Journal of Helminthology

cambridge.org/jhl

Do aspermic (parthenogenetic) *Fasciola* forms have the ability to reproduce their progeny via parthenogenesis?

Y. Ohari[1,2], K. Hayashi[3], Y. Takashima[4,5] and T. 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]Laboratory of Parasitology, Faculty of Veterinary Medicine, Okayama University of Science, 1-3 Ikoinooka, Imabari 794-8555, Japan; [4]The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan and [5]Department of Veterinary Parasitology, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan

Research Paper

Cite this article: Ohari Y, Hayashi K, Takashima Y, Itagaki T (2021). Do aspermic (parthenogenetic) *Fasciola* forms have the ability to reproduce their progeny via parthenogenesis? *Journal of Helminthology* **95**, e36, 1–4. https://doi.org/10.1017/S0022149X21000328

Received: 9 May 2021
Revised: 29 June 2021
Accepted: 30 June 2021

Keywords:
Aspermic *Fasciola* forms; microsatellite marker; parthenogenesis

Author for correspondence:
T. Itagaki,
E-mail: itagaki@iwate-u.ac.jp

Abstract

Across Far East Asia, aspermic *Fasciola* forms are found endemically. They have abnormal spermatogenesis and oogenesis, and it is presumed that their progeny are produced parthenogenetically and clonally. Because of this, they are also termed parthenogenic *Fasciola* forms. Currently, there is no evidence that they do indeed reproduce parthenogenetically and clonally. In this study, the multilocus genetic type (MLG) in 12 microsatellite markers of adult flukes and their subsequent progeny larvae were analysed using two laboratory aspermic *Fasciola* triploid strains. The MLGs of adults and their larvae were identical for all markers evaluated, suggesting that these strains reproduce their progeny clonally. The deviation between theoretical and actual frequency within the larvae genotype of the Fh_6 locus resulted in the inability for self-fertilization within individual adult flukes. These findings strongly suggested that aspermic *Fasciola* forms reproduce their progeny by means of parthenogenesis, possibly gynogenesis.

Introduction

Two *Fasciola* species, *F. hepatica* and *F. gigantica*, have two reproductive stages in their life cycle (Dalton, 1999). The first involves asexual larval multiplication by paedogenesis in the intermediate host snails, and the other is via sexual fertilization for egg production in adults within their definitive animal hosts. However, aspermic (parthenogenetic) *Fasciola* forms that are found widely in eastern and southern Asia have been considered to produce their eggs by parthenogenesis. This means that no fertilization occurs within their definitive host as the forms are diploid and triploid which have abnormal spermatogenesis and oogenesis (Sakaguchi, 1980; Terasaki *et al.*, 1982, 1998). The oocyte chromosomes of triploid aspermic *Fasciola* flukes separate unequally into two daughter nuclei in the first stage of meiosis, and then re-fuse prior to the second meiosis should there be no male pronucleus (Hanna *et al.*, 2016). These findings indicate that aspermic *Fasciola* forms produce eggs with mitosis-like incomplete meiosis, and proliferate clonally with no introgression in the following generations. Aspermic *Fasciola* forms have been shown to have successive generations of progeny, resulting from interspecific hybridization between *F. hepatica* and *F. gigantica*, as these forms possess the genomes of the two species (Hayashi *et al.*, 2018). However, hybridization experiments between the two *Fasciola* species have failed to produce the aspermic *Fasciola* forms, and have therefore been unable to confirm that the forms reproduce parthenogenetically and proliferate clonally (Itagaki *et al.*, 2011). Hermaphroditic trematode species can produce eggs by means of self-fertilization (Trouvé *et al.*, 1996), and the confirmation of whether produced eggs resulted from parthenogenesis or self-fertilization has not been identified in the aspermic *Fasciola* forms. Recently, microsatellite markers for *F. hepatica*, which can trace the mating patterns and analyse the parentage (parent–offspring relationship), have been developed (Cwiklinski *et al.*, 2015), and these markers were available for parthenogenetic (aspermic) *Fasciola* flukes occurring in Japan (Ohari *et al.*, 2021). This study aimed to identify whether two laboratory strains of aspermic *Fasciola* forms reproduce parthenogenetically and clonally utilizing microsatellite markers.

Material and methods

Two laboratory strains of aspermic *Fasciola* forms were used in this study: the strains IWTc2-1 and NRA-4 originated from naturally infected cattle in Iwate prefecture, and sika deer, *Cervus nippon*, in Nara prefecture, Japan, respectively. These strains were triploid, as confirmed by

CAMBRIDGE UNIVERSITY PRESS

counting the chromosome number of their oocytes and spermatocytes, and confirmed aspermic due to no sperm in their seminal vesicles. The molecular markers of the two strains were Fsp1 haplotype (*F. hepatica*-type; GenBank accession no. AB207169) in the mitochondrial NADH dehydrogenase subunit 1 region; the *F. hepatica* and *F. gigantica*-mixed type in the nuclear ribosomal internal transcribed spacer 1, the phosphoenolpyruvate carboxykinase and DNA polymerase delta regions (Ichikawa & Itagaki, 2010; Shoriki *et al.*, 2016); and differed in the multilocus genetic types (MLGs) of the eight microsatellite regions: Fh_1, Fh_2, Fh_5, Fh_6, Fh_10, Fh_11, Fh_14 and Fh_15 (Cwiklinski *et al.*, 2015). Experimental coinfection with IWTc2-1 and NRA-4 strains was carried out using a male Wistar rat (8 weeks old) that were infected orally with ten metacercariae of each of the two strains, and four adult flukes (Adults 1–4) were subsequently recovered from the bile duct 3 months after infection. Experimental infection of the rat (no. A201623) was conducted according to the Guidelines for Animal Experiments of Iwate University, Japan. Of the four adult flukes, two were confirmed IWTc2-1 and the other two were NRA-4 based on the microsatellite analyses. This provided evidence that adult worms of both strains coexisted in the bile duct during the 2 months, and therefore may have mated randomly to each other. Snails (*Austropeplea ollula)* were infected with single miracidia hatched from eggs of individual adults and subsequently maintained at 27°C for a month after exposure. The snails were independently dissected, and 15, 17, 11 and six snails were infected with the larvae from Adults 1, 2, 3 and 4, respectively. The larvae (rediae and cercariae) were collected from individual infected snails and stored in −20°C for microsatellite markers analysis. Total DNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) from the posterior tissues of four adult flukes and the larvae from individual infected snails. The 12 microsatellite DNA regions (Fh_1, Fh_2, Fh_3, Fh_4, Fh_5, Fh_6, Fh_10, Fh_11, Fh_12, Fh_13, Fh_14, Fh_15) (Cwiklinski *et al.*, 2015) were amplified using a post-labelling polymerase chain reaction (PCR) method for multiplexed and multicoloured genotyping, using bar-coded slit tag (BStag) (Shimizu & Yano, 2011). The forward primers were appended with one of the four BStags, F9TAC, F9GAC, F9GTC and F9GCC, in the 5′ end and fluorescent dye-labelled BStag primers, PET-F9TAC, 6-FAM-F9GAC, VIC-F9GTC and NED-F9GCC (Applied Biosystems, Foster City, CA, USA) were used (Ohari *et al.*, 2021). PCR amplification was performed using the KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems, Boston, MA) in a final volume of 5 µl, containing 2.0 ng of extracted DNA, 3 µl of 2× KAPA2G Fast Multiplex Mix, 0.2 pM of each BStag-tailed forward primer, 0.4 pM of each reverse primer, and dye-labelled BStag primer. The PCR cycle was 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, and the subsequent step was for labelling the amplicons and was carried out by three cycles of 94°C for 20 s, 49°C for 10 s and 72°C for 5 s, followed by a final extension at 72°C for 10 min. Subsequently, 2 µl of the PCR product was added to 10.0 µl of Hi-Di Formamide and 0.5 µl of GeneScan LIZ600 size standards (Applied Biosystems), and the mixture was subjected to capillary electrophoresis using an ABI 3500 Genetic Analyzer (Applied Biosystems). The allele size of each locus was confirmed using GeneMapper version 5.0 (Applied Biosystems). The capillary electrophoreses on each microsatellite region for the adults and larvae DNAs were performed at the same time. The MLG of each adult and larvae

Table 1. Multilocus genetic types in 12 microsatellite loci of adults and their larvae of the two aspermic *Fasciola* triploid strains.

			Locus										
Adult fluke code	Strain	No. the larvae	Fh_1	Fh_2	Fh_3	Fh_4	Fh_5	Fh_6	Fh_10	Fh_11	Fh_12	Fh_13	Fh_14	Fh_15
1	IWTc2-1	15	199/-/-	195/228/-	204/-/-	179/187/-	199/224/-	206/209/250	226/232/-	226/-/-	213/-/-	196/208/-	222/231/-	221/-/-
2	IWTc2-1	17	199/-/-	195/228/-	204/-/-	179/187/-	199/224/-	206/209/250	226/232/-	226/-/-	213/-/-	196/208/-	222/231/-	221/-/-
3	NRA-4	11	199/216/-	195/-/-	204/-/-	179/187/-	202/224/-	206/209/215	226/238/-	223/-/-	213/-/-	196/208/-	231/-/-	221/240/-
4	NRA-4	6	199/216/-	195/-/-	204/-/-	179/187/-	202/224/-	206/209/215	226/238/-	223/-/-	213/-/-	196/208/-	231/-/-	221/240/-

-: allele undetected.

https://doi.org/10.1017/S0022149X21000328 Published online by Cambridge University Press

Table 2. Expected and observed frequency of apparent genotype in Fh_6 locus of the larvae produced by adults of aspermic *Fasciola* triploid strains.

Apparent genotype	Expected frequency			Observed frequency
	Selfing or outcrossing in IWTc2-1 adults (32)[a]	Selfing or outcrossing in NRA-4 adults (17)[a]	Random mating in IWTc2-1 and NRA-4 adults (49)a	
206/206	0.89	0.47	1.36	0
209/209	0.89	0.47	1.36	0
215/215	0.00	0.47	0.34	0
250/250	0.89	0.00	0.34	0
206/209	6.22	3.31	9.53	0
206/209/215[b]	0.00	5.67	7.49	17
206/209/215/250	0.00	0.00	1.36	0
206/209/250[c]	10.67	0.00	7.49	32
206/215	0.00	3.31	3.74	0
206/215/250	0.00	0.00	2.04	0
206/250	6.22	0.00	3.74	0
209/215	0.00	3.31	3.74	0
209/215/250	0.00	0.00	2.04	0
209/250	6.22	0.00	3.74	0
215/250	0.00	0.00	0.68	0

[a]The numbers within parenthesis indicate the number of the larvae.
[b]Identical to NRA-4 strain.
[c]Identical to IWTc2-1 strain.

fluke was evaluated based on the combined results of the allele sizes deduced from the 12 loci. The MLGs were compared between adult flukes and their larvae to confirm their clonality. These analyses were conducted using statistical software R (R Development Core Team, 2020) using the *poppr* version 2.8.1 package (Kamvar *et al.*, 2015).

Results and discussion

The MLGs of Adult 1, Adult 2 and their larvae DNAs (15 and 17, respectively) were identical to those of IWTc2-1, and Adult 3, Adult 4 and their larvae DNA (11 and 6, respectively) were identical to those of NRA-4 (table 1). In the Fh_6 locus, Adults 1 and 2 and their larvae exhibited three alleles 206/209/250 (triploid pattern), while Adults 3 and 4 and their larvae displayed 206/209/215 (triploid). On the assumption that triploid adults generated a diploid and a haploid gamete during meiosis, individual adults could generate six gametes (e.g. 206, 209, 250, 206/209, 206/250, 209/250 in IWTc2-1 strain) with a frequency of 1:6, and seven apparent genotypes could be expected in the larvae which were produced by means of selfing and outcrossing in adults of each strain (table 2). As the four adults can generate three gametes (206, 209 and 206/209) with a frequency of 1:6 and six gametes (215, 250, 206/215, 206/250, 209/215 and 206/250) with a frequency of 1:12, and 15 apparent genotypes can be expected in their random mating and selfing (table 2). However, only two genotypes, 206/209/250 and 206/209/215 were detected in the locus by a frequency of 32:17, and no other genotypes were observed (tables 1 and 2).

In aspermic *Fasciola* forms, it is assumed that produce their clonal progeny by utilizing parthenogenesis, meaning no fertilization occurs (Sakaguchi, 1980; Terasaki *et al.*, 1982). Microsatellite DNA markers are useful for elucidating the parentage and mating patterns of sexually reproductive diploid organisms (Flanagan & Jones, 2019). The findings in this study demonstrate that the MLGs in the 12 microsatellite loci of adult flukes and their larvae (progeny) were identical, suggesting that the aspermic *Fasciola* triploid strains reproduce their progeny clonally. Furthermore, the obvious deviation between theoretical and actual frequency observed in the genotype of Fh_6 locus would deny self-fertilization within individual flukes. These findings strongly suggest that the aspermic *Fasciola* triploid strains reproduce their progeny by means of parthenogenesis, possibly gynogenesis. It is considered that aspermic *Fasciola* forms occurred due to hybridization between *F. hepatica* and *F. gigantica* and have survived in evolutionary terms, possibly during hundreds or thousands of years without extinction (Itagaki *et al.*, 2005). The parthenogenetic triploid strains used in this study have been maintained during many successive generations in our laboratory with high developmental and hatching rates of eggs, and high production of metacercariae. Triploid forms of parasitic helminths, which would have the parthenogenetic ability, are also reported in *Paragonimus westermani* fluke (Van Herwerden *et al.*, 1999) and *Spirometra erinaceieuropaei* cestode (Sasada, 1978). It is pertinent to conduct further studies on understanding the survival mechanisms of parasitic triploid forms as it will be of importance for controlling the diseases.

Acknowledgements. We are grateful to the members of the Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University and the Department of Veterinary Parasitology, Gifu University.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

https://doi.org/10.1017/S0022149X21000328 Published online by Cambridge University Press

Conflict of interest. The authors declare none.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

References

Cwiklinski K, Allen K, LaCourse J, Williams DJ, Paterson S and Hodgkinson JE (2015) Characterisation of a novel panel of polymorphic microsatellite loci for the liver fluke, *Fasciola hepatica*, using a next generation sequencing approach. *Infection, Genetics and Evolution* **32**, 298–304.

Dalton, JP (ed.) (1999) *Fasciolosis*. Wallingford, UK, CABI Pub, 544 pp.

Flanagan SP and Jones AG (2019) The future of parentage analysis: From microsatellites to SNPs and beyond. *Molecular Ecology* **28**, 544–567.

Hanna REB, Moffett D, Forster FI, Trudgett AG, Brennan GP and Fairweather I (2016) Fasciola hepatica: A light and electron microscope study of the ovary and of the development of oocytes within eggs in the uterus provides an insight into reproductive strategy. *Veterinary Parasitology* **221**, 93–103.

Hayashi K, Ichikawa-Seki M, Mohanta UK, Shoriki T, Chaichanasak P and Itagaki T (2018) Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, *pepck* and *pold*. *Journal of Veterinary Medical Science* **80**, 98–102.

Ichikawa M and Itagaki T (2010) Discrimination of the ITS1 types of *Fasciola* spp. Based on a PCR–RFLP method. *Parasitology Research* **106**, 757–761.

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.

Itagaki T, Ichinomiya M, Fukuda K, Fusyuku S and Carmona C (2011) Hybridization experiments indicate incomplete reproductive isolating mechanism between *Fasciola hepatica* and *Fasciola gigantica*. *Parasitology* **138**, 1278–1284. Cambridge University Press.

Kamvar ZN, Brooks JC and Grünwald NJ (2015) Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in Genetics* **6**, 208.

Ohari Y, Matsuo K, Yoshida A, Nonaka N, Sato H and Itagaki T (2021) Genetic diversity and population structure analyses based on microsatellite DNA of parthenogenetic Fasciola flukes obtained from cattle and sika deer in Japan. *Parasitology Research* **120**, 1341–1350.

R Development Core Team (2020) *R: A language and environment for statistical computing*. Vienna, Austria, R Foundation for Statistical Computing.

Sakaguchi Y (1980) Karyotype and gametogenesis of the common liver fluke. Fasciola sp. in Japan. *Japanese Journal of Parasitology* **29**, 507–513.

Sasada K (1978) Studies on the chromosomes of parasitic helminthes (II) triploidy and cytological mechanism of parthenogenesis in *Diphyllobothrium erinacei* (cestoda: Diphyllobothriidae). *Japanese Journal of Parasitology* **27**, 547–560.

Shimizu T and Yano K (2011) A post-labeling method for multiplexed and multicolored genotyping analysis of SSR, indel and SNP markers in single tube with bar-coded split tag (BStag). *BMC Research Notes* **4**, 161.

Shoriki T, Ichikawa-Seki M, Suganuma K, *et al.* (2016) Novel methods for the molecular discrimination of *Fasciola* spp. on the basis of nuclear protein-coding genes. *Parasitology International* **65**, 180–183.

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. *The Japanese Journal of Veterinary Science* **44**, 223–231.

Terasaki K, Moriyama-Gonda N and Noda Y (1998) Abnormal spermatogenesis in the common liver fluke (*Fasciola* sp.) from Japan and Korea. *Journal of Veterinary Medical Science* **60**, 1305–1309.

Trouvé S, Renaud F, Durand P and Jourdane J (1996) Selfing and outcrossing in a parasitic hermaphrodite helminth (trematoda, echinostomatidae). *Heredity* **77**, 1–8.

Van Herwerden L, Blair D and Agatsuma T (1999) Genetic diversity in parthenogenetic triploid *Paragonimus westermani*. *International Journal for Parasitology* **29**, 1477–1482.

https://doi.org/10.1017/S0022149X21000328 Published online by Cambridge University Press