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Human fascioliasis emergence in southern Asia: Complete nuclear rDNA spacer and mtDNA gene sequences prove Indian patient infection related to fluke hybridization in northeastern India and Bangladesh

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Pakistan
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ABSTRACT

Fascioliasis is a snail-borne zoonotic disease with impact on the development of human subjects and communities. It is caused by two liver-infecting fasciolid trematode species, the globally-distributed *Fasciola hepatica* and the Africa/Asia-restricted but more pathogenic, larger *F. gigantica*. *Fasciola gigantica* is the cause of endemicity in livestock throughout the warm lowlands from Pakistan to southeastern Asia since old times. Human fascioliasis is emerging in this region at present, with an increase of patient reports. Complete sequences of rDNA ITS-1 and ITS-2 spacers and mtDNA *nad*1 and *cox*1 genes were obtained from fasciolid eggs found in the endoscopic bile aspirate from a patient of Arunachal Pradesh, northeastern India. Egg measurements, pronounced ITS heterozygosity, and pure *F. gigantica* mtDNA haplotypes demonstrate an infection by a recent *F. gigantica*-like hybrid. Sequence identities and similarities with the same DNA markers found in livestock from Bangladesh prove the human-infecting fasciolid to present identical ITSs and *nad*1 haplotypes and only one silent transversion in *cox*1 when compared to a widely-spread combined haplotype in animals. In northeastern India and Bangladesh, human fascioliasis emergence appears linked to increasing livestock prevalences due to: ruminant importation from other countries because of the increasing demand of rapidly growing human populations; numerous livestock movements, including transborder corridors, due to the uncontrolled small-scale household farming practices; and man-made introduction of *F. hepatica* with imported livestock into an area originally endemic for *F. gigantica* leading to frequent hybridization. Sequences, phylogenetic trees, and networks indicate that the origins of intermediate/hybrid fasciolids and factors underlying human infection risk differ in eastern and western South Asia. The emergence scenario in southern China and Vietnam resembles the aforementioned of northeastern India and Bangladesh, whereas in Pakistan it is linked to increasing monsoon rainfall within climate change combined with an impact of an extensive irrigation system. Past human-guided movements of pack animals along the western Grand Trunk Road and the eastern Tea-Horse Road explain the *F. gigantica* mtDNA results obtained. Physicians should be aware about these emerging scenarios, clinical pictures, diagnostic techniques and treatment. Government authorities must appropriately warn health professionals, ensure drug availability and improve livestock control.

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1. Background

Fascioliasis is a parasitic disease caused by trematodes of the genus *Fasciola*. Two fluke species are involved: *F. gigantica* originated in south-eastern Africa and is at present distributed in parts of Africa and Asia, and *F. hepatica* originated in near-eastern Asia and is at present distributed in Europe, Asia, Africa, the Americas and Oceania [1,2]. This is a zoonotic disease, with domestic herbivore mammals as reservoirs (mainly sheep, goats, cattle, buffaloes, equines and Old-World camelids) and therefore associated with worldwide veterinary repercussions due to the great economic losses it induces in livestock [3]. It is also important in medicine because of the disease it causes in humans [4].

Many aspects led the World Health Organization to include fascioliasis among the Neglected Tropical Diseases (NTDs) within the group of foodborne trematodiases [5,6], including: (i) its wide spread and distribution [2]; (ii) symptomatology and pathology, with (iii) more pathogenicity due to the large body size of *F. gigantica* than by the smaller *F. hepatica* [7], together with (iv) underdevelopment consequences in children and rural communities [8], including even (v) early postnatal infections [9], (vi) long-term sequelae even in some treated patients [10], as well as a marked immune-modulation effect during both the first, 3–4-month long, invasive, migratory or acute phase [11] and the subsequent, year-long, biliary, chronic or obstructive phase [12]. Recent studies have, moreover, demonstrated that infection by fasciolid flukes may give rise to severe neurologic and ocular manifestations [4], due to the numerous *Fasciola* plasminogen-binding proteins secreted whether by the liver-infecting adult flukes during the chronic phase [13] or by the migratory juvenile flukes along the initial acute phase [14].

Fascioliasis is a freshwater snail-borne disease, with species of the family Lymnaeidea acting as intermediate hosts or vectors. The species *F. gigantica* is mainly transmitted by usually larger, more aquatic snail species of the *Radix* group preferring warmer lowland areas, whose absence in the Americas explain why this fasciolid never colonized the New World [15]. The other species *F. hepatica* is mainly transmitted by small, more amphibious snail species of the *Galba/Fossaria* group inhabiting cooler environments [2]. A very few lymnaeid species have been proved to be able to transmit both fasciolids, such as the invasive *Pseudosuccinea columella* [16] and the Asiatic *R. viridis* [2].

The zoonotic characteristics of this disease, the dependence of its snail vectors from the environment, and the multidisciplinary factors underlying its transmission complexity and marked epidemiological heterogeneity, explain why a One Health approach is needed to assess fascioliasis [17]. Key aspects include: (i) the very low specificity at mammal host level, (ii) the oligoxenous specificity at snail vector level (only gastropods of the family Lymnaeidae), and (iii) the different ethnographies of people throughout [17].

Additionally, fascioliasis poses the problem of being markedly influenced by global change factors, such as those already highlighted in southern Asia, comprising effects of anthropogenic environmental modifications as e.g. irrigation systems in Pakistan [18], human-guided movements of ruminants throughout Asia [2] and importation/exportation of livestock in Bangladesh [19]. At present, southern Asia is also experiencing a great impact of climate change, mainly related to an increase of monsoon rainfall, which has also been proved to be involved in recent human fascioliasis infections in the Pakistani Punjab [18,20]. In India, the impact of climate change has already been highlighted regarding zoonoses [21] and vector-borne diseases [22].

This climate change impact appears to correlate with the emergence of human infection by *Fasciola* in southern Asia, all in all conforming a complex One Health scenario in need for a multidisciplinary assessment of the disease transmission and epidemiological characteristics, as well as for the design of control measures [17]. The term "tip of the iceberg" was used to emphasize the unexpected detection of two human cases in India in 2012 [23]. The many human infections reported thereafter in this country justify this wake-up call [24–37] and may be interpreted as an increasing human infection risk.

In Asia, both *F. gigantica* and *F. hepatica* occur [2], and hybrid forms have been reported in areas where both fasciolid species and respective specific lymnaeid vector species coexist after long time or a *Fasciola* species has been recently imported into an area where only the other *Fasciola* species was previously present [2,19]. This is why genetic techniques enabling for species classification are recommended within a One Health study of fascioliasis in an endemic area in Asia [17].

To understand which factors underlie the aforementioned recently increasing human infection risk by fascioliasis in India, the present study focuses on the molecular characterization of fasciolid eggs found in a patient from Arunachal Pradesh, in the north-eastern part of this country. For this purpose, the complete sequences of the following DNA makers were used: the two internal transcribed spacers ITS-2 and ITS-1 of the nuclear ribosomal DNA (rDNA) and the protein-coding mitochondrial DNA (mtDNA) genes of the cytochrome *c* oxidase subunit 1 (*cox*1) and the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) and their corresponding protein sequences (COX1 and NAD1). An appropriate analysis of the complete sequences of the same markers obtained from fasciolids infecting different livestock species in the nearby Bangladesh is made for the needed interpretation of the results. A further comparison with materials from Pakistan and data from southern China and Vietnam is made to characterize the epidemiological patterns throughout the wide region of southern Asia. This is the first time that the complete sequences of the four aforementioned DNA markers have been used for the assessment of individually isolated eggs from fasciolid flukes infecting a human.

2. Material and methods

2.1. Patient sample and fluke egg material from India

A 55-year-old lady from Arunachal Pradesh, north-eastern India, with a five-year past history of laparoscopic cholecystectomy, presented with complaints of intermittent right upper quadrant abdominal pain and short febrile episodes for three years. The complete clinical picture of this patient including eosinophilia and ultrasonographic observations, triclabendazole treatment, and subsequent follow-up and recovery, was already described before [23]. Given that stool examination for eggs was negative, fluke eggs were obtained in endoscopically aspirated bile. The numerous eggs found were yellowish brown, ellipsoidal, non-embryonated, with a small operculum, and showing measurements consistent with *Fasciola* flukes.

2.2. Livestock samples and adult fluke material from Bangladesh and Pakistan

Adult flukes of fasciolids were collected directly from the livers of different livestock species in Bangladesh, including sheep, goats, cattle and buffaloes. Rubber-coated forceps were used to avoid any structural damage to the flukes. Details of the specimens of livestock species analyzed and their geographical origins of localities, coordinates and agro-ecological zones were already detailed previously [19].

To understand the unexpected sequencing results, two specimens of *Fasciola* from cattle analyzed in the Pothwar Plateau, northern Punjab province, Pakistan, and their complete sequences of the same four rDNA and mtDNA markers were furthermore selected and included for comparison purposes following the same procedures of DNA extraction, amplification and sequencing detailed below. Unfortunately, no appropriate knowledge about complete sequences of the mtDNA markers needed is nowadays found in the literature on molecular studies published in Pakistan.

2.3. DNA extraction from fasciolid eggs and adults

DNA was extracted from a total of 22 fasciolid eggs obtained from the

bile of the female patient from India (Table 1). Fluke eggs were obtained by filtration of the bile fluid using mineral water, through a column of metal sieves with decreasing pore size (0.5 mm, 0.25 mm, 0.125 mm, 0.1 mm and 0.04 mm) to separate fasciolid eggs from the debris. All eggs trapped on the last sieve were recovered in a Petri dish. After filtration, the eggs were collected from the Petri dish and included in glass vials with clean mineral water for their embryogenesis under conditions of 20 °C/20 °C day/night temperature, 90% relative humidity and a photoperiod of 12 h light/12 h darkness in a climatic chamber (VB-0714, Heraeus-Vötsch, Germany). Later, the eggs already containing a fully developed miracidium were individualized and fixed in 70% ethanol. The DNA was finally extracted from each individual embryonated egg.

DNA was also extracted from a total of 25 adult fasciolids obtained from sheep, goats, cattle and buffaloes collected from the four agro-ecological zones (administrative units) of Bangladesh (Table 1). Fasciolid specimens were washed extensively in physiological saline (0.85% NaCl) to remove blood and bile and finally preserved in ethanol 70% until DNA extraction. A small part of the anterior body region of each adult fasciolid was used for this purpose.

Genomic DNA was extracted and individually processed from each of the 22 embryonated eggs and each of the 25 adult fasciolids (Table 1). Materials were suspended in 400 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate SDS) containing 500 µg/ml Proteinase K (Promega, Madison, WI, USA). The digestion was performed for 2 h at 55 °C, including shaking every 15 min. The phenol-chloroform extraction and ethanol precipitation methods were applied for total DNA isolation. The procedure steps were carried out in accordance with the methods described previously [38,39]. Each DNA pellet was dried and resuspended in 30 µl sterile TE buffer (pH 8.0), and subsequently this suspension was stored at −20 °C until needed.

Table 1
Fasciolid materials analyzed according to their countries, geographical localities and hosts.

Fasciolid samples	Country	Geographical origin	Hosts (number of hosts)
Eggs from bile filtrate (ne = 22)	India	Arunachal Pradesh/ North-East India	Human (1)
Fluke adults from liver dissection (na = 25)	Bangladesh	Chittagong/Cox's Bazar/ Chakaria	Goat (1), Cattle (1), Buffalo (1)
		Sylhet/Sylhet/ Sylhet Sadar	Goat (1), Cattle (1), Buffalo (1)
		Rajshahi/ Naogaon/ Shapahar	Cattle (1), Buffalo (1)
		Chittagong/ Rangamati/ Rangamati Sadar	Cattle (1)
		Rajshahi/ Naogaon/ Naogaon Sadar	Sheep (1), Goat (2)
		Rajshahi/ Naogaon/ Patnitola	Sheep (1), Cattle (1)
		Rangpur/ Lalmonirhat/ Lalmonirhat Sadar	Sheep (1), Goat (1), Cattle (1)
		Dhaka/ Mymensingh/ Mymensingh Sadar	Sheep (1), Goat (1), Cattle (1)
		Rajshahi/Bogra/ Dhunat	Goat (1)
		Khulna/ Jhenaidah/ Jhenaidah Sadar	Sheep (1), Goat (1), Cattle (1)
		Khulna/ Jhenaidah/ Shailkupa	Sheep (1)
Fluke adults from liver dissection (na = 2)	Pakistan	Pothwar Plateau/ Punjab	Cattle (1)

ne = number of eggs from the human patient in India.
na = number of adult specimens from livestock of Bangladesh and Pakistan.

2.4. rDNA and mtDNA PCR amplification and sequencing

The selection of the entire nuclear rDNA internal transcribed spacer region, encompassing the spacers ITS-1 and ITS-2, along with the 5.8S gene, and the two mtDNA genes *nad*1 and *cox*1 were used to define haplotypes of the eggs from India and the flukes from Bangladesh. These combined (rDNA and mtDNA) markers have extensively demonstrated their utility in genetically characterizing *Fasciola* species and strains, both locally and regionally, and have been employed in global analyses to assess the dissemination pathways of fasciolids [1,2,40].

The selected rDNA and mtDNA markers were amplified by PCR for each egg/miracidium and each liver fluke individual. Forward and reverse primers used for the amplification of the complete ITS-1, 5.8S, ITS-2 region, and *nad*1 and *cox*1 genes were those already mentioned previously [1,15,39,41]. PCR amplifications were performed in a Verity-96-well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) using the following programs: one cycle of 2 min at 94 °C, 35 cycles of 1 min at 93 °C, 1 min at 55 °C and 1 min at 72 °C each, preceded by 2 min at 72 °C, and followed by a final cooling at 4 °C, for the rDNA intergenic region; and one cycle of 1 min at 94 °C, 40–42 cycles of 1 min at 93 °C, 1 min at 52–55 °C and 2–3 min at 72 °C each, preceded by 5 min at 72 °C and followed by a final cooling at 4 °C, for the mtDNA *nad*1 and *cox*1 genes.

PCR products were purified using the Ultra Clean™ PCR Clean-up DNA Purification System (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). Final DNA concentration (in µg /ml) and the absorbance at 260/280 nm were determined using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

Every molecular marker underwent sequencing in both forward and reverse directions using the dideoxy chain-termination technique. This sequencing process was carried out utilizing the Taq dye-terminator chemistry kit and conducted on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA), with the PCR primers being employed in the amplification procedure.

2.5. rDNA cloning

Cloning procedures were applied to PCR products whose electropherograms obtained by direct sequencing showed double peaks in relevant positions. These procedures were only applied to fluke specimens from livestock of Bangladesh to verify and clarify heterozygotic sequences [19] and in order to also understand the unexpected double peaks observed in the electropherograms from the eggs of the Indian patient (Fig. 1). DNA markers were cloned with pGEM-T Easy Vector System I (Promega, Madison, WI) and introduced in *Escherichia coli* DH5α competent cells, to confirm the identity of the heterozygous sequences. After the growth of colonies, standard PCR of eight different colonies per sample was performed and individually sequenced. DNA sequencing and sequence analyses of the clones were performed as described above.

2.6. Sequence analyses and haplotype identification

The software Sequencher v. 5.4.6 (Gene Codes Co. MI, USA) was used to edit and assemble the sequences of the ITS-1-5.8S-ITS-2 region, and the *nad*1 and *cox*1 genes, and ClustalW to align them by means of default parameters in MEGA X software [42,43]. Corresponding penalties for gaps were included in pairwise and multiple alignments. Total character differences were used to measure the divergence of the sequences within

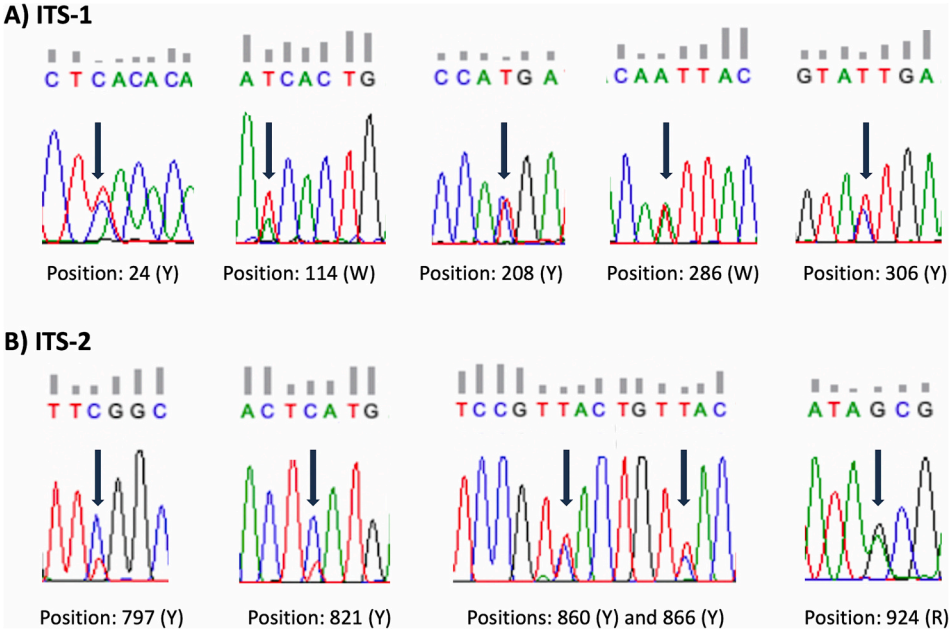

Fig. 1. Detailed parts of the nuclear rDNA intergenic spacer region ITS1–5.8S-ITS2 Sanger sequence chromatogram from the fasciolid eggs obtained in endoscopically aspirated bile from the female patient from Arunachal Pradesh, India, illustrating heterozygosity in the positions differentiating *Fasciola gigantica* from *F. hepatica*: A) ITS-1 parts showing the five heterozygotic positions (indicated by numbers) 24 to 306; B) ITS-2 parts showing the five heterozygotic positions 797 to 924. Position 917 in ITS-2 not included because it is not a mutation but a deletion in one of the alleles (see Fig. 2). The corresponding symbol of IUPAC code for each heterozygotic position is noted in parenthesis.

***Fasciola* species - haplotypes/isolates**	**GenBank Acc. Nos.**	**Country**	**Host**	**Polymorphic sites Intergenic Region (ITS-1, 5.8S, ITS-2)**	
				ITS-1	**ITS-2**
Positions				1223 21080 44866	788888999 923667112 714064974
F. gigantica-1A	AJ853848	Africa[1]	Cattle	TTTAT	TCATTCT-A
F. gigantica-2A	ON661090	Algeria	Sheep	.....	..C....-.
F. gigantica-1/2A*	ON661091	Algeria	Sheep	.....	..M....-.
***F. gigantica*-3A**	OQ064778	Bangladesh,Pakistan	Cattle	.....	C......-.
***FgxFh*-Htz1**	OQ064779	Bangladesh	Sheep,goat,buffalo	YWYWY	YY.YY..-R
***FgxFh*-Htz1**	OQ064779	India	Human	YWYWY	YY.YY..-R
***FgxFh*-Htz2**	OQ064780	Bangladesh	Goat	Y.YW.	C......-R
***FgxFh*-Htz3**	OQ064781	Bangladesh	Cattle	.....	CY.YY..-.
F. hepatica-1A	MG569980	Europe,America[2]	Cattle	CACTC	.T.CC..TG
F. hepatica-2A	MG569978	Spain,America[3]	Cattle	CACTC	.T.CCT.TG
F. hepatica-1/2A**	OQ064782	Ecuador	Sheep	CACTC	.T.CCY.TG
F. hepatica-3A	MK212150	Algeria	Cattle	CACTC	.T.CC.ATG
F. hepatica-674343	KX198626	India	Sheep	CACTC	.T.CC..TG
F. hepatica-65454	KX198627	India	Sheep	CACTC	.T.CC..TG
F. gigantica-APYI2	KT199357	India	Yak	-----	.T.CC..TG
F. sp.-BAPSI	KX467879	India	Sheep	-----	.T.CC..TG
F. hepatica-TSYI5	KT935497	India	Not specified	-----	.T.CC..TG
F. sp.-APZMI	KX509986	India	Not specified	-----	.T.CC..TC
F. gigantica-54343	KX198616	India	Cattle	.....	C......-.
F. gigantica-9687	KX198625	India	Cattle	.....	C......-.
F. gigantica	EF027103	India	Cattle	-----	C......-.
F. gigantica-SHGI7	KU521537	India	Goat	-----	C......-.
F. gigantica-MUBI2	KT199362	India	Buffalo	-----	C......-.
F. gigantica-Shillong	KJ720002	India	Sheep	-----	C......-.
F. gigantica(like)-ZAPCI	KX467878	India	Cattle	-----	C......-.
F. gigantica-SKCI7	KU512806	India	Cattle	-----	C......-.

Fig. 2. Polymorphic sites in the sequence comparison of the complete transcribed spacer region of the nuclear rDNA between the haplotypes of fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan (in bold), and other haplotypes or isolates of *Fasciola hepatica (Fh)*, *F. gigantica (Fg)* and *F.* sp. from GenBank. Sequences and respective haplotypes/isolates vertically ordered according to nucleotide similitudes to facilitate the distinguishing of groupings. Numbers (to be read in vertical) refer to variable positions obtained in the alignment made with MEGA X; . = Identical; – = Indel; - = Not sequenced; Heterozygotic positions represented with the corresponding symbol of IUPAC code for undetermined nucleic acid specification.

[1] Burkina Faso, Niger, N 1 igeria, Senegal, Cameroon.

[2] Spain, France, Poland, Mexico, Venezuela, Peru, Bolivia, Uruguay, Argentina, Ecuador.

[3] Mexico, Bolivia, Uruguay, Ecuador.

* = heterozygotic in position 834/248 not differentiating between F. hepatica and F. gigantica [1].

** = heterozygotic in position 874/288 not differentiating between F. hepatica and F. gigantica [15].

and among each one of the markers. All changes, comprising transitions (ts), transversions (tv) and insertions/deletions (indels), were considered as character states in MEGA X.

In the case of rDNA ITS sequences, a careful inspection of all nucleotide positions in the raw sequence chromatograms allowing for the detection of sequence polymorphisms between *F. gigantica* and *F. hepatica* was done to identify the presence of possible heterozygosity, especially at the polymorphic positions that differentiate between both species (Fig. 1), as previously described [1,15,19]. The ALTER web server [44] was used to condense the aligned sequences into haplotypes, with gaps being considered as variations. Closely related sequences were searched by utilizing the BLASTN programme from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Comparative analyses were performed with complete or almost complete sequences available of ITS-2, ITS-1, *nad*1, and *cox*1 sequences of *F. gigantica, F. hepatica* and undetermined *Fasciola* spp. downloaded from the GenBank.

To identify *Fasciola* species and "pure" or hybrid haplotypes, we use the combined results of the individualized sequencing of each marker for each specimen. In this sense, we defined "pure" haplotypes when ribosomal and mitochondrial markers are in agreement, i.e. all the four markers indicate the same species and therefore allow for a specific classification. We identify hybrids (eggs or adults) when the ITS-1, ITS-2, *nad1,* and *cox*1 markers are read together and the results are discordant in species assignment and/or when heterozygous positions are observed in the ITS-2 and ITS-1 sequences at nucleotide positions that discriminate between the two *Fasciola* species (Fig. 1).

For these comparison purposes, reference sequences used of "pure" haplotypes of *F. gigantica and F. hepatica*, *Fasciola* sp., and hybrids are considered (Figs. 2–6), including:

– 20 sequences of rDNA ITSs of "pure" *F. hepatica* sourced from various regions, such as Europe, the Americas, Algeria and India, and represented by haplotypes including Fh1A (GenBank Accession Nos. MG569980, KX198626, KX198627), Fh2A (MG569978, MG569981), Fh3A (MK212150), and Fh1/2A (OQ064782); 30 sequences of "pure" *F. gigantica* from Africa, Bangladesh and India represented by the haplotypes Fg1A (AJ853848, KX198616, KX198625), Fg2A (ON661090), FG3A (OQ064778) and FgxFh-HtzA (ON661091); and 18 heterozygotic hybrids FgxFh sequences from Bangladesh represented by haplotypes FgxFh-Htz1(OQ064779), FgxFh-Htz2 (OQ064780), and FgxFh-Htz3 (OQ064781).

No.	*Fasciola* spp. haplotypes/isolates	GenBank Acc. Nos.	Country	Host	Nucleotide sequence
	Positions				111111112 1122333367 9235567884 2902346908 6023784093
1	Fg-nad1-1	MT094391	Burkina Faso	Zebu	TTTCGTTGAC GATGCTTAGG
2	**Fg-nad1-20**	OR789889	Pakistan	Cattle	....G.A
3	*Fasciola* sp.	KF543343	China	Cattle	........GT .G...A.G.A
4	**Fg-nad1-17**	OR789886	Bangladesh	Cattle,buffalo	........GT .G...A.G.A
5	**Fg-nad1-17**	OR789886	Bangladesh	Sheep,goat	........GT .G...A.G.A
6	**Fg-nad1-17**	OR789885	India	Human	........GT .G...A.G.A
7	Fsp._ND1-E1	LC012895	India	Cattle	........GT .G...A.G.A
8	Fsp._ND1-E2	LC012896	India	Cattle	........GT .G...A.G.A
9	**Fg-nad1-18**	OR789887	Bangladesh	Buffalo	........TT A....G.G.A
10	**Fg-nad1-19**	OR789888	Bangladesh	Cattle	........TT A....G.G.A
11	Fg_ND1-E6	LC012900	India	Cattle	........TT A....G.G.A
12	Fg_ND1-E11	LC012905	India	Cattle	........TT A....G.G.A
13	Fg_ND1-E12	LC012906	India	Cattle	.....G..TT A....G.G.A
14	Fg_ND1-E4	LC012898	India	Cattle	........GT .....A.G.A
15	Fg_ND1-E3	LC012897	India	Cattle	........GT .....A.G.A
16	*F.hepatica*	AF216697	Australia	Geelong str.	GG.TT.GT.A .GGATGG.A.
17	Fh-nad1-2	MW867318	Spain,Poland	Sheep	GGCTT.AT.A .GGATGG.A.

No.	Nucleotide sequence (continuation)								
	2222222333	3333334444	4444555555	5555555555	5566666666	6666667777	7777777777	7777888888	8
	4567789001	4456771144	6788011112	2222445777	8900123333	4577890112	2455555666	7889012247	9
	6384635366	2519054717	9406924693	5678348469	8409570369	8725796576	7103456235	2578165851	3
1	TTGGTTCTAG	ATGTTGGTGT	ACATCCAGAA	GGTTGTGTGT	GATCTGGGGG	TGTGGGGGTG	CGTTGAATCG	TTATAGGGCG	G
2	G.......GA	G...C.....	.....T....	..GG....A.	.G.T......	C.........	G.GA.GT...	GCT....A..	.
3	G.......GA	G...C.....	G....T....	..GG.C....	.G.T.....A	.......ACA	A..A.GG...	G......A..	.
4	G.......GA	G...C.....	G....T....	..GG.C....	.G.T.....A	.......ACA	A..A.GG...	G......A..	.
5	G.......GA	G...C.....	G....T....	..GG.C....	.G.T.....A	.......ACA	A..A.GG...	G......A..	.
6	G.......GA	G...C.....	G....T....	..GG.C....	.G.T.....A	.......ACA	A..A.GG...	G......A..	.
7	G.......GA	G...C.....	G....T....	..GG------	----------	----------	----------	----------	-
8	G.......GA	G...C.....	GT...T....	..GG------	----------	----------	----------	----------	-
9	G.......GA	G...C.....	.....T....	..GG.C..A.	.G.T......	C.........	G..A.GG...	G......A..	.
10	G.......GA	G...C.....	.....T....	..GG.C..A.	.G.T......	C.........	G..A.GG...	G.........	.
11	G.......GA	G...C.....	.....T....	..GG------	----------	----------	----------	----------	-
12	G.T.....GA	G..CC.....	.....T....	..GG------	----------	----------	----------	----------	-
13	G.T.....GA	G..CC.....	.....T....	..GG------	----------	----------	----------	----------	-
14	G.TA....GA	G.........	.T...T....	.TGG------	----------	----------	----------	----------	-
15	G..A....GA	G.........	.T...T....	.TGG------	----------	----------	----------	----------	-
16	....CGTCTA	.GTG.ATCTG	.TGAT.TTGG	T.AAAAAC.G	A.CA.TATAA	.TCTAAAA.A	TA..AGGCTA	G..GGAT.GT	T
17	.C..CGT.TA	.GTG.ATCTG	.TGAT.TTGG	T.AAAAA..G	A.CACTATAA	.T.TAAAA.A	TA..AGGCTA	G..G.AT.GT	T

Fig. 3. Nucleotide differences found in the complete mtDNA *nad*1 gene sequence between the haplotypes of fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan (country and host in bold) and other reference sequences of *Fasciola hepatica (Fh)*, *F. gigantica (Fg)* and *F.* sp. from GenBank. Haplotypes/isolates in bold = new haplotypes. Sequences and respective haplotypes/isolates vertically ordered according to nucleotide similitudes to facilitate the distinguishing of groupings. Numbers (to be read in vertical) refer to variable positions obtained in the 903 bp-long alignment made with MEGA X; . = Identical; – = Not sequenced.

Fasciola species haplotypes/isolates	GenBank Acc. Nos.	Country	Host	Amino acid sequence
Positions				1 1111111222 2222222 1299990 5777778334 5566899 4782002596 7012562693 2823218
Fg-nad1-1/NAD1-I	MT094391	Burkina Faso	Zebu	FFLFMAGLLG IASSSVLVVL ESVMTVC
Fg-nad1-20/NAD1-IV	OR789889	Pakistan	Cattle	.........S ..F..G...V GAAL...
Fasciola sp.	KF543343	China	Cattle	.........S V.F..G..II GA.....
Fg-nad1-17/NAD1-II	OR789886	Bangladesh	Cattle,buffalo	.........S V.F..G..II GA.....
Fg-nad1-17/NAD1-II	OR789886	Bangladesh	Sheep,goat	.........S V.F..G..II GA.....
Fg-nad1-17/NAD1-II	OR789885	India	Human	.........S V.F..G..II GA.....
Fsp.ND1-E1	LC012895	India	Cattle	.........S V.F..G---- -------
Fsp.ND1-E2	LC012896	India	Cattle	.........S V.F..G---- -------
Fg-nad1-18/NAD1-III	OR789887	Bangladesh	Buffalo	....I....S ..F..G...V GA.....
Fg-nad1-19/NAD1-III	OR789888	Bangladesh	Cattle	....I....S ..F..G...V GA.....
Fg_ND1-E6	LC012900	India	Cattle	....I....S ..F..G---- -------
Fg_ND1-E11	LC012905	India	Cattle	....IS...S ..F..G---- -------
Fg_ND1-E12	LC012906	India	Cattle	...VIS...S ..F..G---- -------
Fg_ND1-E4	LC012898	India	Cattle	.....SS..S ..F..W---- -------
Fg_ND1-E3	LC012897	India	Cattle	......S..S ..F..W---- -------
F.hepatica	AF216697	Australia	Geelong str.	LVFL...VFS .V.CGEMIIF SA..SLF
Fh-nad1-2/NAD1-I	MW867318	Spain,Poland	Sheep	LAFL...VFS .V.CGEMIIF SA..SLF

Fig. 4. Amino acid differences found in the complete mtDNA NAD1 gene protein between the haplotypes of fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan (country and host in bold) and other reference sequences of *Fasciola hepatica (Fh)*, *F. gigantica (Fg)* and *F.* sp. from GenBank. Haplotypes/isolates in bold = new haplotypes. Sequences and respective haplotypes/isolates vertically ordered according to amino acid similitudes to facilitate the distinguishing of groupings. Numbers (to be read in vertical) refer to variable positions obtained in the 301 aa-long alignment made with MEGA X; . = Identical; – = Not sequenced.

Additionally, ten partial sequences (only ITS-2) of *F. gigantica, F. hepatica* and *Fasciola* sp. from India [45,46] were considered for sequence comparisons (Fig. 2).

- 6 complete *nad*1 and *cox*1 gene sequences including the mtDNA genome of *F. hepatica* (Australia: AF216697), and *Fasciola* sp. (China: KF543343), as well as *F. gigantica* from Africa (MT094391; MT094380) and *F. hepatica* from Europe and America (MW867318; MW867324) (Figs. 3–6, respectively).
- 9 partial *nad*1 sequences (LC012900, LC012905, LC012906, LC012898 LC012897, LC012895, LC012896) [47] and two partial *cox*1 sequences (KT334158, KT347282) [46] of *F. gigantica* and *Fasciola* sp. from India, representing different phylogenetic haplogroups and isolates (Figs. 3-6, respectively).

2.7. DNA haplotype code nomenclature

The haplotype nomenclature used is organized by first identifying the species with a two-letter abbreviation (i.e. Fh, Fg) or, in the case of hybrids (Fh/Fg or Fg/Fh, concerning nuclear rDNA before the slash and mtDNA after the slash), followed by the haplotype (H) code, according to the previously proposed combined haplotyping (CH) nomenclature [1,41,48]. According to this nomenclature, ITS-2 haplotypes are defined by numbers, and ITS-1 haplotypes by capital letters.

In the case of hybrid haplotypes detected by heterozygosity, these are indicated by Htz (instead of H) followed by the corresponding number. For example: FgxFh-ITS1-Htz2/Fg-H-nad1–3 refers to a specimen in which the nuclear rDNA ITS1 shows the heterozygotic haplotype 2 (the species abbreviation first noted in FgxFh considers the phenotype, in this case a *F. gigantica*-like specimen) and the mtDNA of the *nad*1 gene shows the haplotype 3 of *F. gigantica*. When the nuclear rDNA marker used is the whole intergenic spacer region (ITS1–5.8S-ITS2) instead of only one of the two spacers, ITSs should be noted instead of ITS1 or ITS2 (example: FgxFh-ITSs-Htz2).

Numbers are also utilized for the nucleotide and protein haplotypes of the mtDNA *nad*1 and *cox*1 genes (protein haplotypes noted in capital letters NAD1 and COX1). Worth mentioning is that haplotype codes are only definitive when the sequences are complete, i.e. full length sequences. When dealing with fragments or incomplete sequences, haplotype codes are considered only provisional.

2.8. Phylogenetic analyses

Two different phylogenetic analyses were performed for each one of the mtDNA markers separately, including all the *nad*1 and *cox*1 haplotypes identified in this study plus appropriately selected reference haplotypes/isolates obtained from GenBank (Figs. 3–6) and the Asiatic representant of the genus *Fascioloides*, *F. jacksoni* (ON713419 and ON733331, respectively) [1]. The data matrix of the *nad*1 and *cox*1 trees included 18 and 14 sequences and 903 and 1554 positions in the final data sets, including the also Asian fasciolid *Fasciolopsis buski* from Vietnam (MF287793 and MF287794, respectively) [49] as outgroup.

The best substitution model selection analysis was run in MEGA X [43], considering the BIC (Bayesian Information Criterion) scores, the AICc (Akaike Information Criterion, corrected) value, the Maximum Likelihood (lnL) value, and the number of parameters (including branch lengths) for each model. The evolutionary history was inferred by using the Maximun Likelihood (ML) method. The initial tree(s) for the heuristic search was automatically obtained using the Nearest-Neighbor-Interchange (NNI) method by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. To assess the reliability of the nodes in the trees, a bootstrap analysis using 1000 replicates was made using the Bootstrap method in MEGA X.

2.9. Haplotype networks

Haplotype networks were generated to depict relationships within the *Fasciola gigantica* clade using samples from India, Bangladesh and Pakistan, plus one sample appropriately selected from China and another from Burkina Faso representing African countries, with PopART 10.2.0.0 software [50]. Networks were constructed with the sequences obtained from *nad*1 and *cox*1 molecular markers, using the median-joining (MJ) inference method in PopART. Hypothetical median sequence vectors (a hypothesized, unsampled sequence that is required to iteratively connect existing sequences within the network) were added to the network to obtain the shortest connection between the data set.

Population genetic statistics including Tajima's D statistic [51], AMOVA [52], nucleotide diversity, segregating sites and parsimony-informative sites were calculated with DnaPv6.12.03 [53].

No.	*Fasciola* spp. haplotypes/ isolates	GenBank Acc. Nos.	Country	Host	Nucleotide sequence
	Positions				111111111 23556799 9123446677 3911249234 6738175817
1	Fg-cox1-1	MT094380	Burkina Faso	Zebu	TAACTGTATA TGTGTGGGGT
2	**Fg-cox1-18**	OR717616	Pakistan	Cattle	...T..G... .....AG
3	*Fasciola* sp.	KF543343	China	Cattle	....C...AG
4	**Fg-cox1-14**	OR717609	Bangladesh	Livestock*	....C...AG
5	**Fg-cox1-15**	OR717610	Bangladesh	Buffalo	......G... ....C...AG
6	**Fg-cox1-16**	OR717611	Bangladesh	Cattle	......G... ....C...AG
7	**Fg-cox1-17**	OR717615	India	Human	....C...AG
8	Fg-MLCC3	KT334158	India	Zebu	---------- ----------
9	Fg-AZCC3	KT347282	India	Zebu	---------- ----------
10	*F. hepatica*	AF216697	Australia	Geelong str.	GGG.GTGGGG GAAT.ATT.G
11	Fh-cox1-5	MW867324	Spain, Poland	Cattle	GGG.GTGGGG GAAT.ATT.G

No.	Nucleotide sequence (continuation)						
	1122222222 7800234444 8317273679	2222333333 5579000234 2534679802	3333333344 4456678900 5913957502	4444445555 0245880011 8049491323	5555555555 1334456677 9154661739	5666666677 8025569900 5951761625	7777777778 1122356680 3736540204
1	GTATGATGCT	GTGTGCTTGG	GAGGTCTAGT	GATGAGGGCT	TATTGAGGTG	AGATTTTTGG	TAGTGATAAG
2	..G.....	.....	....CTG...	A...G.A.T.	.....A.A	...GC.....	.....G..
3	..G.....	..A.....	....CTAT..	A.....T.	..C....A..	...G.CA..A	CG..A..G..
4	..G.....	..A.....	....CTA...	A.....T.	.....A..	...G.C...A	....A..G..
5	..G.....	.....	....CTG...	A.....A.T.	.....A..	...G.C....	.....G..
6	..G.....	...C.....	....CTG...	A.....A.T.	.....A..	...GCC....	.....G..
7	..G.....	..A.....	T...CTA...	A.....T.	.....A..	...G.C...A	....A..G..
8	----------	----------	----------	------A.T.	.....A.A	...GCC....	.....G..
9	----------	----------	----------	----------	----------	--------T.	.....G..
10	AAGGTG.ATG	A...AT.CTA	.GAA.AG.AG	.GGA.A.A.G	GG.CAGA.AT	GAGAG..GT.	.GAC.G..GA
11	AAGGTGCATG	AG..ATG.TA	.GAA.GG.AG	.GGA.A.A.G	GG.CAGA.AT	GAGAG..GT.	.GAA.GC.GA

No.	Nucleotide sequence (continuation)							
	8888999999 2279223345 2867170951	9999000000 6689000111 3649259148	0001111111 2890345888 6054768058	1222222222 9012234778 7981860255	2222233333 8899902556 6706920010	3333333444 6667899112 3581006096	4444444444 3344556889 4739251251	5555 1122 4514
1	GAGTTTGTTA	GTTGAAGTTG	GGTTGTTATG	GTGGTTGTTT	GTTGTTTATT	ATTTTTGTTC	TGGCTGGTTG	CGTG
2	AGA.....	A..A.G....	..AGT.C...	.....C....	.....G.	.....	..AT....C.	...A
3	AGA.CC....	...A.G....	..GGT.C...	.....C....	.....G.	.....T	..A...A.C.	...A
4	AGA.CC....	...A.G....	..GGT.C...	.....C....	.....G.	.....T	..A...A.C.	....
5	AGA.....	A..A.G....	..AGT.C...	.....C....	.....G.	.....	..AT....C.	....
6	AGA..C....	A..A.G....	..AGT.C...	.....C....	.....G.	.....	..AT....C.	....
7	AGA.CC....	...A.G....	..GGT.C...	.....C....	.....G.	.....T	..A...A.C.	....
8	AGA.....	A..A.G....	..--------	----------	----------	----------	----------	----
9	AGAC.G....	A....G....	..GGT-----	----------	----------	----------	----------	----
10	A....ATCCG	.GA.G.AAGA	ATAG.ACGGT	TCATG.ACCG	CGGTCGGT.C	GCGACGACAT	CTA.GT.A.A	TTC.
11	A....GTCCG	.GA.G.AAGA	ATAGAACGGT	TCATG..CCG	CGGTCGGT.C	G.GACGACAT	CT..GT.G.A	TTC.

Fig. 5. Nucleotide differences found in the complete mtDNA *cox*1 gene sequences between the haplotypes of fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan (country and host in bold) and other reference sequences of *Fasciola hepatica (Fh)*, *F. gigantica (Fg)* and *F.* sp. from GenBank. Haplotypes/isolates in bold = new haplotypes. Sequences and respective haplotypes/isolates vertically ordered according to nucleotide similitudes to facilitate the distinguishing of groupings. Numbers (to be read in vertical) refer to variable positions obtained in the 1533 bp-long alignment made with MEGA X; . = Identical; – = Not sequenced; * = Sheep, goat, cattle, and buffalo.

2.10. Sequences in DNA repositories

New sequences of fasciolids from the patient in India and those from livestock in Bangladesh and Pakistan obtained in this study have been deposited in the GenBank Data Library under Accession Nos. OR789885-OR789889; OR717609-OR717611; and OR717615-OR717616. All other sequences used for comparison purposes, whether of pure or hybrid haplotypes of fasciolids, are appropriately identified with their corresponding Accession Nos. according to codes in the GenBank Data Library.

3. Results

3.1. Egg measurements

Fasciolid eggs recovered from the bile of the patient were measured with a calibrated microscope. The variability of length/maximum width measurements of 30 eggs were 157.5–190.0/85.0–107.5 μm (mean

Fasciola species Haplotypes/isolates	GenBank Acc. Nos.	Country	Host	Amino acid sequence
Positions				1111 1111112222 3333444444 4445 123660123 3677881345 3449113555 6670 4415346305 7514292415 1037372478 7998
Fg-cox1-1/COX1-I	MT094380	Burkina Faso	Zebu	IRLIVILSMY GIGTWISFVM IVVIFVCLFI LVRA
Fg-cox1-18/COX1-IV	OR717616	Pakistan	Cattle	.C..... .V.I..... .....V.. ....
Fasciola sp.	KF543343	China	Cattle	.....F ...IR..IA. .....V.. ..C.
Fg-cox1-14/COX1-II	OR717609	Bangladesh	Livestock*	....I..... .....V.. ..C.
Fg-cox1-15/COX1-III	OR717610	Bangladesh	Buffalo	....I..... .....V.. ....
Fg-cox1-16/COX1-III	OR717611	Bangladesh	Cattle	....I..... .....V.. ....
Fg-cox1-17/COX1-II	OR717615	India	Human	....I..... .....V.. ..C.
Fg-MLCC3	KT334158	India	Zebu	---------- --.I..... ...------- ----
Fg-AZCC3	KT347282	India	Zebu	---------- --------.. ...------- ----
F. hepatica	AF216697	Australia	Geelong strain	M.VVIMFPV. S.D..VG..V MMIVVIA.LV VICV
Fh-cox1-5/COX1-I	MW867324	Spain,Poland	Cattle	M.VVIM..V. S.D..VG..V MMIVV.A.LV VICV

Fig. 6. Amino acid differences found in the complete mtDNA *cox*1 gene protein between the haplotypes of fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan (country and host in bold) and other reference sequences of *Fasciola hepatica (Fh)*, *F. gigantica (Fg)* and *F.* sp. from GenBank. Haplotypes/isolates in bold = new haplotypes. Sequences and respective haplotypes/isolates vertically ordered according to amino acid similitudes to facilitate the distinguishing of groupings. Numbers (to be read in vertical) refer to variable positions obtained in the 511 aa-long alignment made with MEGA X; . = Identical; – = Not sequenced; * = Sheep, goat, cattle and buffalo.

168.6/94.3 μm) according to an observer and 160.0–187.5/87.5–102.5 μm (mean 168.6/94.7 μm) according to a second observer.

3.2. Sequences of the rDNA ITS-2 and ITS-1

The 22 fasciolid eggs from the female patient of India provided a unique hybrid haplotype of the ITS1–5.8S-ITS2 region, i.e. an identical nucleotide sequence in all eggs. This haplotype is characterized by presenting heterozygosity in the 5 positions of ITS-1 and also in the 5 positions of ITS-2 that differentiate between *F. hepatica* and *F. gigantica* (Fig. 1), and was identical to the haplotype FgxFh-Htz1 (OQ064779) found in Bangladesh (Fig. 2).

The 25 fluke adult specimens from livestock in Bangladesh furnished two haplotypes of the ITS1–5.8S-ITS2 region. One of them corresponds to "pure" *F. gigantica*, and shows 100% identity with haplotype Fg-3A (OQ064778) and with isolates of *F. gigantica* 54,343 and 9687 (KX198616 and KX198625) from Bangladesh and India, respectively (Fig. 2). This haplotype was present in samples from sheep, goat, cattle, and buffalo and contains a mutation (a C instead of a T) in position 797 of the rDNA ITSs alignment, which does not discriminate between the two *Fasciola* species (Fig. 2). This Fg-3A also shows 100% homology with the partial (only ITS-2) sequences of isolates of *F. gigantica* from different animal hosts of India (Fig. 2). The other haplotype detected in Bangladesh samples from sheep, goat and buffalo was the hybrid haplotype FgxFh-Htz1, which proves to be identical to that found in the fasciolid eggs from the Indian patient.

The five adult fasciolids found in three sheep, one goat and one buffalo belonging to the heterozygotic hybrid haplotype FgxFh-Htz1 (Fig. 2 and Table 2) were, moreover, individually cloned, to further assess their double peak positions in electropherograms obtained through direct sequencing. Additionally, one pure *F. gigantica* specimen from cattle, whose sequence exhibited no double peaks, was employed as a control. A total of 40 rDNA complete intergenic sequences were obtained from the eight different colonies per each of the five aforementioned samples. All eight clone sequences of the control were confirmed to be exclusively *F. gigantica*. The remaining 32 clone sequences were analyzed individually and included: 12 "pure" *F. gigantica* (37.5%) from sheep, goat and buffalo; 5 "pure" *F. hepatica* (15.6%) from sheep and buffalo; and 15 hybrid FgxFh specimens showing mutations of both species (46.9%) from sheep, goat and buffalo.

The intra-individual analysis of the sequences from each of the cloned specimens demonstrates the existence of *Fg, Fh*, and FgxFh clones, both in sheep and buffalo. In goat, only clones of *Fg* and FgxFh clones were obtained and no *Fh* clone was found.

The fasciolid flukes from cattle in Pakistan also provided a unique haplotype whose sequence was identical to the Fg-3A haplotype (Fig. 2).

3.3. Nucleotide and protein sequences of the mtDNA nad1 gene

The mtDNA *nad*1 gene in the fasciolid eggs from the patient of India provided only one haplotype to which the code Fg-nad1–17 was assigned. This haplotype was 903 bp long, contains a 62.7 AT.% content and shows 100% identity with the mtDNA *nad*1 gene of *Fasciola* sp. (KF543343) from China in nucleotide and amino acid sequences, as well as with one partial *nad*1 sequence of *Fasciola* sp.-ND1-E1 from India (LC012895) (Figs. 3 and 4).

The fasciolid adult flukes from livestock in Bangladesh provided three different haplotypes, with the same length of 903 bp and an average AT content of 62.6%. One haplotype obtained in sheep, goat, cattle and buffalo was identical to the Fg-nad1–17 detected in the human patient from India (Fig. 3). The other two haplotypes did not show total homology with any of the previously described haplotypes or isolates, and were consequently in need for new codes: Fg-nad1–18 in buffalo and Fg-nad1–19 in cattle. It should be noted, however, that these complete *nad*1 sequences with new haplotype codes show total homology with the 535-bp partial sequence of the Fg_isolate ND1-E6 (LC012900) from India (Fig. 3).

The fasciolid flukes from cattle in Pakistan provided a unique haplotype (Fg-nad1–20) whose sequence differed from all previously described haplotypes or isolates of *F. gigantica, F. hepatica* or *Fasciola* sp. This new haplotype shows some degree of sequence similitude with Fg-nad1–18 and Fg-nad1–19 haplotypes from Bangladesh, as well as with *Fasciola* sp. (KF543343) (Fig. 3).

The four different *nad*1 haplotypes here described for India, Bangladesh and Pakistan (Fg-nad1–17, Fg-nad1–18, Fg-nad1–19 and Fg-nad1–20), provided only three NAD1 protein haplotypes, namely Fg-NAD1-II and Fg-NAD1-III for India and Bangladesh, and Fg-NAD1-IV for Pakistan. All are 300-aa-long and with start/stop codons of GTG/TAG (Fig. 4). Worth mentioning is that Fg-NAD1-IV only differs by 3 and 5 amino acid changes from Fg-NAD1-III and *Fasciola* sp. or Fg-NAD1-II, respectively (Fig. 4).

The nucleotide and amino acid sequence differences between the *nad*1 haplotypes here described and other reference sequences of *F. gigantica*, *F. hepatica* and *Fasciola* sp. are listed in Figs. 3 and 4. The corresponding 903-bp long alignment contains 802 conserved and 101 variable positions, among which 85 are p-informative and 16 are singleton sites (Figs. 3 and 4).

3.4. Nucleotide and protein sequences of the mtDNA cox1 gene

The mtDNA *cox*1 gene of the fasciolid eggs from the Indian patient provided only one haplotype for all fasciolid eggs, to which the new code Fg-cox1–17 was ascribed. This haplotype was 1533 bp long, contains a 63.8 AT.% content, proved to be different from all previously described

haplotypes, and may be distinguished by a specific mutation in position 345 (T instead of G in *F. gigantica* and *Fasciola* sp.) (Fig. 5).

The fasciolid adult flukes from livestock in Bangladesh provided three different haplotypes with the same length of 1533 bp and an average AT content of 63.7%. After sequence analysis comparisons with other *F. gigantica*, *F. hepatica* and *Fasciola* sp. haplotypes/isolates from Africa; Australia and Europe, as well as with China and India, these three *cox*1 haplotypes proved to be new. The following new codes were created for them: Fg-cox1–14, Fg-cox1–15, and Fg-cox1–16. The haplotype Fg-cox1–14 was the most abundant and detected in samples from sheep, goat, cattle and buffalo (Fig. 5).

The fasciolid flukes from cattle in Pakistan provided a unique haplotype of identical length and 63.9% AT content and which also demonstrated to be new. The new code Fg-cox1–18 was needed. As in the cases of haplotypes from India and Bangladesh, this new haplotype proved to be clearly distant from *F. hepatica* (Fig. 5).

The five different *cox*1 haplotypes described for India, Bangladesh and Pakistan provided only three COX1 protein haplotypes (Fg-COX1-II, Fg-COX1-III and Fg-COX1-IV), with a length of 511 aa and start/stop codons of ATG/TAG in all samples analyzed. The protein haplotypes from India (Fg-COX1-II) and Bangladesh (Fg-COX1-II and Fg-COX1-III) differ between them in only one amino acid change, whereas by 4–5 amino acid changes when compared with *Fasciola* sp. (KF543343) (Fig. 6). The haplotype Fg-COX1-IV generated by samples from Pakistan shows seven amino acid changes when compared to *Fasciola* sp. (KF543343) and only 2–3 to haplotypes from India and Bangladesh (Fig. 6).

The nucleotide and amino acid sequence differences between *cox*1 haplotypes from India, Bangladesh and Pakistan here described and other reference sequences of *F. gigantica*, *F. hepatica* and *Fasciola* sp. are listed in Figs. 5 and 6. The corresponding 1533-bp long alignment contains 1369 conserved and 164 variable positions, among which 140 p-informative and 24 singleton sites (Figs. 5 and 6).

3.5. rDNA and mtDNA combined haplotypes

A summary of the rDNA and mtDNA combined haplotypes detected in individual eggs from the Indian patient and in individual flukes infecting the different livestock species from Bangladesh and Pakistan is detailed in Table 2. These results demonstrate that the ITS1, ITS-2 and *nad*1 haplotypes are the same in both the human case of India and many of the Bangladeshi animals analyzed. Regarding the *cox*1 marker, the Fg-cox1–17 haplotype found in the Indian patient differs from the most common haplotype found in livestock from Bangladesh (Fg-cox1–14) by only one specific silent transversion (T instead of G in position 345). It should be highlighted that this mutation does not discriminate between *F. gigantica, F. hepatica* or *F.* sp. Compared to India and Bangladesh, Pakistan only shares the most common rDNA ITSs haplotype Fg-3A, whereas it presents specific haplotypes for both *nad*1 and *cox*1 (Table 2).

3.6. Phylogenetic trees of the mtDNA genes

In the phylogenetic analysis carried out with the mtDNA *nad*1 data matrix, the ML model best fitting this data-set was Hasegawa-Kishino-Yano, with some sites to be evolutionarily invariable (= 64.19% sites) (HKY + I). The tree with the highest log likelihood (−2607.70) shows two monophyletic clades, including one for *F. gigantica* and another for *F. hepatica*, with the highest bootstrap supports (100% and 100%), respectively (Fig. 7). Inside the clade of *F. gigantica*, all haplotypes from the human case of India and livestock specimens from Bangladesh and Pakistan are included. A clear distribution of these haplotypes into two subclades or branches (well supported, 94%) is observed:

(i) one subclade includes the haplotype Fg-nad1–17 obtained in the Indian patient and in Bangladesh livestock together with other haplotypes found in cattle from India, classified as aspermic specimens, and *Fasciola* sp. from China, clustering together with haplotypes of *F. gigantica* from India (originally noted to belong to a group B), appearing as a sister group;
(ii) the other subclade includes the Fg-nad1–18 and Fg-nad1–19 haplotypes from Bangladesh (cattle and buffalo), clustering together with *F. gigantica* haplotypes representatives of a group A from India (goat, cattle, buffalo); the haplotype Fg-nad1–20 from Pakistan (cattle) appears as a sister group inside this branch.

Outside of these two subclades, but included within the *F. gigantica* clade and with 100% support, the *F. gigantica* haplotype from Africa is found occupying a basal position. The *Fascioloides* species (*Fs. jacksoni*)

Table 2
Combined rDNA and mtDNA haplotypes obtained in fasciolid eggs from the patient in India and fluke adults from livestock in Bangladesh and Pakistan according to their countries, geographical origins and hosts.

Fasciolid samples	Geographical origin	Host	rDNA and mtDNA haplotypes				
			ITSs	*nad*1	NADI	*cox*1	COX1
India (ne = 22)	Arunachal Pradesh/North-East India	Human	FgxFh-Htz1	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–17	Fg-COX1-II
Bangladesh (na = 25)	Chittagong/Cox's Bazar/Chakaria	Goat, cattle, buffalo	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Sylhet/Sylhet/Sylhet Sadar	Buffalo	Fg-3A	Fg-nad1–18	Fg-NAD1-III	Fg-cox1–15	Fg-COX1-III
	Sylhet/Sylhet/Sylhet Sadar	Cattle	Fg-3A	Fg-nad1–19	Fg-NAD1-III	Fg-cox1–16	Fg-COX1-III
	Sylhet/Sylhet/Sylhet Sadar	Goat	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Naogaon/Shapahar	Cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	–	–
	Rajshahi/Naogaon/Shapahar	Buffalo	FgxFh-Htz1	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Chittagong/Rangamati/Rangamati Sadar	Cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Naogaon/Naogaon Sadar	Sheep	FgxFh-Htz1	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Naogaon/Naogaon Sadar	Goat	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Naogaon/Patnitola	Cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Naogaon/Patnitola	Sheep	FgxFh-Htz1	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Sheep, goat	FgxFh-Htz1	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rangpur/Lalmonirhat/Lalmonirhat Sadar	Cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Dhaka/Mymensingh/Mymensingh Sadar	Sheep, goat, cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Rajshahi/Bogra/Dhunat	Goat	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Khulna/Jhenaidah/Jhenaidah Sadar	Sheep,goat,cattle	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
	Khulna/Jhenaidah/Shailkupa	Sheep	Fg-3A	Fg-nad1–17	Fg-NAD1-II	Fg-cox1–14	Fg-COX1-II
Pakistan (na = 2)	Pothwar Plateau/Punjab	Cattle	Fg-3A	Fg-nad1–20	Fg-NAD1-IV	Fg-cox1–18	Fg-COX1-IV

ne = number of eggs from the human patient in India.
na = number of adult specimens from livestock of Bangladesh and Pakistan.

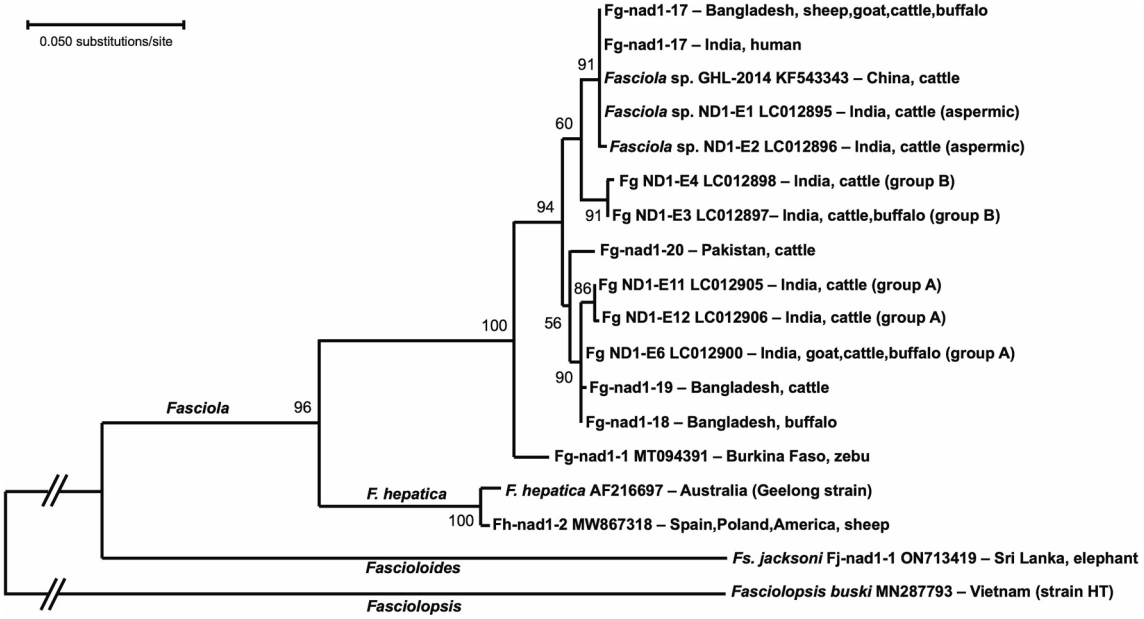

Fig. 7. Phylogenetic tree of species of *Fasciola* and *Fascioloides* based on maximum-likelihood (ML) estimates and reconstructed on mtDNA *nad*1 sequences, rooted using the sequence of the trematode *Fasciolopsis buski* (MF287793) as outgroup (highest log likelihood = −2607.70). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Supports for nodes MEGA X by bootstrap (1000 replicates) with ML parameters (HKY + I). For new Acc. Nos. of Fg-nad1–17,18,19 and 20 see Fig. 3. For notes on aspermic, groups A and B see [47]).

appears in a separate clade, manifesting its evident independence from that of the *Fasciola* clade (Fig. 7).

In the phylogenetic analysis carried out with the mtDNA *cox*1 data matrix, the ML model best fitting this data-set was HKY + G. The tree with the highest log likelihood (−4329.12) was made with a discrete gamma distribution (+*G*, parameter = 0.2132). The ML topology obtained (Fig. 8) coincides completely with that described in the *nad*1 tree, in the large clades that support *F. gigantica*, *F. hepatica* and *Fs. jacksoni*. Concerning the distribution of haplotypes inside the *F. gigantica* clade (100% support), two subclades appear. One subclade (100%) includes the *Fg*-cox1–17 haplotype from the human case in India, the most common haplotype from Bangladesh Fg-cox1–14 and *Fasciola* sp. from China (cattle) clustering together, and another subclade (75%) includes the other two haplotypes from Bangladesh and the haplotype from Pakistan (Fig. 8). The haplotype of *F. gigantica* from Africa occupies a basal position regarding the Asian clade of *F. gigantica*, similarly as in the *nad*1 tree (Fig. 8).

3.7. Haplotype networks

Sequences of *nad*1 obtained from fasciolid eggs and adults from India, Bangladesh and Pakistan, representing the four haplotypes here described, plus other eight reference haplotypes of *F. gigantica* and *Fasciola* sp. included in the *F. gigantica* clade of the phylogenetic tree were analyzed with a median-joining network (MJN) (Fig. 9A). The global haplotype diversity (Hd) was 0. 876 (SD = 0.070), the nucleotide diversity (π) was 0.01143 (SD = 0.0052), the average number of nucleotide differences was 6.114, and the number of polymorphisms was 22, of which 12 were parsimony-informative sites. Tajima's D and Fu's Fs values were − 0.71245 ($P > 0.10$) and = − 0.509, respectively.

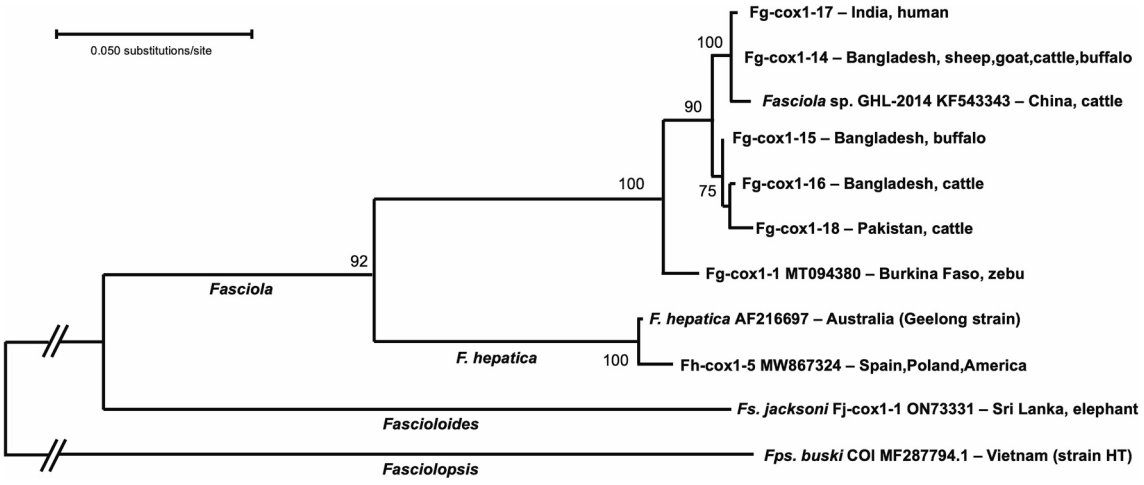

Fig. 8. Phylogenetic tree of species of *Fasciola* and *Fascioloides* based on maximum-likelihood (ML) estimates and reconstructed on mtDNA *cox*1 sequences, rooted using the sequence of the trematode *Fasciolopsis buski* (MF287794) as outgroup (highest log likelihood = −4329.12). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Supports for nodes MEGA X by bootstrap (1000 replicates) with ML parameters (HKY + G). For new Acc. Nos. of Fg-cox1–14,15,16,17 and 18 see Fig. 5.

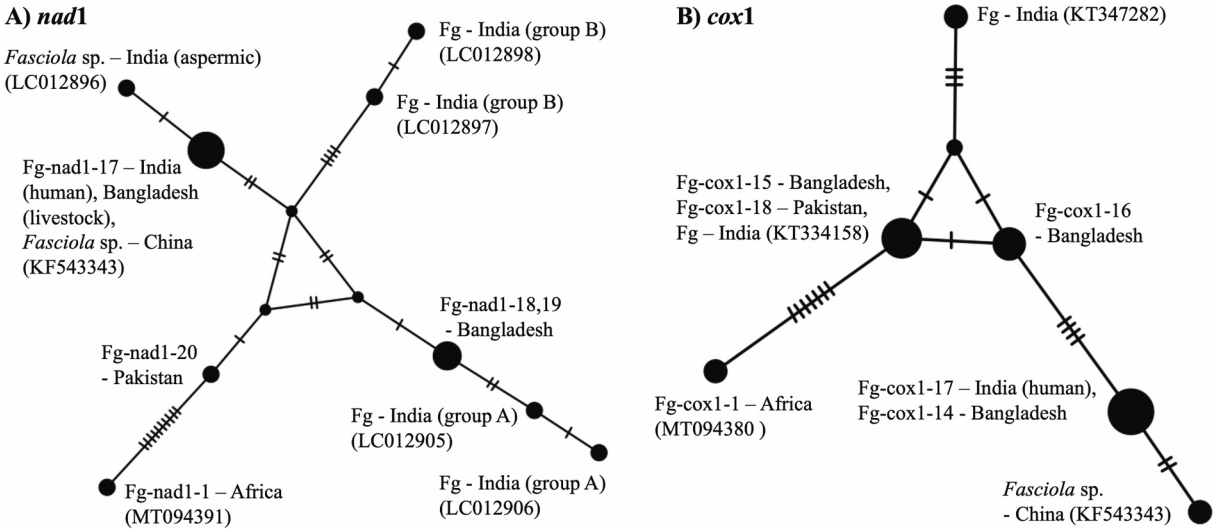

Fig. 9. Median Joininig networks (MJN) constructed with haplotypes of mtDNA genes of *Fasciola* samples from India, Bangladesh and Pakistan and some reference sequences from India and China, according to PopART. Circles are proportional to the number of samples represented for each haplotype. Slashes on branches between nodes indicate mutations. A) MJN of *nad*1 haplotypes (haplotypes included according to Fig. 3); B) MJN of *cox*1 haplotypes (haplotypes included according to Fig. 5).

The *nad*1 MJN distinguishes the haplotype of *F. gigantica* from Africa as the most distant although directly related to the Fg-nad1–20 from Pakistan. The remaining haplotypes appear distributed in three clusters including: (i) Fg haplotypes from Bangladesh and *F. gigantica* from India (group A); (ii) Fg-nad1–17 from the Indian patient and Bangladeshi animals and *Fasciola* sp. from China, plus aspermic *Fasciola* sp. from India; (iii) a last cluster including *F. gigantica* from India (group B).

Sequences of *cox*1 and representing five haplotypes here described plus other four reference haplotypes of *F. gigantica* and *Fasciola* sp. were also analyzed by MJN (Fig. 9B). The global haplotype diversity (Hd) was 0.889 (SD = 0.091), the nucleotide diversity (π) was 0.01301 (SD = 0.0028), the average number of nucleotide differences was 5.11111, and the number of polymorphisms was 15, of which 6 were parsimony-informative sites. Tajima's D and Fu and Li's D values were – 0.63894 ($P > 0.10$) and = – 0.7864, respectively. The *cox*1 MJN also corroborates the long distance between *F. gigantica* from Africa regarding haplotypes from Pakistan, India and Bangladesh. In this network, the haplotype from the Indian patient appears related to Fg haplotypes from Bangladesh animals and *Fasciola* sp. from China.

4. Discussion

4.1. Egg phenotyping for fasciolid diagnosis

The measurements of length and maximum width of the eggs found in the bile filtrate of the female patient from Arunachal Pradesh prove to be intermediate between the size of the eggs of "pure" *F. gigantica* and "pure" *F. hepatica*. Concrete measurements of fasciolid eggs in human stools are as follows: A) *F. gigantica*: 137.2–191.1/73.5–120.0 μm in areas where *F. hepatica* is absent, and 150.9–182.2/85.1–106.2 μm in areas where both fasciolid species are present; B) *F. hepatica*: 100.6–162.2/65.9–104.6 μm in areas where *F. gigantica* is absent, and 106.5–171.5/63.9–95.4 μm in areas where both fasciolid species are present [54,55]. The comparison of these measurements shows an evident morphometric trend towards *F. gigantica* and already suggest that the patient was infected by a *F. gigantica*-like intermediate fasciolid form or hybrid. As well known, the morphometric phenotype of the eggs significantly helps in the specific diagnosis of fasciolids infecting both humans and animals, including hybrid fasciolids [56]. It was also according to egg measurements that another patient in the close Nepal was recently diagnosed as being infected by a *F. gigantica*-like hybrid [57].

In northeastern India and Bangladesh, only two lymnaeid species belonging to the *Radix* group are present, namely *R. acuminata* and *R. luteola*. These two lymnaeid vectors are involved in the transmission of *F. gigantica* [58] and, consequently, the evolutionary vector filter acts favoring the *F. gigantica* genotype [15].

Such a diagnosis after egg size does, moreover, fit well with the sequencing results obtained from the eggs of the Indian patient.

4.2. Nuclear rDNA spacer sequencing

In the rDNA ITS-2 and ITS-1 sequences, the detection of heterozygotic positions manifested by double peaks in the electropherograms obtained by direct sequencing and verified by subsequent cloning, in each one of the positions known to distinguish between the two "pure" *F. gigantica* and "pure" *F. hepatica*, clearly demonstrate that the patient-infecting fasciolid was a fasciolid hybrid. This hybrid may be, moreover, evolutionary catalogued as recent, given that nucleotide homogenization by concerted evolution of the rDNA operon did not yet have sufficient time to apply. Although the time needed for rDNA sequence homogenization by the concerted evolution in fasciolids remains unknown, available data suggest that it may take at least several years in areas where there is stability of disease transmission marked by the lymnaeid vector species present, that is, when a foreign *Fasciola* species is introduced in an area presenting only the other *Fasciola* species [15].

The detection of ITS sequences of both *F. gigantica* and *F. hepatica* coexisting in the rDNA operon in the same host individual evidences not only recent hybridization phenomena, but also crossbreeding between parental specimens which were already hybrids, indicating repeated, superimposed and rapidly evolving hybridization events in the endemic area in question [19].

The fasciolid sequence found in the Indian patient proved to be identical to the same heterozygotic haplotype FgxFh-Htz1 found in sheep, goats and cattle in the nearby Bangladesh (Fig. 2) and widely detected in different localities of this country (Table 2), speaks about a high risk of infection by this heterozygotic haplotype. Additionally, a wide geographical distribution of FgxFh-Htz1 was already recently found in livestock of Bangladesh [19].

It is worth emphasizing that no heterozygotic haplotype was found in the numerous livestock studied throughout similar latitudes in the Punjab of Pakistan (Bargues et al., unpublished), from which material two intermediate fasciolid specimens from cattle were selected and

included as examples for comparison convenience in the present analysis. The convenience for a comparison between eastern South Asia and western South Asia relies on the fact that the epidemiological scenario of fascioliasis in Pakistan pronouncedly resembles that of the Arunachal Pradesh and Bangladesh region, because of: (i) the detection of both *F. gigantica* and *F. hepatica*, (ii) fasciolid infections in humans and animals, and (iii) an evident impact of climate change due to increasing monsoon rainfall [18,20,59].

Results obtained indicate that both (i) the origins of the intermediate or hybrid fasciolid forms and (ii) the factors underlying human infection risks, are different in eastern and western South Asia, although both regions are showing emergence of human fascioliasis at present. In Bangladesh, intermediate or hybrid fasciolid forms have already been reported by both phenotypic methods [60] and molecular methods [19] and the increasing prevalences in livestock and consequent human infection risk have been highlighted [19]. In Pakistan, intermediate or hybrid fasciolid forms have also been found [61] and human fascioliasis emergence has recently been reported [20,59]. It should be here considered that metacercariae of an isolate from a host species are infective for other host species [62–65]. Otherwise said, metacercariae of *Fasciola* species do not show any host specificity and consequently there is no host-specific circulation [17,66]. In southern Asia, this means that fasciolids infecting sheep, goats, cattle and buffaloes are similar sources for human infection. Consequently, the human infection risk in a given locality depends on the number and density of each livestock species and their respective infection rates in the locality in question [17].

4.3. mtDNA gene sequencing

Interestingly, neither total or partial introgression nor heteroplasmy or heterozygotic positions were found in the complete sequences of the two mtDNA genes studied, as a priori could be expected from the eggs found in the Indian patient which show evident phenotypic and genotypic traits of recent hybridization. Similarly, the nucleotide sequences and amino acid sequences of both *nad*1 and *cox*1 in India, Bangladesh and also Pakistan appear to be typical for *F. gigantica*, including variable positions (SNPs) (Figs. 3–6 and Table 2), easily interpretable as local mutations when considering the higher evolving speed of these mtDNA genes regarding that of the nuclear rDNA ITSs in invertebrates in general [67].

The *nad*1 haplotype Fg-nad1–17 found in the Indian patient proved to be the same as one widely distributed in livestock species of Bangladesh. There is consequently no surprise when observing that the female patient of Arunachal Pradesh was infected by this diffused fasciolid haplotype. This was not the case with the *cox*1 haplotype Fg-cox1–17 from the Indian patient eggs which proved to be different from all hitherto detected in livestock in Bangladesh and may suggest a local evolutionary divergence in Arunachal Pradesh, as deduced from only one exclusive silent transversion in position 345 (Fig. 5).

In Pakistan, the livestock fasciolid haplotypes of both *nad*1 and *cox*1 differed from all found in livestock-infecting fasciolids in India and Bangladesh (Figs. 3 and 5). This again indicates a different fasciolid scenario in western South Asia when compared to eastern south Asia.

The mtDNA sequencing results here obtained further demonstrate the existence of shared haplotypes between livestock species and the human patient. This suggests a genetic flow leading to a high human infection risk when dealing on endemic areas where liver fluke endemicity is very high in animals, as observed in Bangladesh [19].

4.4. Analysis of rDNA and mtDNA combined haplotypes

The total nucleotide identity of the complete sequences of the four markers was verified when comparing the sequences obtained in each of the 22 eggs from the Indian patient. The absence of nucleotide variation indicates that all these eggs were produced by selfing of the same hermaphroditic adult fluke, and suggest that the patient was infected by only one liver fluke specimen. Indeed, infection by a single fasciolid worm is a frequent finding in non-human endemic areas. This means that the heterozygotic ITS-1 and ITS-2 were inherent to this fasciolid adult specimen and not the result of hybridization between two fluke specimens inside the patient. Hybridization needs fluke encounter inside a biliary canal, which occurs in massive infections or when many flukes infect the liver of the same host individual. Patient infection by a single worm is also supported by the finding of numerous adult fluke specimens widely infecting livestock in Bangladesh, which show exactly the same heterozygotic haplotype. Moreover, none of the adult flukes infecting livestock in Bangladesh and presenting heterozygotic ITS-1 and ITS-2 proved to be aspermic. Additionally, the clinical picture, the absence of eggs in stools, and the positive response to the triclabendazole treatment with only one 10 mg/kg dose leading to a quick total recovery [23], further support the patient infection by a single adult fluke.

The combination of high heterozygosity rates in rDNA spacers and total absence of any hybridization signal in mtDNA genes within the same egg or fluke adult specimen is an observation which should be highlighted. This indicates that hybridization in fasciolids may also occur in which the nuclear genome is affected without any involvement of the mtDNA genome. Moreover, results suggest that such hybrid specimens including a duplicate nuclear genome belong to a well-established lineage, whose origin has been recent as a consequence of the introduction of *F. hepatica* due to livestock importation in an area of *F. gigantica* transmission.

Interestingly, the epidemiological situation of the rDNA and mtDNA combined haplotypes detected in individual eggs from the Indian patient and in individual flukes infecting the different livestock species from Bangladesh (Table 2) resembles the one recently described in Vietnam.

In a first study in Vietnam, the combination of ITS-2, *nad*1 and *cox*1 was used in the analyses of fasciolids found in humans and ruminants, although only fragments of a short length of 435 bp and 423 bp were obtained for these two mtDNA genes, respectively [68]. In spite of this, the analysis of 12 human patients demonstrated that six patients were infected by introgressed hybrid specimens (flukes showing rDNA of *F. hepatica* and mtDNA of *F. gigantica*) and other two patients were infected by flukes in which the ITS-2 sequences of both *F. hepatica* and *F. gigantica* coexisted within the same specimen

In a more recent study of wider coverage of Vietnam, the combination of ITS-1, ITS-2 and a little bit longer 535-bp-long *nad*1 fragment was used for the same purpose. Among fluke samples from ruminants, 71 were *F. gigantica*, 42 were introgressed and 7 were admixed hybrid *Fasciola* spp. (the term admixed was used to refer to specimens presenting a mixture of both, *F. gigantica* and *F. hepatica* rDNAs). Among 14 flukes infecting humans, 9 proved to be pure *F. gigantica*, 3 were introgressed, and 2 were admixed hybrid *Fasciola* spp. [69].

4.5. Analyses of topologies of phylogenetic trees and networks

The sequences of the mtDNA genes *nad*1 and *cox*1 prove that they have not been affected by hybridization throughout Pakistan-India-Bangladesh. Consequently, the phylogenetic trees obtained for these two mitochondrial markers furnish significant information on the evolution of fasciolids throughout this very wide region.

For the interpretation of the topology of these two trees (Figs. 7 and 8), historical records about very long-term massive movements of pack animals should be considered [2]. Past human-guided movements of livestock indicate that it was up to the warm-lowland-preferring fasciolid species *F. gigantica* to originally colonize Pakistan, whole northern India and Bangladesh by means of the good-transporting caravans including cattle, equines and dromedaries as pack animals and goats and sheep as livestock for subsistence purposes, along the Grand Trunk Road from the Asian Near East and Afghanistan during hundreds of years [2].

Moreover, eastward from Bangladesh, the so-called Tea-Horse Road

was running for good exchange also along many centuries, connecting present-day Bangladesh and northeastern India, with southern China and southeast Asia. All these west-east/east-west movements of livestock underlay pronounced geographical exchanges which are today manifested by the considerable puzzle observed in genetic analyses of livestock throughout the west-east axis of the aforementioned region [2].

The marked heterogeneity of the *nad*1 and *cox*1 haplotypes found along the Pakistan-northern India-Bangladesh-Arunachal Pradesh region in the present study may therefore also be interpreted as a consequence of the past fasciolid exchanges occurred along the west-east/east-west axis of this region due to the pack animal movements carried out throughout the Grand Trunk Road and the Tea-Horse Road.

In both phylogenetic trees obtained from *nad*1 and *cox*1, the clade including sequences of *Fasciola* from Asia shows a highly supported division into two subclades. These two subclades coincide with those designed as haplogroups A and B in a previous study in which a third haplogroup C was considered for aspermic flukes [47]. In the present study, however, the aspermic flukes are included inside the same *nad*1 cluster as the haplotype *Fg*-nad1–17 (India and Bangladesh) and *Fasciola* sp. (China), as indeed none or only one differing mutation appear (see Figs. 3 and 5).

When considering the inclusion of *F. gigantica* from Pakistan and the *Fasciola* sp. from China as geographical markers, we may conclude that the subclade including *F. gigantica* from Pakistan underlie flukes linked to movements of livestock along the western Grand Trunk Road, whereas the other subclade including the *Fasciola* sp. from China should be linked to livestock movements along the eastern Tea-Horse Road. This indicates that the fasciolid infecting the patient from India may be related to old flukes migrating with pack animals along the Tea-Horse Road. Indeed, Arunachal Pradesh lies between two main pack-animal caravan core routes of the Tea-Horse Road: (i) one originating in the tea production zone of Puer, Kumming, and Dali in the Chinese Yunnan province and subsequently traversing the whole present-day state of Assam along the long valley of the river Brahmaputra down to present-day Bangladesh, and (ii) another more northern route from the tea production zone of Yaan along Chengdu and Kangding in Sichuan province of China, westward leading to the Chinese city of Lhasa and subsequently through the corridor between present-day Nepal and Bhutan and the Indian Sikkim state, down to Bangladesh [2].

The role of the Chinese locality of Dali in the first of the aforementioned core routes of the Tea-Horse Road shall be highlighted. Dali played a crucial role of crossroad in the Tea-Horse Road [2] and has recently proved to be an emerging focus of human fascioliasis, including numerous patients [70] infected by *F. gigantica* [71]. When deeply analyzing the size of the fasciolid eggs found in stool samples (144–180 × 73–96 μm) and the body morphology of flukes found in bile ducts of patients (with pronouncedly non-parallel lateral body walls) shown in photographs [70], one may easily conclude that hybrid fasciolids were also involved. This constitutes no surprise, when considering that the Tea-Horse Road connected eastward with Nepal where both *F. hepatica* [72] and *F. gigantica*-like [57] flukes have been reported infecting humans, and northward with the Silk Road along whose cooler routes *F. hepatica* was the main fasciolid [2]. Consequently, this fasciolid emergence in Dali resembles the one here described in eastern India and Bangladesh.

The sequence differences between Bangladesh-Arunachal Pradesh in the east and Pakistan in the west, observed today concerning fasciolid hybrids showing ITS heterozygosity in Bangladesh-Arunachal Pradesh and such a hybridization absence in Pakistan, may be explained by the introduction of *F. hepatica* with livestock imported from countries presenting endemicity by *F. hepatica* into Bangladesh-Arunachal Pradesh in recent times [19]. Once arrived, imported animals are freely released for the recovery of the weight lost during the importation trip according to traditional procedures, usually without following quarantine measures, without looking for their potential liver fluke infection, nor preventatively treated against fascioliasis before being added to local herds [19].

PopART networks based on *nad*1 and *cox*1 appear to be less informative than the phylogenetic tree topologies, although both MJNs coincide in showing a triangular central core, which may be interpreted as the inter-relation/exchanges between the western Grand Trunk Road and the eastern Tea-Horse Road (Fig. 4A, B).

5. Conclusions

Several conclusions reached in the present study represent crucial steps forward within the multidisciplinary One Health analysis about human fascioliasis in southern Asia, including the importance of the animal reservoirs and environmental considerations, as well as key aspects for future action to be undertaken to face the disease emergence in northeastern India and Bangladesh:

A) Although human fascioliasis is at present emerging throughout the wide region of southern Asia, factors underlying this emergence differ in the eastern part when compared to the western part:
   - In northeastern India and Bangladesh, human fascioliasis emergence appears to be linked to increasing liver fluke prevalences in livestock caused by: (i) livestock importation from other fascioliasis endemic countries because of the increasing demand of rapidly growing human populations, (ii) numerous livestock movements, including transborder ones, due to the uncontrolled small-scale household farming management practices and also the aforementioned livestock importation, and (iii) human-made introduction of *F. hepatica* with imported livestock into an area originally endemic of *F. gigantica* leading to local frequent, repetitive and accumulative hybridization phenomena [19], although (iv) an impact of climate change has also been involved at least in eastern-most Uttar Pradesh in north-eastern India where the highest fasciolid infection prevalence in a lymnaeid population (72% in *R. acuminata*) has been recorded [58].
   - In Pakistan, human fascioliasis emergence has been proved to be related to (i) an increasing monsoon rainfall within a climate change phenomenon, together with (ii) an impact of a very large irrigation system. Thus, in the Pakistani Punjab, a yearly prevalence peak is directly caused by rainfall seasonality whereas a second annual prevalence peak is linked to the months of anthropogenic management of irrigation dams, barrages and canals [18].

B) In northeastern India and Bangladesh, physicians and medical/health centres should be made aware about the increasing human infection risk in this region and the need for rapid diagnosis and treatment of patients to diminish the risk of long-term appearing sequelae:
   - In the clinical picture of patients, infection by hybrid fasciolids [23] do not appear to differ from the symptomatology and pathology caused by flukes of "pure" *Fasciola* species [4,73]. Key aspects include: a suspicious clinical picture, usefulness of anamnesis (information about the potential infection source), initial usefulness of liver imaging techniques (ultrasound more easily available in rural centres, CT and MRI in more equipped centres) [74], and the need to avoid misdiagnosis with lithiasis [75,76] or cancer (cholangiocarcinoma or malignancy tumors) to avoid unnecessary surgery [77].
   - For the diagnosis of patients, available parasitological and serological diagnostic techniques have adequately been reviewed [55]. In recently emerging endemic areas, several patients may not shed eggs in stools, as was the case of the female patient of Arunachal Pradesh. In such cases, endoscopy may help in egg finding, but

commercially-available indirect techniques should also be considered, such as coproantigen detection tests whether for individual patients [78] or surveys [79], and also serological tests, which detect the infection even during the initial acute phase [80], and among which several recent ones have proved to be highly specific [81].
- Triclabendazole treatment of a patient infected by *Fasciola* hybrids [23] allows to reach disease cure as successfully as in patients infected by "pure" *Fasciola* species [82]. This drug is also useful for preventive chemotherapy by mass treatments [83].

C) Governmental responsible officers should not only make efforts for diffusion of the appropriate information to warn health professionals, but also undertake steps forward regarding the following three aspects:
   - human health administration officers to accelerate the process for the official registration of triclabendazole for human use (Egaten® from Novartis Pharma, Basel, Switzerland) in both India and Bangladesh [82,84].
   - agriculture administration officers to improve livestock management, concerning quarantine, diagnosis and treatment of animals imported from other countries (mainly those endemic of *F. hepatica*) and control of local ruminant movements and exchanges (although this may be very difficult in the small-scale or familiar market) [19].
   - for animal treatment, drugs different from triclabendazole should be used to avoid the appearance of resistance to this drug [85].

Ethics statement

The Indian female patient was attended in the hospital and clinical departments of the Christian Medical College, Vellore Tamil Nadu, India. All research dealing on animals was performed with the approval of the Evaluation of Projects concerning Animal Research at University of Valencia (Organo Habilitado para la Evaluación de Proyectos de Experimentación Animal de la Universidad de Valencia) (A1263 915,389,140), strictly following the institution's guidelines based on Directive 2010/63/EU. Permission for animal research was additionally obtained from the Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (No. 2015/VSC/PEA/00001 tipo 2). Fasciolid collection was carried out by taking advantage of livestock slaughtering for other purposes. Animal ethics guidelines regarding animal care were strictly adhered.

Consent for publication

All authors read and approved the final version for publication consideration.

Availability of supporting data

All data are presented in the article. The sequence data have been submitted to GenBank and have been included under the following accession Nos: OR789885-OR789889; OR717609-OR717611; and OR717615-OR717616.

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Author contributions

Study concept and design: SMC, MDB; participated in experimental work and its design: PA, GMV, TJJ, SSRA, SAA; participated in field work and its analysis: GMV, TJJ, SSRA, SAA, EHC; local and international coordination, protocols, and logistics: SMC, GMV, SAA, EHC, AFG; analysis and interpretation of data: SMC, MDB; drafting of the manuscript: SMC, MDB; critical revision of the manuscript for important intellectual content and for final approval: SMC, MDB, GMV, TJJ, AFG; obtained project funding: SMC, MDB; principal investigator: SMC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are presented in the article. The sequence data have been submitted to GenBank and have been included under the accession Nos: OR789885-OR789889, OR717609-OR717611, OR717615-OR717616.

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