**NOTE** *Parasitology*

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

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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

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*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 mitochondrial 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-carmine 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

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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 ($\pi$) 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 $\pi$ 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 $\pi$ 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

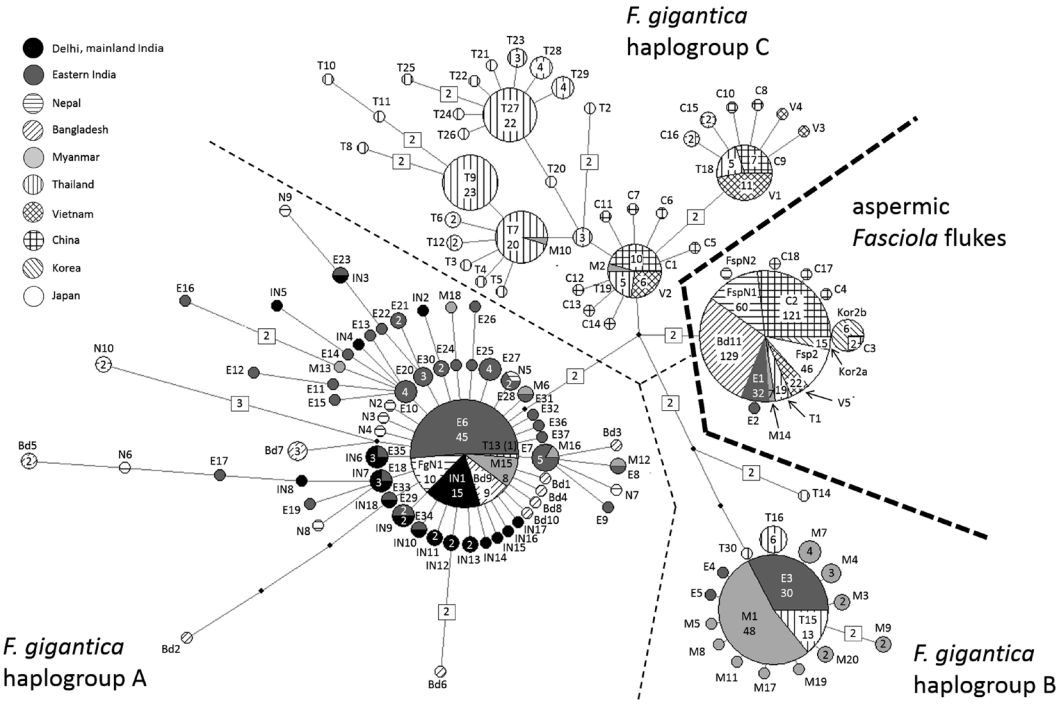


Fig. 1. A median-joining network on the basis of mitochondrial *nad1* haplotypes of *Fasciola gigantica* and aspermic *Fasciola* flukes. *Fasciola* flukes from Delhi are shown in black. A circle indicates a haplotype. Haplotype codes are labeled within or adjacent to the circles. The prefix of each haplotype 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*.

Table 2. Diversity indices for *F. gigantica* populations of the haplogroup a on the basis of the sequence of mitochondrial *nad1* gene

| Populations | N | S | h | Hd ± 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 |

N: number of flukes used for calculation, S: number of substitution sites, h: number of haplotypes, Hd: haplotype diversity, SD: standard deviation, π: nucleotide diversity. Numbers in brackets represent those of the references.

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 the hypothesis. Therefore, further phylogenetic studies using additional *Fasciola* flukes from mainland India are required to understand the spreading route of *F. gigantica*.

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