Veterinary Parasitology 245 (2017) 34–38

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

First genetic characterization of *Fasciola hepatica* in Argentina by nuclear and mitochondrial gene markers

Silvana Carnevale[a,b,*], Jorge Bruno Malandrini[c], María Laura Pantano[a], Claudia Cecilia Soria[c], Rosângela Rodrigues-Silva[d], José Roberto Machado-Silva[e], Jorge Néstor Velásquez[f], Laura Kamenetzky[b,g]

[a] Instituto Nacional de Enfermedades Infecciosas – ANLIS "Dr. Carlos G. Malbrán", Avenida Vélez Sarsfield 563, CP 1281 Ciudad de Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad de Buenos Aires, Argentina
[c] Facultad de Ciencias de la Salud, Universidad Nacional de Catamarca, Maestro Quiroga 1ra, Cuadra, CP 4700 San Fernando del Valle de Catamarca, Argentina
[d] Laboratório de Helmintos Parasitos de Vertebrados, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz. Av. Brasil 4365, Rio de Janeiro, RJ, 21040-360, Brazil
[e] Laboratório de Helmintologia Romero Lascasas Porto, Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro. Av. Prof. Manoel de Abreu, 444, Edifício Piquet Carneiro, quinto andar, Rio de Janeiro, RJ, 20550170, Brazil
[f] Hospital Municipal de Infecciosas "Dr. Francisco Javier Muñiz", Uspallata 2272, CP 1282 Ciudad de Buenos Aires, Argentina
[g] Instituto de Microbiología y Parasitología Médica (IMPaM), UBA-CONICET, Paraguay 2155 piso 13, CP 1121 Ciudad de Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Fasciola hepatica
Phylogenetic analysis
ITS1
*nad*4
*nad*5
*cox*1

ABSTRACT

Fasciola hepatica is a trematode showing genetic variation among isolates from different regions of the world. The objective of this work was to characterize for the first time *F. hepatica* isolates circulating in different regions of Argentina. Twenty-two adult flukes were collected from naturally infected bovine livers in different areas from Argentina and used for DNA extraction. We carried out PCR amplification and sequence analysis of the ribosomal internal transcribed spacer 1 (ITS1), mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunits 4 and 5 (*nad*4 and *nad*5) and mitochondrial cytochrome c oxidase subunit I (*cox*1) genes as genetic markers. Phylogenies were reconstructed using maximum parsimony algorithm. A total of 6 haplotypes were found for *cox*1, 4 haplotypes for *nad*4 and 3 haplotypes for *nad*5. The sequenced ITS1 fragment was identical in all samples. The analyzed *cox*1 gene fragment is the most variable marker and is recommended for future analyses. No geographic association was found in the Argentinean samples.

1. Introduction

Fasciolosis is a worldwide distributed zoonotic disease caused by the trematode parasites *Fasciola hepatica* and *F. gigantica*. *F. hepatica* mainly occurs in Europe, Americas and Oceania, while *F. gigantica* is distributed in Africa and Asia (Torgerson and Claxton, 1999).

The World Health Organization considers fasciolosis as an emerging zoonosis, with estimations reaching from 2.4 million up to 17 million people infected worldwide (Rim et al., 1994; Mas-Coma et al., 1999, 2015). In animals, the infection occurs in cattle, sheep, horses, mules, camels, pigs, deer and goats (Ngategize et al., 1993; Behm and Sangster, 1999). The morbidity and mortality rates vary from one region to another.

In Argentina, a recent study analyzed reports describing 619 human cases, involving 13 provinces, mostly in high altitudes of the Andes (97.7%) concentrated in Córdoba, Catamarca, San Luis and Mendoza (Mera y Sierra et al., 2011). Animal fasciolosis has an extensive area, which stretches from the northern end of our territory to central Patagonian region and from the foothills to the Atlantic coast, being endemic in most regions of the country (Olaechea, 1994). The most important hosts are cattle and sheep but the parasite is also found in goats, horses, pigs, deer and American camelids.

Several studies have genetically characterized *Fasciola* spp. isolates from different geographic areas including countries from Europe (Spain, Italy, Russia, Belarus, Ukraine, Bulgaria, Armenia, Azerbaijan, Georgia, and Turkey), Africa (Egypt, Mauritania, Niger, Nigeria, Tunisia, Algeria, South Africa and Zimbabwe), America (Uruguay, Peru and USA), Asia (Turkmenistan, Japan, Vietnam, Iran, India, Nepal, Indonesia, Bangladesh, Korea and China), and Australia (Huang et al., 2004; Semyenova et al., 2006; Alasaad et al., 2007; Vara-Del Río et al., 2007; Le et al., 2008; Ali, 2008; Li et al., 2009; Nguyen et al., 2009; Peng et al., 2009; Farjallah et al., 2009, 2013; Amor et al., 2011a,b; Ichikawa and Itagaki, 2012; Moazeni et al., 2012; Shu et al., 2012; Elliott et al., 2014; Mohanta et al., 2014; Shoriki et al., 2014; Mucheka

* Corresponding author at: Av. Vélez Sarsfield 563, CP 1281 Ciudad de Buenos Aires, Argentina.
E-mail address: jorgeysilvana@speedy.com.ar (S. Carnevale).

http://dx.doi.org/10.1016/j.vetpar.2017.08.006
Received 29 July 2017; Received in revised form 7 August 2017; Accepted 8 August 2017

et al., 2015; Amer et al., 2016; Reaghi et al., 2016; Hayashi et al., 2015, 2016a,b; Ichikawa-Seki et al., 2016, 2017). Although there have been an increased number of reports showing genetic variation within and among *Fasciola* populations, these approaches have not been employed in Argentina. Our group described the first *F. hepatica* haplotype in Argentina (Carnevale et al., 2013). The goal of this work was to characterize for the first time *F. hepatica* isolates derived from different geographic locations in Argentina by sequence analysis of the ribosomal internal transcribed spacer 1 (ITS1), mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunits 4 and 5 (*nad*4 and *nad*5) and mitochondrial cytochrome c oxidase subunit I (*cox*1) genes as genetic markers.

2. Materials and methods

2.1. *Fasciola individuals*

Adult flukes of *F. hepatica* were collected from the bile ducts of 5 naturally infected cattle in different areas from Argentina and kept at −20 °C in 70% ethanol until DNA extraction. A total of 5 isolates were selected from each animal for analysis, in one case fewer numbers were used resulting in a total of 22 worms analyzed. *F. hepatica* used were from Ancasti, Catamarca (5 specimens), Tatón, Catamarca (5), Rosario de la Frontera, Salta (7), and Esquel, Chubut (5). The samples were labeled as the name of the parasite (Fh), followed by abbreviation of the collection site (city/province), number of the infected animal (numbered consecutively in each province) and capital letter for each fluke.

2.2. DNA extraction and amplification

The ethanol in each sample was allowed to evaporate for a minimum of 2 h before the genomic DNA was extracted from individual flukes by using a NucleoSpin DNA Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufactureŕs instructions. The analysis of purified DNA was performed by electrophoresis in ethidium bromide-stained 1% agarose gel; the intensity was compared to the standards of Low DNA Mass Ladder (Invitrogen) and quantified with the Quantity One Software (Bio-Rad). DNA fragments of each target region were amplified by PCR in a total volume of 50 µl. The PCR mixture consisted of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% (v/v) Nonidet P40, 100 µM (each) dNTP, 2.5 mM MgCl2, 0.1 µM of each primer and 1.0 U of *Taq* DNA polymerase (Fermentas International Inc.). The template used for each reaction corresponds to 1 ng of purified DNA from each fluke.

The ITS1 region (representing the complete sequence of ITS1 and partial sequence of both 18 S and 5.8 S rDNA regions) was amplified according to Itagaki et al. (2005a) with the primer set ITS1-F (5′-TTGCGCTGATTACGTCCCTG-3′) and ITS1-R (5′-TTGGCTGCGCTCTTCATCGAC-3′) in order to obtain a fragment of 639 bp. Reaction cycles consisted of an initial denaturing step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 90 s, 53 °C for 90 s and 72 °C for 120 s with final extension at 72 °C for 10 min.

In order to amplify a portion of 399 bp of the *cox*1 gene, the primer set JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) (Bowles et al., 1992) was employed. A 463-bp fragment of the *nad*4 gene was amplified using the primers ALF (5′-AGATGTCTATCCTTCCTT-3′) and ALR (5′-ACTACCACAATATGTGCC-3′) (Ai et al., 2011). For the nad5 gene, a portion of 347 bp was amplify with the primers nad5F (5′-GCTATGCGCGCTCCTACTCCTGTTA-3′) and nad5R (5′-CTAGAACCAGACTGCCTCATCAAAT-3′) (Ai et al., 2011). The PCR procedure for the mitochondrial markers consisted of 30 s of denaturation at 94 °C, followed by 30 s of annealing (55 °C for *cox*1, 50 °C for *nad*4, 60 °C for *nad*5) and 30 s of extension at 72 °C, for 30 cycles. An initial step of 5 min at 94 °C and a final step of 5 min at 72 °C were included.

5 µl of amplicons were run on ethidium bromide-stained 1.5% agarose gels and visualized under UV illumination. Amplicons were sequenced in both directions employing the same primers used in the PCR.

2.3. Sequencing and phylogenetic analysis

The PCR products were automatically sequenced by a commercial sequencing service (Macrogen, Korea). DNA sequences were analyzed using BioEdit and ClustalX softwares. Both strands were sequenced for every sample analyzed. Sequences from ITS1 and mitochondrial *nad4*, *nad5* and *cox1* genes from *F. hepatica*, *F. gigantica* and *Paragonimus westermani* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/). They were analyzed by MaGat software and redundant sequences were removed. The sequences obtained were used to assemble consensus sequences with CAP contig software and they were manually edited with BioEdit (v7.1.3). Molecular marker sequences were concatenated and multiple alignments were carried out with ClustalX software. Phylogenetic trees were constructed by Maximum Likelihood algorithm implemented in MEGA software v6 with 1000 bootstrap replicates and *P. westermani* as outgroup. A representative sequence of each haplotype was deposited in GenBank.

3. Results

3.1. DNA profile of Fasciola adults

DNA from each of 22 adult parasites was obtained from 3 region of Argentina, 10 from Catamarca, 7 from Salta and 5 from Chubut. Using *Fasciola*-derived primers, the DNA fragments of three mitochondrial gene regions (*cox*1, *nad*4 and *nad*5) and one partial nuclear gene (ITS1) were amplified from each sample.

For each DNA region, no size variation was detected on agarose gel. The length of nucleotide sequences determined were 307 bp in *cox*1, 365 bp in *nad*4, 252 in *nad*5 and 619 bp in ITS1.

As shown in Table 1, all the sequences of the adults were compared with the known sequences of *Fasciol*a spp. and grouped by identity. A

Table 1
Fasciola hepatica sequences obtained from Argentinean samples.

Isolate ID[a]	Molecular marker[b]				Haplotype ID
	*cox*1	*nad*4	*nad*5	ITS	
FhTCa1A	4	1	1	1	Fh_4_1_1
FhTCa1B	4	4	1	1	Fh_4_4_1
FhTCa1C	1	4	3	1	Fh_1_4_3
FhTCa1D	4	4	1	1	Fh_4_4_1
FhTCa1E	2	4	3	1	Fh_2_4_3
FhACa2A	1	4	1	1	Fh_1_4_1
FhACa2B	3	4	2	1	Fh_3_4_2
FhACa2C	4	2	1	1	Fh_4_2_1
FhACa2D	4	2	1	1	Fh_4_2_1
FhACa2E	6	4	1	1	Fh_6_4_1
FhRSa1A	4	4	1	1	Fh_4_4_1
FhRSa1B	5	4	1	1	Fh_5_4_1
FhRSa2A	4	3	1	1	Fh_4_3_1
FhRSa2B	1	4	3	1	Fh_1_4_3
FhRSa2C	1	4	3	1	Fh_1_4_3
FhRSa2D	4	4	1	1	Fh_4_4_1
FhRSa2E	4	3	1	1	Fh_4_3_1
FhECh1A	3	4	2	1	Fh_3_4_2
FhECh1B	4	4	1	1	Fh_4_4_1
FhECh1C	7	3	1	1	Fh_7_3_1
FhECh1D	3	4	2	1	Fh_3_4_2
FhECh1E	4	4	1	1	Fh_4_4_1

[a] The samples were labeled as the name of the parasite (Fh), followed by abbreviation of the collection site (city/province), number of the infected animal (numbered consecutively in each province) and capital letter for each fluke. TCa: Tatón, Catamarca; ACa: Ancasti, Catamarca; RSa: Rosario de la Frontera, Salta; ECh: Esquel, Chubut.

[b] Numbers indicate different haplotype sequence.

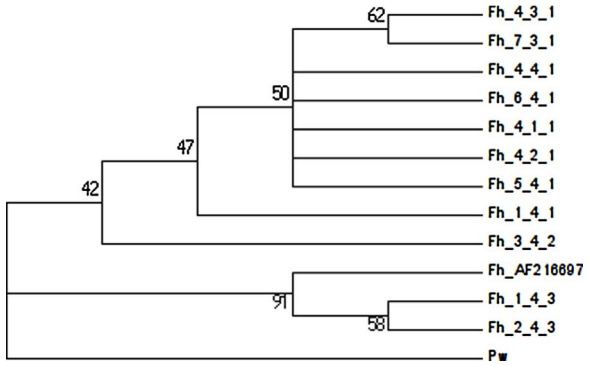

Fig. 1. Phylogenetic relationships of *F. hepatica* isolates from Argentina using the mtDNA sequences (*cox*1, *nad*4 and *nad*5) from the *Fasciola* adults conducted with Maximum Likelihood algorithm implemented in MEGA software v6. Bootstrap values (in percentage) above 40% from 1000 replicates are shown. *Paragonimus westermani* (Pw) was used as outgroup.

total of 7 haplotypes were found for *cox*1, 4 haplotypes for *nad*4 and 3 haplotypes for *nad*5. The overall intraspecific nucleotide variations were 98.90% for *cox*1, 98.97% for *nad*4, and 98.40% for *nad*5 (supplementary tables). For the *cox*1, polymorphic sites were mainly at the first and third codon positions, and no substitutions were found in the second positions. Also, all nucleotide substitution were synonymous, generating the same aminoacid sequence. Haplotype Fh-cox1-7, corresponding to a specimen from Chubut province, presented a polymorphism that had not been previously reported at the GenBank.

For *nad*4, polymorphic sites were also mainly at the first and third codon positions, and 2 non-synonymous substitution were found, one each in haplotype 1 and haplotype 4. The resulting aa changes were M/I^{84} and L/V^{99}, respectively, among the 121 NAD 4 aa analyzed. Neither one was described before (are absent in Genbank entries).

For *nad*5, there were changes in the second and third codon positions showing one non synonymous substitution in haplotype 3 (F/C^{12}) among the 82 NAD 5 aa analyzed. Interestingly, the same substitution was found in a *F. hepatica* isolate from sheep and cattle from Niger (Ai et al., 2011; GU121093 and GU121093 GenBank accession numbers). The ITS1 fragment sequenced was identical in all samples.

3.2. Phylogenetic analyses

Using the mtDNA sequences (*cox*1, *nad*4 and *nad*5) from the *Fasciola* adults we conducted Maximum Likelihood analyses. The bootstrapped phylogenetic tree obtained is shown in Fig. 1. Samples with haplotypes Fh_1_4_3 and Fh_2_4_3 grouped together with *F. hepatica* previously described (GenBank accession number AF216697) while samples with other haplotypes were found in different nodes. No geographic association was found (Fig. 2). Similar phylogenetic tree topology was obtained when Neighbor-Joining method was implemented (Supplementary Fig. S1).

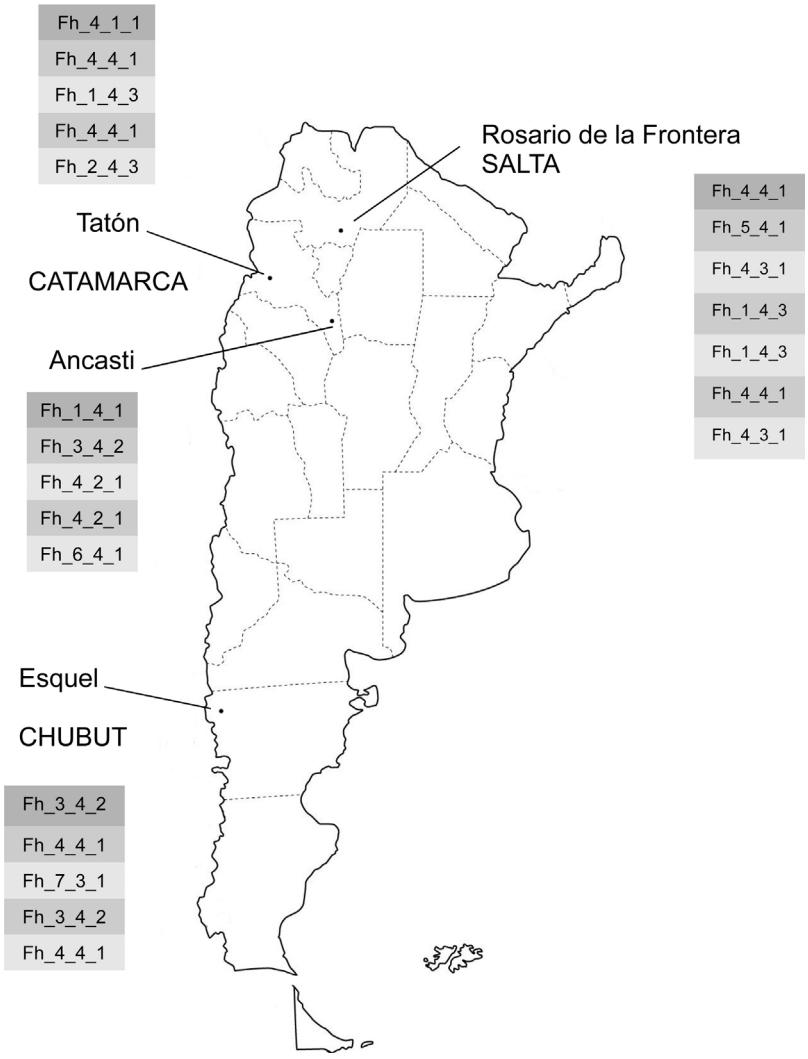

Fig. 2. Geographic distribution of *F. hepatica* isolates in Argentina. *F. hepatica* used were from Ancasti and Tatón, Catamarca province, Rosario de la Frontera, Salta province, and Esquel, Chubut province.

4. Discussion

This is the first report that characterizes adult specimens of *F. hepatica* infecting cattle from different regions of Argentina on the bases of sequences of ITS1 and mitochondrial DNA regions. These sequences have shown to be reliable genetic markers for species-level identification and genetic differentiation of *Fasciola* (Itagaki et al., 2005a,b, 2009; Semyenova et al., 2006; Nguyen et al., 2009; Mas-Coma et al., 2009; Amer et al., 2011; Mohanta et al., 2014).

The sequence of the ITS1 region in Argentinean flukes consisted of 619 bp without variable nucleotide positions and confirmed that all the individuals analyzed belonged to the single haplotype of *F. hepatica* (FhITS1) which is in accordance to Ichikawa-Seki et al. (2016) confirming the absence of hybrids in the region of South America. A similarity of 100% was previously shown between isolates of *F. hepatica* from different regions and hosts (Ali et al., 2008; Farjallah et al., 2009; , 2013; Peng, 2009; Rokni et al., 2010; Shalaby et al., 2013).

Regarding the mitochondrial sequences as useful markers, Walker et al. (2007) showed that genetic diversity exists among and within *F. hepatica* populations from cattle and sheep. Semyenova et al. (2006) found genetic variation in eastern European and western Asian populations of *F. hepatica* and revealed the existence of two well-defined lineages with two main haplotypes and a number of shared divergent haplotypes among the examined *F. hepatica* populations.

The absence of polymorphic sites at the second codon positions for *cox*1 observed in our samples is in concordance with previous results analyzing specimens from Eastern Europe, western Asia and Africa (Semyenova et al., 2006; Ai et al., 2011). Changes at any of the three codon positions for *nad*4 and *nad*5 detected in our samples had been previously reported (Ai et al., 2011).

Ai et al. (2011) found that among the three mtDNAgenes, *cox*1, *nad*4 and *nad*5, sequence variation in *nad*5 was higher compared to those of *cox*1 and *nad*4, and *nad*4 sequences exhibited more variability than the *cox*1 when analyzing samples of *F. hepatica, F. gigantica* and the intermediate form. For our samples, the *cox*1 gene fragment analyzed was the most variable marker and should be considered for future analysis.

The phylogenetic trees showed that the distribution of haplotypes revealed small genetic geographical variation but did not show significant geographical association, in concordance with previous results that analyzed samples of *F. hepatica* from different countries (Ai et al., 2011). Further studies using more *F. hepatica* samples from different host species should be analyzed in order to determine the presence or not of a significant genetic variability in *F.* hepatica.

The phylogenetic analysis based on partial sequences of the mitochondrial genes *cox*1, *nad*4 and *nad*5 and the nuclear gene ITS1 from the Argentinean samples confirm for the first time by molecular methods that all the specimens belong to the species *F. hepatica*. Moreover, this is the first description of the different haplotypes circulating in Argentina, including that first reported in the world. The genetic characterization of *F. hepatica* present in different regions from Argentina will be a useful tool to understand the disease in heterogeneous epidemiological situations.

Acknowledgments

We thank to Dr. Marcela Larroza from INTA Bariloche and Dr. Olga Edith Feres Escándar from Rosario de la Frontera, for kindly providing adult flukes from Chubut and Salta, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.08.006.

References

Ai, L., Weng, Y.B., Elsheikha, H.M., Zhao, G.H., Alasaad, S., Chen, J.X., Li, J., Li, H.L., Wang, C.R., Chen, M.X., Lin, R.Q., Zhu, X.Q., 2011. Genetic diversity and relatedness of *Fasciola* spp. isolates from different hosts and geographic regions revealed by analysis of mitochondrial DNA sequences. Vet. Parasitol. 181, 329–334.

Alasaad, S., Huang, C.Q., Li, Q.Y., Granados, J.E., García-Romero, C., Pérez, J.M., Zhu, X.Q., 2007. Characterization of *Fasciola* samples from different host species and geographical localities in Spain by sequences of internal transcribed spacers of rDNA. Parasitol. Res. 101, 1245–1250.

Ali, H., Ai, L., Song, H.Q., Ali, S., Lin, R.Q., Seyni, B., Issa, G., Zhu, X.Q., 2008. Genetic characterization of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. Parasitol. Res. 102, 1021–1024.

Amer, S., Dar, Y., Ichikawa, M., Fukuda, Y., Tada, C., Itagaki, T., Nakai, Y., 2011. Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS1 and ITS2) and mitochondrial (NDI and COI) gene markers. Parasitol. Int. 60, 5–12.

Amer, S., ElKhatam, A., Zidan, S., Feng, Y., Xiao, L., 2016. Identity of *Fasciola* spp. in sheep in Egypt. Parasit. Vectors 9, 623.

Amor, N., Farjallah, S., Salem, M., Lamine, D.M., Merella, P., Said, K., Ben Slimane, B., 2011a. Molecular characterization of *Fasciola gigantica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. Exp. Parasitol. 129, 127–136.

Amor, N., Halajian, A., Farjallah, S., Merella, P., Said, K., Ben Slimane, B., 2011b. Molecular characterization of *Fasciola* spp. from the endemic area of northern Iran based on nuclear ribosomal DNA sequences. Exp. Parasitol. 128, 196–204.

Behm, C.A., Sangster, N.C., 1999. Pathology, pathophysiology and clinical aspects. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, Walling Ford, UK, pp. 185–224.

Bowles, J., Blair, D., McManus, D.P., 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54, 165–173.

Carnevale, S., Cabrera, M.G., Cucher, M.A., di Risio, C.A., Malandrini, J.B., Kamenetzky, L., Alazraqui, M., Etchart, C.B., Pantano, M.L., Velásquez, J.N., 2013. Direct, immunological and molecular techniques for a fasciolosis survey in a rural area of San Luis, Argentina. J. Parasit. Dis. 37, 251–259.

Elliott, T., Muller, A., Brockwell, Y., Murphy, N., Grillo, V., Toet, H.M., Anderson, G., Sangster, N., Spithill, T.W., 2014. Evidence for high genetic diversity of NAD1 and COX1 mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. Vet. Parasitol. 200, 90–96.

Farjallah, S., Sanna, D., Amor, N., Ben Mehel, B., Piras, M.C., Merella, P., Casu, M., Curini-Galletti, M., Said, K., Garippa, G., 2009. Genetic characterization of *Fasciola hepatica* from Tunisia and Algeria based on mitochondrial and nuclear DNA sequences. Parasitol. Res. 105, 1617–1621.

Farjallah, S., Ben Slimane, B., Piras, C.M., Amor, N., Garippa, G., Merella, P., 2013. Molecular characterization of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers. Exp. Parasitol. 135, 471–478.

Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Singh, T.S., Shoriki, T., Sugiyama, H., Itagaki, T., 2015. Molecular phylogenetic analysis of *Fasciola* flukes from eastern India. Parasitol. Int. 64, 334–338.

Hayashi, K., Ichikawa-Seki, M., Allamanda, P., Wibowo, P.E., Mohanta, U.K., Sodirun Guswanto, A., Nishikawa, Y., 2016a. Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from western Java Indonesia. Parasitol. Int. 65, 424–427.

Hayashi, K., Mohanta, U.K., Neeraja, T., Itagaki, T., 2016b. Molecular characterization of *Fasciola gigantica* in Delhi, India and its phylogenetic relation to the species from South Asian countries. J. Vet. Med. Sci. 78, 1529–1532.

Ichikawa, M., 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.

Ichikawa-Seki, M., Ortiz, P., Cabrera, M., Hobán, C., Itagaki, T., 2016. Molecular characterization and phylogenetic analysis of *Fasciola hepatica* from Peru. Parasitol. Int. 65, 171–174.

Ichikawa-Seki, M., Tokashiki, M., Opara, M.N., Iroh, G., Hayashi, K., Kumar, U.M., Itagaki, T., 2017. Molecular characterization and phylogenetic analysis of *Fasciola gigantica* from Nigeria. Parasitol. Int. 66, 893–897.

Itagaki, T., Kikawa, M., Sakaguchi, K., Shimo, J., Terasaki, K., Shibahara, T., Fukuda, K., 2005a. Genetic characterization of parthenogenetic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. Parasitology 131, 679–685.

Itagaki, T., Kikawa, M., Terasaki, K., Shibahara, T., Fukuda, K., 2005b. 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.

Itagaki, T., Sakaguchi, K., Terasaki, K., Sasaki, O., Yoshihara, S., 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.

Le, T.H., De, N.V., Agatsuma, T., Thi Nguyen, T.G., Nguyen, Q.D., McManus, D.P., Blair, D., 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. Int. J. Parasitol. 38, 725–730.

Li, Q.Y., Dong, S.J., Zhang, W.Y., Lin, R.Q., Wang, C.R., Qian, D.X., Lun, Z.R., Song, H.Q., Zhu, X.Q., 2009. Sequence-related amplified polymorphism, an effective molecular approach for studying genetic variation in *Fasciola* spp. of human and animal health significance. Electrophoresis 30, 403–409.

Mas-Coma, S., Esteban, J.G., Bargues, M.D., 1999. Epidemiology of human fascioliasis: a review and proposed new classification. Bull. WHO 77, 340–346.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis with a global overview on disease transmission, epidemiology, evolutionary

genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2015. *Fasciola* and *Fascioplopsis*. In: Xiao, L., Ryan, U., Feng, Y. (Eds.), Biology of Foodborne Parasites. CRC Press, Boca Raton, pp. 111–130.
Mera y Sierra, R., Agramunt, V.H., Cuervo, P., Mas-Coma, S., 2011. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. Parasit. Vectors 4, 104. http://dx.doi.org/10.1186/1756-3305-4-104.
Moazeni, M., Sharifiyazdi, H., Izadpanah, A., 2012. Characterization of *Fasciola hepatica* genotypes from cattle and sheep in Iran using cytochrome C oxidase gene (CO1). Parasitol. Res. 110, 2379–2384.
Mohanta, U.K., Ichikawa-Seki, M., Shoriki, T., Katakura, K., 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.
Mucheka, V.T., Lamb, J.M., Pfukenyi, D.M., Mukaratirwa, S., 2015. DNA sequence analyses reveal co-occurrence of novel haplotypes of *Fasciola gigantica* with *F. hepatica* in South Africa and Zimbabwe. Vet. Parasitol. 214, 144–151.
Ngategize, P.K., Bekele, T., Tilahun, G., 1993. Financial losses caused by ovine fasciolosis in the Ethiopian highlands. Trop. Anim. Health. Prod. 25, 155–161.
Nguyen, T.G., Van De, N., Vercruysse, J., Dorny, P., Le, T.H., 2009. Genotypic characterization and species identification of *Fasciola* spp. with implications regarding the isolates infecting goats in Vietnam. Exp. Parasitol. 123, 354–361.
Olaechea, F.V., 1994. Epidemiologia y control de *Fasciola hepatica* en la Argentina. In: Nari, A., Fiel, C. (Eds.), Enfermedades Parasitarias De Importancia Económica En Bovinos. Editorial Agropecuaria, Hemisferio Sur SRL, Montevideo, Uruguay, pp. 213–232.
Peng, M., Ichinomiya, M., Ohtori, M., Ichikawa, M., Shibahara, T., Itagaki, T., 2009. Molecular characterization of *Fasciola hepatica*, *Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. Parasitol. Res. 105, 809–815.
Reaghi, S., Haghighi, A., Harandi, M.F., Spotin, A., Arzamani, K., Rouhani, S., 2016. Molecular characterization of *Fasciola hepatica* and phylogenetic analysis based on mitochondrial (nicotiamide adenine dinucleotide dehydrogenase subunit I and cytochrome oxidase subunit I) genes from the North-East of Iran. Vet. World 9, 1034–1038.
Rim, H.J., Farag, H.F., Sornmani, S., Cross, J.H., 1994. Food-borne trematodes: ignored or emerging? Parasitol. Today 10, 207–209.
Rokni, M.B., Mirhendi, H., Mizani, A., Mohebali, M., Sharbatkhori, M., Kia, E.B., Abdoli, H., Izadi, S., 2010. Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. Exp. Parasitol. 124, 209–213.
Semyenova, S.K., Morozova, E.V., Chrisanfova, G.G., Gorokhov, V.V., Arkhipov, I.A., Moskvin, A.S., Movsessyan, S.O., Ryskov, A.P., 2006. Genetic differentiation in eastern European and western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial nad1 and cox1 genes. J. Parasitol. 92, 525–530.
Shalaby, I., Gherbawy, Y., Banaja, A., 2013. Molecular characterization of *Fasciola* species isolated from imported sheep in Taif region (Saudi Arabia). Trop. Biomed. 30, 15–26.
Shoriki, T., Ichikawa-Seki, M., Devkota, B., Rana, H.B., Devkota, S.P., Humagain, S.K., Itagaki, T., 2014. Molecular phylogenetic identification of *Fasciola* flukes in Nepal. Parasitol. Int. 63, 758–762.
Shu, F.F., Lv, R.Q., Zhang, Y.F., Duan, G., Wu, D.Y., Li, B.F., Yang, J.F., Zou, F.C., 2012. Characterization of *Fasciola* samples by ITS of rDNA sequences revealed the existence of *Fasciola hepatica* and *Fasciola gigantica* in Yunnan Province, China. J. Parasitol. 98, 889–890.
Torgerson, P., Claxton, J., 1999. Epidemiology and control. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, Walling Ford, UK, pp. 113–149.
Vara-Del Río, M.P., Villa, H., Martinez-Valladares, M., Rojo-Vázquez, F.A., 2007. Genetic heterogeneity of *Fasciola hepatica* isolates in the northwest of Spain. Parasitol. Res. 101, 1003–1006.
Walker, S.M., Prodohl, P.A., Fletcher, H.L., Hanna, R.E., Kantzoura, V., Hoey, E.M., Trudgett, A., 2007. Evidence for multiple mitochondrial lineages of *Fasciola hepatica* (liver fluke) within infrapopulations from cattle and sheep. Parasitol. Res. 101, 117–125.