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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

New insight into genetic variation and haplotype diversity of *Fasciola hepatica* from Algeria

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Abstract

The liver fluke *Fasciola hepatica* is the main cause of fasciolosis in North Africa leading to significant economic losses and public health problems. In this study, the ribosomal internal transcribed spacer (ITS), cytochrome c oxidase I (COI), the mitochondrial region spanning the COI-trnT-rrnL, and the NADH dehydrogenase subunit I (NADI) markers were used to characterize *Fasciola* flukes from Algeria. *Fasciola* appeared widespread from the east to the west of Algeria. Among 1701 sampled cattle from 8 Algerian provinces, 5% were infected. Using morphological and morphometric analysis, one morphotype of *Fasciola* was observed. Nuclear ITS marker indicated that all collected flukes belong to *F. hepatica*. Multiple alignments of ITS dataset revealed two haplotypes, one described here for the first time. Analysis of molecular variance (AMOVA) of mitochondrial markers revealed weak population structure in Algeria. Mismatch distributions, neutrality tests, and median-joining network analysis all were compatible with a recent expansion of Algerian *F. hepatica* population. Fasciolosis appeared common in Algerian cattle, it seems that the absence of control strategy coupled to the favorable Mediterranean climate may lead to a reconstruction and dispersion of its populations. This study provides important results concerning the genetic characterization and variability of *F. hepatica* in Algeria as well as the significant role of cattle importation in shaping its dispersal route worldwide.

Keywords *Fasciola hepatica* · Algeria · Genetic characterization · ITS · NADI · COI · COI-trnT-rrnL

Introduction

The liver flukes, *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1856, are the cause of fasciolosis in humans and livestock (Mas-Coma and Bargues 1997). *Fasciola gigantica* has been reported in tropical areas, while *F. hepatica* is characterized by a worldwide distribution (Mas-Coma and Bargues 1997). Fasciolosis causes important economic losses (liver condemnation at slaughter, decreased milk production, mortality) (Nyindo and Lukambagire 2015). The World Health Organization estimated that 2.4 million people are infected worldwide, with the highest prevalence in developing countries (Mehmood et al. 2017).

Traditional species identification is based on phenotypic characters of the adult worms, such as body width and length (Valero et al. 2018). However, several studies have revealed that morphological differences might be biased because of the presence of intermediate forms (Shafiei et al. 2014). Moreover, Dosay-Akbuluta et al. (2005) showed a greater intraspecific variation

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between *Fasciola* spp. specimens from different hosts than the interspecific one.

Molecular classification has so far attested to be the method of choice, providing a clear differentiation between the two *Fasciola* spp. and the intermediated forms (hybrid and introgressed). Genetic characterization of *Fasciola* spp. can be carried out using several markers as the ribosomal internal transcribed spacer (ITS), phosphoenolpyruvate carboxykinase (pepck), DNA polymerase delta (pold), cytochrome c oxidase I (COI), and the NADH dehydrogenase (NAD) genes (Itagaki et al. 2005; Farjallah et al. 2009; Mas-Coma et al. 2009; Amor et al. 2011; Hayashi et al. 2016; Bargues et al. 2017; Carnevale et al. 2017; Ichikawa-Seki et al. 2017; Rouhani et al. 2017; Sarkari et al. 2017). In 2012, Le et al. proposed a duplex PCR based on the mitochondrial region COI-trnT-rrnL as alternative to differentiate between species within overlapping geographic regions. Mitochondrial markers are also useful for studying population genetic structure. Hayashi et al. (2015, 2016) explored the dispersal routes of *F. gigantica* in Southeast Asia using mitochondrial NADI dataset and revealed the significant role of the host movement in shaping its genetic structure.

F. hepatica is widely distributed in North Africa (Mekroud et al. 2004; Farjallah et al. 2009; Amor et al. 2011; Ouchene-Khelifi et al. 2018). Egypt is still the only North African country where both *Fasciola* spp. and their aspermic hybrids have been described (Amer et al. 2016).

In Algeria, fasciolosis is very common, especially within the humid northern region favorable for the survival of the intermediate host, the snail *Galba truncatula* Müller, 1774 (Mekroud et al. 2004; Righi et al. 2016). The prevalence of fasciolosis has been reported to vary from 6.5 to 18.2% in sheep and from 9.1 to 27% in cattle (Mekroud et al. 2004; Ouchene-Khelifi et al. 2018). Ouchene-Khelifi et al. (2018) reported the condemnation of over 3000 infected liver in El Tarf region, which correspond to an economic loss of 60,000 euros.

Despite the impact of fasciolosis in Algeria, only one study restricted to a single locality has assessed the genetic diversity of *Fasciola* flukes collected from sheep (Farjallah et al. 2009). Therefore, the objective of the present study is to investigate the genetic variability of *Fasciola* samples, recovered from different Algerian provinces, based on several molecular markers (ITS, COI-trnT-rrnL, NADI, and COI). It is the first time that a study will include several regions from the eastern to the western part of Algeria. In addition, data dealing with the prevalence and the morphometric variability of *Fasciola* samples in the studied localities will be provided.

Materials and methods

Study area

The study was carried out in eight provinces of northern Algeria: Bejaïa, Souk-Ahras, Batna, Médéa, Tissemsilt, Tiaret, Tlemcen, and Ain-Temouchent (Fig. 1; Table 1). Samples from Bejaïa province were collected from 12 different districts (Fig. 1; Table 1). The studied area is characterized by a Mediterranean climate, and it is divided by two geographic barriers, the Tell and the Sahara Atlas. North Algeria is characterized by a cold winter and warm summer with significant rainfall. These conditions are advantageous for the transmission of *Fasciola*.

Fasciola samples and morphometric analysis

After post-mortem examination of 1701 cattle by veterinary officers, adult flukes were collected from 87 infected livers from January to December 2017 (Table 1). Samples were collected in plastic jars and then washed in PBS.

The body length (BL) and body width (BW) were measured using digital calipers prior to photography (Ashrafi et al. 2006). Body length to width ratio (BL/BW) was also calculated for each specimen. One-way analysis of variance (ANOVA), implemented in SPSS 18.0 (SPSS, Chicago, IL), was conducted in order to determine whether there was any significant morphometric variation between flukes isolated from different localities.

Localities were divided into two groups, eastern, including Bejaïa, Souk-Ahras, and Batna, and western, including Médéa, Tissemsilt, Tiaret, Tlemcen, and Ain-Temouchent.

DNA extraction, amplification, and sequencing

One to three adult flukes were selected randomly from each host for molecular analysis, for a total of 140 specimens. Samples were stored in 70% ethanol and preserved at -20 °C. Genomic DNA was extracted from a portion of the lateral zone of the adult fluke using Wizard Genomic DNA Purification kit (Promega, USA) according to the manufacturer's recommendations. For amplification, 1 μL of genomic DNA was added in a 25 μL reaction mix containing, 1 U of MyTaq DNA polymerase (Bioline, Memphis, USA), 5 μL 5× MyTaq buffer, 10 pmol of each primer, and ddH_2O. The primers BD1 and BD2 were used for the amplification of ITS region including ITS-1, 5.8S rDNA and ITS-2, FhF and FHR for COI-trnT-rrnL (Le et al. 2012), Ita 10 and Ita 2 for NADI (Itagaki et al. 2005), and JB3 and JB4 for COI (Bowles et al. 1992). Reaction cycles consisted of an initial denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 90 s, 55 °C (for COI) or 53 °C (for NADI), 52 °C (COI-trnT-rrnL) or 62 °C (for ITS) for 90 s, and 72 °C for 120 s with a final extension at 72 °C for 10 min. During amplifications, negative control was included to assess possible contamination. All the PCR products were sequenced using forward primers at Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

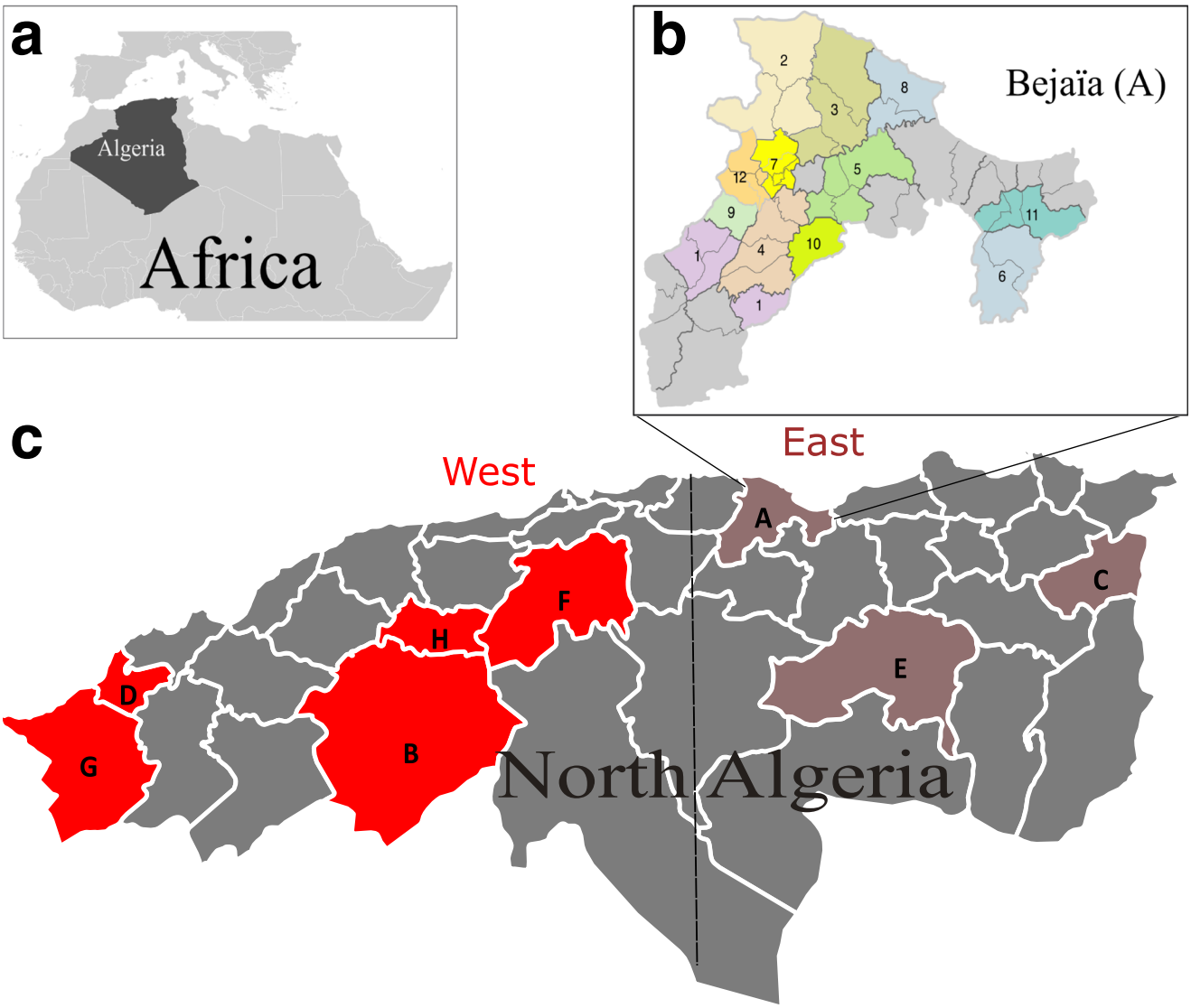

Fig. 1 Map of Algeria (**a**). Bejaïa (**b**). North Algeria (**c**). Provinces: Bejaïa (A), Tiaret (B), Souk-Ahras (C), Ain-Temouchent (D), Batna (E), Médéa (F), Tlemcen (G), and Tissemsilt (H). Districts: Akbou (1) Adekar (2), El Kseur (3), Seddouk (4), Amizour (5), Kherrata (6), Sidi-Aïch (7), Béjaïa (8), Ouzellaguen (9), Beni Maouche (10), Darguina (11), and Chemini (12)

Data analysis

Seaview 3.2 (Galtier et al. 1996) was used to perform multiple alignments for each genetic marker. The sequences of the two mitochondrial genes were translated into amino acids to check for possible amplification of pseudogenes. Representative sequences of *F. hepatica*, *F. gigantica*, and the aspermic *Fasciola* available in GenBank were included in the three datasets (ITS, COI, and NADI) (Table 2).

Haplotype (Hd) and nucleotide (π) diversities were estimated and the mismatch distribution for the two mitochondrial markers was constructed using DnaSP v 5.10 (Librado and Rozas 2009).

Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) were performed to evaluate the assumption of selective neutrality of mtDNA sequences and population mutation-drift equilibrium.

In addition, the genetic structure of *F. hepatica* in Algeria was estimated via a spatial analysis of molecular variance (AMOVA) implemented in Arlequin 3.5 (Excoffier et al. 2005). The studied populations were grouped according to their origin, eastern and western populations. Levels of significance of statistics characterizing variation at different hierarchical levels were assessed through 10,000 permutations.

The evolutionary relationships between mitochondrial haplotypes were analyzed for both markers NADI and COI, by a median-joining network (Bandelt et al. 1999) constructed with Network 5.0 (Fluxus Technology Ltd.).

The best-fitting models of molecular evolution applied to the datasets in the phylogenetic reconstructions were inferred

Table 1 Number of cattle slaughtered and prevalence of livers infected with fascioliasis from Algeria. Codes used for each province or district are given in parentheses

Province	District	N. examined	N. infected	Prevalence (%)
Bejaïa (A)	Akbou (1)	95	10	10.5
	Adekar (2)	77	8	10.4
	El Kseur (3)	81	4	4.9
	Seddouk (4)	65	4	6.2
	Amizour (5)	96	6	6.3
	Kherrata (6)	61	7	11.5
	Sidi-Aïch (7)	106	5	4.7
	Béjaïa (8)	83	2	2.4
	Ouzellaguen (9)	87	4	4.6
	Beni Maouche (10)	80	3	3.8
	Darguina (11)	73	5	6.8
	Chemini (12)	187	6	3.2
Tiaret (B)		122	3	2.45
Souk-Ahras (C)		18	4	22.3
Ain-Temouchent (D)		113	5	4.4
Batna (E)		30	2	6.7
Médéa (F)		88	3	3.4
Tlemcen (G)		111	3	2.7
Tissemsilt (H)		128	3	2.3
Total		1701	87	5.1

with jModeltest 2.1.8 (Darriba et al. 2012), based on the Akaike information criterion (Akaike 1974).

Neighbor joining (NJ) and maximum likelihood (ML) analyses were carried out to infer the relationships among NADI and COI haplotypes using Seaview 3.2 and RAxML (Stamatakis 2006), respectively. *Fascioloides magna* (EF535001, EF534997) was included as outgroup. In both tree constructions, bootstrap support was evaluated by 1000 pseudoreplicates. A consensus topology was generated using 50% of the resulting trees.

Results

The overall prevalence of fasciolosis in cattle from northern Algeria was 5.1% ($n = 87$). Comparing provinces, prevalence varied from 2.3% (Tissemsilt) to 22.0% (Souk-Ahras) (Table 1). Concerning Bejaïa province, the overall prevalence was 5.8% and between districts, it varied from 3.2% (Chemini) to 11.5% (Kherrata). All flukes were leaf shaped presenting oblique body angle with apparent shoulders, morphological characters specific to *F. hepatica*. One-way ANOVA showed no significant difference between eastern and western samples for all parameters (body width, body length, and ratio body length body width ratio) (Supplementary materials Table 1).

Molecular analysis

ITS fragments sequencing yielded sequence of a length of about 959 bp. Annotation of ITS was inferred by multiple alignments with *Fasciola* spp. sequences available in GenBank. This genetic marker includes a partial 18S fragment of 41 bp, whole ITS-1, and 5.8S sequences, 435 bp and 137 bp respectively, and whole ITS-2 sequence of 346 bp (Table 2).

Sequences of multiple alignment confirmed the conservation of the 18S and 5.8S rDNA within *Fasciola* sp. ITS-1 sequences were 100% identical to those of *F. hepatica* previously published (Itagaki et al. 2005; Mekroud et al. 2004; Farjallah et al. 2009; Amor et al. 2011; Ouchene-Khelifi et al. 2018). *F. hepatica* differed from *F. gigantica* in five substitutions (Table 2). Alignment of ITS-2 sequences showed ten variable sites within *Fasciola*. Comparing *F. hepatica* sequences, two different substitutions generating 3 haplotypes were observed (Table 2). The first substitution, transition, at the position 892 (C/T) differentiates between the common haplotype, FhITS2-H1, described worldwide and the second one, FhITS2-H2 (accession number = MK212149), previously reported in Spain, Algeria, and recently in South America (Alasaad et al. 2007; Farjallah et al. 2009; Valero et al. 2018). The second substitution was a transversion located at the position 935 (T/A), indicating the existence of a new haplotype, FhITS2-H3 (accession number = MK212150), reported here for the first time. Among the 140 Algerian *F. hepatica*, 95 samples belonged to the common haplotype FhITS2-H1, having a "T" at the position 935, and 35 belong to the new haplotype FhITS2-H3, having an "A" at this position. Within the eastern localities, both haplotypes (45% FhITS2-H1, 55% FhITS2-H3) were observed. However, FhITS2-H1 appeared more abundant in western Algeria (86%). Haplotype diversity (H) varied from 0.503 in the eastern to 0.239 in the western.

According to AMOVA outputs, most variations within populations explained the observed variation for COI (95%), COI-trnT-rrnL (81%), and NADI (86%). AMOVA revealed that 11.39%, 14.31%, and 12.76% of the total variation of COI, COI-trnT-rrnL, and NADI, respectively, was among groups (Table 3).

Amplification of COI-trnT-rrnL yielded products of 1031 bp specific to *F. hepatica* (Le et al. 2012). Alignment of the nucleotide sequences generated eight haplotypes (accession numbers = MK372236–43) differing in eight sites. Nucleotide diversity was low for COI-trnT-rrnL ($\pi = 0.0017$); however, haplotype diversity was high (Hd = 0.802). Median-joining network analysis showed a star-like pattern. The common haplotype FhCOI-trnT-rrnL-H1 (accession number = MK372236) occupied a basal position in the network (Fig. 2a) and was shared by all localities ($n = 37$,

Table 2 Details of *Fasciola* ITS sequences from Algeria and other countries

		ITS1					ITS2											
		50	140	234	312	332	823	847	874	886	892	935	943	950	957	958		
F. gigantica	Niger	T	T	T	A	T	C	C	T	T	C	T	–	A	A	T	AM900371	Ali et al. (2008)
	Egypt	T	T	T	A	T	C	C	T	T	C	T	–	A	T	A	EF612472–84	Lotfy et al. (2008)
	China	T	T	T	A	T	C	C	T	T	C	T	–	–	T	A	KF543340	Liu et al. (2014)
F. hepatica	Niger	C	A	C	T	C	T	T	C	C	C	T	T	G	T	A	AM900370	Ali et al. (2008)
	Egypt	C	A	C	T	C	T	T	C	C	C	T	T	G	T	A	EF612468	Lotfy et al. (2008)
	Spain	C	A	C	T	C	T	T	C	C	C	T	T	G	T	A	AM709498	Alasaad et al. (2007)
	Spain	C	A	C	T	C	T	T	C	C	T	T	T	G	T	A	AM709621	Alasaad et al. (2007)
	Bolivia	C	A	C	T	C	T	T	C	C	T	T	T	G	T	A	MG569981	Valero et al. (2018)
	Spain	C	A	C	T	C	T	T	C	C	T	T	T	G	T	A	MG569978	Valero et al. (2018)
	Bolivia	C	A	C	T	C	T	T	C	C	C	T	T	G	T	A	MG569977	Valero et al. (2018)
	Mexico	C	A	C	T	C	T	T	C	C	T	T	T	G	T	A	MG569976	Valero et al. (2018)
	Tunisia	C	A	C	T	C	T	T	C	C	C/T	T	T	G	T	A	GQ231546	Farjallah et al. (2009)
	Algeria	C	A	C	T	C	T	T	C	C	C/T	T	T	G	T	A	GQ231547	Farjallah et al. (2009)
	China	C	A	C	T	C	T	T	C	C	C	T	T	G	T	A	KX856340	Ai et al. (2017)
	Algeria	C	A	C	T	C	T	T	C	C	C	A/T	T	G	T	A	MK212149-50	Present study

39%). Haplotype FhCOI-trnT-rrnL-H3 (accession number = MK372238) was specific to Bejaïa. The remaining haplotypes differ in a single mutation step and were detected in more than three localities (Table 4; Fig. 2a).

Analysis of partial COI sequences (391 bp) yielded seven haplotypes (FhCOI-H1–FhCOI-H7, accession numbers = MK212142–48) differing in six sites (Table 5). COI dataset showed low nucleotide ($\pi = 0.0024$) and haplotype diversity (Hd = 0.428). Median-joining network analysis generated a star-like pattern (Fig. 2b). The majority of Algerian *F. hepatica* appeared carrying the haplotype FhCOI-H1 occupying a basal position in the network. The largest proportion (70%) of flukes belonging to this predominant haplotype was from Bejaïa. The remaining haplotypes appeared diverging from FhCOI-H1 at least in one nucleotide. Haplotypes FhCOI-H2 to FhCOI-H6 were observed only in Bejaïa and FhCOI-H5 and FhCOI-H6 were unique. Haplotype FhCOI-H7 was detected in two localities, Tiaret and Ain-Temouchent,

Table 3 Analysis of molecular variance (AMOVA)

	Source of variation	Sum of squares	Variance components	% variation	*P* value
COI-trnT-rrnL	Among groups	7.706	0.03725 Va	4.495	0.030
	Among populations within groups	4.287	0.01155 Vb	1.394	0.013
	Within populations	163.776	0.77989 Vc	94.111	0.000
	Total	175.770	0.82868		
NADI	Among groups	0.933	0.01881 Va	12.76	0.17595
	Among populations within groups	0.829	0.00155 Vb	1.05	0.0567
	Within populations	11.186	0.12712 Vc	86.19	0.000
	Total	12.948	0.14748		
COI	Among groups	1.406	0.03649 Va	11.39	0.04106
	Among populations within groups	0.903	− 0.02068 Vb	− 6.46	0.0315
	Within populations	26.491	0.30449 Vc	95.07	0.000
Total		28.800	0.32030		

Percentage of variation explained by different hierarchical levels for COI and NADI loci considering two groupings in *F. hepatica*

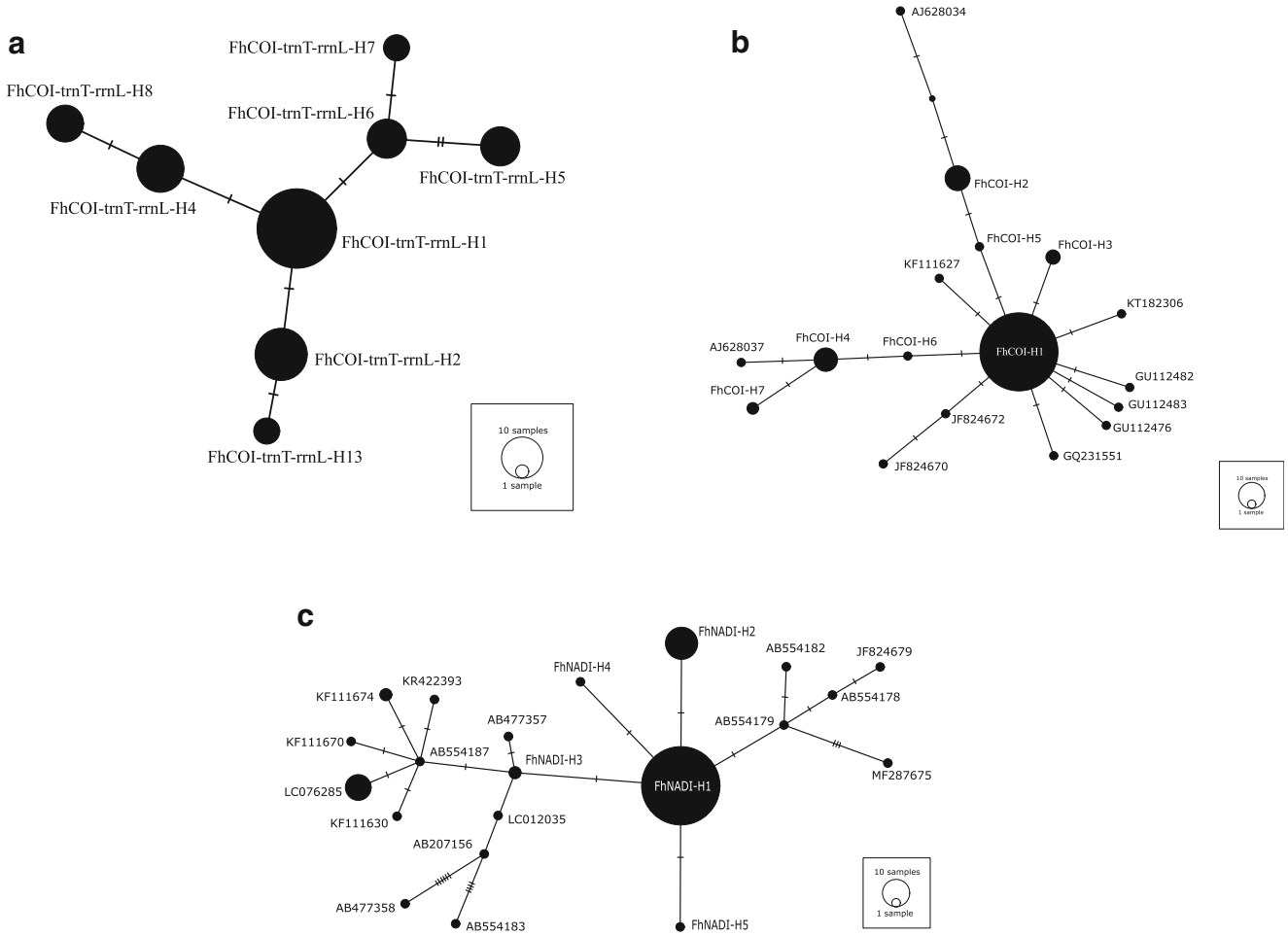

Fig. 2 Haplotype network obtained from COI-trnT-rrnL (**a**), COI (**b**), and NADI (**c**) sequences. The area of each circle is proportional to the haplotype frequency, and each branch represents one mutation

in western Algeria. After adding representative sequences of *F. hepatica* from different countries to COI dataset, the network pattern was the same (Fig. 2b). This confirms the close relationships among *F. hepatica* haplotypes.

Table 4 Details of *Fasciola* COI-trnT-rrnL sequences from Algeria and other countries

Marker	Location	Accession number	Reference	Haplotype
COI	Algeria (all localities)	MK372236	Present study	FhCOI-trnT-rrnL-H1
	Algeria (all localities)	MK372237	Present study	FhCOI-trnT-rrnL-H2
	Algeria (A)	MK372238	Present study	FhCOI-trnT-rrnL-H3
	Algeria (all localities)	MK372239	Present study	FhCOI-trnT-rrnL-H4
	Algeria (all localities)	MK372240	Present study	FhCOI-trnT-rrnL-H5
	Algeria (all localities)	MK372241	Present study	FhCOI-trnT-rrnL-H6
	Algeria (all localities)	MK372242	Present study	FhCOI-trnT-rrnL-H7
	Algeria (all localities)	MK372243	Present study	FhCOI-trnT-rrnL-H8
	Egypt	KU058263	Arafa et al. (2018)	
	Egypt	KU058264	Arafa et al. (2018)	
	Japan	AP017707	Coghlan et al. (2019)	
	Australia	AF216697	Le et al. (2000)	

Provinces: Bejaïa (A), Tiaret (B), Souk-Ahras (C), Ain-Temouchent (D), Batna (E), Médéa (F), Tlemcen (G), and Tissemsilt (H)

Table 5 Details of *Fasciola* COI sequences from Algeria and other countries

Marker	Location	Accession number	Reference	Haplotype
COI	Algeria (all localities)	MK212142	Present study	FhCOI-H1
	Algeria (Mostaganem northwest of Algeria)	GQ231549	Farjallah et al. (2009)	FhCOI-H1
	Tunisia	GQ231548	Farjallah et al. (2009)	FhCOI-H1
	Egypt	KX470584	Lotfy et al. (2008)	FhCOI-H1
	Egypt	AB510491	Omar et al. (2013)	FhCOI-H1
	South Africa	KT182300, 04, 05	Mucheka et al. (2015)	FhCOI-H1
	France	AJ628039	Dong et al. (unpublished)	FhCOI-H1
	Turkey	GQ121276	Simsek	FhCOI-H1
	Spain	KF111618	Martínez-Valladares and Rojo-Vázquez (2014)	FhCOI-H1
	Algeria Bejaïa	MK212143	Present study	FhCOI-H2
	Algeria Bejaïa	MK212144	Present study	FhCOI-H3
	Algeria Bejaïa	MK212145	Present study	FhCOI-H4
	South Africa	KT182260	Mucheka et al. (2015)	FhCOI-H4
	Tunisia	GQ231550	Farjallah et al. (2009)	FhCOI-H4
	Australia	AF216697	Let et al. 2000	FhCOI-H4
	China	AJ628035, 38	Dong et al. (unpublished)	FhCOI-H4
	Algeria Bejaïa	MK212146	Present study	FhCOI-H5
	Algeria Bejaïa	MK212147	Present study	FhCOI-H6
	Algeria Tiaret–Ain-Temouchent	MK212148	Present study	FhCOI-H7
	Tunisia	GQ231551	Farjallah et al. (2009)	
	Zimbabwe	KT182306	Mucheka et al. (2015)	
	France	GU112476	Ai et al. (2011)	
	Italy	JF824670, 72	Farjallah et al. (2013)	
	Spain	KF111627	Martínez-Valladares and Rojo-Vázquez (2014)	
	Japan	AP017707	Coghlan et al. (2019)	
	china	AJ628034	Dong et al. (unpublished)	
	USA	GU112482-3	Ai et al. (2011)	

The 140 partial NADI sequences (525 bp) included four variable sites and generated five haplotypes (FhNADI-H1–FhNADI-H5 (accession numbers = MK212137–41)) (Table 6; Fig. 2c). FhNADI-H1 was the most common haplotype (80%) and included samples from all localities, 77% from Bejaïa. FhNADI-H2 included exclusively samples from Bejaia; FhNADI-H3 was observed only in Tlemcen and Souk-Ahras; and FhNADI-H4 and FhNADI-H5 were unique to Ain-Temouchent and Batna, respectively. Nucleotide and haplotype diversities were very low ($\pi = 0.0006$, Hd = 0.313).

Reference haplotypes from Egypt, Uruguay, Iran, and Spain were closely related to FhNADI-H1 while sequences from Egypt, Italy, France, Iran, Japan, Australia, and China were similar to FhNADI-H2. Two additional haplogroups showed independent segregation, one mutation, from FhNADI-H1. The first haplogroup included spermic flukes from Egypt, Italy, and Brazil. The second included more divergent *F. hepatica* from Spain, Poland, Ireland, Japan, China, and Peru (Table 6; Fig. 2c)

Neutrality tests Tajima's D and Fu's FS showed significant negative values (Supplementary materials Table 2), indicating an excess of rare polymorphisms in the studied population. Mismatch distributions for both mitochondrial markers were L-shaped unimodal and supported the assumption of a sudden expansion model (Fig. 3a–c).

The optimum evolutionary models selected by jModelTest were GTR + I, HKY, and GTR for COI, NADI, and COI-trnT-rrnL genes, respectively. Both phylogenetic analyses showed similar topologies for COI, NADI, and COI-trnT-rrnL. Only the ML trees are shown (Fig. 4a–c). Node support values within the text consist of ML bootstrap values. Two well-supported sister groups corresponding to *F. hepatica* and *F. gigantica* were observed. Algerian flukes belonged to a monophyletic group including all *F. hepatica*. In NJ (Supplementary materials Figure 1a–c) and ML trees, bootstrap values for this clade were higher than 80% for COI and 99% for COI-trnT-rrnL and NADI. Within *F. hepatica* clade, all nodes were weakly supported.

Table 6 Details of *Fasciola* NADI sequences from Algeria and other countries

Marker	Location	Accession number	Reference	Haplotype
NAD1	Algeria Bejaïa	MK212137	Present study	FhNADI-H1
	Italy	JF824675	Farjallah et al. (2013)	FhNADI-H1
	France	KJ200623	Wannasan et al. (2014)	FhNADI-H1
	Egypt	AB554177	Amer et al. (2011)	FhNADI-H1
	Egypt	LC076285	Amer et al. (2016)	FhNADI-H1
	Iran	GQ175362	Moazeni et al. (2012)	FhNADI-H1
	Japan	LC228620	Ohari et al. (2017)	FhNADI-H1
	China	AB477363	Peng et al. (2009)	FhNADI-H1
	Australia	AF216697	Le et al. (2000)	FhNADI-H1
	Australia	AB207155	Itagaki et al. (2005)	FhNADI-H1
	Algeria all localities	MK212138	Present study	FhNADI-H2
	Egypt	AB554187, 92	Amer et al. (2011)	FhNADI-H2
	China	AB477357	Peng et al. (2009)	FhNADI-H2
	Uruguay	AB207154	Itagaki et al. (2005)	FhNADI-H2
	Iran	GQ356033	Moazeni et al. (2012)	FhNADI-H2
	Algeria Tlemcen, Ain-Temouchent	MK212139	Present study	FhNADI-H3
	Algeria Ain-Temouchent	MK212140	Present study	FhNADI-H4
	Algeria Batna	MK212141	Present study	FhNADI-H5
	Egypt	LC076240, 85	Amer et al. (2016)	
	Egypt	AB554178, 79, 82, 83, 87	Amer et al. (2011)	
	Italy	JF8246779	Farjallah et al. (2013)	
	Ireland	AB207156	Itagaki et al. (2005)	
	Poland	KR422393	Januszkiewicz et al. (2015)	
	Brazil	MF287675	Labruna et al. 2018	
	Japan	LC012035	Ichikawa-Seki et al. (2017)	
	China	AB477357, 58	Peng et al. (2009)	
	Spain	KF111630, 64, 67, 7474	Martínez-Valladares and Rojo-Vázquez (2014)	
	Peru	LC070669	Ichikawa-Seki et al. (2016)	

Discussion

Fasciolosis is a water-borne parasitic zoonosis causing significant economic losses worldwide (Nyindo and Lukambagire 2015). The control of such infections requires epidemiological studies and a prior genetic characterization.

Previous studies have reported that fasciolosis is very common in Algeria (Mekroud et al. 2004; Righi et al. 2016). The overall prevalence observed here in cattle was lower than previous published values in Jilel area (27.0%) (Mekroud et al. 2004) and El Tarf (26.7%) (Ouchene-Khelifi et al. 2018). Only in Souk-Ahras, prevalence was high and reached 22.3%. Ouchene-Khelifi et al. (2018) reported a similar value from El Tarf region. Among the remaining provinces and districts, values ranged between 2.3 and 11.5%. This apparent reduction of prevalence could be associated to several causes, as a change of the biotope, the application of control plans, or a significant drop in host number (Mas-Coma et al. 2008). Further, the use of anthelmintics can explain the observed low prevalence.

Present results are comparable to previous epidemiologic studies from different countries. The overall prevalence was 9.77% in Egypt (ElTahawy et al. 2017) and 12.6% in Tunisia (Hamed et al. 2014). In France, Meissonnier and Mage (2007) reported a fluctuation of the prevalence between 3.7 to 10.8%.

In the present work, both morphometric and molecular analysis of *Fasciola* flukes, collected from eight Algerian provinces, revealed the existence of the single species *F. hepatica*. Actually, using morphometric analysis, one single morphotype was observed. Several studies considered that the ratio body length/body is an appropriate character differentiating *F. hepatica* from *F. gigantica* (Ashrafi et al. 2006; El-Rahimy et al. 2012; Asadian et al. 2013; Aryaeipour et al. 2017). Farjallah et al. (2009) were the first to use molecular markers to characterize Algerian (Mostaganem Province) flukes from sheep. Here, a single ITS-1 haplotype was observed within the eight studied province. The nuclear sequence ITS-1 is a consistent phylogenetic marker allowing the characterization of *Fasciola* species (Itagaki et al. 2005;

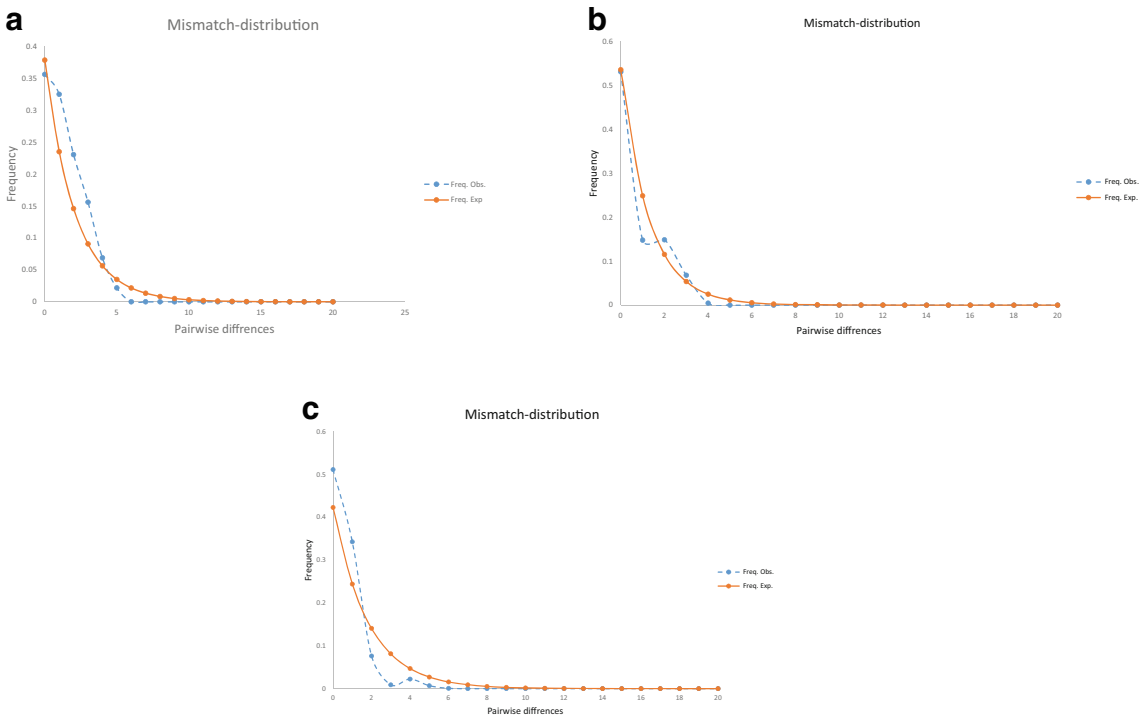

Fig. 3 Mismatch distribution to test the expansion of *F. hepatica* isolates. The number of nucleotide differences between pairs of sequences is indicated along the *X*-axis, and their frequency along the *Y*-axis. COI-trnT-rrnL (**a**), COI (**b**), and NADI (**c**)

Farjallah et al. 2009; Rokni et al. 2010; Amor et al. 2011). Among the studied samples, the haplotype FhITS2-H2 was not observed. It was previously reported in Spain (Alasaad et al. 2007), Algeria (Farjallah et al. 2009), and recently in South America (Valero et al. 2018). However, the new FhITS2-H3 haplotype is described for the first time, as the result of a polymorphism at the position 935 (T/A). This haplotype probably spread throughout Algeria because of movements of infected animals between provinces via nomadism or transhumance systems (Gabli et al. 2015).

Mitochondrial marker results were consistent with previous finding (Itagaki et al. 2005; Farjallah et al. 2009; Amor et al. 2011; Carnevale et al. 2017; Rouhani et al. 2017; Sarkari et al. 2017; Valero et al. 2018) insofar as all Algerian haplotypes belonged to a monophyletic clade—*F. hepatica*. Both COI and NADI datasets showed low nucleotide and haplotype diversity, with seven and five haplotypes for COI and NADI, respectively. However, COI-trnT-rrnL showed low nucleotide diversity and high haplotype diversity. This pattern usually indicates a recent bottleneck followed by rapid population growth (Grant and Bowen 1998).

Median-joining network analysis generated star-like patterns. Common haplotypes (FhCOI-trnT-rrnL-H1, FhCOI-H1, and FhNADI-H1), with basal position within both networks, are probably the source of dispersion of *F. hepatica* through the country, especially within the humid northern region. Moreover, COI and NADI haplotypes appear shared with several countries (Tunisia, Egypt, South Africa, Turkey, France, Italy, and Spain), suggesting that they may have a common origin (Le et al. 2000; Itagaki et al. 2005; Lotfy et al. 2008; Farjallah et al. 2009, 2013; Amor et al. 2011; Simsek et al. 2011; Omar et al. 2013; Martínez-Valladares and Rojo-Vázquez 2014; Wannasan et al. 2014; Mucheka et al. 2015; Valero et al. 2018). Remaining references from Tunisia, France, Italy, Spain, China, Japan, and USA were derived from common haplotypes with one to three nucleotide substitutions. These results suggest that imported cattle might have a significant impact to the entry of *F. hepatica* into Algeria. Srairi et al. (2013) stated that Algerian authorities have ensured the annual supply of imported cattle as a response to the demographic expansion. The majority of cattle are imported from France and Spain, with 40,000/year each.

NJ and ML showed low bootstrap values within the clade of *F. hepatica*, confirming the close relationship between *F. hepatica* haplotypes from different origins.

AMOVA revealed a weak population structure in Algeria. In addition, L-shaped unimodal mismatch distributions for COI, NADI, and COI-trnT-rrnL were observed and star-like patterns for median-joining network analysis, both compatible with population expansions. Negative values of neutrality tests point to the same results. During the last decades, several reports indicated the foot-and-mouth disease (FMD) as the key factor affecting several

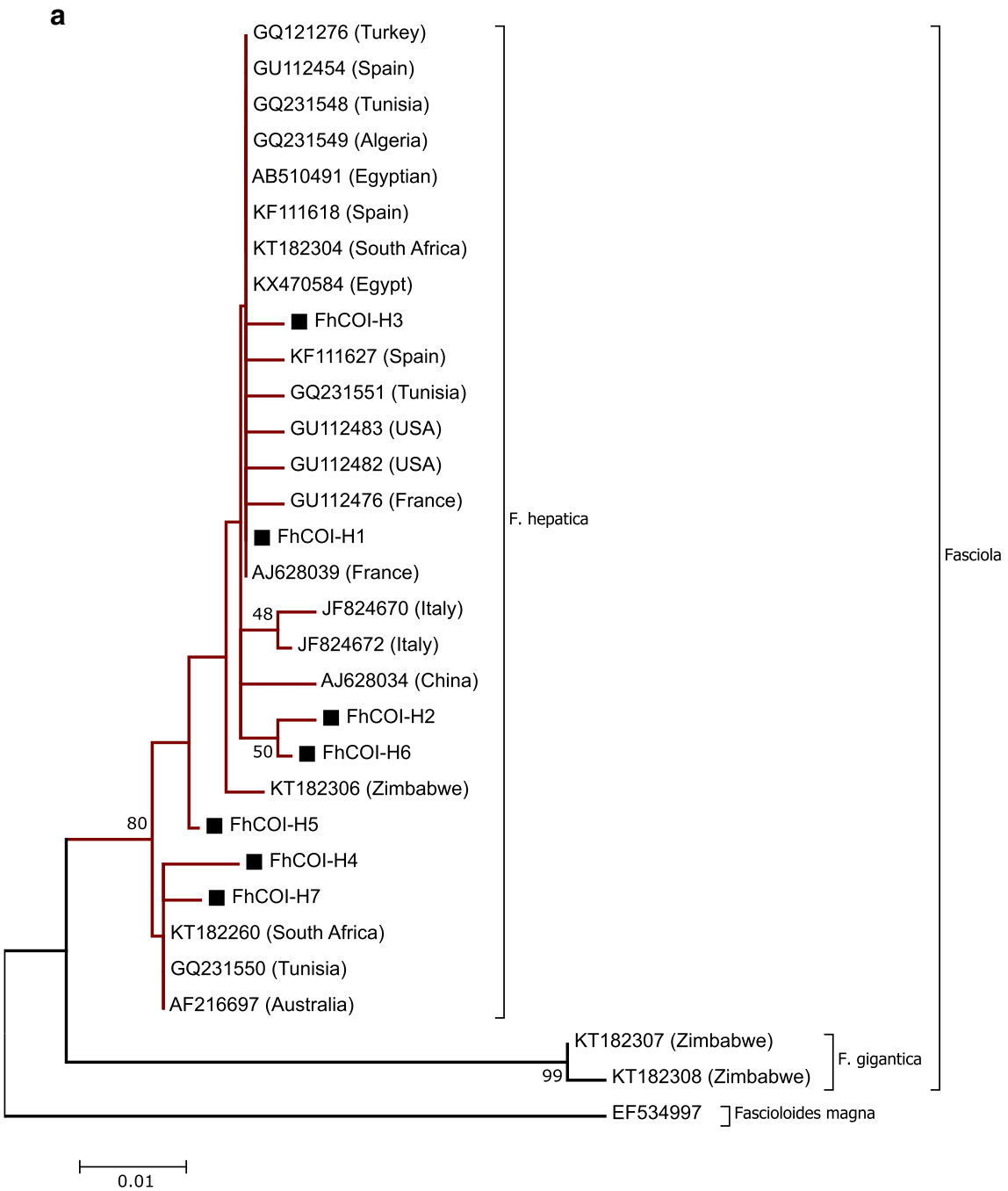

Fig. 4 Consensus trees constructed with ML method showing phylogenetic relationships among *F. hepatica and F. gigantica*. Phylogenies inferred from COI (**a**), NADI (**b**), and COI-trnT-rrnL (**c**) sequences. Numbers shown at nodes of branches are bootstrap values. *Fascioloides magna* (COI, NADI, and COI-trnT-rrnL) was used as outgroup

species farms (bovine, sheep, and goat) in Algeria (Samuel et al. 1999, Kardjadj 2018). In fact, after 2014, an outbreak of FMD destroyed 30% of Algerian cattle. The drop in host species population size is clearly a source of the observed decrease of genetic variability of the parasite. The observed population expansions as well as the reported prevalence could be correlated to the absence of a control strategy in Algeria.

Genetic diversity and expansion of *F. hepatica* population could also be influenced by the intermediate host, *Galba truncatula*, where clonal expansion of *Fasciola* occurs (Beesley et al. 2017). Vilas et al. (2012) confirmed the potential of the intermediate host to disseminate metacercariae with identical genotype in pasture. They also found the same multilocus genotype of *Fasciola* shared between definitive hosts (cattle and sheep). When studying cattle and sheep,

Fig. 4 (continued)

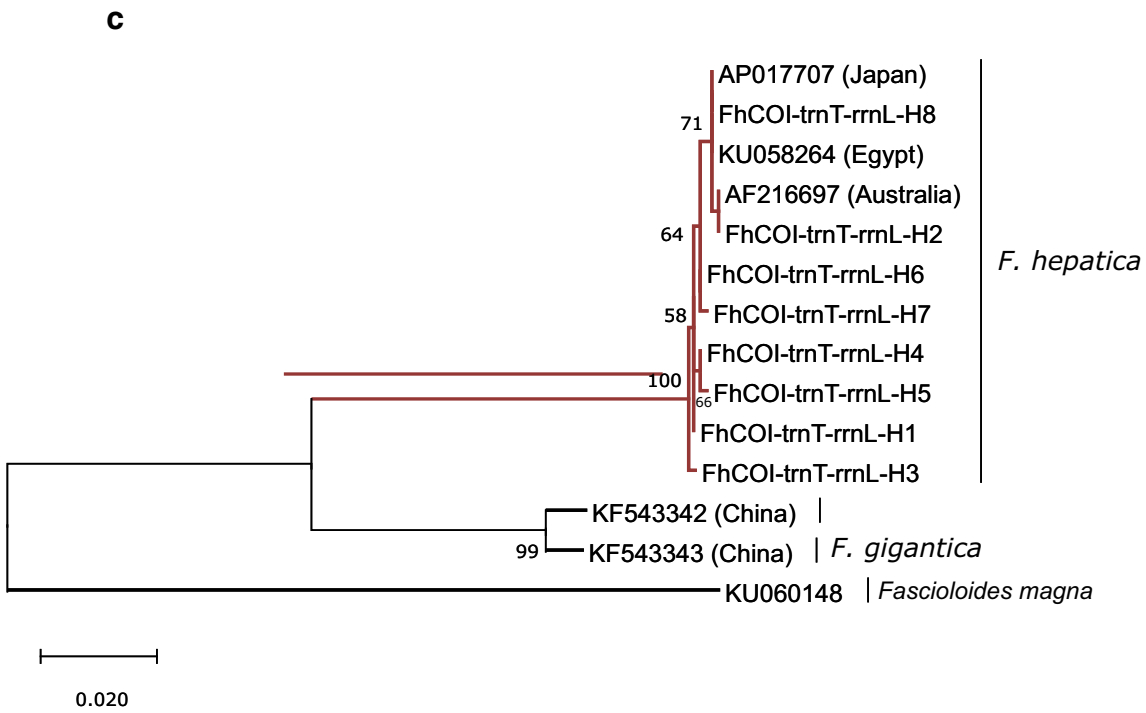

Fig. 4 (continued)

Beesley et al. (2017) found no significant difference in *Fasciola* diversity. *Fasciola* has wide host range which may allow the maintenance of its genetic diversity in wild animals (Beesley et al. 2017).

The present study provides important results concerning the genetic characterization and variability of *F. hepatica* in Algeria as well as the significant role of cattle importation in shaping the dispersal route of *F. hepatica* worldwide. Fasciolosis appeared common in Algerian cattle from the east to the west. Despite Algerian *Fasciola* flukes suffered from recent FMD outbreak, it seems that the absence of control strategy coupled to the favorable Mediterranean climate may lead to a reconstruction and dispersion of its populations.

Algeria is the largest African country; consequently, further studies of *Fasciola* flukes from different hosts and provinces are essential to better understand their genetic history and to develop specific control measures.

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