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Molecular characterization of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers

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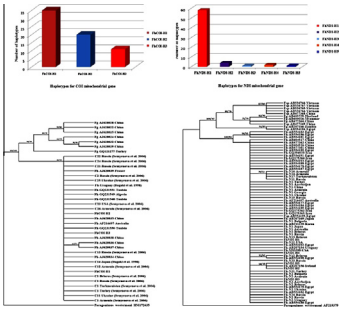
HIGHLIGHTS

- Characterization of F. hepatica based on the ITS1 and 2, the CoxII and NDI genes.
- FhITS-H1 the most frequent haplotype of F. hepatica species from Sardinia.
- Phylogenetic trees showed reliable grouping among F. hepatica from Sardinia.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study is to investigate for the first time the genetic diversity of samples identified morphologically as *Fasciola hepatica* (Platyhelminthes: Trematoda: Digenea) (n = 66) from sheep and cattle from two localities of Sardinia and to compare them with available data from other localities by partial sequences of the first (ITS-1), the 5.8S, and second (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) genes, the mitochondrial cytochrome c oxidase subunit I (COI), and nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1) genes. Comparison of the sequences from Sardinia with sequences of *Fasciola* spp. from GenBank confirmed that all samples belong to the species *F. hepatica*. The nucleotide sequencing of ITS rDNA showed no nucleotide variation in the ITS-1, 5.8S and ITS-2 rDNA sequences among all Sardinian samples, comparing with two ITS-2 haplotypes in standard *F. hepatica*, showing a substitution C/T in 20 position 859, reported previously from Tunisia, Algeria, Australia, Uruguay and Spain. The present study shows that in Sardinian sheep and cattle there is the most frequent haplotype (FhITS-H1) of *F. hepatica* species from South Europe. Considering NDI sequences, the phylogenetic trees showed reliable grouping among the haplotypes of *F. hepatica* from Sardinia and the mitochondrial lineage I, including the main N1 haplotype, observed previously from Europe (Russia, Belarus, Ukraine and Bulgaria), Armenia, West Africa (Nigeria), America (Uruguay and USA), Asia (Turkey, Japan, and China), Georgia, Turkmenistan, Azerbaijan and Australia. Furthermore, common haplotypes FhCOI-H1 and FhCOI-H2 of *F. hepatica* from Sardinia also corresponded mostly to the first lineage including the main C1 haplotype reported previously from Eastern European and Western Asian populations, they belonged just to a phylogenically distinguishable clade, as *F. hepatica* from Australia, France, Turkey, Uruguay, Russia, Armenia, Ukraine, Belarus, Turkmenistan, USA, Tunisia and Algeria, indicating that this is the main haplotype involved in the spread of *F. hepatica* throughout all continents.

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1. Introduction

The two species commonly recognized as the causative agents of fascioliasis in domestic and wild animals and humans are *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Platyhelminthes: Trematoda: Digenea). Fasciolosis is considered the most important helminth infection of ruminants in tropical countries, involved in considerable socioeconomic problems (Spithill and Dalton, 1998). Several studies have shown that *F. hepatica* occurs in temperate areas, *F. gigantica* mainly in tropical zones, and both species overlap in subtropical areas (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2005, 2009; Ashrafi et al., 2006). Recent estimates suggest that more than 90 million people are at risk of fascioliasis with 2.4–17 million individuals infected (Keiser and Utzinger, 2009).

F. hepatica is of European origin, but its geographical distribution has expanded over the last five centuries as a result of global colonisations by Europeans, and the associated continual export of livestock (Mas-Coma et al., 2003). In the last decades, several infections with *F. hepatica* in domestic ruminants have been noted in Europe (Torgerson and Claxton, 1999). These prevalences are 5% in Italy (Poglayen et al., 1995), 10% in Great Britain (Taylor, 1989), 29.5% in Spain (Gonzalez-Lanza et al., 1989), and ranged from 11.2% to 25.2% in central France (Mage et al., 2002).

Despite the importance of *F. hepatica*, the knowledge on its population structure and genetic diversity is limited, and most studies on *Fasciola* spp. have concentrated on interspecific differences. In fact, the two species and their intermediate forms can be discriminated by sequences of the first (ITS-1), the 5.8S and second (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA), 28S ribosomal ribonucleic acid (rRNA) (Adlard et al., 1993; Itagaki and Tsutsumi, 1998; Marcilla et al., 2002; Itagaki et al., 2005a,b; Le et al., 2008; Ichikawa and Itagaki, 2010; Amor et al., 2011a,b; Ai et al., 2011), 18S rRNA (Karimi, 2008), mitochondrial NADH dehydrogenase I (NDI) and cytochrome c oxidase I (COI) genes (Hashimoto et al., 1997; Itagaki et al., 2005a).

To date, some studies have addressed *F. hepatica* intraspecific genetic variability, which is useful for the understanding of speciation, host–parasite interactions, and the origin of drug resistance. Random amplified polymorphic DNA (RAPD) markers were used previously to quantify the genetic diversity in *F. hepatica*. These studies showed that the majority of variance occurred within, rather than between, hosts and that it was also greater within than between populations (Semyenova et al., 2003). Semyenova et al. (2006) have also shown the potential utilization of the mitochondrial genome to develop intraspecific markers to discriminate between *F. hepatica* infrapopulations. In fact, mitochondrial genes NDI and COI were found to be informative, and their sequences have been analyzed for differentiation of Eastern European and Western Asian populations of liver fluke (Semyenova et al., 2006). Among them 13 (ND1) and 10 (COI) haplotypes have been identified. The analysis of the distribution of these haplotypes has revealed two main lineages and although one of them has been suggested to be of Asian origin, both of them have been found in European populations (Semyenova et al., 2006).

The nuclear ribosomal DNA is particularly useful for molecular studies because it is highly repeated and contains variable regions flanked by more conserved regions (Hillis and Dixon, 1991). A part of 28S rDNA region was used to determine genetic heterogeneity of *F. hepatica* isolates in Spain (Vara-Del Río et al., 2007), showing that there was nucleotide variation at one position (corresponding to 105th nucleotide from 28S rDNA) including heterozygous specimens. The first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA) which occurs between the 18S, 5.8S, and 28S coding regions, have been used for diagnostic purposes at the level of species. ITS-2 polymorphisms have been analyzed for liver flukes worldwide (Mas-Coma et al., 2009). Within populations of *F. hepatica* one main widespread genotype and three derivate genotypes typical for particular geographic areas have been defined (Semyenova et al., 2005). Several studies for ITS-2 has established again one major widespread genotype, revealing only one derivate genotype based on a single nucleotide polymorphism from Uruguay, Spain, Tunisia and Algeria (Itagaki and Tsutsumi, 1998; Alasaad et al., 2007; Farjallah et al., 2009).

Non-coding regions of mtDNA (LNR, SNR) have been studied as well. The observed polymorphisms and differences in structural features have been suggested to be associated with the divergence of *F. hepatica* haplogroups (Korchagina et al., 2009). Then, Teofanova et al. (2011) reported clear distinguishable liver fluke populations in far Northern and far Southern regions of Eastern Europe, using different molecular markers, revealing genotypic differences between Greek, Bulgarian and Polish liver fluke populations.

From different European countries, previous studies have characterised genetically *F. hepatica* using molecular techniques, and there are several studies dealing with the genetic characterization of *Fasciola* spp. from France, Spain, Corsica, Ireland, Eastern European (Russia, Belarus, Ukraine, Bulgaria, Armenia) and Turkey (Huang et al., 2004; Semyenova et al., 2005, 2006; Alasaad et al., 2007), but there are no reports characterizing *Fasciola* sp. from Sardinia. The aim of the present work is to describe the molecular characterisation of *F. hepatica* from sheep and cattle from Sardinia, to assess their phylogenetic analysis, and to investigate the genetic variability, in geographically isolated liver fluke populations from Sardinia, African and Asian countries, by sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) and the mitochondrial cytochrome c oxidase subunit I (COI) and nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1) genes.

2. Materials and methods

2.1. Parasites

Adult trematodes ($n = 66$) were collected at necropsy during slaughter inspection from the cattle liver from Valledoria (coastal municipality in North Sardinia) in February 2009, and from sheep from Paulilatino (internal municipality in the middle of Sardinia) in April 2009. Flukes were morphologically identified as *F. hepatica* according to existing keys and descriptions (Ashrafi et al., 2006; Periago et al., 2006), and fixed in 70% ethanol until DNA extraction. Their codes and geographical origins are shown in Table 1.

2.2. DNA extraction, polymerase chain reaction amplification, purification and sequencing

Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer (10 mM Tris, 1 mM EDTA) and kept at −20 °C until use. The polymerase chain reaction (PCR) was carried out in 25 μl of total volume, contained 1 μl of DNA solution (20–40 ng), 2.5 U AmpliTaq Gold (Applera), 10 mM Tris–HCl (pH = 8.3), 50 mM KCl (Applied Biosystems), 3 mM $MgCl_2$ (Promega), 1 mM of dNTPs (dCTP, dGTP, dATP, dTTP; Promega) and 0.25 μM of each primer.

The DNA region comprising ITS-1, 5.8S rDNA and ITS-2 (ITS) was amplified by polymerase chain reaction using primers BD1 (forward: 5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (reverse: 5′-TATGCTTAAATTCAGCGGGT-3′) (Luton et al., 1992). The conserved primers, Ita 8 (forward; 5′-ACGTTGGATCATAAGCGTGT-3′) and Ita

Table 1
Comparison of the ITS sequences of *F. hepatica* from two localities of Sardinia with those from different hosts and geographical locations. Haplotypes of *F. hepatica* specimens observed in this study are at the bottom of table.

Species	Country	Variable sites of ITS region																Accession number/codes of samples
		ITS-1					ITS-2											
		5	95	189	267	287	205	216	229	268	274	282	296	325	332	339	340	
F. gigantica	Thailand	T	T	T	A	T	–	–	–	–	–	–	–	–	–	–	–	AB514854
	Korea	T	T	T	A	T	–	–	–	–	–	–	–	–	–	–	–	AB211238
	Japan	T	T	T	A	T	–	–	–	–	–	–	–	–	–	–	–	AB207146
	Egypt	T	T	T	A	T	–	–	–	–	–	–	–	–	–	–	–	EF612470
	China	T	T	T	A	T	–	–	–	–	–	–	–	–	–	–	–	AB477355
F. hepatica	Korea	C	A	C	T	C	–	–	–	–	–	–	–	–	–	–	–	AB211236
	Japan	C	A	C	T	C	–	–	–	–	–	–	–	–	–	–	–	AB207145
	Ireland	C	A	C	T	C	–	–	–	–	–	–	–	–	–	–	–	AB514850
	China	C	A	C	T	C	–	–	–	–	–	–	–	–	–	–	–	AB514852
	India	C	A	C	T	C	–	–	–	–	–	–	–	–	–	–	–	EF198867
F. gigantica	Egypt	–	–	–	–	–	T	T	C	T	T	C	T	–	A	T	C	EF612482
	Indonesia	–	–	–	–	–	C	T	C	T	T	C	T	–	A	T	C	AB010977
	Japan	–	–	–	–	–	C	T	C	T	T	C	T	–	A	T	C	AB207151
	China	–	–	–	–	–	C	T	C	T	T	C	T	–	A	A	T	AJ557569
F. hepatica	Turkey	–	–	–	–	–	T	T	T	C	C	C	T	T	G	T	A	FJ593632
	Turkey	–	–	–	–	–	T	T	T	C	C	C	T	T	G	T	A	FJ467927
	France	–	–	–	–	–	T	T	T	C	C	C	T	T	G	A	T	AJ557567
	Japan	–	–	–	–	–	T	T	T	C	C	C	T	T	G	T	A	AB207150
	China	–	–	–	–	–	T	T	T	C	C	C	T	T	G	A	T	AJ557568
	Spain	–	–	–	–	–	T	T	T	C	C	T	T	T	G	T	A	AM707030
F. gigantica	Burkina Faso	T	T	T	A	T	T	T	C	T	T	C	T	–	A	T	A	AJ853848
	Kenya	T	T	T	A	T	T	T	C	T	T	C	T	–	A	T	A	EF612472; EF612484
	Vietnam	T	T	T	A	T	C	C	C	T	T	C	T	–	A	T	A	AB385614; EU260063
	Zambia	T	T	T	A	T	T	T	C	T	T	C	C	–	A	T	A	AB514855; AB010976
	Niger	T	T	T	A	T	C	T	C	T	T	C	T	–	A	A	T	AM900371
F. hepatica	Australia	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	AB207140; AB207148
	Egypt	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	EF612468; EF612479
	Spain	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	AM709648; AM709498
	Tunisia	C	A	C	T	C	T	T	T	C	C	T	T	T	G	T	A	GQ231546
	Tunisia	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	GQ231547
	Uruguay	C	A	C	T	C	T	T	T	C	C	T	T	T	G	T	A	AB514848; AB010974
	Niger	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	AM850107; AM900370
Fasciola sp.	Japan	Y	W	Y	W	Y	Y	Y	Y	Y	Y	C	T	T	G	T	A	AB514867; AB207153
F. hepatica	Sardinia (present study)																	
	Valledoria	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	FhBM01–56
	Paulilatino	C	A	C	T	C	T	T	T	C	C	C	T	T	G	T	A	FhPL01–10

9 (reverse: 5′-CCTCATCCAACATAACCTCT-3′), were used to amplify the COI gene and Ita 10 (5′-AAGGATGTTGCTTTGTCGTGG-3′) and Ita 2 (5′-GGAGTACGGTTACATTCACA-3′) for NDI (Itagaki et al., 2005a).

PCR amplification was performed in an Amplitron® PCR System II (Thermolyne) programmed for one cycle of 3 min at 94 °C, 45 cycles of 40 s at 94 °C, 45 s at 55 °C or 53 °C (depending on the primer, 55 °C ITS, 53 °C COI and NDI) and 1 min and 40 s at 72 °C each. At the end, a post-treatment for 5 min at 72 °C and a final cooling at 4 °C were performed.

A negative control (no DNA) was included in all PCR amplifications. Five millilitres of the amplification products were visualized on 1% ethidium-bromide-stained agarose gels to check the quality of amplification.

The PCR products of ribosomal DNA were purified using the commercial kit NucleoSpin Extract (Macherey–Nagel) according to the manufacturer's instructions.

2.3. Sequencing and phylogenetic construction

The purified products of ITS-1, ITS-2 rDNA, NDI and COI were sequenced using an external sequencing core service (Macrogen Inc., World Meridian Center 908, 60–24 Gasan-dong, Gumchun-gu Seoul, Korea). The GenBank Blast program was used for ITS rDNA, NDI and COI comparisons. Sequences were analyzed using Chromas 2.13 software and aligned with published sequences ITS rDNA, NDI and COI of the different Digenea subclass by ClustalW multiple alignments (Thompson et al., 1994) with the default gap and extension penalties used by this program.

COI and NDI sequences were entered in the MEGA for construction of the phylogenetic trees using maximum parsimony (MP) (Tamura et al., 2007). Branch support was given using 1000 bootstrap replicates in MEGA (Hillis and Bull, 1993). PhyML (Guindon and Gascuel, 2003) was used in order to estimate maximum likelihood (ML) phylogenies. We used 1000 bootstrap replicates to assess confidence in the inferred relationships and assign bootstrap support levels to each clade in the maximum likelihood tree. Specific identification was confirmed by comparison with known sequences of the corresponding species in GenBank. COI and NDI sequences, from *Paragonimus westermani*, were used as out-groups to confirm monophyly of each *Fasciola* species.

3. Results

3.1. Genotypic characterization based on the ITS rDNA marker

The 66 ITS PCR products were subjected to direct sequencing giving products 929 bp long and deposited in GenBank (accession Nos. JF824666–JF824669). The sequence was composed of the

complete ITS-1 sequence of 436 bp, the complete 5.8S sequence of 137 bp and the complete ITS-2 sequence of 356 bp, for all samples. Comparison of ITS-1 and ITS-2 sequences of the Sardinian *F. hepatica* samples examined in the present study with those of *F. hepatica* and *F. gigantica* and the "intermediate *Fasciola*" from GenBank confirmed that all the individuals analysed belonged to the single species *F. hepatica* (FhITS1 and FhITS2).

When comparing ITS-1 sequences obtained, the single haplotype of *F. hepatica* (FhITS1) differed from *F. gigantica* haplotype (FgITS1) in five polymorphic sites in positions 5, 95, 189, 267 and 287, including three transitions and two tranversions (Table 1).

While there was no nucleotide variation in the ITS-2 sequences among the 66 *F. hepatica* samples from Sardinia, the published ITS-2 sequences have two haplotypes differing in only one mutation at position 282: haplotype 1 has "C" (FhITS2-H1), whereas haplotype 2 has "T" (FhITS2-H2) (Table 1). According to the sequences deposited in GenBank, the haplotype distribution showed geographical overlap in several countries and areas: FhITS2-H1 in Niger (AM900370), Spain (AM709498), Japan (AB207150), Turkey (FJ593632, FJ467927), Egypt (EF612479), Australia (AB207148), and Sardinia (Table 1); FhITS2-H2 in Spain (AM707030), Uruguay (AB010974), Tunisia (GQ231546) and Algeria (Table 1).

When comparing ITS-2 sequences, the single haplotype of *F. hepatica* (FhITS2) with the most frequent haplotype of *F. gigantica* (FgITS2A: AJ853848, EF612482, EF612484), five polymorphic sites differed between the two species: four transversion in positions 229, 268, 274 and 332, and one indel in position 325 (Table 1).

The single haplotype of *F. hepatica* (FhITS2) observed in the present study differed, also, from *F. gigantica* from China (AJ557569), Indonesia (AB010977), Japan (AB207151) and Niger (AM900371) in 205 nucleotide position including T–C transition. This haplotype of *F. hepatica* (FhITS2) differed to with *F. gigantica* specimens from Vietnam (EU260063) in two C–T transition at 205 and 216 positions (Table 1).

Two additional transversions in positions 339 and 340, inverted relatively to all obtained ITS-2 sequences, were found in *F. gigantica* sequences from Niger (AM900371) and China (EU260079), and also *F. hepatica* sequences from France (AJ557567) and China (AJ557568) (Table 1).

3.2. Genotypic characterization based on mitochondrial (COI and NDI) gene markers

COI sequences (439 bp) of the 66 *F. hepatica* specimens from Sardinia contained 3 variable sites and yielded 3 haplotypes represented by FhCOI-H1 to FhCOI-H3 (accession Nos. JF824670–JF824674). All *F. hepatica* specimens had high pairwise percentage of mitochondrial COI sequences to *F. hepatica* varying between 98% and 100%. This indicates that the specimens from both localities of Sardinia are maternally linked to *F. hepatica*. The sequence analysis showed FhCOI-H1 for 34 specimens, FhCOI-H2 for 21 specimens, and FhCOI-H3 for 11 specimen, respectively, and they were more closely related to *F. hepatica* from China, France, Tunisia, Algeria, Australia, Uruguay, Japan, Ukraine, Turkey, Russia, Belarus, Armenia and Turkmenistan (AJ628038, AJ628035, AJ628037, AJ628036, AJ628034, AJ628039, GQ231548, GQ231551, GQ231550, GQ231549, AF216697, Itagaki et al., 1998; Semyenova et al., 2006). The most common haplotype FhCOI-H1 was detected in both localities investigated, and the nucleotide sequence was identical to that of the C1 haplotype of *F. hepatica* (Semyenova et al., 2006) shown from Belarus, Russia, Turkmenistan, Turkey and Armenia. The sequence of FhCOI-H3, observed in Valledoria matched with the sequence of the *F. hepatica* haplotype from Tunisia, Australia and China (GQ231550, AF216697, and AJ628035). Sequences of FhCOI-H2, detected in both localities, corresponded mostly with the sequences of the haplotype C1 of *F. hepatica* from Belarus, Russia, Turkmenistan, Turkey and Armenia, and were identical to those of the C26 haplotype from Armenia (Semyenova et al., 2006).

Partial NDI sequences (527 bp) was determined for *F. hepatica* specimens from Sardinia and it was found to include 5 variable sites. On the basis of the sequences, flukes were classified into 5 haplotypes, that were very similar to the sequences of *F. hepatica* (FhNDI-H1–FhNDI-H5; accession Nos. JF824675–JF824680). The haplotype FhNDI-H1 was completely identical to that of the haplotype N1 (Semyenova et al., 2006) and those obtained from Russia, Belarus, Ukraine, Bulgaria, Armenia, Azerbaijan, Turkey, Georgia, China, Japan, Korea, Egypt and Australia (Semyenova et al., 2006; AB211239, AB207169, AB554184, AB554177 and AF216697). Homology search for haplotypes FhNDI-H2 to FhNDI-H5 showed about 99% similarities with closest sequences of *F. hepatica* from Egypt, Ireland, USA and Uruguay (AB554182, AB554192, AB554179, AB554180, AB207156, M93388 and AB207154; Garey and Wolstenholme, 1989; Itagaki et al., 2005a; Amer et al., 2011), and different haplotypes (N2, N21, N23 and N24) from Europe and Asia (Semyenova et al., 2006).

3.3. Phylogenetic analyses

Phylogenetic trees were obtained by comparing the sequences of *F. hepatica* and available COI and NDI sequences of other fasciolid species (Figs. 1 and 2). Phylogenetic analyses using the various distance and character methods showed a similar topology of the trees obtained. Bootstrapping of the COI and NDI sequences with maximum likelihood (ML) and maximum parsimony revealed significant support for the clade containing *F. hepatica*, *F. gigantica* and *P. westermani* (Figs. 1 and 2).

All the Sardinian isolates fell into the *F. hepatica* cluster, based on COI and NDI data. Based on COI sequences, phylogenetic analysis indicated that members of the *F. hepatica* group, from Sardinia clustered with the sequences of *F. hepatica* (AF216697, AJ628035, AJ628036, AJ628037, AJ628038, GQ231550), and the haplotype C1 reported by Semyenova et al. (2006) (Fig. 1). Although, the reference COI sequences of *F. gigantica* are placed in a separate group (Fig. 1).

Considering NDI sequences, the phylogenetic tree (Fig. 2) indicated reliable grouping, with a bootstrap support value of 100% (ML) and 99% (MP), among different specimens of *F. hepatica* from Egypt, Europe (mainly Ireland, Russia, Belarus, Ukraine and Bulgaria), America (Uruguay and USA), Asia (Turkey, Japan, Korea and China), Armenia, Georgia, Turkmenistan, Azerbaijan and Australia (Fig. 2). Although the sequences of *F. gigantica* from Africa (Zambia and Egypt) and Asia (China, Vietnam, Myanmar and Thailand) are placed in a separate group (Fig. 2). Both *Fasciola* species are clearly distinguished, and remain so in analyses using other fasciolids as outgroups (Figs. 1 and 2).

4. Discussion

In the present study, adult specimens of *F. hepatica* from sheep and cattle from two localities of Sardinia were characterized by sequencing of the ITS, COI and NDI regions; in fact, previous studies have shown that these sequences provide reliable genetic markers for the accurate differentiation and identification of *Fasciola* spp. (Itagaki and Tsutsumi, 1998; Agatsuma et al., 2000; Huang et al., 2004; Itagaki et al., 2005a,b, 2009; Amer et al., 2011; Amor et al., 2011a,b).

The analyses confirmed that all the sequences from the two host species and localities are identical to those of previously published for *F. hepatica* selected as references (Bargues and Mas-Coma, 2005; Ali et al., 2008; Lotfy et al., 2008; Le et al., 2008; Itagaki et al., 2009; Farjallah et al., 2009; Peng et al., 2009; Rokni et al., 2010; Amor et al., 2011a,b).

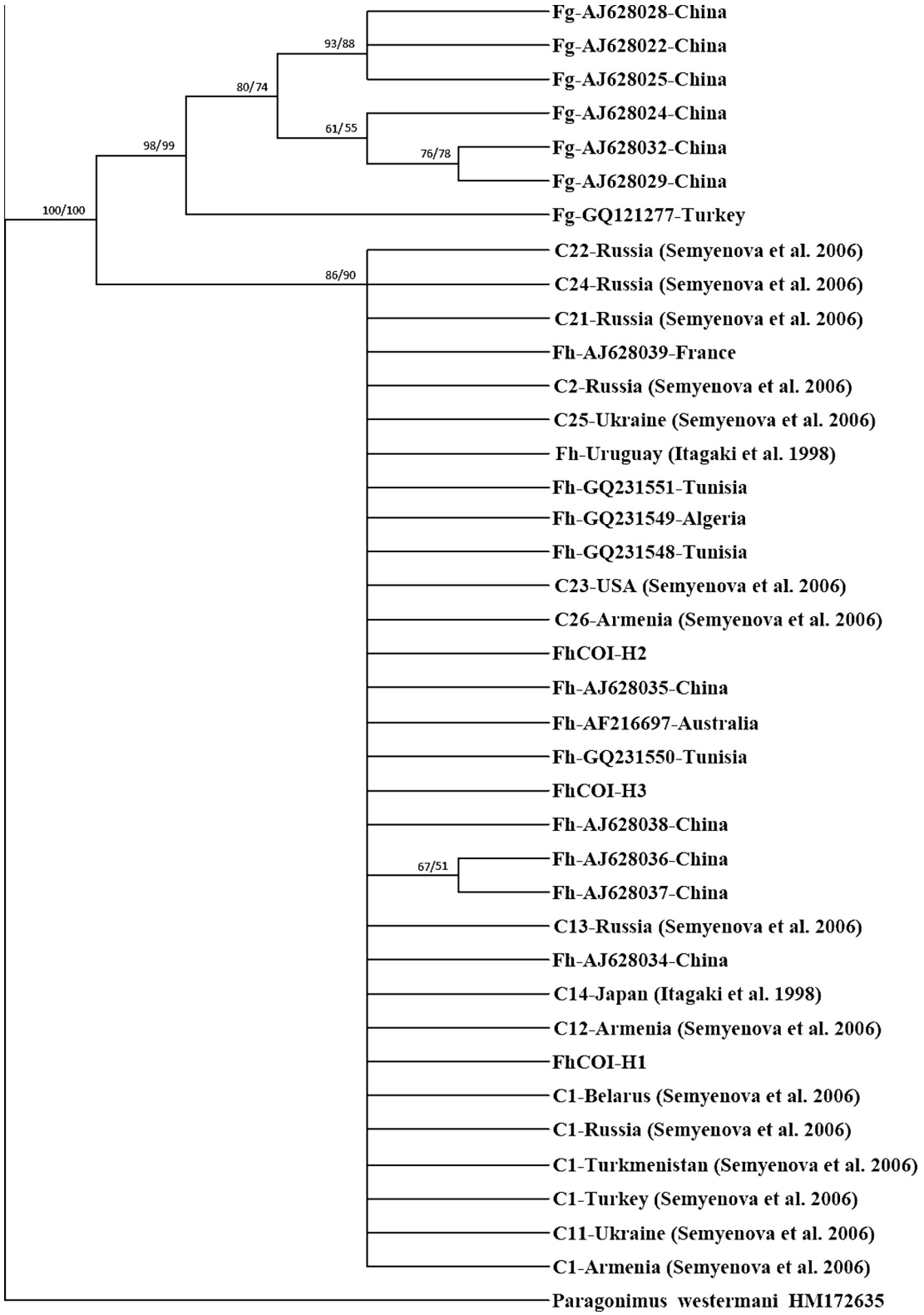

Fig. 1. The phylogenetic relationships of *F. hepatica* from sheep and cattle from Sardinia and other representative isolates (*F. gigantica*, *F. hepatica* and *Fasciola* sp.) from different localities, from COI sequences estimated by Maximum Likelihood (ML) and Maximum Parsimony (MP). Phylogenetic trees were obtained by using PhyML (Guindon and Gascuel, 2003) and MEGA 4.0 (Tamura et al., 2007) with bootstrap values of 1000 replicates set. Sequences from specimens isolated in different hosts from Sardinia are coded as FhCOI-H1–FhCOI-H3. The numbers at the nodes represent the support values in the following order: ML/MP.

Previously Alasaad et al. (2007) using ITS sequencing reported that specimens of *Fasciola* from different localities in the Iberian Peninsula in different hosts belong to *F. hepatica*. From France, Huang et al. (2004) also reported the occurrence of *F. hepatica* by ITS-2 ribosomal DNA sequence, whereas, Simsek et al. (2011) identified for the first time COI–RFLP patterns as either *F. hepatica* or *F. gigantica* from Turkey.

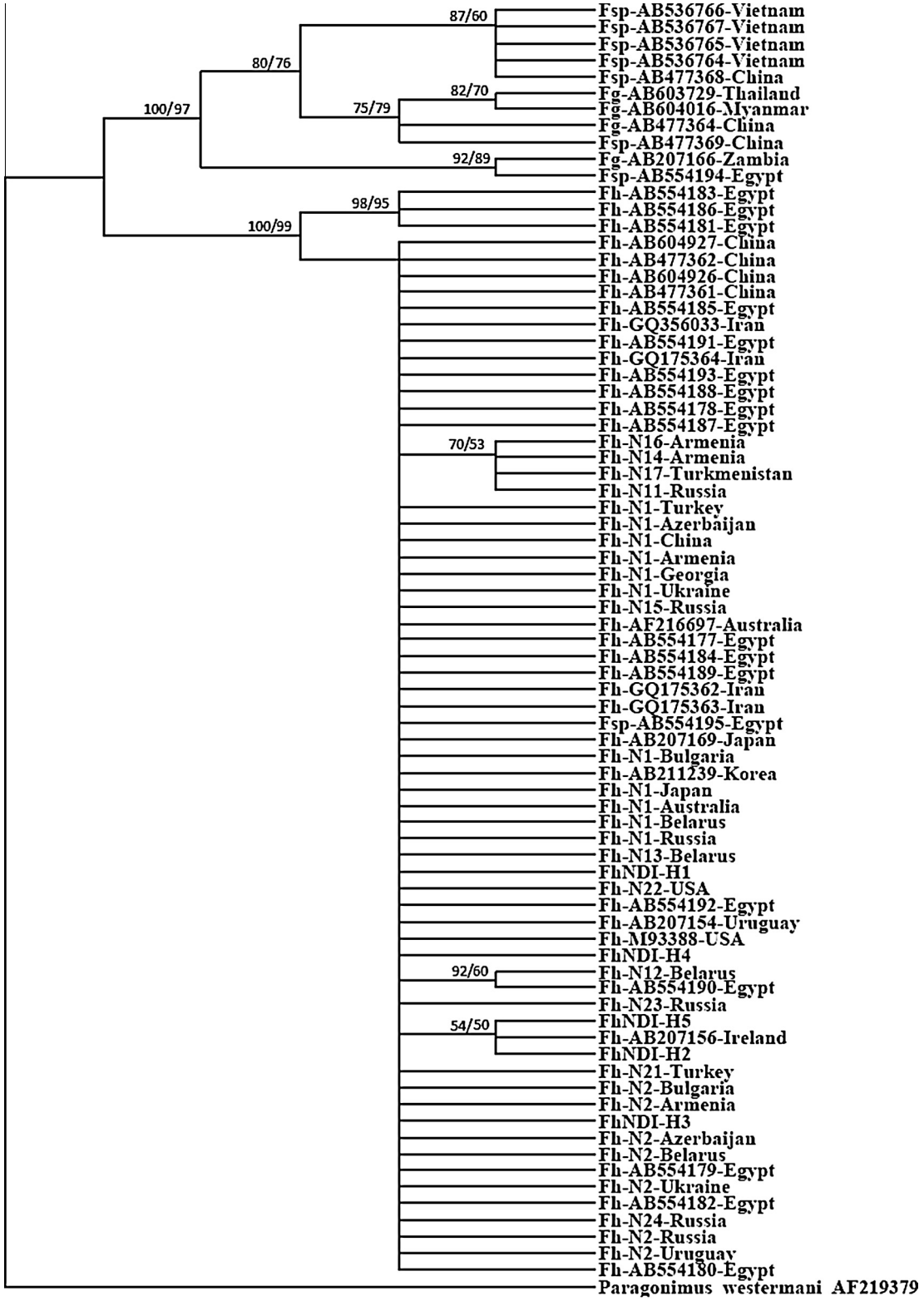

Fig. 2. The phylogenetic relationships of *F. hepatica* from Sardinia and other representative isolates (*F. gigantica*, *F. hepatica* and *Fasciola* spp.) from different localities, from NDI sequences estimated by Maximum Likelihood (ML) and Maximum Parsimony (MP). Phylogenetic trees were obtained by using PhyML (Guindon and Gascuel, 2003) and MEGA 4.0 (Tamura et al., 2007) with bootstrap values of 1000 replicates set. Sequences from specimens isolated in different hosts from Sardinia are coded as FhNDI-H1–FhNDI-H5. The numbers at the nodes represent the support values in the following order: ML/MP.

The ITS-1, 5.8S, and ITS-2 rDNA sequences of *F. hepatica* obtained from Sardinia showed no nucleotide variations and were identical, but the comparisons with ITS2 sequences of *F. hepatica* from other localities showed nucleotide differences at least in one position.

The sequences of the ITS rDNA reported in the present study match with the most frequent haplotype (FhITS-H1) of *F. hepatica*. In fact, the most frequent ITS-2 haplotype (FhITS2-H1) showed a widespread distribution, indicating that this is the main haplotype involved in the spread of *F. hepatica* from Spain (Alasaad et al.,

2007), Australia (Le et al., 2008), Iran (Bargues et al., 2002), Japan (Itagaki et al., 2005b), Korea (Agatsuma et al., 2000), Poland (Mas-Coma et al., 2009), Ukraine, Russia, Armenia, Turkmenistan, Belarus (Semyenova et al., 2005), Vietnam (Le et al., 2008), Egypt (Periago, 2004), Tunisia, Algeria (Farjallah et al., 2009) and Niger (Ali et al., 2008). The second most frequent ITS-2 haplotype of *F. hepatica* (FhITS2-2) differed by a transition in position 282 of the alignment of the two species, but appeared to be less common, being reported from Spain (Alasaad et al., 2007), Australia (Adlard et al., 1993), Uruguay (Itagaki and Tsutsumi, 1998), Tunisia and Algeria (Farjallah et al., 2009). These findings suggest that the above mentioned variants of *F. hepatica*, occurring in isolated countries, may have a common origin, and that they have spread recently throughout these countries because of movement of infected animals. Moreover, it is interesting to note that the *F. hepatica* sequences from France and China (Huang et al., 2004), include two additional transversions in position 341 and 342 near the 3′ end, inverted relatively to all other available sequences, that are claimed by original authors as a sequencing error (Le et al., 2008).

Previously, Semyenova et al. (2006) analysed the distribution of both NDI and COI haplotypes, revealing the existence of 2 well-defined lineages with 2 main haplotypes. The first lineage included the main N1–C1 haplotype, which was found in Australia, China, Georgia, Turkey, Armenia, Azerbaijan, and in all European populations (from Russia, Belarus, Ukraine and Bulgaria). The second lineage was found in all European populations and in populations from Armenia and Azerbaijan (Semyenova et al., 2006). Considering NDI sequences, the phylogenetic trees showed reliable grouping among the haplotypes of *F. hepatica* from Sardinia and the mitochondrial type (N1-lineage I) previously reported by Semyenova et al. (2006) from Europe (Russia, Belarus, Ukraine and Bulgaria), Armenia, West Africa (Nigeria), America (Uruguay and USA), Asia (Turkey, Japan, and China), Georgia, Turkmenistan, Azerbaijan and Australia. As well as, the common haplotypes FhCOI-H1 and FhCOI-H2 of *F. hepatica* from Sardinia corresponded mostly to the first lineage, including the main C1 haplotype previously reported by Semyenova et al. (2006), they belonged just to a phylogenically distinguishable clade, as *F. hepatica* from Australia, France, Turkey, Uruguay, Russia, Armenia, Ukraine, Belarus, Turkmenistan, USA, Tunisia and Algeria, indicating that this is the main haplotype involved in the spread of *F. hepatica* throughout all continents (Semyenova et al., 2006; Mas-Coma et al., 2009; Itagaki et al., 2009; Nguyen et al., 2009).

All the haplotypes of liver flukes from Sardinia were of mitochondrial origin of *F. hepatica*, suggesting the specific maternal linkage inherited from *F. hepatica* in this Island. These results are in contrast to the findings in Turkey, Egypt, China, Niger, Korea and Japan that showed two maternal lineages of *Fasciola* i.e., *F. hepatica* and *F. gigantica* (Itagaki et al., 2005a,b, 2009; Peng et al., 2009; Amer et al., 2011; Simsek et al., 2011). Based on nuclear and mitochondrial markers, the phylogenetic trees showed that the distribution of haplotypes within *Fasciola* spp. revealed geographical variation but did not show significant geographical association. In fact, groups of multiple closely related genotypes of *F. hepatica* from Europe, Africa and several parts of Asia are broadly sympatric and a shallow geographical specialization of genotypes was also found for *F. gigantica*. Such pattern is expected for species with high gene flow, whose populations have not been sundered by long-term biogeographic barriers (Avise, 2000).

The genetic characterization of *F. hepatica* present in Sardinia is useful to achieve the basic information necessary for the field control of this parasite and may have implications for the diagnosis and control of the disease. To better understand the genetic variability and population genetic structure of *F. hepatica* in Sardinia and in other neighbouring areas a wide range of isolates from different hosts and geographical localities and the use of more variable genetic markers are needed; in fact, studies applying rDNA and mtDNA markers are necessary to the understanding of a disease which causes important public health problems worldwide and that involves very heterogeneous epidemiological situations and transmission patterns (Mas-Coma et al., 2009). Such molecular epidemiology baseline will help in designing global control measures and local interventions.

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