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Polymorphism of internal transcribed spacer 2 (ITS-2) sequences and genetic relationships between *Fasciola hepatica* and *F. gigantica*

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Abstract

Nucleotide sequences of internal transcribed spacer 2 (ITS-2) of nuclear DNA were obtained from 58 adult worms *Fasciola hepatica* and *F. gigantica* of naturally infected cattle and sheep in Russia, Ukraine, Belarus, Armenia, Uzbekistan, Turkmenistan and Tajikistan. No variation was observed between 43 liver flukes *F. hepatica* from Russia, Belarus, Ukraine, Armenia and Turkmenistan. Only one specimen from Armenia had a single unique transversion C-G (0.3% variation). *F. gigantica* from Turkmenistan, Tajikistan and Uzbekistan differed at four nucleotide transitions (1.1% variations). For comparative purpose, the ITS-2 sequences of two species from Europe, Africa, Asia, America, Australia and Oceania were used and evolution history of ITS-2 sequences of *Fasciola* species was reconstructed with statistical parsimony network (SPN) method. The relationships between *F. hepatica* and *F. gigantica* from different regions were discussed.

Key words

Fasciola hepatica, *F. gigantica*, second internal transcribed spacer (ITS-2), genetic relationships

Introduction

Two species of liver fluke *Fasciola hepatica* (L., 1758) and *Fasciola gigantica* (Cobbold, 1855) (Trematoda, Fasciolidae) are obligate parasitic trematodes and major pathogens of livestock. *F. hepatica* has a cosmopolitan distribution, whereas *F. gigantica* is presented in the tropical and subtropical regions of Africa and Asia. It can be assumed that *F. hepatica* is of European origin, with the snail *Lymnaea* (*Galba*) *truncatula* as the original intermediate host, while *F. gigantica* appears to be linked to lymnaeids of the *Radix* group in Africa and Asia (Mas-Coma *et al*. 1999, Bargues *et al*. 2001).

The identification of these closely related species based on morphological characters can be difficult especially in local areas, where two species coexist and can interbreed giving hybrids (Lotfy and Hillyer 2003). However, recent advances in molecular biology, in particular, the amplification of specific DNA regions via the polymerase chain reaction and improved direct sequencing techniques, may allow closely related species to be distinguished by comparing their DNA.

Adlard *et al*. (1993) determined partial nucleotide sequences of the ribosomal second internal transcribed spacer (ITS-2) for several isolates of *F. hepatica* and *F. gigantica* from Australia, New Zealand, Hungary, Mexico, Indonesia, Malaysia and Japan. It was shown that the *F. hepatica* and *F. gigantica* sequences differed at six nucleotide sites. More recently, ITS-2 sequences were used to characterize diploid and triploid Japanese, Korean and Chinese worms of the genus *Fasciola* and to compare them with *F. hepatica* and *F. gigantica* (Hashimoto *et al*. 1997, Itagaki and Tsutsumi 1998, Agatsuma *et al*. 2000, Huang *at al*. 2004).

In the present study we determined nucleotide sequences of the ITS-2 in worms from populations of *F. hepatica* and *F. gigantica* of different regions in East Europe, Central Asia and Caucasus and compare them with the previously obtained sequences of *F. hepatica* and *F. gigantica* to estimate the level

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of intraspecific variation and genetic relationships between these species.

Materials and methods

Fifty eight adult worms of *F. hepatica* and *F. gigantica* were obtained from bile ducts of naturally infected cattle and sheep during 2000–2003 at the slaughterhouses in Russia (Moscow region and Mordovia), Ukraine (Sumy region), Belarus (Brest region), Armenia (Echmiatdzin, Artaschat, Gugar, Erevan regions) and Uzbekistan (Kara-Kalpak region). Samples of Turkmenistan (Kirovsk and Bairam-Aly regions) and Tajikistan (Seradzh region) were collected in 1986.

Genomic DNA was extracted from whole frozen flukes or flukes preserved in 70% ethanol as described by Morozova *et al*. (2004). Complete ITS-2 sequences were determined after PCR amplification as reported by Itagaki *et al*. (1998). The PCR products were sequenced directly using the sequencing kit ABI PRISM® BigDye™ Terminator v. 3.1 in the sequencer ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, U.S.A.). Sequence alignment was conducted by eye and the evolution history of ITS-2 sequences of *Fasciola* species was reconstructed with statistical parsimony network (SPN) using program TCS 1.18 (Clement *et al*. 2000).

Results

ITS-2 nucleotide sequences were obtained for 58 specimens from seven geographical isolates of two species. The length of whole ITS-2 region from *F. hepatica* and *F. gigantica* is 362 and 361 bp, respectively. The average G+C content was close to 50%. Few substitutions were noted in ITS-2 from each species (Table I). No variation was observed between worms from the same location and between 43 liver flukes *F. hepatica* from Russia, Belarus, Ukraine, Armenia and Turkmenistan. Only one specimen (0.3%) from Armenia had the single transversion C-G at 217 nucleotide position.

Three isolates of *F. gigantica* (n = 14) from Turkmenistan, Tajikistan and Uzbekistan differed at four nucleotide position (1.1% variable sites). Transition T-C was detected at 334 nucleotide position in single worms from Turkmenistan and Uzbekistan. The same two substitutions (at 425 and 468 position) were found in one worm from Turkmenistan. Transition C-T at 455 position was revealed in one Turkmenian and two Tadjik flukes. The *F. hepatica* and *F. gigantica* sequences differ at 3–9 nucleotide sites (0.8–2.5%) and five of which (including one gap) can be diagnostic.

For comparative purpose, the ITS-2 sequences of *Fasciola* species from several countries were obtained from original articles and GenBank (Table I). SPN for ITS-2 sequences shows the number of nucleotide differences between known *F. hepatica* (H1-H4) and *F. gigantica* (G5-G14) genotypes (Fig. 1). Two nested clades yielded significant association between genotypes and species status. The genotypes of *F. hepatica* or *F. gigantica* associate with one of two distinguished clades A or B. The small A group consists of one main genotype H1 found almost everywhere and two one-step (H2, H4) and one two-step genotypes (H3). The latter three genotypes are characteristic only for Armenia, America, China and France, respectively.

The association between genotypes of *F. gigantica* (clade B) is more complex. The first known sequence from Zambia (G9) turned to be the most close to *F. hepatica* genotypes H1-H4. The second known Zambian G10 was found to be connected to different extent with all Asian genotypes. Among the latter, one can distinguish two most often found G5 and G6 revealed in most of all regions. Despite the single differences between the genotypes G13 and G14, they are more closely related to each other and to the G10 from Africa (one step) than to those from Asia (two or more steps). G7 from Turkmenistan is apparently a derivate between the sequences from Iran and Africa (G13, G10).

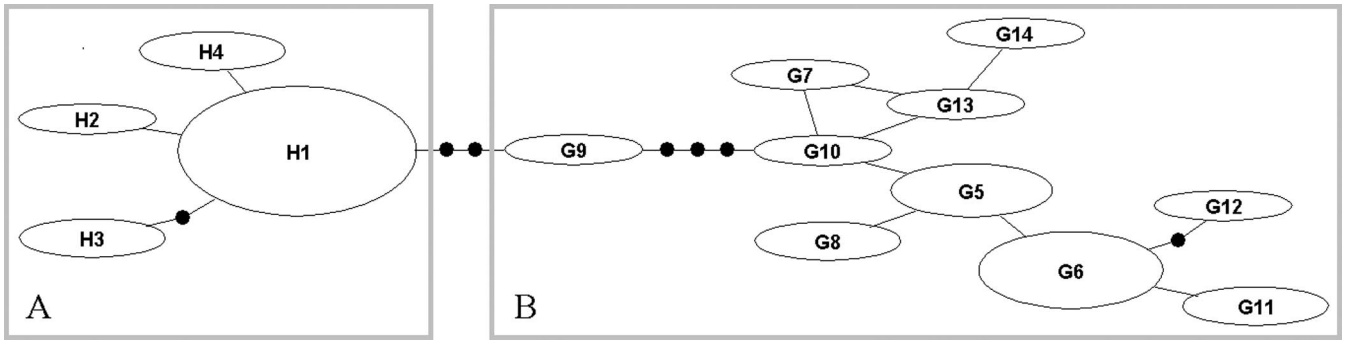

Fig. 1. Statistical parsimony network (SPN) showing the number of nucleotide differences between *F. hepatica* (Group A, H1-H4) and *F. gigantica* (Group B, G5-G14) genotypes derived from ITS-2 sequences. Each branch between two genotypes indicates a single mutational step with black circles representing inferred genotypes. The area of each oval is proportional to the number of regions where this genotype was found

Table I. The distribution of ITS-2 polymorphic sites from different populations of *Fasciola hepatica* obtained from different countries

Species and geographical region	Genotypes	ITS-2 polymorphic sites													N	References	Accession No.
		217	334	345	358	397	403	411	425	451	455	461	468	469			
F. hepatica*	H1	C	T	T	T	C	C	C	T	T	C	G	T	A		1	
Russia	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	8		
Belarus	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	4		
Ukraine	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	3		
Turkmenistan	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	1		
Armenia	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	27		
	H2	G	.	.	.	.	.	.	.	.	.	.	.	.	1		
Hungary	*H1*	.	.	.	.	.	.	.	.	.	.	.	?	?	?	2	
Poland	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	11	3	
Spain	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	?	4	AJ272053
France	H3	.	.	.	.	.	.	.	.	.	.	.	A	T	5	4	AJ557567
Australia	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	
	H1	?	.	.	.	.	.	.	.	.	.	.	.	.	1	6	
	H1	.	.	.	.	.	.	.	.	.	.	.	?	?	?	2	
New Zealand	*H1*	.	.	.	.	.	.	.	.	.	.	.	?	?	?	2	
Egypt	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	?	5	
China	H3	.	.	.	.	.	.	.	.	.	.	.	A	T	30	4	AJ557568
Korea	*H1*	?	.	.	.	.	.	.	.	.	.	.	.	.	1	6	
Iran	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	?	5	
Uruguay	H4	.	.	.	.	.	.	T	.	.	.	.	.	.	3	7	AB010974
Mexico	H4	.	.	.	.	.	.	T	.	.	.	.	.	.	?	2	
Bolivia	H1	.	.	.	.	.	.	.	.	.	.	.	.	.	?	5	
F. gigantica																	
Turkmenistan	G5	.	.	.	C	T	T	.	.	–	.	A	.	.	2		
	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	1		
	G7	.	.	.	C	T	T	.	C	–	.	A	C	.	1		
	G8	.	.	.	C	T	T	.	.	–	T	A	?	.	1		
		?	?	?	?	?	?	.	?	–	.	A	.	.	1		
Uzbekistan	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	2		
	G5	.	.	.	C	T	T	.	.	–	.	A	.	.	1		
		.	?	.	C	T	T	.	.	–	.	A	.	.	1		
		.	?	.	C	T	T	.	.	–	?	A	.	.	1		
Tadjikistan	G5	.	.	.	C	T	T	.	.	–	.	A	.	.	1		
	G8	.	.	.	C	T	T	.	.	–	T	A	.	.	1		
	G8	?	?	?	?	T	T	.	.	–	T	A	.	.	1		
Zambia	G9	.	.	.	A	.	.	.	C	–	.	.	.	.	1	7	AB010975
	G10	.	.	.	C	T	T	.	C	–	.	A	.	.	1		AB010976
Indonesia	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	2	7	AB010977
	G6	?	C	.	C	T	T	.	.	–	.	A	.	.	1	6	
	G6	?	C	.	C	T	T	.	.	–	.	A	?	?	?	2	
Malaysia	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	2	1	
	G6	.	C	.	C	T	T	.	.	–	.	A	?	?	?	2	
Japan	*G11*	?	C	C	C	T	T	.	.	–	.	A	?	?	?	2	
	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	2	1	
	G6	?	C	.	C	T	T	.	.	–	.	A	.	.	1	6	
Korea	G11	?	C	C	C	T	T	.	.	–	.	A	.	.	1	6	
		?	C	?	C	T	T	.	.	–	?	?	?	?	1		
		?	?	.	?	.	.	.	.	–	?	?	?	?	1		
China	G12	.	C	.	C	T	T	.	.	–	.	A	A	T	20	4	AJ557569
Iran	G13	?	.	.	C	T	T	.	C	–	.	A	A	?	1	5	
	G14	?	.	.	C	T	G	.	C	–	.	A	A	?	1		
Egypt	G6	.	C	.	C	T	T	.	.	–	.	A	.	.	?	5	

*Nucleotides and the site numbers are given following Hashimoto *et al*. (1997) (the sequence of *F. hepatica* from Australia (537 bp). ? – missing data. In italics given are the putatively designated genotypes with missing data. References: 1 – Hashimoto *et al*. 1997; 2 – Adlard *et al*. 1993; 3 – Artigas *et al*. 2004; 4 – Huang *et al*. 2004; 5 – Periago *et al*. 2004; 6 – Agatsuma *et al*. 2000; 7 – Itagaki *et al*. 1998.

Discussion

The comparison of ITS-2 sequences from worms of different hosts (cattle, sheep, European bison) and of different countries indicate that only 4 polymorphic sites can be found in 18 geographical localities of *F. hepatica* (Table I). It means that there exists a high species-specific homogeneity of ITS-2 sequences. The majority of Asiatic and American genotypes of *F. hepatica* demonstrate complete homology with the genotypes of European origin and with genotypes from Australia and New Zealand. Only one substitution was found in the genotype of a single worm in Armenia, Uruguay and Mexico (0.3%). Unexpectedly, two identical substitutions (0.6%) in ITS-2 region were identified from flukes in China and France. In contrast, the highest level of intraspecies variability was found in *F. gigantica* populations. With some exceptions, 1 substitution (0.3%) was observed in most populations and 4 substitutions (1.1% variable sites) were revealed in Zambia and Turkmenistan. The difference between African (G9 and G10) and Asian isolates averaged at 3–4 sites (0.8–1.1%).

Nested clade analysis discriminated the *Fasciola* sequences into two separate clades, one for *F. hepatica* (A) and other for *F. gigantica* (B) (Fig.1). The distribution of haplotypes within species showed geographical variation but did not show significant geographical association. The net of multiple closely related genotypes of *F. gigantica* from Asia are broadly sympatric and shallow geographical specialization of genotypes was found for *F. hepatica* as well. Such pattern is expected for species with high gene flow whose populations have not been sundered by long-term biogeographic barriers (Avise 2000).

In that way we can predict that the *F. gigantica* populations differentiated in Asian region giving several lineages. One of them is mainly spread in South-Eastern Asia (G6, G11, G12), and the second lineage can be found in Central Asia. The latter could migrate to Africa where the separation of two *Fasciola* species took place. *F. hepatica* could enter Europe from Africa through Central Asia as well as from South-Eastern Asia. In addition, the present populations of *F. gigantica* from Central Asia may be the results of natural (recent or ancient) hybridization between ancestral lineages of genotypes, which originated from Asian and/or African ancestors. The existence of sexual and asexual reproduction of *Fasciola* species (Itagaki *et al*. 1995, Lotfy and Hillyer 2003) and the occurrence of polyploidization events may have been a source of such lineage differentiation. Lack of data on genotypic diversity of *Fasciola* species in Africa and India makes it difficult to perform fine-scale phylogeographic analysis of extant liver fluke populations and does not allow the origin of regional populations to be unambiguously determined. Further studies with additional molecular markers are needed to determine the population structure and divergence between the two close related trematodes.

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