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NOTE

Parasitology

First report of *Fasciola* larva infection in *Galba truncatula* (Müller, 1774) (Gastropoda, Lymnaeidae) occurring in the natural environment in Hokkaido, Japan

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ABSTRACT. In Hokkaido, Japan, wild sika deer are highly infected with *Fasciola* flukes, suggesting that the flukes complete their life cycle via intermediate host snails and definitive host animals occurring in the natural environment. However, infected snails have been found only in cattle farms contaminated with fasciolosis. This study reports the first *Fasciola* larva infection in *Galba truncatula* snails occurring in the Shoro and Atsuma rivers in the natural environment. Molecular analysis revealed that the *nad1* haplotype of the larvae was consistent with that of *Fasciola* adults obtained from sika deer in Hokkaido. These results indicated that *Fasciola* flukes complete their life cycle via *G. truncatula* and sika deer occurring in the natural environment.

KEY WORDS: *Fasciola* fluke, *Galba truncatula*, Hokkaido, larval infection, *nad1*

Fasciolosis is one of the most important parasitic diseases of domestic ruminants [2]. Indeed, the annual economic losses related to this disease have been estimated at over three billion US dollars [3]. Humans and wild animals are also affected by the disease. The disease is caused by *Fasciola* spp. which parasitize the bile ducts of the definitive hosts. *Fasciola* flukes need freshwater snails of the family Lymnaeidae as intermediate hosts to complete their life cycle [9].

In Japan, the prevalence of *Fasciola* flukes has decreased gradually in cattle [15], whereas it remains high in wild sika deer (*Cervus nippon*) [8, 11, 13]. Molecular genetic analyses have revealed that the flukes detected in cattle and wild sika deer have an identical haplotype, Fsp1, in mitochondrial NADH dehydrogenase subunit 1 (*nad1*), suggesting that the Fsp1 flukes complete their life cycle in both cattle farms and natural environments endemic for fasciolosis in Hokkaido [5, 14]. The intermediate host of *Fasciola* flukes in Hokkaido, Japan has been confirmed as *Galba truncatula* (=*Lymnaea truncatula*) [20] based on the experimental [7] and natural infection [12]. Although naturally infected snails have been detected in cattle farms endemic for fasciolosis, they have yet to be found in the natural environment. In this study, we found *Fasciola* larvae, for the first time, in *G. truncatula* snails occurring in the natural environment in Hokkaido, Japan and analyzed their molecular properties. These results may enhance our understanding of the infection dynamics of the flukes in wild sika deer.

Lymnaeid snails were collected in September 2015 from streams of the Shoro River in Shiranuka, Kushiro District (43°09′00.6″N 144°00′13.7″E) and the Atsuma River in Atsuma, Iburi District (42°43′57.2″N 142°00′33.0″E) in Hokkaido (Fig. 1). These sites are located in over 50 meters away from close pasture areas which are surrounded by fences, and in the upper reaches of the areas. The snails were maintained in plastic containers with moistened tissue papers and stored at −30°C until dissection. The snails were identified based on their shell morphology as described previously [10]. To confirm the presence of trematode larvae, the frozen snails were thawed at room temperature, and the soft body and shell were separated to observe their midgut and hermaphroditic glands under a stereomicroscope. The detected trematode larvae were used for DNA extraction. Total DNA was extracted from the larvae obtained from in each infected snail using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at −20°C until use. Polymerase chain reaction (PCR) was preformed using the primer set, Ita 10 and Ita 2, to amplify the *nad1* region of *Fasciola* flukes [5]. The PCR amplicons were

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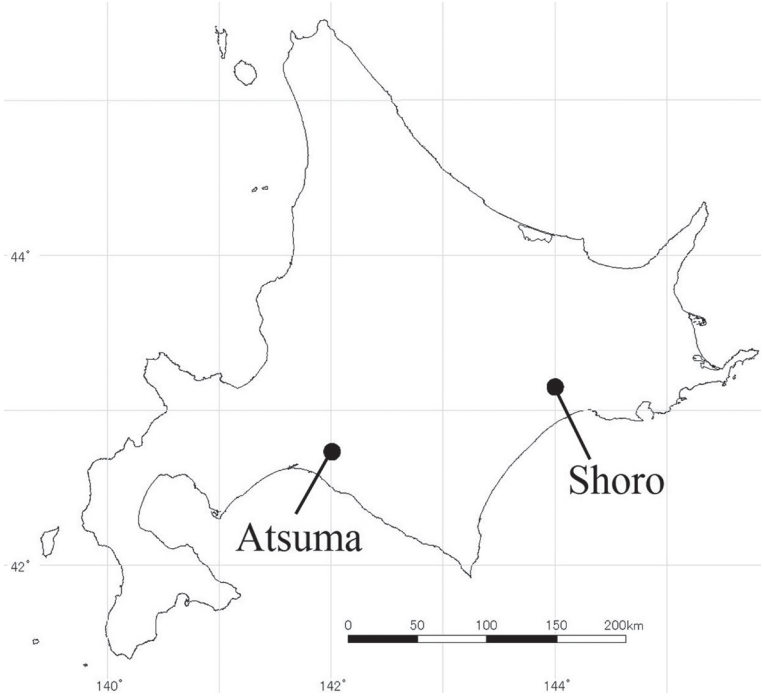

Fig. 1. Map of Hokkaido showing the sampling sites.

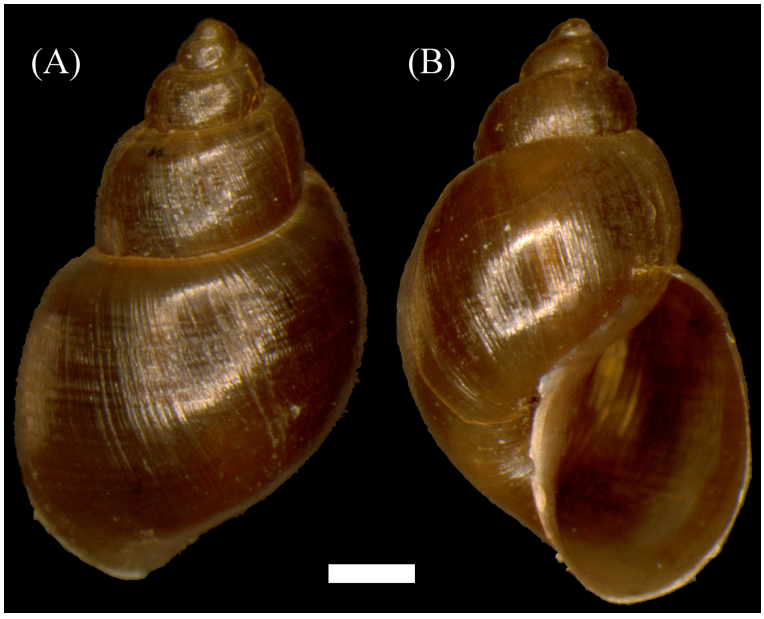

Fig. 2. Shell of *Galba truncatula* from Shoro, Hokkaido, Japan. (A) dorsal view. (B) ventral view. The white scale bar represents 1 mm.

verified on 1.8% agarose gel in TAE buffer, and then, purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Subsequently, direct sequencing was performed in both directions with the same primers used for PCR, using a BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, U.S.A.) in an ABI 3500 Genetic Analyzer (Applied Biosystems). The resulting sequences were initially assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan). Sequence alignment and haplotype detection were performed using Clustal W in MEGA 6.0 [18, 19].

Twenty-one and ten snails were collected from the Shoro and Atsuma rivers, respectively, and all the snails were identified as *G. truncatula* (Fig. 2). Trematode larvae were detected from the midgut glands of nine (42.86%) and one (10.00%) snails from Shoro and Atsuma, respectively. Partial *nad1* sequences (535bp) were generated from the pooled larvae of each infected snail. The

sequences showed no variation and were registered in GenBank (accession no. LC228620). The sequences were identical to that of the Fsp1 haplotype (AB207169) found in adult *Fasciola* flukes in Hokkaido [5, 14].

Although *Fasciola* infection has previously been detected in snails in the cattle farms contaminated with fasciolosis [12], this is the first report of *Fasciola* larva infection in *G. truncatula* occurring in the natural environment in Japan. Furthermore, the prevalence detected in this study was high, particularly in the Shoro River (42.86%), and almost equal to that detected in *Lymnaea* snails collected from farm areas in Japan where fasciolosis was highly endemic in the 1900s [1, 12, 16, 17, 21]. Moreover, the prevalence of *Fasciola* in sika deer is also high in Hokkaido [8, 11, 13]. These findings indicate that these areas of the Shoro and Atsuma rivers possibly represent sites of infection for wild sika deer in the natural environment, even though sika deer may be infected in artificial pasture areas because the animals are sometimes invaded in the area and feed on there [6].

The molecular study revealed that the Fsp1 haplotype was detected from naturally occurring *G. truncatula* as the intermediate host, as well as from wild sika deer [5, 14]. Sika deer is the only wild animal reported as final host of *Fasciola* flukes in Hokkaido, although wild mountain hare, *Lepus timidus ainu* and some rodent species also occur in this district. Furthermore, the sites where infected snails were collected were isolated from pasture areas. Lymnaeid snails could migrate to the direction of water flow but rarely to the opposite direction [4]. Therefore, it is quite unlikely that the snails infected in pasture areas migrate to theses collection sites. Thus, the snails collected at these sites seem to be unaffected by *Fasciola* originated in domestic cattle. These results suggest that the Fsp1 population of *Fasciola* completes its life cycle in naturally occurring *G. truncatula* snails and wild sika deer. Additional prevalence surveys of the naturally occurring intermediate host snails from diverse areas may provide a clearer picture of the dynamics of *Fasciola* infection in sika deer in Japan.

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