Veterinary Parasitology 268 (2019) 21–31

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Fasciola spp. in Armenia: Genetic diversity in a global context

Sargis Aghayan[a,1], Hasmik Gevorgian[b,c,1], Dennis Ebi[d], Hripsime A. Atoyan[a], Francis Addy[e], Ute Mackenstedt[d], Thomas Romig[d], Marion Wassermann[d,*]

[a] Chair of Zoology, Yerevan State University, Yerevan, Armenia
[b] Scientific Center of Zoology and Hydroecology, NAS RA, Yerevan, Armenia
[c] National Institute of Health, Moh Ra, Yerevan, Armenia
[d] Department of Parasitology, University of Hohenheim, Stuttgart, Germany
[e] Department of Biotechnology, University for Development Studies, Faculty of Agriculture, Tamale, Ghana

ARTICLE INFO

Keywords:
Fasciola hepatica
Fasciola gigantica
Fasciolosis
Prevalence
Genetic diversity
Armenia

ABSTRACT

Fasciolosis, a food- and waterborne infection caused by the trematodes *Fasciola hepatica* and *F. gigantica*, is recognized by WHO as a neglected zoonotic disease. Whereas *F. hepatica* is distributed worldwide in cooler climates, *F. gigantica* occurs mainly in the tropics of Africa and Asia. The southern Caucasus, with Armenia, is one of the most northern regions where both species occur and may produce hybrids. In this study, livestock in central Armenia was surveyed for fasciolosis, the causative species were determined and the genetic diversity of both species was estimated. Total prevalence in sheep (1794), cattle (324) and goats (9) was 21.2%, 15.7% and 44.4%, respectively. After morphological identification and sequencing of a mitochondrial (nad1) and a nuclear marker gene (28S rRNA), 62 collected specimens were allocated to *F. hepatica* (n = 55) and *F. gigantica* (n = 7). Intraspecific diversity was evaluated for the complete nad1 gene, resulting in 29 haplotypes of *F. hepatica* and six haplotypes of *F. gigantica*. Diversity was higher among *F. gigantica* than *F. hepatica* in the Armenian sample set, a difference that was confirmed analyzing available sequences for both species worldwide. Maximum genetic distance between haplotypes in global networks was 49 nucleotide steps for *F. gigantica* compared to 15 for *F. hepatica*. In the available sample sets, *F. hepatica* showed higher diversity in western Asia and the Middle East compared to Europe and eastern Asia, while for *F. gigantica* loosely structured clusters comprising mainly western/southern Asian and African haplotypes could be identified. A distinct clade comprising haplotypes from Zambia was basal in the phylogenetic tree. Biogeographical implications of these data are discussed.

1. Introduction

Human fasciolosis, a food- and waterborne infection caused by the trematodes *Fasciola* spp., is recognized as a neglected zoonotic disease by the World Health Organization (2007). The genus contains two species infecting livestock and humans: *Fasciola hepatica* is generally distributed in temperate regions worldwide (including cooler parts of the tropics), *F. gigantica* is mainly present in tropical and subtropical regions of Africa and Asia (World Health Organization, 2007). In some regions (e.g. the Mediterranean and western Asia), the ranges of both species overlap, and hybrid populations are known to occur (Agatsuma et al., 2000; Mas-Coma et al., 2009; Peng et al., 2009). Both species have similar two-host life cycles that involve ruminants and a range of other mammals (including humans) as definitive hosts, and lymnaeid snails as intermediate hosts. A third species, *F. nyanzae*, a parasite of hippopotamus, is not known to be zoonotic (Dinnik and Dinnik, 1961). The same applies to *F. jacksoni* of the Asian elephant, whose taxonomic placement in the genus *Fasciola* has been challenged (Caple et al., 1978; Heneberg, 2013).

While the public health impact of human fasciolosis has only been acknowledged recently (Ashrafi et al., 2014; Mas-Coma et al., 2014, 2018), both *F. hepatica* and *F. gigantica* are well-known causes of morbidity and mortality in ruminant livestock. The annual economic loss in animal production was estimated to exceed 3.2 billion US$ worldwide (Mehmood et al., 2017). There is an apparent need for control strategies to manage these trematodes in endemic regions, but inadequate knowledge of the disease situation and varying transmission dynamics in the different regions hinder control progress. Precise determination of the species and their intraspecific variability as well as epidemiological information from the various endemic regions are required to

* Corresponding author.
E-mail addresses: marion.wassermann@email.de, marion.wassermann@uni-hohenheim.de (M. Wassermann).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.vetpar.2019.02.009
Received 18 October 2018; Received in revised form 11 February 2019; Accepted 23 February 2019

assess infection risks and design effective management strategies, because ecological requirements of the two species differ. *F. gigantica* is adapted to truly aquatic snail hosts (e.g. *Radix auricularia* in Eurasia, *R. natalensis* in Africa) and is therefore bound to the presence of permanent water bodies. In contrast, *F. hepatica* uses the amphibious species *Galba truncatula* (in addition to a range of aquatic lymnaeids), whose ability to persist in seasonal wetlands by aestivating in dry mud drastically enlarges the range of habitats suitable for transmission of this parasite. The requirement for different temperatures during larval development may also separate transmission localities of the two species even in regions of range overlap (Mas-Coma et al., 2009).

The present study was conducted in Armenia, which is situated in one of the areas where both *F. hepatica* and *F. gigantica* have been recorded from livestock (Mas-Coma et al., 2009), but where very few data are available on frequency and genetic identity of these parasites (Semyenova et al., 2006, 2005). Armenia, like neighbouring areas in western Asia, is a region of particular interest for the study of livestock parasites, because it is close to the 'fertile crescent', where domestication of most livestock species had occurred 11,000 to 10,000 years ago (Zeder, 2008), and where eggs of *F. hepatica* were recently identified in approximately 1500 years old paleofaeces (Askari et al., 2018). If this is true, then a high proportion of the ancestral genetic polymorphism of the original wildlife-transmitted parasites should have been preserved in the secondary livestock cycles which dominate today.

Objectives of the study were (1) to estimate prevalence of fasciolosis in different species of livestock from different regions of Armenia in order to establish baseline data on the disease burden, (2) to characterize the causative parasites and (3) to evaluate the diversity of the two species in the context of the global genetic structure, based on mitochondrial and nuclear marker genes.

2. Materials and methods

2.1. Detection, sampling and morphological identification of Fasciola spp.

The presence of *Fasciola* spp. was investigated in six regions of central Armenia (Table 1). Livers from 2127 animals (1794 sheep, 324 cattle and 9 goats) were obtained from slaughterhouses and local markets, and dissected for the presence of *Fasciola* flukes. For all animals from slaughterhouses, the province of origin is known, while samples obtained at local markets around Yerevan may have originated from either Kotayk, Ararat or Gegharkunik.

For molecular analyses, a total of 73 flukes were collected from 65 animals (50 sheep, 11 cattle and 4 goats) in three of the regions (Kotayk, Ararat and Gegharkunik). Flukes were washed in physiological saline and subsequently stored in 70% ethanol at room temperature.

All flukes were examined for morphological characteristics. A tentative allocation to species was done using body shape and ration length: width. As these simple criteria do not allow reliable separation (Periago et al., 2006), the species allocation was confirmed by molecular diagnosis.

2.2. DNA isolation

To obtain DNA, a tissue piece of approximately 0.5 mm^3 was excised from each fluke and lysed in 30 µl of 0.02 M NaOH for 20 min at 99 °C. The lysates were centrifuged and the supernatant was used as DNA template source for the following PCRs (Addy et al., 2017).

2.3. Amplification and sequencing of marker genes

2.3.1. Nuclear 28S ribosomal RNA (rRNA) gene

The first approach to discriminate the *Fasciola* species was an RFLP-PCR of a partial *28S rRNA* gene fragment with the restriction enzyme AvaII according to Marcilla et al. (2002). Due to ambiguous banding patterns (data not shown) all fragments (approx. 520 bp) were sequenced.

2.3.2. Mitochondrial NADH dehydrogenase subunit 1 gene

The second investigated gene, the mitochondrial NADH dehydrogenase subunit 1 (*nad1*), was chosen for the high number of available GenBank entries from different geographic locations and in sufficient fragment lengths. For the amplification of the complete *nad1* gene (903 bp) a nested PCR was performed using newly designed primers. The primer pair for the first PCR was, forward 5′-TTT AAT TTA AGA TGT GTG CTC TGC-3′ and reverse 5′-ATA TCA CAG TAA CCT GCT AAC GC-3′ and for the nested PCR was, forward 5′-CGA GCG TTC GGT GGA GG-3′ and reverse 5′-GAC CTC TAA CCC CCA AAG CTA G-3′. All PCRs were performed in 50 µl reaction mixtures containing 10 mM Tris − HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs, 1.25 U Ampli-Taq Polymerase (Applied Biosystems) and 2 µl crude lysate or 1 µl of first PCR product. The conditions during amplification of all PCRs were as follows: initial denaturation step at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 60 s, followed by a final elongation step at 72 °C for 5 min. PCR amplicons were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim-Germany) and sequenced (GATC Biotech AG, Konstanz-Germany).

Nucleotide sequence data reported in this paper are available in GenBank™/DDBJ/EMBL databases under accession nos. **MG972375-MG972409**.

2.4. Sequence analyses, phylogenetic trees and haplotype networks

DNA sequences were analyzed and edited using GENtle software v. 1.9 (Manske M., 2003, University of Cologne, Germany). The sequences of the complete nad1 gene were compared with the reference sequence for *F. hepatica* (**AF216697**) and *F. gigantica* (**NC024025**), respectively (Le et al., 2000; Liu et al., 2014), which differ at 76 nucleotide positions. The readable length of the *28S rRNA* gene was approximately 520 bp and included 4 nucleotide sites that had been described as being suitable for the discrimination of *F. hepatica* from *F. gigantica* (Marcilla et al., 2002). Reference sequences were **AJ440788** (*F. hepatica*) and

Table 1
Prevalence estimates of fascioliasis in sheep, cattle and goats of central Armenia.

Region (altitude m a.s.l.*)	Sheep			Cattle			Goat		
	n	n infected	%	n	n infected	%	n	n infected	%
Aragatsotn (900–1500 m a.s.l.)	226	21	9.3	40	3	7.5	–	–	–
Armavir (800–1000 m a.s.l.)	348	65	18.7	55	7	12.7	–	–	–
Ararat (800–1200 m a.s.l.)	408	152	37.3	84	19	22.6	–	–	–
Gegharkunik (1900–2200 m a.s.l.)	242	43	17.8	49	7	14.3	–	–	–
Kotayk (1200–2000 m a.s.l.)	381	83	21.8	72	13	18.1	9	4	44.4
Vayots Dzor (1000–1500 m a.s.l.)	189	17	9.0	24	2	8.3	–	–	–
Total	**1794**	**381**	**21.2**	**324**	**51**	**15.7**	**9**	**4**	**44.4**

* m a.s.l. = meter above sea level.

Table 2
Accession Numbers of sequences used for phylogenetic and haplotype analyses.

Fasciola hepatica				*Fasciola gigantica*			
Country	No.	Acc. No	Reference	Country	No.	Acc. No	Reference
China	−1	**AB477362**	Peng et al. (2009)	Myanmar	−1	**AB604007**	Hayashi et al. (2018)
	−2	**AB477361**	"		−2	**AB604008**	"
	−3	**AB477360**	"		−3	**AB604009**	"
	−4	**AB477359**	"		−4	**AB604010**	"
	−5	**AB477358**	"		−5	**AB604011**	"
	−6	**AB477357**	"		−6	**AB604012**	"
	−7	**AB604926**	Ichikawa et al., unpublished		−7	**AB604013**	"
	−8	**AB604927**	"		−8	**AB604014**	"
	−9	**AB604929**	"		−9	**AB604015**	"
	−10	**AB604930**	"		−10	**AB604016**	"
Egypt	−1	**AB554192**	Amer et al. (2011)	India	−1	**LC128314**	Hayashi et al., unpublished
	−2	**AB554191**	"		−2	**LC128315**	"
	−3	**AB554190**	"		−3	**LC128316**	"
	−4	**AB554189**	"		−4	**LC128317**	"
	−5	**AB554188**	"		−5	**LC128318**	"
	−6	**AB554187**	"		−6	**LC128320**	"
	−7	**AB554186**	"		−7	**LC128321**	"
	−8	**AB554185**	"		−8	**LC128322**	"
	−9	**AB554184**	"		−9	**LC128323**	"
	−10	**AB554182**	"		−10	**LC128319**	"
Iran	−1	**MF628264**	Maozeni et al., unpublished	Iran	−1	**KX063830**	Raeghi et al., unpublished
	−2	**MF628266**	"		−2	**KF992227**	Shafiei et al., unpublished
	−3	**MF628263**	"		−3	**KX712320**	Raeghi et al., unpublished
	−4	**MF628262**	"		−4	**KX036358**	"
	−5	**GQ175364**	Sharifi et al., unpublished		−5	**KX036357**	"
	−6	**GQ356033**	Maozeni et al., unpublished		−6	**KX036356**	"
	−7	**KF992222**	Shafiei et al., unpublished		−7	**KX036355**	"
	−8	**KF992226**	"		−8	**KX021289**	"
	−9	**KX021281**	Raeghi et al., unpublished		−9	**KX021288**	"
	−10	**KX021280**	"	Egypt	−1	**AB554176**	Amer et al. (2011)
Italy	−1	**JF824680**	Farjallah et al. (2013)		−2	**AB554174**	"
	−2	**JF824679**	"		−3	**AB554173**	"
	−3	**JF824678**	"		−4	**AB554172**	"
	−4	**JF824677**	"		−5	**AB554171**	"
	−5	**JF824676**	"		−6	**AB554169**	"
Poland	−1	**KR422395**	Norbury et al., unpublished		−7	**AB554167**	"
	−2	**KR422393**	"		−8	**AB554166**	"
	−3	**KR422392**	"		−9	**AB554164**	"
	−4	**KR422390**	"		−10	**AB554159**	"
	−5	**KR422397**	"	Ghana	−1	**MF490242**	Addy et al. (2017)
Reference (Australia)	−1	**AF216697**	Le et al. (2000)		−2	**MF490243**	"
					−3	**MF490244**	"
					−4	**MF490245**	"
					−5	**MF490247**	"
				Nigeria	−1	**LC142771**	Ichikawa-Seki et al. (2017)
					−2	**LC142770**	"
					−3	**LC142769**	"
					−4	**LC142768**	"
					−5	**LC142767**	"
					−6	**LC142766**	"
					−7	**LC142765**	"
					−8	**LC142764**	"
					−9	**LC142763**	"
					−10	**LC142762**	"
				Zambia	−1	**AB207165**	Itagaki et al. (2005)
					−2	**AB207167**	"
					−3	**AB207166**	"
					−4	**AB207164**	"
					−5	**AB207163**	"
					−6	**AB983833**	Ichikawa-Seki et al., unpublished
					−7	**AB983837**	"
					−8	**AB983836**	"
					−9	**AB983832**	"
					−10	**AB983834**	"
				Reference (China)	−1	**NC024025**	Liu et al. (2014)
					−2	**KF543342**	"

AJ439739 (*F. gigantica*) (Marcilla et al., 2002).

Intraspecific genetic variations (haplotypes) were analyzed for the *nad1* gene using TCS software v.1.8 (Clement et al., 2000). The statistical parsimony network was drawn with the same software with a fixed connection limit at 125 steps including the reference sequences for *F. hepatica*, *F. gigantica* and *F. jacksoni* (**KX787886**, Le et al.,

Table 3
Species identification of the *Fasciola* fluke samples.

Sample	Region[#]	Host species	Species identification								
			morphological[●]	molecular[●]							
				28S rRNA gene					*nad1* gene		
				91[⊗]	116[⊗]	269[⊗]	533[⊗]	*F.hep/F.gig**	species	haplotype	AccNos
F. hepatica according to *nad1* analysis											
1	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	**MG972375**
2	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
3	A	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh01	"
4	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh01	"
5	A	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh01	"
6	K	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh01	"
7	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
8	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
9	K	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh01	"
10-1	G	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh01	"
10-2	G	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh01	"
11	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
12-1	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
13	G	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
14	K	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh02	**MG972376**
15	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh02	"
16	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh02	"
17	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh03	**MG972377**
18	G	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh03	"
19	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh03	"
20-1	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh03	"
21	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh04	**MG972378**
22	G	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh04	"
23	G	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh05	**MG972379**
24	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh06	**MG972380**
25	A	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh07	**MG972381**
26	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh08	**MG972382**
27	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh09	**MG972383**
28	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh10	**MG972384**
29	A	sheep	*F.gig*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh11	**MG972385**
30	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh12	**MG972386**
31	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh13	**MG972387**
32	G	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh14	**MG972388**
33	G	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh15	**MG972389**
34	G	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh16	**MG972390**
12-2	A	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh17	**MG972391**
35	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh18	**MG972392**
36	K	sheep	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh19	**MG972393**
37	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh20	**MG972394**
38	K	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh21	**MG972395**
20-2	A	sheep	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh22	**MG972396**
39-1	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh23	**MG972397**
39-2	K	sheep	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh24	**MG972398**
40	K	goat	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	**MG972375**
41	K	goat	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
42	K	goat	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh01	"
43	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
44	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh01	"
45-1	U	cattle	*F. hep*	*F. gig*	*F. hep*	*F. hep*	*F. hep*	3/1	***F. hep***	Fh02	**MG972376**
46	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh02	"
47	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh25	**MG972399**
48-1	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh26	**MG972400**
48-2	U	cattle	*F.gig*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh27	**MG972401**
49	U	cattle	*F. hep*	*F. hep*	*F. hep*	*F. hep*	*F. hep*	4/0	***F. hep***	Fh28	**MG972402**
50	U	cattle	*F. hep*	DP	*F. hep*	*F. hep*	*F. hep*	3/1*	***F. hep***	Fh29	**MG972403**
F. gigantica according to *nad1* analysis											
51	K	sheep	*F. hep*	*F. gig*	*F. gig*	DP	*F. gig*	1/3*	***F.gig***	Fg01	**MG972404**
52	A	sheep	*F.gig*	*F. gig*	*F. gig*	DP	*F. gig*	1/3*	***F.gig***	Fg02	**MG972405**
53	K	sheep	*F. hep*	*F. gig*	*F. gig*	DP	*F. gig*	1/3*	***F.gig***	Fg03	**MG972406**
54-1	K	sheep	*F.gig*	*F. gig*	*F. gig*	*F. hep*	*F. gig*	1/3	***F.gig***	Fg04	**MG972407**
54-2	K	sheep	*F.gig*	*F. gig*	*F. gig*	*F. hep*	*F. gig*	1/3	***F.gig***	Fg05	**MG972408**
55	U	cattle	*F.gig*	*F. gig*	*F. gig*	DP	*F. gig*	1/3*	***F.gig***	Fg01	**MG972404**
45-2	U	cattle	*F.gig*	*F. gig*	*F. gig*	DP	*F. gig*	1/3*	***F.gig***	Fg06	**MG972409**

[#]Geographic origin of the samples; A = Ararat, K = Kotayk, G = Gegharkunik, U = unknown, but either A, K or G.
[●]*F. hep = F. hepatica*; *F. gig = F. gigantica.*
[⊗]Position of the four nucleotide exchanges between *F. hepatica* and *F. gigantica* starting with the first nucleotide of AccNo. **AJ440788** (Marcilla et al., 2002).
*Indicates sequences where at one position a doublepeak (DP) either referring to *F. hepatica* or *F. gigantica*, respectively, was detected.

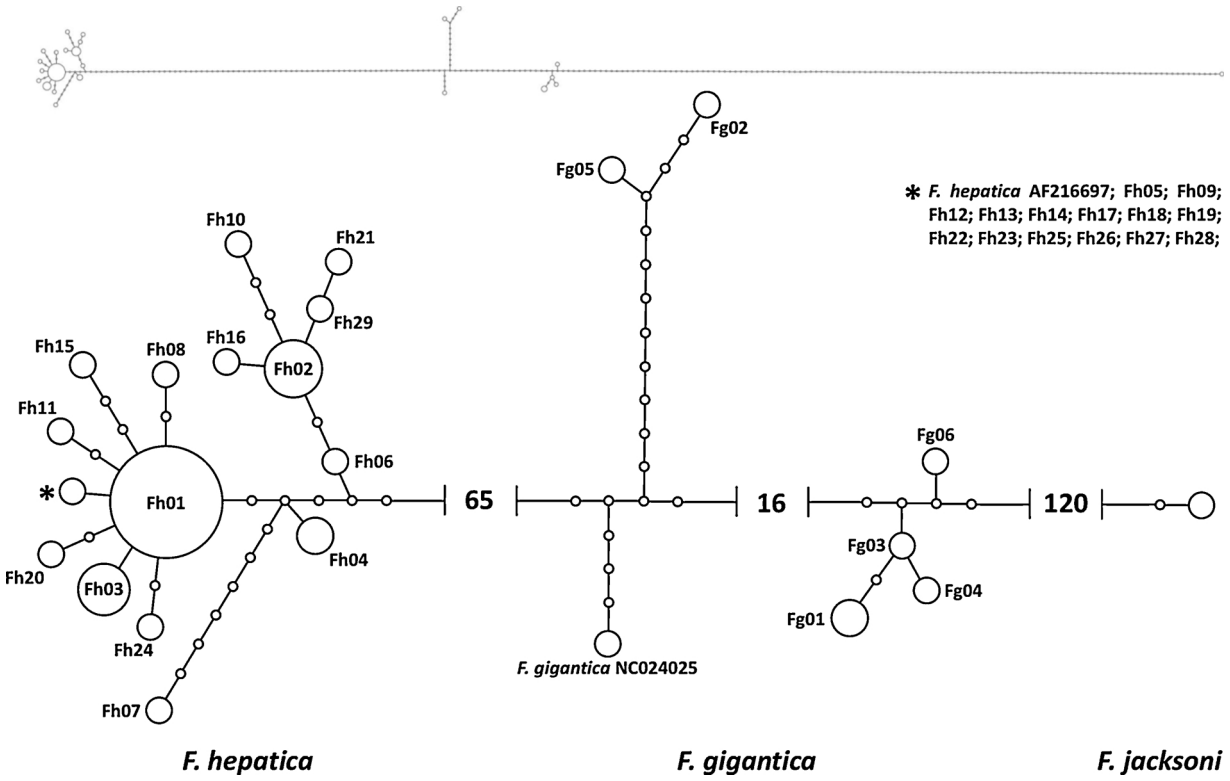

Fig. 1. Haplotype network based on the complete *nad1* gene (904 bp), including all samples from Armenia together with reference sequences of *F. hepatica*, *F. gigantica* and *F. jacksoni*. Circle sizes (area) are proportional to the number of samples belonging to a specific haplotype. The circle with the asterisk represents 15 distinct haplotypes (including the reference sequence), all one mutational step from the central haplotype Fh01. The smallest unnamed circles are hypothetical intermediate haplotypes, not found in this study. The small figure (above the detailed network) shows all intermediate haplotypes for better visual presentation of the genetic distances.

unpublished).

The phylogenetic analysis was based on maximum likelihood and calculated using MEGA 7 software (Kumar et al., 2016) with the substitution model HKY + G and tested with 1000 bootstrap replications. *Fasciola jacksoni* and *Fasciolopsis buski* (**NC030528**, Ma et al., unpublished) were used as outgroups. From the Armenian sample set, only one sample of each haplotype was included.

The *nad1* haplotypes of *F. hepatica* and *F. gigantica* found in Armenia were compared with published haplotypes from other regions worldwide. For this purpose, the nad1 sequences were shortened to the length of previously published sequences (535 bp), a haplotype network was drawn using the TCS software v. 1.8 and a maximum likelihood analysis was carried out with MEGA 7. The GenBank sequences used for the analyses are listed in Table 2.

3. Results

3.1. Prevalence of Fasciola spp.

Prevalence estimates for fasciolosis in 2127 slaughtered animals are presented in Table 1. Total prevalence across all host species and regions was 20.5% (436/2127). Infection was more frequent in sheep (21.2%–381/1794) compared to cattle (15.7%–51/324), while 4 out of only 9 examined goats were positive. Geographical spread of prevalence was similar for sheep and cattle, the most affected province being Ararat with 37.3% and 22.6% infected sheep and cattle, respectively, followed by Kotayk, Armavir and Gegharkunik. Least affected were Aragatsotn and Vayots Dzor with < 10% prevalence in both species.

3.2. Species identification

Out of 73 collected flukes, 62 (from 55 host individuals) yielded all targeted gene sequences and could be determined. Morphologically, 55 flukes corresponded to *F. hepatica*, 7 flukes to *F. gigantica*. *Nad1* haplotypes fell into two clearly separable clusters, where 53/55 *F. hepatica* morphotypes grouped in the *F. hepatica* cluster, and 5/7 *F. gigantica* morphotypes grouped in the *F. gigantica* cluster (Table 3). In all of the 62 flukes, the majority (3–4 of 4) nucleotide sites of the *28S rRNA* gene that were used to distinguish *F. hepatica* and *F. gigantica* (Marcilla et al., 2002) showed the alleles consistent with the results of the *nad1* analysis (including the four specimens with the diverging morphological appearance). The flukes were therefore diagnosed according to the sequence data, which resulted in 55 specimens of *F. hepatica* and 7 specimens of *F. gigantica*. Only in 8 of the 55 *F. hepatica* all four *28S rRNA* marker alleles were in agreement, while in nucleotide position 91 the majority of specimens showed double peaks (n = 31) indicating heterozygosity, or showed the allele typical for *F. gigantica* (n = 16). Of the 7 specimens typed as *F. gigantica*, none showed agreement in these 4 positions. Alleles in nucleotide position 269 were either heterozygous (double peaks) (n = 5) or showed the allele typical for *F. hepatica* (n = 2) according to Marcilla et al. (2002) (Table 3).

Of the 55 *F. hepatica* flukes, 43 were recovered from sheep, 9 from cattle and 3 from goats. *F. gigantica* originated from sheep (n = 5) and cattle (n = 2). Where more than one fluke was collected from an individual host animal (5 sheep and 2 cattle), only in one case did the sequences of 2 flukes show complete identity. In case of one cattle, a mixed infection with *F. hepatica* and *F. gigantica* was observed (Table 3).

Geographically, *F. hepatica* came from Kotayk (n = 17), Ararat (n = 20) and Gegharkunik (n = 9), *F. gigantica* from Kotayk (n = 4) and Ararat (n = 1). The geographic origin of the remaining 9 *F. hepatica* and

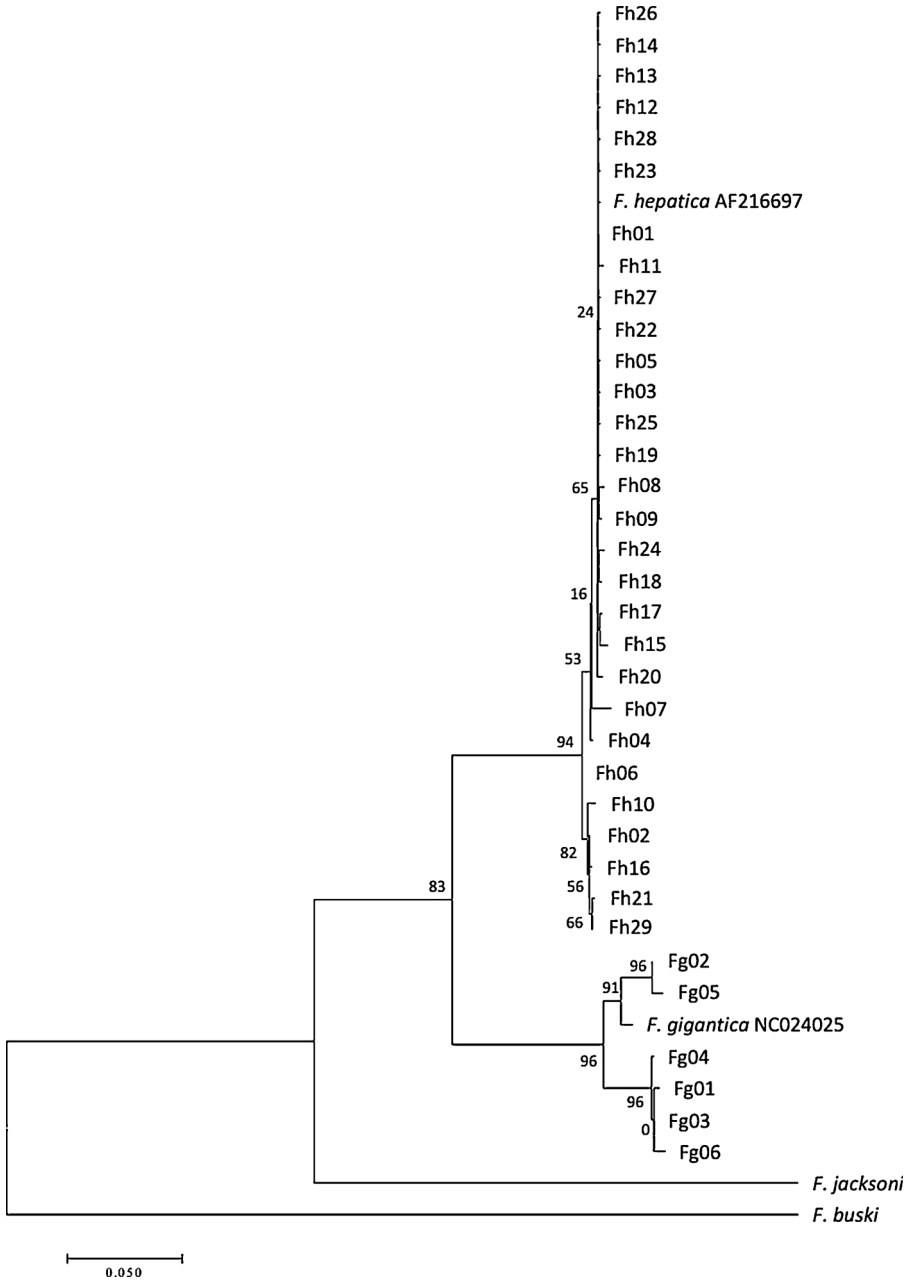

Fig. 2. Phylogenetic relationship of all Armenian *Fasciola hepatica* and *Fasciola gigantica* haplotypes, based on a maximum likelihood analysis of the complete *nad1* gene (904 bp). *Fasciola jacksoni* and *Fasciolopsis buski* are outgroups.

2 *F. gigantica* samples could not be defined more precisely, except that they originate from one of these three provinces.

3.3. Phylogenetic and haplotype analyses of the nad1 gene

Intraspecific genetic variation analysis of the complete *nad1* gene (904 bp) of the Armenian sample set resulted in 29 haplotypes (among 55 flukes) of *F. hepatica* and six haplotypes (among 7 flukes) of *F. gigantica* (Table 3). A parsimony network with all samples, reference sequences for both species and, in addition, the reference sequence of *F. jacksoni* was created (Fig. 1). For the phylogenetic analysis *Fasciolopsis buski as a more basal member of the Fasciolidae* (Lotfy et al., 2008) was included as outgroup (Fig. 2). The haplotype network of *F. hepatica* was dominated by a central haplotype (Fh01) and closely related variants arranged in star-like manner, that represented 19 Armenian samples and included the Chinese reference sequence **AF216697**; in addition, the network contained some more distantly related haplotypes (Fig. 1). In clear contrast, the few samples of *F. gigantica* showed a far higher genetic diversity and more complex network structure than *F. hepatica*, with the most distantly related haplotypes separated by 32 theoretical mutation steps. For comparison *F. jacksoni* was included to visualize the distances between the species.

To place the Armenian network topology of both species in a global perspective, additional haplotype networks and phylogenetic trees were created that included additional sequences from different geographic regions available in GenBank. Reduction of the sequence length from 903 to 535 bp was necessary to allow a comparison with the published sequences. This resulted in a decline from 29 to 23 Armenian *F. hepatica* haplotypes, while the haplotype number remained identical for *F. gigantica*. The resulting *F. hepatica* network shows three clusters, where samples from China were confined to two of them (around haplotypes Fh01 and Fh04), and samples from Europe to the third (around

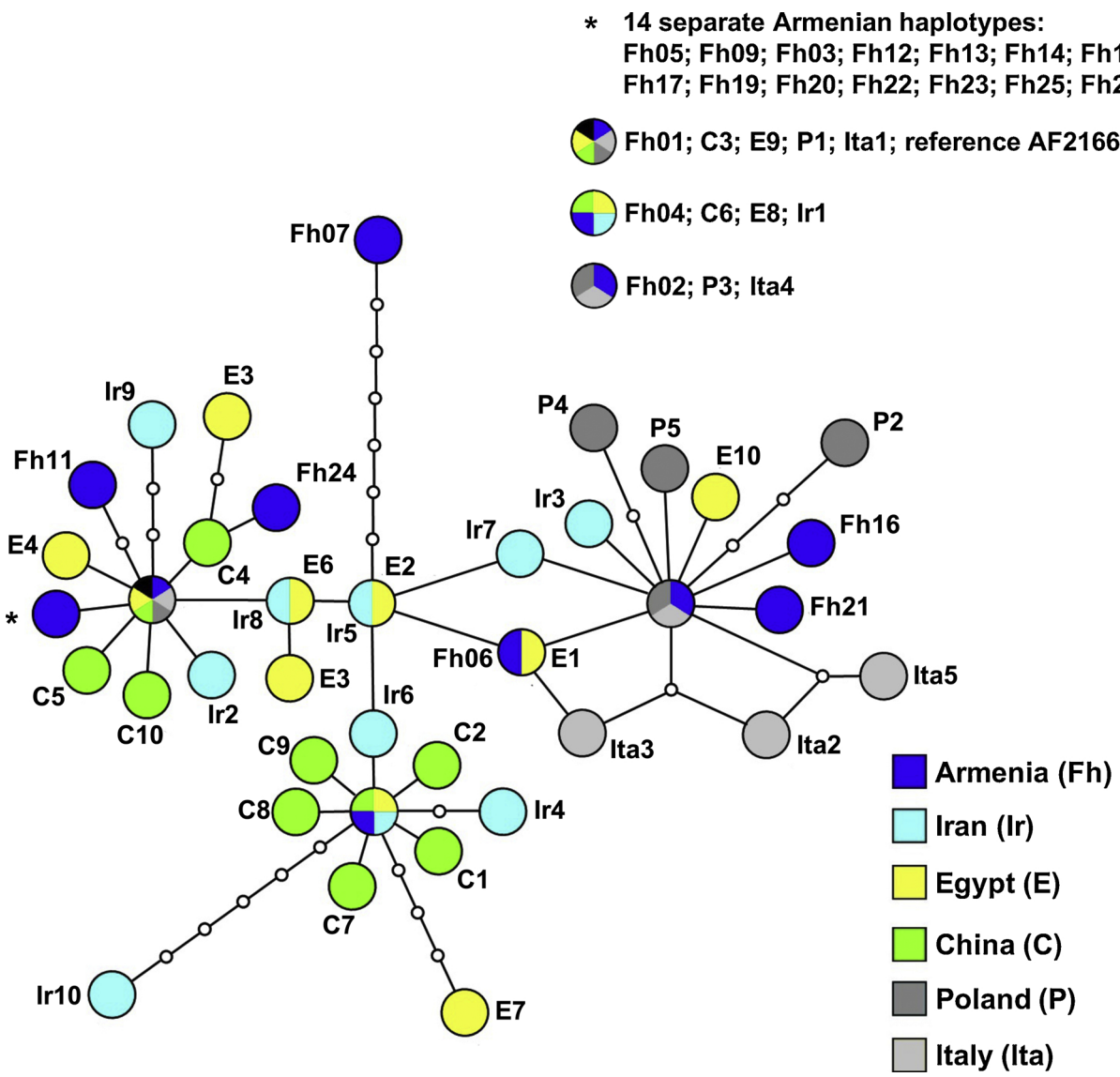

Fig. 3. Haplotype network of *Fasciola hepatica* based on a 535 bp fragment of the *nad1* gene. The frequency of samples belonging to individual haplotypes is not indicated. The circle with the asterisk represents 14 distinct haplotypes, all one mutational step from the central haplotype (1). The smallest unnamed circles are hypothetical intermediate haplotypes, not found in this study. The letters in the haplotype codes indicate their geographic origin; all haplotypes listed under the central haplotypes 1, 2 and 3, respectively, are identical.

haplotype Fh02), with the exception of haplotype Fh01 itself, which could be found in Europe as well. Samples from Armenia, Iran and Egypt were, interestingly, distributed both in the 'European' and 'East Asian' clusters (Fig. 3). These clusters are also apparent in the phylogenetic tree (Fig. 4).

The haplotype network of *F. gigantica* shows a far higher diversity and complexity, with various clusters corresponding to the geographical origin of the samples (Fig. 5). These include a closely related South / East Asian cluster, and a loosely connected group of clusters, that contains all samples from northern, western and southern Africa. Haplotypes from Armenia and Iran are either distributed in intermediate positions between African and Asian clusters, or, in case of some haplotypes, seemingly derive from one the 'African' clusters. This geographical arrangement is likewise clearly visible in the phylogenetic tree (Fig. 6), where an Asian clade is separated from the mainly African intermixed with some western Asian haplotypes. A rather divergent cluster consists exclusively of samples from Zambia. This group is already clearly separated within the haplotype network (Fig. 5), and, interestingly, occupies a basal position in the phylogenetic tree (Fig. 6).

4. Discussion

Data on the frequency and identity of livestock parasites, obtained at slaughtering facilities, often suffer uncertainties concerning the precise origin of the host animals due to movement of livestock within countries and across borders. In the case of Armenia, livestock imports from foreign countries are minimal. For cattle, out of 590,585 animals present in Armenia in 2018, only 2398 animals were imported, mainly for breeding purposes (State Service for Food Safety, Ministry of Agriculture, unpublished). Sheep are not imported for slaughter, except for animals from Russia to be slaughtered in Armenia and destined for meat export to Iran. Such animals are kept separate, slaughtered with different religious requirements and were not included in this study. Our samples were from slaughtering facilities that serve a limited geographical area (province, or group of neighbouring provinces). However, no attempt was made to locate the origin of animals within a province, whose borders therefore serve as sampling grid.

The present survey data confirm previous reports of central Armenia as a high-endemicity region for livestock fasciolosis with 21.2% of 1794

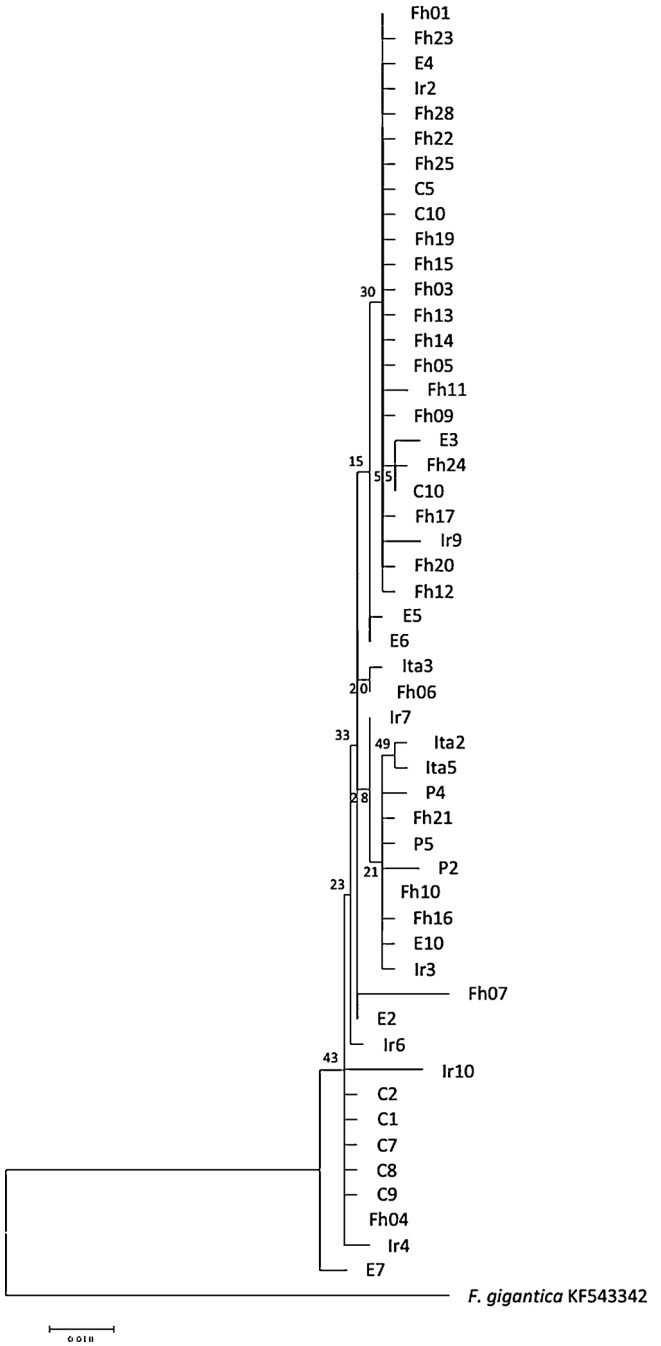

Fig. 4. Phylogenetic analyses of the genetic variance within the 535 bp long fragment of the *nad1* gene from *Fasciola hepatica* based on maximum likelihood, with *F. gigantica* as outgroup. For the geographic origin of the haplotypes see Fig. 3.

sheep and 15.7% of 324 cattle found infected. These figures are considerably lower compared to previous surveys with > 50% infection in sheep (Badeyan et al., 2001; Tonakanyan, unpublished). This can be explained by the fact that, in our survey, the majority of sheep were under one year of age with correspondingly lower prevalence. Within the country, there was considerable variation of prevalence with Ararat province as the most heavily affected part of Armenia (37.3% infection in sheep, 22.6% in cattle). This province includes the Ararat plains, an area with extensive natural wetlands that support the lifecycle of *Fasciola* spp. by providing suitable habitats for intermediate host snails. In contrast, the low-prevalence provinces of Aragatsotn and Vayots Dzor are characterized by mountainous landscape and steppe vegetation with a far lower proportion of wetland habitats. Overall, these prevalence data from Armenia are at a similar level as in neighboring areas of Iran (Daryani et al., 2006; Eslami et al., 2009; Mas-Coma, 2004; Moghaddam et al., 2004). In most studies sheep and cattle are the most frequently infected livestock animals, while goats (whose browsing behavior puts them at lower risk of ingesting metacercariae) are usually far less frequently infected (Ashrafi, 2015). Our results seem to be contradictory (four out of nine examined goats infected), but this is likely due to the low number of sampled animals. There are no data available on human fasciolosis in Armenia both in publications and unpublished data banks (Gevorgian, unpublished). However, human cases have been frequently reported from neighboring countries like Iran, Georgia and Turkey (Ashrafi, 2015; Moghaddam et al., 2004; Yilmaz and Gödekmerdan, 2004; Zirakishvili and Potshverija, 2009), which suggests that the lack of data from Armenia is due to non-reporting by the diagnosing institutions. The public health system in Armenia should therefore be sensitized to the presence of this zoonosis.

Armenia is situated in a region of overlapping geographic ranges of *F. hepatica* and *F. gigantica*, which are known to hybridize. Although our analysis of gene sequences confirmed the preliminary initial morphological species allocation in 58 of 62 flukes, the nuclear sequences did not give unequivocal results. Based on the alleles of four nucleotide sites of the *28S rRNA* gene, which had been described as species-diagnostic, only 8 of the 62 flukes belonged 100% to either species (all *F. hepatica*). Of the 55 flukes allocated to *F. hepatica*, 16 showed the corresponding *F. gigantica*-allele and 31 showed double peaks, all at nucleotide site 91. Of the 7 flukes allocated to *F. gigantica*, 5 showed the corresponding *F. hepatica*-allele and 2 showed double peaks, all at nucleotide site 269. Double peaks in electropherograms of a recombinant gene like the *28S rRNA* is an indication of heterozygosity resulting from hybridization between the two taxa, while homozygote alleles typical for other species indicate previous genetic introgression. However, to confirm hybridizations within the Armenian sample set, additional nuclear marker genes like ITS1 or the DNA polymerase delta (*pold*) will have to be analyzed. Such signs of cross-breeding between the two species have been observed earlier in nuclear genes and indicate the close relationship between the two species, whose geographical ranges are assumed to have come into contact rather recently as a result of movement of livestock (Agatsuma et al., 2000; Itagaki et al., 2005; Mas-Coma et al., 2009; Peng et al., 2009).

Intraspecific genetic variation of the *nad1* gene was high in both species with 29 haplotypes among the 55 *F. hepatica* specimens and six haplotypes among the seven *F. gigantica* specimens. Reducing the sequence length to 535 bp, the number of haplotypes was 23/55 and 6/7, respectively. This is the highest genetic diversity of *F. hepatica* and *F. gigantica* in the Middle East / West Asian region reported so far. Previous studies from Armenia and Iran reported six haplotypes among 30 *F. hepatica* samples (based on a 745 *cox1-nad1*concatenated fragment) and eight haplotypes among 90 *F. hepatica* samples (based on a 535 bp *nad1* fragment), respectively (Reaghi et al., 2016; Semyenova et al., 2006).

Looking at the calculated parsimony network of the Armenian *Fasciola* spp. haplotypes, the highest number of *F. hepatica* samples (18 of 55) belonged to one haplotype (Fh01) that forms the center of a cluster including the majority (21) of *F. hepatica* haplotypes found in Armenia; only 8 haplotypes are in more distant position, although clearly belonging to the *F. hepatica* cluster. In contrast, the (admittedly few) *F. gigantica* samples grouped loosely in three rather distant clusters, indicating a greater genetic diversity than *F. hepatica*.

Analyzing the position of 'Armenian' haplotypes in a global *F. hepatica* network, all haplotypes from Europe (except the ubiquitous Fh01) form a separate cluster around haplotype Fh02, while haplotypes from East Asia are exclusively found in two different clusters around haplotypes Fh01 and Fh04. In contrast, haplotypes found in western Asia and the Middle East (Armenia, Iran, Egypt) are distributed throughout the global network, indicating a far higher genetic diversity

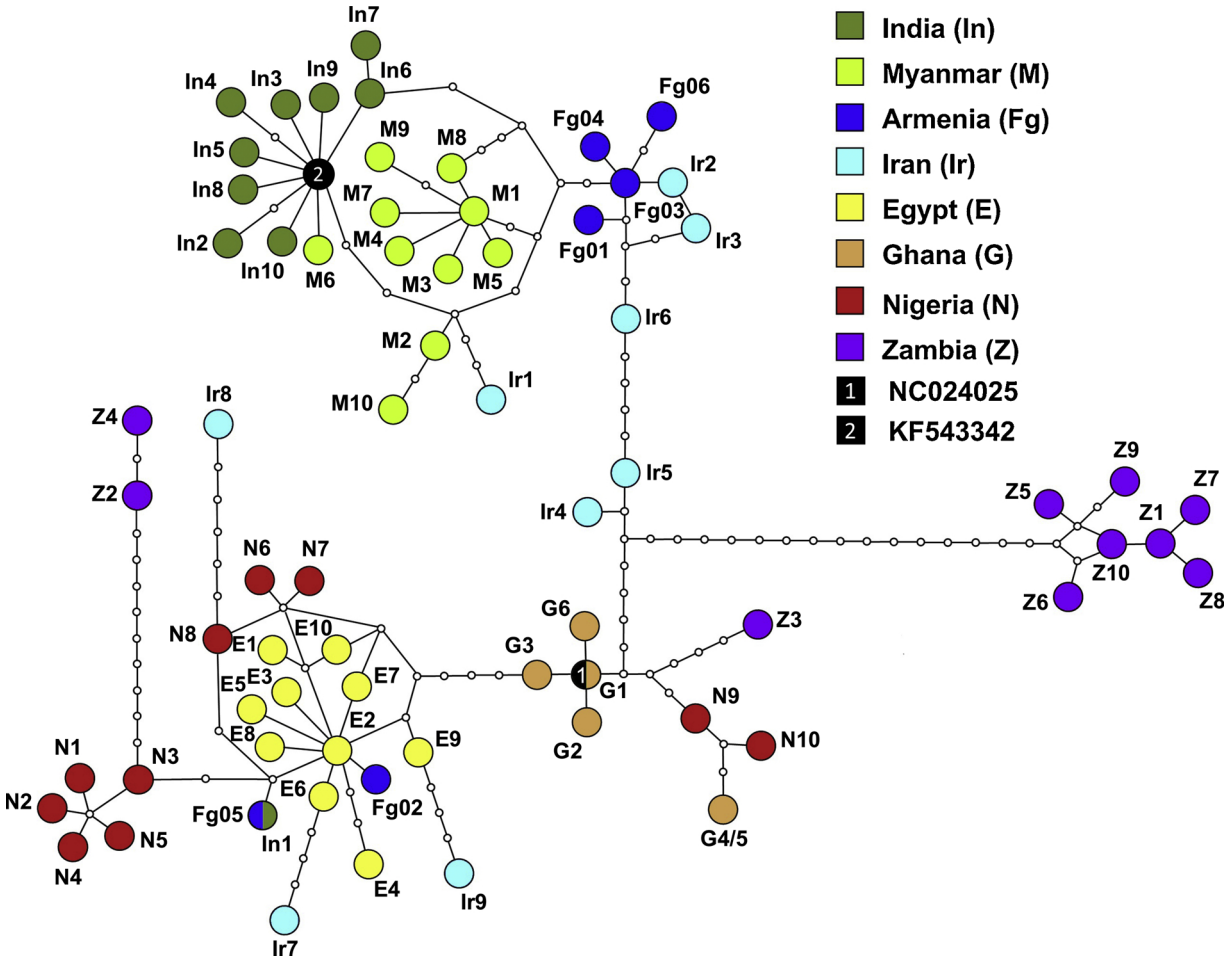

Fig. 5. Haplotype network of *Fasciola gigantica* based on a 535 bp long fragment of the *nad1* gene. The frequency of samples belonging to individual haplotypes is not indicated. The smallest unnamed circles are hypothetical intermediate haplotypes, not found in this study. The letters in the haplotype codes indicate their geographic origin.

in that region compared to Europe and eastern Asia. The low number of sequences available for this analysis does not allow further conclusions, but a higher genetic diversity in areas around the Middle East would be in line with the history of livestock domestication in the 'fertile crescent' (Zeder, 2008). A higher proportion of the genetic polymorphism of the parasite (that initially evolved in wild animals ancestral to livestock) would have been preserved there, part of the diversity having been lost during livestock movements to eastern Asia, Europe and, hence, to other continents during the period of European settlement (Mas-Coma et al., 2009; Valero et al., 2018).

The origin of the species *F. hepatica* in temperate Eurasia is supported by its close adaptation to the snail host *Galba truncatula*, which had initially a European distribution (although by now it is present almost globally due to human activities) (Mas-Coma, 2005), and the minimum temperature needed for larval development, which is far lower for *F. hepatica* than for in its tropical sister species (Malone et al., 1998).

The global *nad1* haplotype network of *F. gigantica* confirms the higher genetic diversity, which was already apparent among the Armenian haplotypes. The maximum genetic distances between two *F. gigantica* haplotypes, measured using hypothetical mutational steps, was 49 compared to 15 in the global *F. hepatica* network. The high intraspecific genetic variability of *F. gigantica* has been demonstrated before in various studies with African or Asian specimens (Chaichanasak et al., 2012; Hayashi et al., 2015; Ichikawa-Seki et al., 2017); we could show that this high level of intraspecific variance is also true for specimens collected in one of the most northern geographic range of the *F. gigantica* distribution, indicating a prolonged history of introductions that is also visible from the positions of western Asian haplotypes in the global haplotype network. There, as with *F. hepatica*, geographic associations are clearly visible. Most haplotypes found in Armenia group together with haplotypes known from southern Asia and Iran, while haplotypes from western Africa from a cluster together with those known from Egypt. An interesting cluster is formed by a group of isolates from Zambia, which are rather distant to both other clusters. As these Zambian isolates also form the basal clade in the phylogenetic tree, it is intriguing to speculate about a phylogeographic origin of the species in southern (possibly eastern) Africa, followed by gradual expansion into northern/western Africa and hence, via the Middle East, to Asia. This is in agreement with the detailed hypothesis forwarded by Mas-Coma et al. (2009) and contradicts previous suggestions of an Asian origin of *F. gigantica* (Lotfy et al., 2008). However, the comparatively low number of isolates available for this study does not allow further conclusions. As *F. gigantica* parasitizes a large range of wild African ruminant species – probably its original hosts (Mas-Coma et al., 2009) – more comprehensive studies on flukes from African wildlife will certainly be the key to gain further insights into the geographical origin of *F. gigantica*.

It is intriguing to see, that, after centuries of livestock trade, such geographical clustering of genetic variants is still recognizable in the case of parasites that predominantly infect livestock, although translocations of isolates can be assumed by the 'appearance' of single haplotypes in clusters of geographically distant samples. Further data will be required to resolve the global genetic structure of these

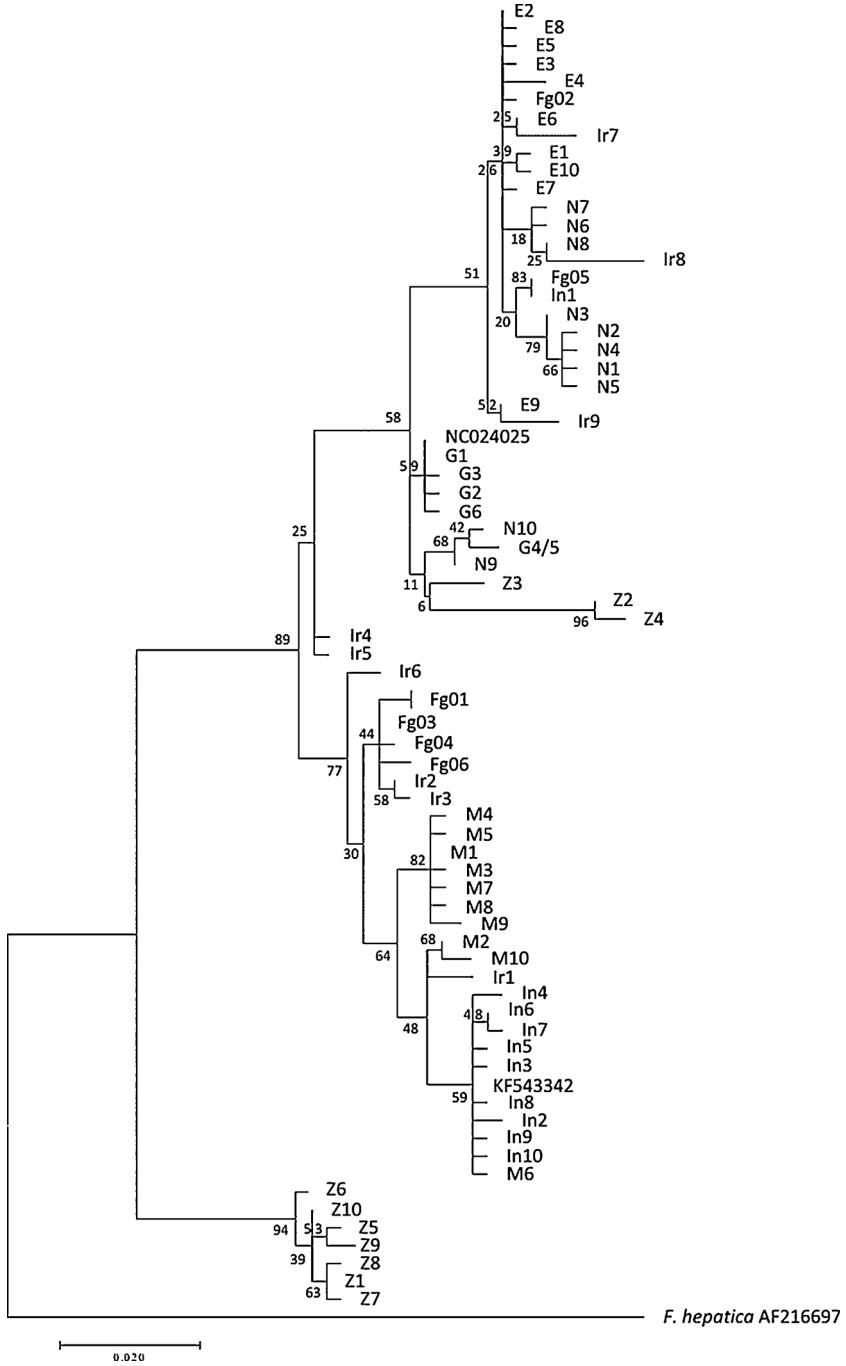

Fig. 6. Phylogenetic analyses of the genetic variance within the 535 bp long fragment of the *nad1* gene from *Fasciola gigantica* based on maximum likelihood with *F. hepatica* as outgroup. For the geographic origin of the haplotypes see Fig. 5.

parasites, based on epidemiologically stringent sampling strategies in terms of samples size, sample independence and geographical spacing.

Conflict of interest

None.

Acknowledgements

This study was financially supported by the Bundesministerium für Bildung und Forschung (BMBF) (Germany), grant 01DK14012 ('Zoonotische Infektionskrankheiten im Südkaukasus: integrative Forschung für Prävention und Bekämpfung'). The field work was partly supported by the RA MES State Committee of Science (Armenia), in the frames of the research project № 16YR-1F077.

References

Addy, F., Wassermann, M., Banda, F., Mbaya, H., Aschenborn, J., Aschenborn, O., Koskei, P., Umhang, G., De La Rue, M., Elmahdi, I.E., Mackenstedt, U., Kern, P., Romig, T., 2017. Genetic polymorphism and population structure of *Echinococcus ortleppi*. Parasitology 144, 450–458.

Agatsuma, T., Arakawa, Y., Iwagami, M., Honzako, Y., Cahyaningsih, U., Kang, S.Y., Hong, S.J., 2000. Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. Parasitol. Int. 49, 231–238.

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.

Ashrafi, K., 2015. The status of human and animal fascioliasis in Iran: a narrative review article. Iran. J. Parasitol. 10, 306–328.
Ashrafi, K., Bargues, M.D., O'Neill, S., Mas-Coma, S., 2014. Fascioliasis: a worldwide parasitic disease of importance in travel medicine. Travel Med. Infect. Dis. 12, 636–649.
Askari, Z., Mas-Coma, S., Bouwman, A.S., Boenke, N., Stöllner, T., Aali, A., Rezaiian, M., Mowlavi, G., 2018. *Fasciola hepatica* eggs in paleofaeces of the Persian onager *Equus hemionus onager*, a donkey from Chehrabad archaeological site, dating back to the Sassanid Empire (224-651 AD), in ancient Iran. Infect. Genet. Evol. 62, 233–243.
Badeyan, G., Boyakhchyan, G., Postoyan, S., Hakobyan, V., Nazaretyan, G., Hovasapyan, N., 2001. About epizootological situation on fasciolosis among livestock in Armavir region of Armenia (in Russian). Proceeding of National Scientific Conference on Zoology, May 30-31, Yerevan. pp. 41–42.
Caple, I.W., Jainudeen, M.R., Buick, T.D., Song, C.Y., 1978. Some clinico-pathologic findings in elephants (*Elephas maximus*) infected with *Fasciola jacksoni*. J. Wildl. Dis. 14, 110–115.
Chaichanasak, P., Ichikawa, M., Sobhon, P., 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.
Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1659.
Daryani, A., Alaei, R., Arab, R., Sharif, M., Dehghan, M.H., Ziaei, H., 2006. Prevalence of liver fluke infections in slaughtered animals in Ardabil Province, northwestern Iran. J. Anim. Vet. Adv. 5, 408–411.
Dinnik, J.A., Dinnik, N.N., 1961. On the morphology, and life history of *Fasciola nyanzae* Leiper, 1910 from the hippopotamus. J. Helminthol. Suppl 53–62.
Eslami, A., Hosseini, S.H., Meshgi, B., 2009. Animal Fasciolosis in North of Iran. Iran. J. Public Health 38, 132–135.
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., Mohanta, U.K., Shoriki, T., Chaichanasak, P., Itagaki, T., 2018. Hybrid origin of Asian aspermic *Fasciola* flukes is confirmed by analyzing two single-copy genes, pepck and pold. J. Vet. Med. Sci. 80, 98–102.
Heneberg, P., 2013. Phylogenetic data suggest the reclassification of *Fasciola jacksoni* (Digenea: Fasciolidae) as Fascioloides jacksoni comb. nov. Parasitol. Res. 112, 1679–1689.
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., 2005. Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. Parasitology 131, 679–685.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Le, T.H., Blair, D., Agatsuma, T., Humair, P.F., Campbell, N.J., Iwagami, M., Littlewood, D.T., Peacock, B., Johnston, D.A., Bartley, J., Rollinson, D., Herniou, E.A., Zarlenga, D.S., McManus, D.P., 2000. Phylogenies inferred from mitochondrial gene orders – a cautionary tale from the parasitic flatworms. Mol. Biol. Evol. 17, 1123–1125.
Liu, G.-H., Gasser, R.B., Young, N.D., Song, H.-Q., Ai, L., Zhu, X.-Q., 2014. Complete mitochondrial genomes of the "intermediate form" of *Fasciola* and *Fasciola gigantica*, and their comparison with *F. hepatica*. Parasit. Vectors 7 (150). https://doi.org/10.1186/1756-3305-7-150.
Lotfy, W.M., Brant, S.V., DeJong, R.J., Le, T.H., Demiaszkiewicz, A., Rajapakse, R.P.V.J., Perera, V.B.V.P., Laursen, J.R., Loker, E.S., 2008. Evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae). Am. J. Trop. Med. Hyg. 79, 248–255.
Malone, J.B., Gommes, R., Hansen, J., Yilma, J.M., Slingenberg, J., Snijders, F., Nachtergaele, F., Ataman, E., 1998. A geographic information system on the potential distribution and abundance of *Fasciola hepatica* and *F. gigantica* in east Africa based on Food and Agriculture Organization databases. Vet. Parasitol. 78, 87–101.
Marcilla, A., Bargues, M.D., Mas-Coma, S., 2002. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. Mol. Cell. Probes 16, 327–333.
Mas-Coma, S., 2004. Chapter 19: human fascioliasis. In: Cotruvo, J.A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D.O., Craun, G.F., Fayer, R., Gannon, V.P.J. (Eds.), Waterborne Zoonoses: Identification, Causes and Control. World Health Organization, London, pp. 305–322.
Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. J. Helminthol. 79, 207–216.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. Chapter 2. *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., Bargues, M.D., Valero, M.A., 2014. Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario. Parasitology 141, 1918–1946.
Mas-Coma, S., Bargues, M.D., Valero, M.A., 2018. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. Parasitology 145, 1665–1699.
Mehmood, K., Zhang, H., Sabir, A.J., Abbas, R.Z., Ijaz, M., Durrani, A.Z., Saleem, M.H., Rehman, M.U., Iqbal, M.K., Wang, Y., Ahmad, H.I., Abbas, T., Hussain, R., Ghori, M.T., Ali, S., Khan, A.U., Li, J., 2017. A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. Microb. Pathog. 109, 253–262.
Moghaddam, A.S., Massoud, J., Mahmoodi, M., Mahvi, A.H., Periago, M.V., Artigas, P., Fuentes, M.V., Bargues, M.D., Mas-Coma, S., 2004. Human and animal fascioliasis in Mazandaran province, northern Iran. Parasitol. Res. 94, 1–9.
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.
Periago, M.V., Valero, M.A., Panova, M., Mas-Coma, S., 2006. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). Parasitol. Res. 99, 368–378.
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.
Semyenova, S.K., Morozova, E.V., Vasilyev, V.A., Gorokhov, V.V., Moskvin, A.S., Movsessyan, S.O., Ryskov, A.P., 2005. Polymorphism of internal transcribed spacer 2 (ITS-2) sequences and genetic relationships between *Fasciola hepatica* and *F. gigantica*. Acta Parasitol. 50, 240–243.
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.
Valero, M.A., Bargues, M.D., Calderón, L., Artigas, P., Mas-Coma, S., 2018. First phenotypic and genotypic description of *Fasciola hepatica* infecting highland cattle in the state of Mexico, Mexico. Infect. Genet. Evol. 64, 231–240.
World Health Organization, 2007. Action against Worms. 2007 ed.. World Health Organization.
Yilmaz, H., Gödekmerdan, A., 2004. Human fasciolosis in Van province, Turkey. Acta. Trop. 92, 161–162.
Zeder, M.A., 2008. Domestication and early agriculture in the Mediterranean Basin: origins, diffusion, and impact. Proc. Natl. Acad. Sci. 105, 11597–11604.
Zirakishvili, L.M., Potshverija, S.O., 2009. Some questions on epidemiology and epizootology of fasciolosis in Georgia (in Russian). Russ. J. Parasitol. 1, 38–42.