Development of novel DNA marker for species discrimination of *Fasciola* flukes based on the fatty acid binding protein type I gene

Emi Okamoto¹, Michiyo Tashiro¹, Pedro Ortiz², Uday Kumar Mohanta³, Cristian Hobán², César A. Murga-Moreno², José M. Angulo-Tisoc⁴ and Madoka Ichikawa-Seki¹*

Abstract

**Background:** Multiplex polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) for nuclear phosphoenolpyruvate carboxykinase (*pepck*) and polymerase delta (*pold*), respectively, have been used to differentiate *Fasciola hepatica*, *F. gigantica*, and hybrid *Fasciola* flukes. However, discrimination errors have been reported in both methods. This study aimed to develop a multiplex PCR based on a novel nuclear marker, the fatty acid binding protein type I (*FABP*) type I gene.

**Methods:** Nucleotide sequence variations of FABP type I were analyzed using DNA samples of *F. hepatica*, *F. gigantica*, and hybrid *Fasciola* flukes obtained from 11 countries in Europe, Latin America, Africa, and Asia. A common forward primer for *F. hepatica* and *F. gigantica* and two specific reverse primers for *F. hepatica* and *F. gigantica* were designed for multiplex PCR.

**Results:** Specific fragments of *F. hepatica* (290 bp) and *F. gigantica* (190 bp) were successfully amplified using multiplex PCR. However, the hybrid flukes contained fragments of both species. The multiplex PCR for FABP type I could precisely discriminate the 1312 *Fasciola* samples used in this study. Notably, no discrimination errors were observed with this novel method.

**Conclusions:** Multiplex PCR for FABP type I can be used as a species discrimination marker in place of *pepck* and *pold*. The robustness of the species-specific primer should be continuously examined using a larger number of *Fasciola* flukes worldwide in the future since nucleotide substitutions in the primer regions may cause amplification errors.

**Keywords:** *Fasciola*, Multiplex PCR, Genotyping, FABP type I

Background

Fasciolosis causes huge economic losses to the livestock industry in endemic areas [1, 2]. *Fasciola hepatica* and *F. gigantica* are well-known causative agents of this disease. Both species have normal spermatogenic abilities and reproduce bisexually by fertilization. In contrast, the hybrid *Fasciola* flukes of the two species have been reported in many Asian countries [3]. Both diploids and triploids have been reported in hybrid *Fasciola* flukes [4, 5]. Because hybrid flukes harbor a meiotic disorder that affects spermatogenesis, they probably reproduce parthenogenetically [5]. Therefore, it is important to precisely discriminate hybrid flukes from *F. hepatica* and *F. gigantica*.
gigantica because they are speculated to have stronger viability than the two species [6].

Multiplex polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (RFLP) for nuclear phosphoenolpyruvate carboxykinase (pepck) and polymerase delta (pold), respectively, can differentiate Fasciola spp. by the fragment patterns of F. hepatica (Fh), F. gigantica (Fg), and the hybrid (both Fh and Fg: Fh/Fg) [7]. The existence of the Fh/Fg type in the two nuclear markers suggests that hybrid Fasciola flukes are descendants originating from the hybridization of F. hepatica and F. gigantica [3, 6].

Although discrimination errors in the fragment pattern analysis of the multiplex PCR for pepck have been reported in F. hepatica isolates from Afghanistan [8], Algeria [9], Ecuador [10], and Spain [11], subsequent nucleotide sequencing of DNA fragment of pepck enabled precise species identification. Regarding pold, discrimination errors were observed in F. gigantica isolates from Nigeria [12]. A single-nucleotide substitution at the recognition site of the restriction enzyme was identified as the cause of the error in PCR-RFLP [12].

Fatty acid binding protein (FABP) type I of Fasciola flukes encoded in the nuclear DNA has multifunctional roles, such as immune modulation and anthelmintic sequestration [13]. Moreover, the messenger RNA (mRNA) sequence of FABP type I is available in the DNA databank [13]. This study analyzed the nucleotide sequence variations of FABP type I in F. hepatica, F. gigantica, and hybrid Fasciola flukes. Then, a multiplex PCR for FABP type I was developed and applied to 1312 Fasciola spp. from 11 countries in Asia, Africa, Europe, the Near and Middle East, and Latin America. The novel multiplex PCR for FABP type I was proven to be a useful marker in place of pepck and pold for precise species discrimination of Fasciola spp.

Methods

Fasciola samples

A total of 1312 Fasciola flukes (470 F. hepatica, 609 F. gigantica, and 233 hybrid Fasciola) from 11 countries (Afghanistan, Algeria, Peru, Spain, Indonesia, Malaysia, Nigeria, Pakistan, Uganda, Japan, and Bangladesh) [8, 9, 11, 12, 14–20] were used in the present study. Fragment analyses of nuclear pepck and pold and the nucleotide sequencing of mitochondrial nad1 have been performed in previous studies [8, 9, 11, 12, 14–20]. Discrepancies between pepck and pold were observed among 7, 19, 6, 27, and 15 Fasciola isolates from Afghanistan, Algeria, Peru, Spain, and Nigeria, respectively. All available information on the Fasciola samples is summarized in Table 1.

Some of the analyses for pepck and pold were conducted in the present study. Briefly, a small portion of the vitelline glands from the posterior part of each fluke was used for DNA extraction using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), following the

**Table 1** Nuclear marker profiles of Fasciola flukes used in this study

| Species   | Country          | Sample number | FABP type I (multiplex PCR) | pepck (multiplex PCR) | pold (PCR–RFLP) |
|-----------|------------------|----------------|-----------------------------|------------------------|-----------------|
| F. hepatica | Afghanistan [8] | 92             | 92 Fh 0 Fg 0 Fh/Fg          | 85 Fh 0 Fg 7 Fh/Fg    | 92 Fh 0 Fg 0 Fh/Fg |
|           | Algeria [9]      | 68             | 68 Fh 0 Fg 0 Fh/Fg          | 49 Fh 1 Fg 18 Fh/Fg   | 49 Fh 1 Fg 18 Fh/Fg |
|           | Peru [14]        | 114            | 114 Fh 0 Fg 0 Fh/Fg         | 108 Fh 1 Fg 5 Fh/Fg   | 108 Fh 1 Fg 5 Fh/Fg |
|           | Spain [11]       | 196            | 196 Fh 0 Fg 0 Fh/Fg         | 169 Fh 1 Fg 26 Fh/Fg  | 169 Fh 1 Fg 26 Fh/Fg |
| Subtotal  |                  | 470            | 470 Fh 0 Fg 0 Fh/Fg         | 411 Fh 3 Fg 56 Fh/Fg  | 411 Fh 3 Fg 56 Fh/Fg |
| F. gigantica | Indonesia [15] | 60             | 0 Fh 60 Fg 0 Fh/Fg          | 0 Fh 60 Fg 0 Fh/Fg    | 0 Fh 60 Fg 0 Fh/Fg |
|           | Malaysia [20]    | 36             | 0 Fh 36 Fg 0 Fh/Fg          | 0 Fh 36 Fg 0 Fh/Fg    | 0 Fh 36 Fg 0 Fh/Fg |
|           | Nigeria [12]     | 172            | 0 Fh 172 Fg 0 Fh/Fg         | 0 Fh 172 Fg 0 Fh/Fg   | 0 Fh 172 Fg 0 Fh/Fg |
|           | Pakistan [17]    | 49             | 0 Fh 49 Fg 0 Fh/Fg          | 0 Fh 49 Fg 0 Fh/Fg    | 0 Fh 49 Fg 0 Fh/Fg |
|           | Uganda [18]      | 292            | 0 Fh 292 Fg 0 Fh/Fg         | 0 Fh 292 Fg 0 Fh/Fg   | 0 Fh 292 Fg 0 Fh/Fg |
| Subtotal  |                  | 609            | 0 Fh 609 Fg 0 Fh/Fg         | 0 Fh 609 Fg 0 Fh/Fg   | 0 Fh 609 Fg 0 Fh/Fg |
| Hybrid    | Japan [16]       | 201            | 0 Fh 201 Fg 0 Fh/Fg         | 0 Fh 201 Fg 0 Fh/Fg   | 0 Fh 201 Fg 0 Fh/Fg |
|           | Bangladesh [c]   | 32             | 0 Fh 32 Fg 0 Fh/Fg          | 0 Fh 32 Fg 0 Fh/Fg    | 0 Fh 32 Fg 0 Fh/Fg |
| Subtotal  |                  | 233            | 0 Fh 233 Fg 0 Fh/Fg         | 0 Fh 233 Fg 0 Fh/Fg   | 0 Fh 233 Fg 0 Fh/Fg |
| Total     |                  | 1312           | 470 Fh 609 Fg 233 Fh/Fg     | 411 Fh 612 Fg 289 Fh/Fg | 470 Fh 594 Fg 248 Fh/Fg |

*a* Seventy-eight Fasciola flukes were used in the previous study. The remaining samples were analyzed in the present study

*b* The results of pold were obtained in the present study

*c* Analyzed in the present study
manufacturer's protocols, and stored at –20 °C until further use. Fragments of *pepck* were amplified using a multiplex PCR assay with Fh-*pepck*-F (5′-GATTGCAACGGTACGGTGGTTAGC-3′), Fg-*pepck*-F (5′-AAAGTCTTCTATCCCAGACGAAG-3′), and Fcmn-*pepck*-R (5′-CGAAGATATTTGCATCAAAATCCC-3′) primers based on a previous study [7]. PCR amplicons were electrophoresed on 1.8% agarose gels for 30 min to detect fragment patterns for *F. hepatica* (approximately 500 bp), *F. gigantica* (approximately 240 bp), or hybrid (both fragments). The fragments of *pold* were analyzed using the PCR-RFLP assay described in a previous study [7]. The PCR products were amplified using Fasciola-*pold*-F1 (5′-GCTAACATTGCATCAAGAG-3′) and Fasciola-*pold*-R1 (5′-ATCGCATTCGATCAAAGGCTC-3′) and subsequently digested with *Alu* I enzyme (Toyobo, Osaka, Japan) at 37 °C for 3 h. The resulting products were electrophoresed on 1.8% agarose gels for 30 min to detect fragment patterns for *F. hepatica* (approximately 700 bp), *F. gigantica* (approximately 500 bp), or hybrid (both fragments).

**Sequence determination of FABP type I**

A primer set, FABP type I-F (5′-CACGATGGCTGAATTTGTGGT-3′) and FABP type I-R (5′-AAATTATTAGGGTGAG-3′), was designed based on the mRNA sequence of FABP type I generated from *F. hepatica* (accession no. M95291) [13].
PCRs were performed for *F. hepatica* isolates from Peru, and *F. gigantica* isolates from Uganda in a 25 μl reaction mixture containing 2 μl template DNA, 0.2 μM of each primer, 1 U of Gflex polymerase (Takara Bio, Shiga, Japan), and the manufacturer’s supplied reaction buffer. Thermal conditions included an initial denaturation step at 94 °C for 60 s, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 180 s. Fragments

Fig. 2 Maximum-likelihood tree of FABP type I genotypes. Sequences obtained with Clo-F and Clo-R primers were used in the tree. Bootstrap values > 60% are shown for the tree node. No suitable outgroups were available in the DNA databank. Abbreviations of the names of countries where each genotype was detected are mentioned on the tree. AFG: Afghanistan; DZA: Algeria; PER Peru; ESP: Spain; IDN: Indonesia; MYS: Malaysia; NGA: Nigeria; PAK: Pakistan; UGA: Uganda; JPN: Japan; BGD: Bangladesh. Red, blue, and green indicate the regions where *F. hepatica*, *F. gigantica*, and hybrid *Fasciola* used in the present study were collected.
of approximately 3000 bp were amplified and purified using the NucleoSpin Gel and PCR Clean-up kit (MACHEREY–NAGEL, Düren, Germany) and then directly sequenced from both directions to obtain the preliminary sequences of FABP type I. An inner primer set, FABP type I-2F (5′-CTGGTTGATGGAGAAGGA-3′) and FABP type I-2R (5′-ACTCGTCTGTTCACCG-3′), was generated to amplify partial FABP type I gene in *F. hepatica* (1951 bp) and *F. gigantica* (1961 bp), respectively. PCR conditions were almost the same as that described above, except for the annealing temperature, 55 °C. The nucleotide sequences of the PCR amplicons were determined precisely.

Another inner primer set, Clo-F (5′-CCATTGGTT TATAATAACTTCC-3′) and Clo-R (5′-ACTCTATT TCTCCATCTTCC-3′), which could amplify an intron of FABP type I, was designed to examine nucleotide variations between the primer regions (*F. hepatica*: 567 or 568 bp; *F. gigantica*: 566 or 567 bp) (Fig. 1). Sequence determination between Clo-F and Clo-R was performed for approximately 5% of *F. hepatica* and *F. gigantica* as well as 10 hybrid flukes selected from each country, and flukes with different *nad1* haplotypes were selected as much as possible to ensure variations in the samples (Additional file 1: Table S1). PCs were performed in a 25 μl reaction mixture containing 2 μl template DNA, 0.4 mM of each dNTP, 0.3 μM of each primer (Clo-F and Clo-R), 1 U of KOD FX Neo (Toyobo, Osaka, Japan), and the manufacturer’s supplied reaction buffer. Thermal conditions included an initial denaturation step at 94 °C for 120 s, followed by 35 cycles of 95 °C for 10 s, 55 °C for 30 s, and 68 °C for 30 s. PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), cloned into the pUC118 Hind II/BAP vector (Takara Bio), and sequenced. Two clones were analyzed for *F. hepatica* and *F. gigantica*, whereas four clones (two for *F. hepatica* genotype and two for *F. gigantica* genotype) were analyzed for the hybrid *Fasciola* fluke (Additional file 1: Table S1). The obtained sequences were aligned to construct a maximum likelihood (ML) tree using MEGA 10.0.5 software [21]. For ML tree construction, all sites were selected in the gaps/missing data treatment, and the T92+I model was used.

### Multiplex PCR

A primer set for multiplex PCR was designed using the resulting sequences of Clo-F and Clo-R. FABP type I-CommF (5′-GCGGTTCTGAGTGTTGTGGTT-3′) is a common primer for *F. hepatica* and *F. gigantica*, whereas FABP type I-FhR (5′-TGACGAACGCTTACCTT CGAG-3′) and FABP type I-FgR (5′-CAATACCTTCACA CCACCCAG-3′) are specific to *F. hepatica* (length of the amplicon: 287 bp) and *F. gigantica* (189 bp), respectively (Fig. 1). PCR amplification was performed in 10 μl reaction mixtures containing 0.5 μl template DNA, 0.1 μM of each dNTP, 0.2 μM of each primer, 0.01 U of Go Taq DNA Polymerase (Promega, Madison, WI, USA), and the manufacturer’s supplied reaction buffer. The PCR conditions included an initial denaturation step at 95 °C for 120 s, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and a final extension step at 72 °C for 5 min. PCR amplicons were electrophoresed on 1.8% agarose gels and visualized using ethidium bromide staining. Multiplex PCR was then applied to all 1312 flukes (Table 1).

### Results and discussion

The nucleotide sequences of PCR amplicons generated by FABP type I-2F and R for *F. hepatica* (1951 bp) and *F. gigantica* (1961 bp) were deposited in the DNA data bank of Japan (DDBJ) under accession numbers LC718926 and LC718927, respectively. The shorter nucleotide sequences of 24 *F. hepatica*, 31 *F. gigantica*, and 20 hybrid *Fasciola* flukes amplified using Clo-F and Clo-R were determined by cloning analysis (Additional file 1: Table S1). As a result, 10 genotypes (FABP type I-Fh1 to Fh10) were detected from *F. hepatica*, and 34 genotypes (FABP type I-Fg1 to Fg34) were detected from *F. gigantica*. Moreover, 12 *F. hepatica* (FABP type I-Fh1 and from FABP type I-Fh11 to Fh21) and 11 *F. gigantica* genotypes (FABP type I-Fg1 and from FABP type I-Fg35 to Fg44) were found in the hybrid *Fasciola* flukes. They were deposited in the DDBJ under accession numbers LC718928–LC718992 (Additional file 1: Table S1). FABP type I-Fh1 was detected in both *F. hepatica* and the hybrid *Fasciola* (Additional file 1: Table S1). FABP type I-Fg1 was found in both *F. gigantica* and the hybrid flukes (Additional file 1: Table S1). These observations may
Fig. 4 Nucleotide variations in the primer region of the multiplex PCR. a Nucleotide variations of FABP type I-CommF region. *Fasciola hepatica* genotypes. b Nucleotide variations of FABP type I-CommF region. *F. gigantica* genotypes. c Nucleotide variations of FABP type I-FhR. d Nucleotide variations of FABP type I-FgR.
indicate an ancestor-descendant relationship. However, geographical distribution of the FABP type I genotypes among the 11 countries was not clear (Fig. 2).

The FABP type I genotypes obtained in this study were clearly divided into the clades of *F. hepatica* and *F. gigantica* (Fig. 2). Therefore, the nucleotide variations of FABP type I are sufficient to distinguish *F. hepatica* and *F. gigantica* genotypes and therefore can be regarded as a useful molecular discrimination marker. The species-specific primers developed for multiplex PCR successfully generated specific fragments of *F. hepatica* (approximately 290 bp) and *F. gigantica* (approximately 190 bp), whereas hybrid flukes had both fragment patterns (Fig. 1 and 3).

No mutation was found in the FABP type I-ConnF primer region of *F. hepatica*, whereas a single-nucleotide mutation was found in the four *F. gigantica* genotypes (Fig. 4a and b). Similarly, no mutation was detected in the FABP type I-FhR region, but one to two nucleotide substitutions were observed in five *F. gigantica* genotypes in the FABP type I-FgR region (Fig. 4c and d). However, these mutations did not interfere with the DNA amplification of the multiplex PCR in this study because no ambiguous or variant fragments were detected (Table 1).

Previous studies observed discrepancies in 7, 19, 6, and 27 *F. hepatica* isolates from Afghanistan, Algeria, Peru, and Spain, respectively. They displayed the Fg or Fh/Fg type in the pepck (Table 1). However, in this study, all of them displayed Fh fragment patterns in the multiplex PCR for FABP type I, and there was no discrepancy when compared with the results of pold (Table 1). Moreover, the 15 *F. gigantica* from Nigeria showed Fg type in the multiplex PCR for FABP type I, which coincided with the results of pepck, even though they displayed an Fh/Fg-like fragment pattern in the pold (Table 1). Therefore, the novel multiplex PCR for FABP type I proved to be a useful marker to replace pepck and pold.

**Conclusions**

We successfully developed a novel multiplex PCR based on FABP type I using 1312 *Fasciola* flukes from 11 countries. Although discrimination errors occurring in pepck and pold were completely resolved by fragment analysis of FABP type I (Table 1), the robustness of the species-specific primer should be examined continuously in the future using a larger number of *Fasciola* flukes worldwide as nucleotide variations were detected in the primer regions.

**Abbreviations**

FABP type I: Fatty acid binding protein type I; PCR: Polymerase chain reaction; PCR-RFLP: PCR-restriction fragment length polymorphism; pepck: Nuclear phosphoenolpyruvate carboxykinase; pold: Polymerase delta; mRNA: Messenger RNA.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05538-7.

**Acknowledgements**

Not applicable.

**Author contributions**

All authors have made substantial contributions to the study conception, EO, molecular analysis, and drafting of the manuscript; MT molecular analyses; PO, sampling; UKM, sampling; MI designed the study and substantially revised the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported by a Grant-in-Aid for Scientific Research (C) from MEXT KAKENHI (grant number 21K05954) and the Tohoku Initiative for Fostering Global Researchers for Interdisciplinary Sciences (TI-FRIS) of MEXT’s Strategic Professional Development Program for Young Researchers.

**Availability of data and materials**

The nucleotide sequences obtained in this study are available under accession nos. LC718926 to LC718992.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1 Iwate University, Morioka, Japan. 2 Universidad Nacional de Cajamarca, Cajamarca, Peru. 3 Sher-E-Bangla Agricultural University, Dhaka, Bangladesh. 4 Universidad Nacional Mayor de San Marcos, Cusco, Peru.

**Received:** 22 July 2022  **Accepted:** 12 October 2022  **Published online:** 20 October 2022

**References**

1. Kaplan RM. *Fasciola hepatica*: a review of the economic impact in cattle and considerations for control. Vet Ther. 2001;2:40–50.

2. Mas-Coma S, Barques MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol. 2005;35:1255–78.

3. Hayashi K, Ichikawa-Seki M, Mohanta UK, Shoriki T, Chaichanasak P, Itagaki T. Hybrid origin of Asian aspermic Fasciola flukes is confirmed by analyzing two single-copy genes, pepck and pold. J Vet Med Sci. 2018;80:98–102. https://doi.org/10.1292/jvms.17-0406.

4. Terasaki K, Noda Y, Shibahara T, Itagaki T. Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, pepck and pold. J Vet Med Sci. 2018;80:98–102. https://doi.org/10.1292/jvms.17-0406.

5. Terasaki K, Itagaki T, Shibahara T, Noda Y, Moriyama-Gonda N. Comparative study of the reproductive organs of Fasciola groups by optical microscopy. J Vet Med Sci. 2001;63:735–42. https://doi.org/10.1292/jvms.63.735.

6. Ichikawa-Seki M, Peng M, Hayashi K, Shoriki T, Mohanta UK, Shibahara T, et al. Nuclear and mitochondrial DNA analysis reveals that hybridization between *Fasciola hepatica* and *Fasciola gigantica* occurred in China.
7. Shoriki T, Ichikawa-Seki M, Suganuma K, Naito I, Hayashi K, Nakao M, et al. Novel methods for the molecular discrimination of Fasciola spp on the basis of nuclear protein-coding genes. Parasitol Int. 2016;65:180–3. https://doi.org/10.1016/j.parint.2015.12.002.

8. Thang TN, Hakim H, Rahimi RR, Ichikawa-Seki M. Molecular analysis reveals expansion of Fasciola hepatica distribution from Afghanistan to China. Parasitol Int. 2019;72:101930. https://doi.org/10.1016/j.parint.2019.101930.

9. Laatamna AE, Tashiro M, Zakbi Z, Chibout Y, Megrane S, Mebarka F, et al. Molecular characterization and phylogenetic analysis of Fasciola hepatica from high-plateau and steppe areas in Algeria. Parasitol Int. 2021;80:102234. https://doi.org/10.1016/j.parint.2020.102234.

10. Kasahara S, Ohari Y, Jin S, Calvopina M, Takagi H, Sugiyama H, et al. Molecular characterization revealed Fasciola specimens in Ecuador are all Fasciola hepatica, none at all of Fasciola gigantica or parthenogenic Fasciola species. Parasitol Int. 2021;80:102215. https://doi.org/10.1016/j.parint.2020.102215.

11. Thang TN, Vázquez-Prieto S, Vilas R, Paniagua E, Ubeira FM, Ichikawa-Seki M. Genetic diversity of Fasciola hepatica in Spain and Peru. Parasitol Int. 2020;76:102100. https://doi.org/10.1016/j.parint.2020.102100.

12. Ichikawa-Seki M, Tokashiki M, Opara MN, Iroh G, Hayashi K, Kumar UM, et al. Molecular characterization and phylogenetic analysis of Fasciola gigantica from Nigeria. Parasitol Int. 2017;66:893–7. https://doi.org/10.1016/j.parint.2016.10.010.

13. Ichikawa-Seki M, Ortiz P, Cabrera M, Hobán C, Itagaki T. Molecular characterization and phylogenetic analysis of Fasciola hepatica from Peru. Parasitol Int. 2016;65:171–4. https://doi.org/10.1016/j.parint.2015.11.010.

14. Hayashi K, Ichikawa-Seki M, Allamanda P, Wibowo PE, Mohanta UK, Guswanto A, et al. Molecular characterization and phylogenetic analysis of Fasciola gigantica from western Java. Indonesia Parasitol Int. 2016;65:424–7.

15. Ichikawa-Seki M, Shiroma T, Kariya T, Nakao R, Ohari Y, Hayashi K, et al. Molecular characterization of Fasciola flukes obtained from wild sika deer and domestic cattle in Hokkaido, Japan. Parasitol Int. 2017;66:519–21. https://doi.org/10.1016/j.parint.2017.04.005.

16. Rehman ZU, Tashibu A, Tashiro M, Rashid I, Ali Q, Zahid O, et al. Molecular characterization and phylogenetic analyses of Fasciola gigantica of buffaloes and goats in Punjab, Pakistan. Parasitol Int. 2021;82:102288. https://doi.org/10.1016/j.parint.2021.102288.

17. Vudriko P, Echodu R, Tashiro M, Oka N, Hayashi K, Ichikawa-Seki M. Population structure, molecular characterization, and phylogenetic analysis of Fasciola gigantica from two locations in Uganda. Infect Genet Evol. 2022;104:105359. https://doi.org/10.1016/j.meegid.2022.105359.

18. Mohanta UK, Ichikawa-Seki M, Shoriki T, Kataura K, Itagaki T. Characteristics and molecular phylogeny of Fasciola flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. Parasitol Res. 2014;113:2493–501.

19. Ichikawa-Seki M, Hayashi K, Tashiro M, Khadijah S. Dispersal direction of Malaysian Fasciola gigantica from neighboring Southeast Asian countries inferred using mitochondrial DNA analysis. Infect Genet Evol. 2022;105:105373.

20. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9. https://doi.org/10.1093/molbev/msy096.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.