Hybrid origin of Asian aspermic Fasciola flukes is confirmed by analyzing two single-copy genes, pepck and pold

Kei HAYASHI1,2), Madoka ICHIKAWA-SEKI1,2)*, Uday Kumar MOHANTA1,3), Takuya SHORIKI1), Panngian CHAICHANASAK1,4) and Tadashi ITAGAKI1,2)

1)Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, Ueda, 3-18-8, Morioka 020-8550, Japan
2)Department of Pathogenetic Veterinary Science, United Graduate School of Veterinary Science, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan
3)Department of Microbiology and Parasitology, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaik 1207, Bangladesh
4)Faculty of Veterinary Medicine, Mahanakorn University of Technology, 140 Cheum-Sampan Road, Nong Chok, Bangkok 10530, Thailand

ABSTRACT. Nuclear gene markers, phosphoenolpyruvate carboxykinase (pepck) and DNA polymerase delta (pold), have been developed for precise discrimination of Fasciola flukes instead of internal transcribed spacer 1. In this study, these two genes of 730 Fasciola flukes from eight Asian countries were analyzed. The results were compared with their mitochondrial NADH dehydrogenase subunit 1 (nad1) lineages for obtaining a definitive evidence of the hybrid origin of aspermic Fasciola flukes. All the flukes categorized into the aspermic nad1 lineages possessed both the fragment patterns of F. hepatica and F. gigantica (mixed types) in pepck and/or pold. These findings provide clear evidence for the hybrid origin of aspermic Fasciola lineages and suggest that “aspermic Fasciola flukes” should hereafter be called “hybrid Fasciola flukes”.

KEY WORDS: Asia, Fasciola, hybrid, pepck, pold

Fasciolosis is a parasitic disease responsible for liver disorders in ruminant hosts and leads to a reduction in livestock productivity. The two well-known causative agents of fasciolosis are Fasciola hepatica and Fasciola gigantica. The former species is distributed mainly in Europe, America and Oceania, while the latter is distributed mainly in Asia and Africa [19]. The two species reproduce bisexually thorough fertilization. Mature spermatozoa of the species are ejaculated from their seminal vesicles, which serve as temporary storage for self-produced sperm [18]. In addition to the two species, aspermic Fasciola flukes, which contain only few or no spermatozoa in their seminal vesicles, have been reported in Asian countries [3, 18]. Not only diploid but also triploid flukes were reported from aspermic Fasciola flukes [11].

The nucleotide sequence of the ribosomal internal transcribed spacer 1 (ITS1) has been employed so far for molecular characterization of Fasciola flukes [1, 3, 5–7, 9–11, 14, 15]. Three ITS1 types (ITS1-Fh, Fg and mixed type) were distinguished using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method [4]. The Fh and Fg types have identical fragment patterns to F. hepatica and F. gigantica, respectively. Therefore, the spermic species have been identified based on their ITS1 types [1, 3, 4, 6, 7, 10, 14, 15]. However, species identification cannot be performed using ITS1 when the spermatogenic status of a fluke is unclear because both the ITS1-Fh and ITS1-Fg types are found also in aspermic flukes [4]. The ITS1-mixed type has both the fragment patterns of the two species that were detected in aspermic Fasciola flukes [5–7, 9–11, 14, 15], suggesting that the aspermic flukes are hybrids between F. hepatica and F. gigantica [4]. However, the ITS1 has hundreds of copies organized as tandem repeats with highly recombinogenic and unstable characteristics [13], and it is therefore, unsuitable to provide reliable evidence of natural hybridization.

Phylogenetic studies based on the nucleotide sequence of the mitochondrial NADH dehydrogenase subunit 1 (nad1) have revealed that the nad1 haplotypes of F. hepatica and F. gigantica are well diversified [8]. In contrast, the nad1 haplotypes of aspermic Fasciola flukes displayed uniform characteristics, which originated from the two different maternal lineages, F. hepatica and F. gigantica [11]. The two maternal lineages, which are described as “aspermic Fh” and “aspermic Fg” in this study, contain respective major haplotypes together with some derivative haplotypes. This indicates the maternal ancestors of aspermic flukes

*Correspondence to: Ichikawa-Seki, M.: madoka@iwate-u.ac.jp
©2018 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. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)
HYBRID ORIGIN OF ASPERMIC FASCIOLA SP.

were *F. hepatica* or *F. gigantica* with the major haplotypes [8]. So far, the spermatogenic status, ITS1 type and nad1 haplotypes have been reported for 730 *Fasciola* flukes from eight Asian countries [1, 3, 5–7, 9–11, 14, 15]. However, contradictions concerning species discrimination were observed for 11 flukes from Eastern India, Myanmar and Bangladesh [3, 7, 14], because inconsistent results of the nad1 lineages and spermatogenic status were of the flukes could not be resolved by analyzing ITS1.

Recently, the single-copy nuclear gene markers, *pepck* and *pold*, which encode phosphoenolpyruvate carboxykinase and DNA polymerase delta, respectively were developed [16]. The fragment patterns of *F. hepatica*, *F. gigantica* and the mixed fragment of both the species were distinguished by both *pepck* and *pold* based on multiplex PCR and PCR-RFLP methods, respectively [16]. In this study, the fragment patterns of *F. hepatica* in *pepck* and *pold* were described as “pepck-Fh type” and “pold-Fh type”, respectively. Similarly, the fragment patterns of *F. gigantica* and those of both the species were described as “pepck-Fg type”, “pold-Fg type”, “pepck-mixed type” and “pold-mixed type”. Single-copy genetic markers are suitable for detecting evidence of hybridization. Interestingly, all the aspermic *Fasciola* flukes analyzed previously by *pepck* and *pold* were determined as hybrids even though they possessed the ITS1-Fh or ITS1-Fg type [16]. However, the number of samples was insufficient to draw a conclusion regarding the hybrid origin in the aspermic flukes. In this study, 730 *Fasciola* flukes from eight Asian countries [1, 3, 5–7, 9–11, 14, 15] were reanalyzed using *pepck* and *pold* markers to obtain a definitive evidence for the origin of aspermic *Fasciola* flukes as well as to confirm the reliability of the markers.

A total of 730 *Fasciola* flukes from eight Asian countries (Japan, Korea, Vietnam, Thailand, Myanmar, India, Bangladesh and Nepal) were examined in this study (Fig. 1). The spermatogenic status, ITS1 types, and mitochondrial nad1 haplotypes of the flukes were reported in previous studies [1, 3, 5–7, 9–11, 14, 15] (Table 1 and S1). Ploidy of the 42 flukes from Japan and Vietnam were also reported in the previous studies (Table S1) [10, 11]. Unfortunately, ploidy of the remaining flukes could not be analyzed in the previous studies because the flukes were not fixed in ethanol-acetic acid, essential for the ploidy analysis.

The *pepck* region was amplified from genomic DNA by using a multiplex PCR with Fh-pepck-F (5’-GATTGCACCGTTAGGTTAGC-3’), Fg-pepck-F (5’-AAAGTTTTCTATCCGAGAAG-3’) and Fcmn-pepck-R

Fig. 1. Species discrimination of *Fasciola* flukes from eight Asian countries based on the analysis of *pepck* and *pold* genes. White and black circles denote *F. gigantica* and hybrid *Fasciola* flukes, respectively. The circle sizes are proportional to the number of flukes, and the actual numbers are labeled in the circles.
Table 1. Profiles of *Fasciola* flukes from eight Asian countries

| Country       | Species a) | Number of flukes | Sperm in seminal vesicles a) | Nuclear DNA types | Mitochondrial nad1 b) lineages |
|---------------|------------|------------------|------------------------------|-------------------|-------------------------------|
| Japan         | Aspermic *Fasciola* flukes | 5 | mixed mixed | Fh | aspermic Fh |
|               |            | 1 | mixed mixed Fh | aspermic Fg |
|               |            | 4 | mixed N.D. | Fh | aspermic Fh |
|               |            | 1 | N.D. mixed Fh | aspermic Fh |
|               |            | 33 | mixed mixed Fg | aspermic Fg |
|               |            | 2 | mixed N.D. Fg | aspermic Fg |
|               |            | 2 | mixed mixed mixed | aspermic Fh |
|               |            | 2 | mixed mixed mixed | aspermic Fg |
| Subtotal      |            | 50 b) |                      |                   |                               |
| Korea         | Aspermic *Fasciola* flukes | 1 | mixed mixed | Fh | aspermic Fh |
|               |            | 4 | mixed mixed Fh | aspermic Fg |
|               |            | 2 | mixed mixed Fg | aspermic Fg |
|               |            | 10 | mixed mixed mixed | aspermic Fh |
|               |            | 1 | N.D. mixed mixed | aspermic Fh |
|               |            | 15 | mixed mixed mixed | aspermic Fg |
| Subtotal      |            | 33 |                      |                   |                               |
| Vietnam       | Aspermic *Fasciola* flukes | 2 | mixed mixed | Fh | aspermic Fg |
|               |            | 1 | mixed mixed Fg | aspermic Fg |
|               |            | 1 | mixed N.D. Fg | aspermic Fg |
|               |            | 12 | mixed mixed mixed | aspermic Fg |
|               |            | 5 | mixed N.D. mixed | aspermic Fg |
|               | *F. gigantica* | 15 | + Fg Fg Fg | *F. gigantica* |
|               |            | 3 | + Fg N.D. Fg | *F. gigantica* |
| Subtotal      |            | 39 b) |                      |                   |                               |
| Thailand      | Aspermic *Fasciola* flukes | 17 | mixed mixed Fg | aspermic Fg |
|               |            | 2 | mixed N.D. Fg | aspermic Fg |
|               | *F. gigantica* | 122 | + Fg Fg Fg | *F. gigantica* |
|               |            | 4 | + Fg N.D. Fg | *F. gigantica* |
| Subtotal      |            | 145 b) |                      |                   |                               |
| Myanmar       | Aspermic *Fasciola* flukes | 7 | mixed mixed mixed | aspermic Fg |
|               | *F. gigantica* | 79 | + Fg Fg Fg | *F. gigantica* |
|               |            | 1 c) | - Fg Fg Fg | *F. gigantica* |
| Subtotal      |            | 87 b) |                      |                   |                               |
| Eastern India | Aspermic *Fasciola* flukes | 29 | mixed mixed Fg | aspermic Fg |
|               | *F. gigantica* | 115 | + Fg Fg Fg | *F. gigantica* |
|               |            | 6 c) | + Fg Fg Fg | *F. gigantica* |
|               |            | 3 | + Fg N.D. Fg | *F. gigantica* |
| Subtotal      |            | 157 |                      |                   |                               |
| Bangladesh    | Aspermic *Fasciola* flukes | 86 | mixed mixed Fg | aspermic Fg |
|               | *F. gigantica* | 29 | mixed mixed mixed | aspermic Fg |
|               |            | 1 c) | + mixed mixed Fg | aspermic Fg |
|               |            | 1 c) | + mixed mixed mixed | aspermic Fg |
|               | *F. gigantica* | 19 | + Fg Fg Fg | *F. gigantica* |
|               |            | 2 c) | - Fg Fg Fg | *F. gigantica* |
| Subtotal      |            | 138 b) |                      |                   |                               |
| Nepal         | Aspermic *Fasciola* flukes | 61 | mixed mixed mixed | aspermic Fg |
|               | *F. gigantica* | 20 | + Fg Fg Fg | *F. gigantica* |
| Subtotal      |            | 81 |                      |                   |                               |
| Total         |            | 730 |                      |                   |                               |

a) Spermatic status, ITS1 types and nad1 haplotypes were analyzed in previous studies; Japan [7, 9], Korea [5], Vietnam [11], Thailand [1], Myanmar [6], Eastern India [3], Bangladesh [14] and Nepal [15]. b) The numbers for some of the subtotals do not completely match those from previous reports because some DNA samples were exhausted. c) Flukes possessing inconsistent characters for spermatogenesis status, ITS1 genotypes and nad1 haplotypes.

“Fh” and “Fg” represent *F. hepatica* and *F. gigantica* band patterns, respectively. “mixed” represents a both band pattern for Fh and Fg types. “aspermic Fh” and “aspermic Fg” represent nad1 haplotypes of aspermic *Fasciola* flukes whose maternal ancestry is *F. hepatica* and *F. gigantica*, respectively. N.D., not detected. a) Spermatic status, ITS1 types and nad1 haplotypes were analyzed in previous studies; Japan [7, 9], Korea [5], Vietnam [11], Thailand [1], Myanmar [6], Eastern India [3], Bangladesh [14] and Nepal [15]. b) The numbers for some of the subtotals do not completely match those from previous reports because some DNA samples were exhausted. c) Flukes possessing inconsistent characters for spermatogenesis status, ITS1 genotypes and nad1 haplotypes.
(5'-CGAAAATTATGGCATCAATGGG-3') primers, and the fragment patterns were distinguished on 1% agarose gel [16]. The pold region was analyzed by PCR-RFLP [16]. Briefly, the pold products amplified by Fasciola-pold-F1 (5'-GCTAACTTATCTGCTTACACGTGGACA-3') and Fasciola-pold-R1 (5'-ATCGCATTCGATCAAAGCCCTCCCATG-3') primers were digested with the AluI restriction enzyme (Roche, Mannheim, Germany), and then the fragment patterns were distinguished on 1.8% agarose gel.

In this study, the pepck genotyping yielded two DNA types, namely pepck-Fg type (F. gigantica) and pepck-mixed type (hybrid between F. hepatica and F. gigantica). Similarly, pold genotyping yielded pold-Fg type, and pold-mixed type. The newly obtained results of the pepck and pold genotyping were combined with the previous results of the spermatogenetic status, ITS1 types and mitochondrial nad1 haplotypes (Table 1) [1, 3, 5–7, 9–11, 14, 15]. The nad1 haplotypes are divided into three haplogroups, “F. gigantica”, “aspermic Fh” and “aspermic Fg” [8]. The first haplogroup consists of haplotypes detected from populations of F. gigantica, whereas the second and third haplogroups include haplotypes of aspermic Fasciola flukes whose maternal ancestors are F. hepatica and F. gigantica, respectively. Detailed information about each fluke is summarized in Supplementary Table (Table S1).

Although the ITS1 region for all the flukes was adequately amplified in the previous studies [1, 3, 5–7, 9–11, 14, 15], the pepck and pold regions for two and 28 of the flukes, respectively were not amplifiable in the present study (Table 1). This observation is probably related to differences in the copy numbers of the target genes since ITS1 is a multi-copy gene, it was more easily amplified than pepck and pold, single-copy genes in many eukaryotic species [2, 12]. Additionally, the small sizes of the pepck amplicons (241 bp and 509 bp or 510 bp) made them more readily generated than the pold amplicons (844 bp) [16].

The discrimination of Fasciola species based on spermatogenetic status, ITS1 and nad1 haplotypes has produced contradictory results for 11 Fasciola flukes analyzed in previous studies. Indeed, nine aspermic flukes from Myanmar, eastern India and Bangladesh appeared to have no spermatogenic ability and were actually thought to be F. gigantica because their nad1 haplotypes were not included in “aspermic Fg” but were included in “F. gigantica” [3, 7, 14] (Table 1). In the present study, these flukes showed the pepck-Fg type and the pold-Fg, and were confirmed as F. gigantica (Table 1). Additionally, one fluke from Bangladesh, appeared to retain its spermatogenic ability and possessed the ITS-Fg type, was regarded as an aspermic Fasciola fluke because it was included in the “aspermic Fg” haplogroup in the nad1 gene [14]. Here, this fluke displayed the pepck-mixed type and pold-mixed type (Table 1). Similarly, another fluke from Bangladesh, appeared to retain its spermatogenic ability and possessed the ITS1-mixed type and the nad1 haplotype of “aspermic Fg” [14], also showed the mixed types in both pepck and pold. In summary, all the Fasciola flukes belonging to the “aspermic Fh” or “aspermic Fg” haplogroups in nad1 showed the mixed types in both pepck and pold regardless of their ITS1 types (Table 1). These results strongly suggest that Asian aspermic Fasciola flukes originated through the hybridization between F. hepatica and F. gigantica, and should now be called “hybrid Fasciola flukes” instead of “aspermic Fasciola flukes” as having been proposed by Ichikawa-Seki et al. [8]. “Aspermic” seems no longer an adequate term because the two hybrid flukes from Bangladesh [14] (Table 1) retained sperm in their seminal vesicles.

Although the ploidy of almost all the flukes was unknown in this study, the 25 triploids as well as the 1 diploid aspermic flukes from Japan and Vietnam showed the mixed types in both pepck and pold (Table S1). Since triploid flukes can never occur in a single hybridization, the most possible origin of a triploid may be through the fertilization of a parthenogenetically produced egg (diploid) by the sperm of a male from the bisexual ancestor [17]. According to this theory, triploid would also be called as “hybrid” in a broad sense. Actually, triploid flukes were successfully produced by an experimental hybridization between hybrid diploid and F. hepatica (unpublished results).

In this study, all the Fasciola flukes belonging to “F. gigantica” haplogroup in nad1 displayed pepck-Fg type and pold-Fg type, and were therefore confirmed as F. gigantica. These findings revealed that the results of the pepck and pold analyses were completely consistent with those of the nad1 linesages. Therefore, pepck and pold were proved as potential markers for precise discrimination of F. hepatica, F. gigantica and hybrid Fasciola flukes. Accurate discrimination of Fasciola flukes is very important because hybrid Fasciola flukes are thought to have superior fecundity to F. gigantica [14]. Hybrid Fasciola flukes were predominant in Nepal (75.3%), Bangladesh (84.8%), South Korea (100%), Japan (100%) and Vietnam (53.8%) (Fig. 1), and are therefore needed a further attention to monitor their dispersal route in Asian countries.

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

REFERENCES
1. 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]
2. Friedlander, T. P., Regier, J. C. and Mitter, C. 1994. Phylogenetic information content of five nuclear gene sequences in animals: initial assessment of character sets from concordance and divergence studies. Syst. Biol. 43: 511–525. [CrossRef]
3. 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]
4. Ichikawa, M. and Itagaki, T. 2010. Discrimination of the ITS1 types of Fasciola spp. based on a PCR-RFLP method. Parasitol. Res. 106: 757–761. [Medline] [CrossRef]
5. Ichikawa, M. and Itagaki, T. 2012. 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. J. Vet. Med. Sci. 74: 899–904. [Medline] [CrossRef]
6. Ichikawa, M., Iwata, N. and Itagaki, T. 2010. DNA types of aspermic Fasciola species in Japan. J. Vet. Med. Sci. 72: 1371–1374. [Medline]

doi: 10.1292/jvms.17-0406
7. Ichikawa, M., Bawn, S., Maw, N. N., Htun, L. L., Thein, M., Gyï, A., Sunn, K., Katahara, 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]

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

9. 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]

10. 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]

11. 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]

12. Loeb, L. A. and Monnat, R. J. Jr. 2008. DNA polymerases and human disease. *Nat. Rev. Genet.* **9**: 594–604. [Medline] [CrossRef]

13. Miyazaki, T. and Kobayashi, T. 2011. Visualization of the dynamic behavior of ribosomal RNA gene repeats in living yeast cells. *Genes Cells* **16**: 491–502. [Medline] [CrossRef]

14. Mohanta, U. K., Ichikawa-Seki, M., Shoriki, T., Katahara, 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]

15. 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]

16. 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]

17. Simon, J. C., Delmotte, F. and Rispe, C. 2003. Phylogenetic relationships between parthenogens and their sexual relatives: the possible routes to parthenogenesis in animals. *Biol. J. Linn. Soc. Lond.* **79**: 151–153. [CrossRef]

18. Terasaki, K., Akahane, H., Habc, S. and Moriyama, N. 1982. The geographical distribution of common liver flukes (the genus Fasciola) with normal and abnormal spermatogenesis. *Nippon Juigaku Zasshi* **44**: 223–231. [Medline] [CrossRef]

19. Torgerson, P. and Claxton, J. 1999. Epidemiology and control. pp. 113–149. In: *Fasciolosis* (Dalton, J. P. ed.), CABI Publishing, New York.