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ORIGINAL PAPER

Genetic characterization of *Fasciola hepatica* from Tunisia and Algeria based on mitochondrial and nuclear DNA sequences

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Abstract Fasciolosis caused by *Fasciola* spp. (Platyhelminthes: Trematoda: Digenea) is considered the most important helminth infection of ruminants in tropical countries, causing considerable socioeconomic problems. In the present study, samples identified morphologically as *Fasciola hepatica* from sheep and cattle from different geographical locations of Tunisia and Algeria were genetically characterised by sequences of the first (ITS-1), the 5.8S and second (ITS-2) Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) and mitochondrial Cytochrome c Oxidase subunit I (COI) gene. Comparison of the ITS and COI sequences of the North African samples with sequences of *Fasciola* spp. from GenBank confirmed that all samples from Tunisia and Algeria samples belong to a single species, namely *F. hepatica*. Several specimens from Tunisia and Algeria showed a substitution C/T in position 859 in the ITS-2 sequences, previously reported from Spain, suggesting that the above mentioned variant may have a common origin and spread recently throughout the three countries because of movement of infected animals. This is the first molecular characterization of *F. hepatica* in North Africa which provides a foundation for further studies on *Fasciola* spp. in Tunisia and Algeria.

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Introduction

The digenean trematodes *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Platyhelminthes: Trematoda: Digenea) are common liver flukes of a range of species of animals and have a global geographical distribution (Spithill and Dalton 1998). Previous studies have shown that *F. hepatica* occurs in temperate areas and *F. gigantica* mainly in tropical zones, and both species may overlap in subtropical areas (Mas-Coma et al. 2005).

Fasciolosis caused by *Fasciola* spp. is considered the most important helminth infection of ruminants in tropical countries, involved in considerable socioeconomic problems (Spithill and Dalton 1998). The infection with *Fasciola* spp. represents a major human health problem in diverse parts of Africa such as Egypt, Zambia, Kenya, Algeria, Zimbabwe, Tanzania and Nigeria (Haseeb et al. 2002; Lotfy et al. 2002; Mekroud et al. 2004; Keyyu et al. 2006; Mungube et al. 2006; Pfukenyi et al. 2006; Phiri et al. 2007; Ali et al. 2008), and recently, human infection cases with *F. hepatica* have been documented from southwest Tunisia, with prevalence infection of 6.6% (Hammami et al. 2007).

The intermediate hosts of *Fasciola* spp. are different species of snails: *F. hepatica* has been found on *Bulinus truncatus*, *Galba truncatula* and *Lymnaea truncatula* from North and Southwest of Tunisia, *G. truncatula* from Algeria and Morocco (Hammami and Ayadi 2008; Hamed et al. 2009; Hammami et al. 2007; Mekroud et al. 2004; Khallaayoune et al. 1991), *F. gigantica* on *G. truncatula* from Egypt and *Lymnaea natalensis* from Mali (Dar et al. 2003; Tembely et al. 1995).

F. hepatica and *F. gigantica* can generally be distinguished on the basis of their morphology (Ashrafi et al. 2006), but the use of molecular methods and markers are necessary to distinguish between species and intermediate forms (Marcilla et al. 2002). Several studies have previously genetically characterised both *Fasciola* spp. from different countries using molecular techniques (Marcilla et al. 2002; Huang et al. 2004; Le et al. 2007; Alasaad et al. 2007; Ali et al. 2008; Li et al. 2009), while there are no studies dealing with the genetic characterisation of *Fasciola* spp. from North-Africa. Therefore, the aim of the present work is devoted to characterise *Fasciola* spp. samples from Tunisia and Algeria from different definitive host animals and geographical localities by sequencing the region spanning the Internal Transcribed Spacers (ITS)1, the 5.8S and the ITS2 of nuclear ribosomal DNA (rDNA) and the mitochondrial Cytochrome c Oxidase I (COI) gene.

Materials and methods

Parasites

Adult trematodes (N=65) were collected from the livers of infected sheep and cattle at necropsy from five geographical locations in Tunisia and Algeria between July 2008 and March 2009.

Individual worms were washed extensively in physiological saline solution, identified morphologically as *F. hepatica* according to existing keys and descriptions of Periago et al. (2006) and fixed in 70% ethanol until extraction of genomic DNA. Their codes, host species and geographical origins are listed in Table 1.

Table 1 Haplotype of *Fasciola* specimens observed in this study, their geographical locations and host. *N*: number of parasites analysed; Fhep: *F. hepatica*

Geographical origins	Host species	Haplotype		Number
		ITS	COI	
Tunisia				
Beja	Cattle Sheep	Fhep	Fhep	15
Tunis	Sheep	Fhep	Fhep	15
Monastir	Cattle Sheep	Fhep	Fhep	09
Gafsa	Sheep	Fhep	Fhep	11
Algeria				
Mostaganem	Sheep	Fhep	Fhep	15
Total	Cattle/Sheep	Fhep	Fhep	65

DNA extraction and polymerase chain reaction amplification

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 of Euroclone® Taq DNA Polymerase, 1× reaction buffer, 2 mM of $MgCl_2$, 0.4 µM of both forward and reverse primers and 200 µM of dNTPs mix.

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). Two conserved primers, Ita 8 (forward; 5′-ACGTTGGATCATAAGCGTGT-3′) and Ita 9 (reverse: 5′-CCTCATCCAACATAACCTCT-3′) (Itagaki et al. 2005), were used to amplify the COI gene.

PCR amplification was performed in a MJ PTC-100 Thermal Cycler (MJ research) 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 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. Electrophoresis runs were performed on 2% agarose gels, made using 0.5× TBE buffer and stained with ethidium bromide (10 mg/ml), at 4 V/cm for 20 min. One hundred *bp* ladder (DNA Molecular Weight Marker XIV, Roche®) was used as reference for the bands with each marker.

Sequencing analysis

PCR products, purified by ExoSAP-IT (USB Corporation, under licence from GE Healthcare) following manufacturer's instructions, were sequenced using an external sequencing core service (Macrogen Inc.,World Meridian Center 908, 60-24 Gasan-dong, Gumchun-gu Seoul, Korea).

Sequences obtained were aligned using the software BioEdit 7.0.9.0 (Hall 1999) with previously published *Fasciola* ITS and COI sequences, respectively [GenBank accession numbers: AM900370, AM900371, AM709649, FJ593632, EF612468, AB207141-AB207148, EF612481, AJ853848, EF612472-EF612484, AB300704, AJ628039 and AJ628037 (Ali et al. 2008; Alasaad et al. 2007; Lotfy et al. 2008; Itagaki et al. 2005)].

Results

Analysis of the ITS regions

The ITS fragment amplified from each sample using primers BD1 and BD2 was expected to be approximately 1,000 bp in length. The 65 ITS PCR products were subjected to direct sequencing giving products 918 bp long.

The sequence was composed of the complete ITS-1 sequence of 435 bp, complete 5.8S sequence of 137 bp and complete ITS-2 sequence of 346 bp. Comparison of sequences of the Tunisian and Algerian *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 samples examined represented one single *Fasciola* species, namely *F. hepatica* (Fhep, Table 1).

While there was no nucleotide variation in the ITS-1 and 5.8S rDNA among the 65 *F. hepatica* samples, two different ITS-2 sequences were defined for the examined Tunisian and Algerian *F. hepatica* samples, differing at one nucleotide (0.28%, one of 346) in the ITS-2 (Table 2). They were deposited in the GenBank™ under accession numbers GQ231546 and GQ231547 (Table 2). Indeed, out of 65 specimens identified as *F. hepatica*, 58 isolates (89.23%) yielded 100% homology with *F. hepatica* sequences selected as reference, while seven isolates (10.76%) showed minor variation by differing in one nucleotide, i.e. C/T in position 859 in isolates from sheep and cattle from Tunisia (n=6, 85.71%) and Algeria (n=1, 14.28%).

Analysis of the COI gene

A subgroup of five individuals (one per population) identified as being *F. hepatica* using the ITS sequence were also characterised by obtaining partial COI gene sequences (439 bp), which revealed two haplotypes (GQ231548 and GQ231551) that differ at one site.

These data confirmed the identification of the Tunisian and Algerian samples as *F. hepatica* when compared to

Table 2 Comparison of the ITS sequences of *Fasciola* from North Africa (Tunisia and Algeria) with those from different hosts and geographical locations

Species	Locality	Variable sites of ITS region													Accession number
		ITS 1					ITS 2								
		9	99	193	271	291	782	806	845	851	859	909	916	917	
F. gigantica	Niger	T	T	T	A	T	C	C	T	T	C	A	A	T	AM900371
	Burkina Faso	T	T	T	A	T	T	C	T	T	C	A	T	A	AJ853848
	Kenya	T	T	T	A	T	T	C	T	T	C	A	T	A	EF612472-EF612484
F. hepatica	Niger	C	A	C	T	C	T	T	C	C	C	G	T	A	AM900370
	Spain	C	A	C	T	C	T	T	C	C	C	G	T	A	AM709649
		C	A	C	T	C	T	T	C	C	T	G	T	A	AM709621
	Turkey	–	–	–	–	–	T	T	C	C	C	G	T	A	FJ593632
	Egypt	C	A	C	T	C	T	T	C	C	C	G	T	A	EF612468
	Ireland	C	A	C	T	C	T	T	C	C	C	G	T	A	AB207141-AB207148
	Iran	–	–	–	–	–	T	T	C	C	C	G	T	A	EF612481
Fasciola spp.	Tunisia: *F. hepatica*	C	A	C	T	C	T	T	C	C	C/T	G	T	A	GQ231546
	Algeria: *F. hepatica*	C	A	C	T	C	T	T	C	C	C/T	G	T	A	GQ231547

published sequences (AB300704, AJ628039 and AJ628037).

Discussion

In Asia and Africa, *F. hepatica* and *F. gigantica* appear to be sympatric (Mas-Coma et al. 2005), and this makes it difficult to identify morphologically each species (Alasaad et al. 2007). Previous studies in Africa have shown that *F. gigantica* mainly occurs in Burkina Faso, Senegal, Kenya, Zambia and Mali (Tembely et al. 1995; Mungube et al. 2006; Periago et al. 2006; Phiri et al. 2007), while *F. hepatica* has been reported from Morocco (Khallaayoune et al. 1991), and both species have been observed from Egypt and Niger (Haridy et al. 2007; Lotfy et al. 2008). From Tunisia and Algeria, the presence of *F. hepatica* was detected in domestic ruminants using serology: Tunisia, 14.3% of cattle, 35–55% of sheep and 68% of goats (Jemli et al. 1991; Hammami et al. 2007) and Algeria, 6.3–27.3% of cattle (Mekroud et al. 2004).

In the present study, adult specimens of *F. hepatica* from Tunisia and Algeria were characterised on the base of sequences of ITS and COI regions since 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. 2005).

The ITS sequences obtained confirmed that the sequences of *F. hepatica* from sheep and cattle from Tunisia and Algeria were identical to those of previously published *F. hepatica* (Itagaki et al. 2005; Alasaad et al. 2007; Ali et al. 2008; Lotfy et al. 2008). The ITS sequence analysis revealed a close relationship of the Tunisian and Algerian isolates of *F. hepatica* with those from Niger, Turkey, Egypt, Ireland and Iran (Ali et al. 2008; Lotfy et al. 2008; Erensoy et al. 2009). A polymorphism previously detected in Spain (Alasaad et al. 2007) was found among several individuals from Tunisia and Algeria.

It has been shown that this sequence variation was not related to host species and/or geographical origins of the samples (Alasaad et al. 2007). These findings suggest that the above mentioned variant of *F. hepatica* occurring in Tunisia, Algeria and Spain may have a common origin and spread recently throughout the three countries because of movement of infected animals.

The economic exchanges among these countries have been significant in the past and in the present, and animal migrations and exchanges may have occurred long before among the North African and European countries (Gil et al. 2009).

The present study is the first molecular characterization of *F. hepatica* on animals from North Africa, using ITS and COI as genetic markers. Genetic characterization of *Fasciola* species present in Tunisia and Algeria is a useful tool to achieve the basic information necessary for the field control of this parasite and have implications for the diagnosis and control of the disease they cause. Other investigations, using this method, are needed for further genetic analysis of a wider range of isolates from different host species in order to better understand the genetic structure of *F. hepatica* populations and their transmission dynamics in these and in the neighbouring African countries.

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