Molecular characterization of *Fasciola gigantica* in Delhi, India and its phylogenetic relation to the species from South Asian countries

Kei HAYASHI^{1,2)}, Uday K. MOHANTA^{1,2)}, Tambireddy NEERAJA³⁾ and Tadashi ITAGAKI^{1,2)*}

¹⁾Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka 020–8550, Japan

²⁾Department of Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan

³⁾Department of Aquatic Animal Health Management Sri Venkateswara Veterinary University College of Fishery Science, Muthukur 524 344 SPSR Nellore, Andhra Pradesh, India

(Received 10 March 2016/Accepted 24 May 2016/Published online in J-STAGE 11 June 2016)

ABSTRACT. The aim of this study was to phylogenetically analyze *Fasciola gigantica* (*F. gigantica*) from mainland India and to reveal the expansion history of *F. gigantica* in the Indian subcontinent. We analyzed 40 *Fasciola* flukes that were collected from Delhi, in the Indian mainland, and identified them as *F. gigantica* by using nucleotide analyses of the nuclear phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) genes. Based on the nucleotide sequence of mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene, the flukes had 18 haplotypes. The haplotypes were classified under haplogroup A, which is predominant in the *F. gigantica* of South Asia. The population genetics of haplogroup A revealed that Delhi population showed higher π value than eastern India population. These results suggest that *F. gigantica* of haplogroup A might have spread from the west to the east in India along with the artificial migration of the domestic Zebu cattle, *Bos indicus*.

KEY WORDS: Delhi, *Fasciola gigantica*, India, *nad1*, phylogeny

doi: 10.1292/jvms.16-0126; *J. Vet. Med. Sci.* 78(9): 1529–1532, 2016

Fasciola hepatica (*F. hepatica*) and *Fasciola gigantica* (*F. gigantica*) are well-known causative agents of fasciolosis in livestock and human being [12]. *F. hepatica* is mainly distributed in temperate zones, whereas *F. gigantica* is present in tropical zones [20]. Both of these species contain mature spermatozoa in their seminal vesicles for fertilization [19]. In contrast, aspermic *Fasciola* flukes, which contain few or no spermatozoa in the seminal vesicles, have been reported in Asia [19]. These *Fasciola* flukes have been identified based on their spermatogenetic status and the nucleotide sequence of nuclear ribosomal internal transcribed spacer 1 (ITS1) [1, 9, 14]. However, some *Fasciola* flukes have shown inconsistent results in these analyses [15]. Recently, novel nuclear single copy markers, phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) genes, have been developed for precise discrimination of *F. hepatica*, *F. gigantica* and aspermic *Fasciola* flukes through multiplex polymerase chain reaction (PCR) and/or PCR-restriction fragment length polymorphism (RFLP) methods [18].

Molecular phylogenetic analysis and elucidation of propagation route of *Fasciola* species are important for the epidemic prevention of fasciolosis. *F. gigantica* populations in Asia have been divided into three haplogroups, A, B and C based on the nucleotide sequences of the mi-

tochondrial NADH dehydrogenase subunit 1 (*nad1*) gene [6]. Haplogroup A is the predominant population in Nepal, Bangladesh and eastern India on the Indian subcontinent, whereas haplogroups B and C have been distributed mainly in Southeast Asia [6]. Zebu cattle, *Bos indicus*, which is one of the definitive hosts of *Fasciola* flukes in Asia, have been domesticated in the Indus Valley, in the western area of the Indian subcontinent, and then spread throughout the subcontinent [5]. From those findings, we hypothesized that *F. gigantica* of haplogroup A originated in the Indus Valley, and the anthropogenic movements of domesticated Zebu cattle might have been involved in the spreading of the haplogroup [6]. In order to verify this hypothesis and to reveal the expansion history of *F. gigantica* throughout the Indian subcontinent, we analyzed *Fasciola* flukes collected from mainland India, which occupies a majority of the Indian subcontinent, using molecular phylogenetic analysis.

A total of 40 *Fasciola* flukes (1–8 flukes per host) were collected from the bile ducts of 11 buffalos at slaughterhouses in Delhi, India in December 2014 (Table 1). The flukes were fixed in 70% ethanol and transported to the laboratory for further studies. Their seminal vesicles were removed under a stereomicroscope, stained with hematoxylin-carmin solution and then observed under an optical microscope to determine the existence of sperm [19].

Total DNA was extracted from each fluke with a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at –20°C until use. The flukes were identified on the basis of nuclear ITS1, *pepck* and *pold*. The ITS1 and *pold* were analyzed by a PCR-RFLP method. Briefly, PCR amplicons were obtained with the primer sets: ITS1-F and ITS1-R

*CORRESPONDENCE TO: ITAGAKI, T., Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, 3–18–8 Ueda, Morioka 020–8550, Japan. e-mail: itagaki@iwate-u.ac.jp

©2016 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

Table 1. The mitochondrial *nad1* haplotype and spermatic status in seminal vesicles of *Fasciola gigantica* from Delhi

Haplotype	Accession no.	Number of flukes	Sperm in seminal vesicles
ND1-IN1	LC128314	15	+
ND1-IN2	LC128315	1	+
ND1-IN3	LC128316	1	+
ND1-IN4	LC128317	1	+
ND1-IN5	LC128318	1	+
ND1-IN6	LC128319	3	+
ND1-IN7	LC128320	3	+
ND1-IN8	LC128321	1	+
ND1-IN9	LC128322	2	+
ND1-IN10	LC128323	1	+
ND1-IN11	LC128324	2	+
ND1-IN12	LC128325	1	+
ND1-IN12	LC128325	1	-
ND1-IN13	LC128326	2	+
ND1-IN14	LC128327	1	+
ND1-IN15	LC128328	1	+
ND1-IN16	LC128329	1	+
ND1-IN17	LC128330	1	+
ND1-IN18	LC128751	1	+
Total		40	

for ITS1 [9] and *Fasciola*-pold-F1 and *Fasciola*-pold-R1 for *pold* [18], and were digested using restriction enzymes *Rsa*I for ITS1 and *Alu*I for *pold*. The fragment patterns of the amplicons were distinguished on 1.8% agarose gels. The *pepck* region was amplified by a multiplex PCR with the primers, Fh-pepck-F, Fg-pepck-F and Fcmn-pepck-R, and the fragment patterns were distinguished on 1.0% agarose gels [18]. The *nad1* fragment was amplified with the primers, Ita 10 and Ita 2 [9], and directly sequenced in both directions with Ita 10 and Ita 2 primers using a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, U.S.A.) on an ABI 3500 Genetic Analyzer (Applied Biosystems). The resulting sequences were initially assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan), and the haplotypes were distinguished using GENETYX ver. 10 (Genetyx Co.).

The median-joining network that was inferred from the *nad1* haplotypes was generated using Network 4.6.1.2 software [2]. The frequencies of the reference haplotypes were cited from our previous studies in Japan [7, 9], South Korea [10], China [16], Vietnam [11], Thailand [4], Myanmar [8], eastern India [6], Bangladesh [15] and Nepal [17]. The frequency data of 129 *Fasciola* flukes that were collected in China remain unpublished. For the *F. gigantica* populations belonging to haplogroup A, haplotype diversity (*Hd*) and nucleotide diversity (π) were calculated using DnaSP 5.1 [13].

A total of 39 *Fasciola* flukes held plenty of sperm in their seminal vesicles (spermic). The flukes displayed the *F. gigantica* fragment pattern (Fg) in ITS1, *pepck* and *pold*. Thus, these flukes were identified as *F. gigantica*. The remaining one fluke did not contain any sperm in the seminal vesicle (aspermic). However, the fluke also displayed Fg in ITS1,

pepck and *pold*, and therefore, the fluke was identified as *F. gigantica* as well (Table 1). Hence, all of the *Fasciola* flukes analyzed in this study were identified as *F. gigantica* on the basis of their molecular markers [18]. No fluke displayed the hybrid fragment pattern between *F. hepatica* and *F. gigantica* (Fh/Fg), which is a recognizable characteristic of aspermic *Fasciola* flukes.

The partial nucleotide sequences (535 bp) of the *nad1* region displayed 19 substitution sites yielding 18 haplotypes, ND1-IN1 to ND1-IN18 (GenBank accession numbers: LC128314–LC128330 and LC128751) (Table 1). All of the *nad1* haplotypes belonged to *F. gigantica* haplogroup A, which has been primarily detected on the Indian subcontinent. No haplotype was included in the haplogroup of aspermic *Fasciola* flukes (Fig. 1). The predominant haplotype of *F. gigantica* from Delhi was ND1-IN1, which had a nucleotide sequence identical to the haplotypes of *F. gigantica* from Nepal (Fg-ND1-N1) [16], Bangladesh (Fg-ND1-Bd9) [15], Myanmar (ND1-M15) [7], Thailand (ND1-T13) [3] and eastern India (ND1-E6) [5]. ND1-IN2 to ND1-IN18 were colony haplotypes, which had one to three nucleotide substitutions from ND1-IN1 (Fig. 1).

No aspermic *Fasciola* flukes were detected among the samples collected in Delhi, though aspermic *Fasciola* flukes have been reported in Nepal, Bangladesh and eastern India on the Indian subcontinent [6, 15, 17]. *Nad1* haplotype of one aspermic fluke, which displayed Fg fragment patterns in the ITS1, *pepck* and *pold*, was also included in the *F. gigantica* haplogroup A, not in the haplogroup of aspermic *Fasciola* flukes. Therefore, the fluke is considered *F. gigantica* that may have lost spermatogenetic ability due to aging or other unknown causes. *F. gigantica* with no sperm in their seminal vesicles was also detected in Myanmar [8], Bangladesh [15] and eastern India [6]. Aspermic *Fasciola* flukes were predominant in Bangladesh (86.0%) [15] and Nepal (75.3%) [17], while the prevalence was low in eastern India (24.8%) [6]. Aspermic *Fasciola* flukes are thought to have originated in China and have been introduced into these countries [15–17], and thus, these results suggest that the flukes have not yet dispersed extensively throughout India. However, to verify this phenomenon, further analysis of *Fasciola* flukes from other localities in India is required.

All of the *nad1* haplotypes of *F. gigantica* collected from Delhi were categorized into haplogroup A. Similarly, *F. gigantica* haplotypes from the Indian subcontinent, Nepal [17], Bangladesh [15] and eastern India [6] also belonged to haplogroup A. In contrast, the haplotypes collected from Southeast Asia, Thailand and Myanmar belonged to haplogroups B and C in addition to haplogroup A [4, 8]. Moreover, the π value of haplogroup A was the highest in the Nepal (0.00366), followed by Bangladesh (0.00362), Delhi (0.00255), eastern India (0.00242) and Myanmar (0.00225) populations (Table 2). Genetic diversity in the latest population generally decreases with distance from its geographical origin [3, 5, 21]. These π values suggest that *F. gigantica* populations of haplogroup A might have been spread from the west to the east in India. Additionally, zebu cattle have been domesticated in the Indus Valley and then

Fig. 1. A median-joining network on the basis of mitochondrial *nad1* haplotypes of *Fasciola gigantica* and aspermic *Fasciola* flukes. *Fasciola* flukes from Delhi 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 1 was omitted. Numbers on each circle and node indicate the number of flukes and substitution sites, respectively. Circles and nodes without labeled numbers indicate that the number is only one. Small, dark circles on the node represent median vectors. A thick dashed line separates *F. gigantica* from aspermic *Fasciola* sp., and thin dashed lines divide the three haplogroups of *F. gigantica*.

Populations	N	S	h	Hd $\pm$ SD	π
Delhi	40	19	18	0.853 ± 0.052	0.00255
Eastern India [6]	91	32	32	0.751 ± 0.050	0.00242
Nepal [18]	20	16	10	0.758 ± 0.101	0.00366
Bangladesh [16]	20	15	10	0.832 ± 0.075	0.00362
Myanmar [8]	13	7	6	0.641 ± 0.150	0.00225

spread throughout Indian subcontinent from the west to the east [5], and therefore, the *F. gigantica* populations in India might have spread along with the anthropogenic movement of the ruminant [6, 15, 17]. However, the π value of the Delhi population was smaller than that of Nepal and Bangladesh, although Delhi is located in the west than the two countries. This inconsistency might be caused by using *Fasciola* samples collected from a single locality (Delhi) in vast mainland of India. Moreover, the number and geographical source of the flukes analyzed in this study are inadequate to confirm

ACKNOWLEDGMENTS. This study was supported in part by the Grants-in-Aid for Science Research (B) and (C) (grant nos. 23405044 and 24580420) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

1. Adlard, R. D., Barker, S. C., Blair, D. and Cribb, T. H. 1993. Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). *Int. J. Parasitol.* **23**: 423–425. [\[Medline\]](#) [\[CrossRef\]](#)
2. Bandelt, H. J., Forster, P. and Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**: 37–48. [\[Medline\]](#) [\[CrossRef\]](#)
3. Beja-Pereira, A., Caramelli, D., Lalueza-Fox, C., Vernesi, C., Ferrand, N., Casoli, A., Goyache, F., Royo, L. J., Conti, S., Lari, M., Martini, A., Ouragh, L., Magid, A., Atash, A., Zsolnai, A., Boscato, P., Triantaphylidis, C., Ploumi, K., Sineo, L., Mallegni, F., Taberlet, P., Erhardt, G., Sampietro, L., Bertranpetit, J., Barbujani, G., Luikart, G. and Bertorelle, G. 2006. The origin of European cattle: evidence from modern and ancient DNA. *Proc. Natl. Acad. Sci. U.S.A.* **103**: 8113–8118. [\[Medline\]](#) [\[CrossRef\]](#)

4. Chaichanasak, P., Ichikawa, M., Sobhon, P. and 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. [Medline] [CrossRef]
5. Chen, S., Lin, B. Z., Baig, M., Mitra, B., Lopes, R. J., Santos, A. M., Magee, D. A., Azevedo, M., Tarroso, P., Sasazaki, S., Ostrowski, S., Mahgoub, O., Chaudhuri, T. K., Zhang, Y. P., Costa, V., Royo, L. J., Goyache, F., Luikart, G., Boivin, N., Fuller, D. Q., Mannen, H., Bradley, D. G. and Beja-Pereira, A. 2010. Zebu cattle are an exclusive legacy of the South Asia neolithic. *Mol. Biol. Evol.* **27**: 1–6. [Medline] [CrossRef]
6. Hayashi, K., Ichikawa-Seki, M., Mohanta, U. K., Singh, T. S., Shoriki, T., Sugiyama, H. and Itagaki, T. 2015. Molecular phylogenetic analysis of *Fasciola* flukes from eastern India. *Parasitol. Int.* **64**: 334–338. [Medline] [CrossRef]
7. Ichikawa, M., Iwata, N. and Itagaki, T. 2010. DNA types of aspermic *Fasciola* species in Japan. *J. Vet. Med. Sci.* **72**: 1371–1374. [Medline] [CrossRef]
8. Ichikawa, M., Bawn, S., Maw, N. N., Htun, L. L., Thein, M., Gyi, A., Sunn, K., Katakura, K. and 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. [Medline] [CrossRef]
9. Itagaki, T., Kikawa, M., Sakaguchi, K., Shimo, J., Terasaki, K., Shibahara, T. and Fukuda, K. 2005. Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology* **131**: 679–685. [Medline] [CrossRef]
10. Itagaki, T., Kikawa, M., Terasaki, K., Shibahara, T. and Fukuda, K. 2005. 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. [Medline] [CrossRef]
11. Itagaki, T., Sakaguchi, K., Terasaki, K., Sasaki, O., Yoshihara, S. and 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. [Medline] [CrossRef]
12. Keiser, J. and Utzinger, J. 2005. Emerging foodborne trematodiasis. *Emerg. Infect. Dis.* **11**: 1507–1514. [Medline] [CrossRef]
13. Librado, P. and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452. [Medline] [CrossRef]
14. Marcilla, A., Bargues, M. D. and Mas-Coma, S. 2002. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Mol. Cell. Probes* **16**: 327–333. [Medline] [CrossRef]
15. Mohanta, U. K., Ichikawa-Seki, M., Shoriki, T., Katakura, K. and 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. [Medline] [CrossRef]
16. Peng, M., Ichinomiya, M., Ohtori, M., Ichikawa, M., Shibahara, T. and 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**: 809–815. [Medline] [CrossRef]
17. Shoriki, T., Ichikawa-Seki, M., Devkota, B., Rana, H. B., Devkota, S. P., Humagain, S. K. and Itagaki, T. 2014. Molecular phylogenetic identification of *Fasciola* flukes in Nepal. *Parasitol. Int.* **63**: 758–762. [Medline] [CrossRef]
18. Shoriki, T., Ichikawa-Seki, M., Suganuma, K., Naito, I., Hayashi, K., Nakao, M., Aita, J., Mohanta, U. K., Inoue, N., Murakami, K. and Itagaki, T. 2016. Novel methods for the molecular discrimination of *Fasciola* spp. on the basis of nuclear protein-coding genes. *Parasitol. Int.* **65**: 180–183. [Medline] [CrossRef]
19. Terasaki, K., Akahane, H., Habe, S. and Moriyama, N. 1982. The geographical distribution of common liver flukes (the genus *Fasciola*) with normal and abnormal spermatogenesis. *Jpn. J. Vet. Sci.* **44**: 223–231. [Medline] [CrossRef]
20. Torgerson, P. and Claxton, J. 1999. Epidemiology and control. pp. 113–149. In: Fasciolosis. (Dalton, J. P. ed.), CABI publishing, New York.
21. Troy, C. S., MacHugh, D. E., Bailey, J. F., Magee, D. A., Loftus, R. T., Cunningham, P., Chamberlain, A. T., Sykes, B. C. and Bradley, D. G. 2001. Genetic evidence for Near-Eastern origins of European cattle. *Nature* **410**: 1088–1091. [Medline] [CrossRef]