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Molecular evidence shows that the liver fluke *Fasciola gigantica* is the predominant *Fasciola* species in ruminants from Pakistan

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Abstract

Fascioliasis is an important disease affecting livestock, with great costs to producers worldwide. It has also become a serious issue for human populations in some endemic areas as an emerging zoonotic infection. There are two *Fasciola* species of liver fluke responsible for this disease, which occur worldwide, *Fasciola hepatica* and *Fasciola gigantica*. Identifying these two species on the basis of adult or egg morphology requires specialist knowledge due to the similarity of characters, and may misidentify putative intermediate or hybrid forms. In this study we sequenced the internal transcribed spacer 2 (ITS-2) rDNA of liver flukes collected from multiple species of hosts from seven localities in the Punjab and Baluchistan provinces of Pakistan, to determine the distribution of these two species. All 46 flukes processed in this study, collected from seven sites, showed the rDNA ITS-2 genotype corresponding to *F. gigantica*, contradicting previous reports, based on adult and egg morphology, that both species are present in Pakistan, with *F. hepatica* being the more common.

Introduction

Trematodes of the genus *Fasciola* are the common liver flukes of a range of species of animals and have a global distribution (Spithill & Dalton, 1998). A number of snail species serve as intermediate hosts. Mammals of various species serve as definitive hosts, with ruminants being the most important ones (Urquhart & Armour, 1996). Animals become infected with *Fasciola* following the ingestion of contaminated infective metacercariae. The parasite penetrates the intestinal wall and moves to the liver, causing perforations in the capsule and extensive haemorrhage to the parenchyma. The adult trematodes reside in the bile ducts of infected animals (Urquhart & Armour, 1996). The annual economic losses associated with fasciolosis stem from mortality (mild to heavy), cost of diagnosis and treatment, condemned livers, reduced milk yield, fertility disorders and reduced meat production (Rokni *et al.*, 2010; Hossain *et al.*, 2011).

Fasciolosis is considered to be an important helminth infection of ruminants that causes significant economic losses (Spithill & Dalton, 1998). Recognized as an emerging food-borne zoonosis in many parts of the

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world (Qureshi *et al.*, 2005; Freites *et al.*, 2009; Karahocagil *et al.*, 2011; Mera y Sierra *et al.*, 2011), human fasciolosis has now been included among neglected tropical diseases described by the World Health Organization (WHO) in 2008 (press statement). It is estimated that 2.4 million people are infected and the number of people at risk is more than 180 million worldwide (Haseeb *et al.*, 2002).

Several species have been described within the genus *Fasciola*, but only two species, *F. hepatica* and *F. gigantica*, are commonly recognized as taxonomically valid species occurring in domestic animals and humans (Itagaki *et al.*, 1998). Several studies have shown that *F. hepatica* occurs in temperate regions (Garippa, 2009; Ichikawa & Itagaki, 2010; Farjallah *et al.*, 2013) and *F. gigantica* in tropical areas (Amor *et al.*, 2011a); however, both species overlap in subtropical areas, along with intermediate genotypes (Agatsuma *et al.*, 2000; Marcilla *et al.*, 2002; Huang *et al.*, 2004; Ichikawa & Itagaki, 2010; Rokni *et al.*, 2010).

Pakistan has an agriculture-based economy, with livestock being an integral part. *Fasciola* infections have been reported in Pakistan. Several of these reports suggested that only *F. hepatica* is commonly found in small and large ruminants (Ijaz *et al.*, 2007; Iqbal *et al.*, 2007; Gadahi *et al.*, 2009; Akhtar *et al.*, 2012; Shahzad *et al.*, 2012; Ashraf *et al.*, 2014) and three reports indicated the presence of both *F. hepatica* and *F. gigantica* species in small ruminants (Afshan *et al.*, 2013) and large ruminants (Ahmed, 2005; Kakar *et al.*, 2011). However, all of these previous reports were based on egg and adult morphology, with no molecular confirmation of species identity. Sequences of the internal transcribed spacers (ITS-2) of ribosomal DNA provide reliable genetic markers to differentiate between *F. hepatica* and *F. gigantica,* and can detect proposed intermediate genotypes (Adlard *et al.*, 1993; Marcilla *et al.*, 2002; Huang *et al.*, 2004; Ai *et al.*, 2010; Ichikawa & Itagaki, 2010; Rokni *et al.*, 2010; Amor *et al.*, 2011a; Le *et al.*, 2012). The present study is the first to confirm species identity of *Fasciola* from ruminants in Pakistan using the ITS-2 genetic marker. Our results suggest, in contrast to previous morphologically based studies, that *F. gigantica* is the predominant species of *Fasciola* in the Punjab and Baluchistan provinces of Pakistan. No evidence for the presence of *F. hepatica* was found in this study.

Materials and methods

Fluke collection and the isolation of genomic DNA

We chose to study several different regions in Pakistan, where we anticipated *Fasciola* spp. to be prevalent. Adult flukes were harvested on necropsy from the liver of ruminants collected from five city abattoirs located in the Punjab province and two city abattoirs in the Baluchistan province. Fourteen infected livers from individual hosts were transported on ice from abattoirs to the laboratory, and the extraction was performed by dissection to reveal the flukes in the biliary ducts of the livers.

A minimum of one and maximum of eight flukes (46 in total) were collected from 14 infected livers, which were considered to be 14 separate populations. In the case of Baluchistan province, four populations (F23S, F22S, F21S and F19G) were obtained from the Quetta abattoir (30°N, 67°E) and one population (F20G) from Mastoung abattoir (30°N, 67°E). In the case of Punjab province, three populations (F13G, F12 and F11G) were obtained from Rawalpindi abattoir (33°N, 73°E), three populations (F14C, F16G and F17B) from Multan abattoir (30°N, 71°E), one population (F6C) from Sahiwal abattoir (31°N, 71°E), one population (F18G) from RY Khan abattoir (28°N, 70°E) and one population (F15B) from DG Khan abattoir (30°N, 71°E).

Individual flukes were washed extensively in phosphate-buffered solution (PBS) and preserved with 70% ethanol at −80°C. For DNA extraction, a small piece of tissue (~2 mg) was removed from each fluke and rinsed in distilled water (dH_2O) twice for 5 min each. Tissue sections were then lysed in lysis buffer and protinease K (10 mg/ml, New England BioLabs, Ipswich, Massachusetts, USA). Lysis buffer contained 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.045% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin and dH_2O in 50-ml volumes. Samples were lysed in 50 μl for 98 min at 60°C followed by 15 min at 94°C, then stored at −20°C until the polymerase chain reactions (PCRs) were performed.

Molecular analysis of rDNA ITS-2 from Fasciola *spp.*

A 490–743 bp fragment of the ITS-2 rDNA region was amplified from individual adult fluke lysates using universal forward primers complementary to the 5.8S rDNA coding sequence (5′-GGTGGATCACTCGGCTCGTG-3′) and reverse primer complementary to the 28S rDNA coding sequence (5′-TTCCTCCGCTTAGTGATATGC-3′). These primers were designed by modifying previously reported primers (Adlard *et al.*, 1993). Reaction mixtures comprised a final volume of 25 μl, containing final concentrations of 1×Thermopol reaction buffer (New England BioLabs), 2 mM $MgSO_4$, 100 μM deoxynucleoside triphosphates (dNTPs), 0.1 μM forward primer and reverse primer, and 1.25 U *Taq* DNA polymerase at 5000 U/ml (New England BioLabs). Thermocycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 56°C for 60 s and 72°C for 60 s, with a final extension of 72°C for 5 min.

PCR products were cleaned using Omega BioTek Micro Elute Cycle Pure Kit (D6293-02; Omega Bio-Tek, Norcross, Georgia, USA) and the same amplification primers were used to sequence both strands using an Applied Biosystems 3730Xl genetic analyser (Burlington, Ontario, Canada). Both strands of rDNA ITS-2 sequences from each individual fluke were assembled, aligned and edited to remove primers on both ends using Geneious Pro 5.4 software (Drummond *et al.*, 2012). Sequences showing 100% base pair similarity were grouped into haplotypes using the CD-HIT Suite software (Huang *et al.*, 2010). These haplotypes were then aligned with *F. hepatica*, *F. gigantica* and reported intermediate *Fasciola* species rDNA ITS-2 genes previously used to determine inter- and intraspecific variation between and within *Fasciola* species, and this alignment was used in the following phylogenetic analysis.

Phylogenetic analysis of the rDNA ITS-2 from Fasciola *spp.*

Haplotype sequences and references were imported into MEGA 6 (Tamura *et al.*, 2013) and used to determine

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the appropriate model of nucleotide substitution to be used for building the phylogeny. A phylogenetic tree of the haplotypes was reconstructed using maximum likelihood (ML) in MEGA 6 after determining the appropriate model of substitution (Tamura *et al.*, 2013) from the rDNA ITS-2 sequence data. According to Bayesian information criterion, the best model was the Kimura 2 model (K2 + G). This model of substitution was used with parameters estimated from data. Branch supports were obtained by 1000 bootstraps of the data. The most probable ancestral node was determined by rooting the networks to a closely related outgroup, in this case a *Fascioloides magna* (EF534994) sequence.

Results

Adult worms were collected from the livers of ruminant hosts from abattoirs across two provinces of Pakistan (3 sheep, 7 goats, 2 cattle and 2 buffalo). In all cases, the size and gross morphology of the worms were typical of *Fasciola* spp. Since it was previously confirmed that the rDNA ITS-2 genotype reliably distinguishes *F. gigantica* and *F. hepatica* (Adlard *et al.*, 1993), this was further used to determine the species of *Fasciola* present. Between 1 and 8 worms from each individual host were sequenced for the rDNA ITS-2 (46 worms sequenced in total) and aligned with 13 sequences from *F. hepatica* (accession nos: AB207148, AJ557568, EF612479, AJ557567, AB207150, AM900370, AM707030, AM709498, GQ231546, GQ231547, FJ467927, FJ593632, AB010974), ten from *F. gigantica* (accession nos: AJ853848, AJ557569, EF612482, AB010977, AB207151, EF612484, AM900371, EU260063, AB010975, AB010976) and seven intermediate sequences (AB207150, EU260064, EU260066, EU260067, EU260068, EU260069, EU260071). All sequences in the alignment were trimmed to 343 bp, the length of the shortest sequence available that still contained all the informative sites.

This alignment showed five interspecific variable nucleotide positions, which is consistent with the previously studied rDNA ITS-2 of *F. hepatica* and *F. gigantica* species. According to the alignment, four of these are nucleotide substitutions at positions 231, 270, 276 and 334, and the final mutation is an insertion in *F. hepatica* at position 324. Two sequences did not conform to this pattern. The first is from Zambia (accession no.: AB010975), which contained members of both *F. gigantica* and *F. hepatica* interspecific nucleotide positions (table 1). The second is from Vietnam and was identified as an intermediate species (accession no.: EU260069) and was consistent with all *F. hepatica* interspecific positions except for the inserted T at position 324 (table 1). In addition to confirming the five species-specific fixed single nucleotide polymorphisms (SNPs), these rDNA ITS-2 sequences identified six sites that showed intraspecific variation within *F. hepatica* and *F. gigantica* (positions 207, 218, 284, 298, 341 and 342), which is also consistent with the previously studied rDNA ITS-2 of *F. hepatica* and *F. gigantica* species (table 1).

The ten rDNA ITS-2 sequences from *F. gigantica* selected from the public databases from different geographical regions had six unique haplotypes (Fg-H1, Fg-H3, Fg-H7, Fg-H8, Fg-H9 and Fg-H10). The 13 rDNA ITS-2 sequences from *F. hepatica* populations selected from the databases from different geographical regions had three haplotypes (Fh-H2, Fh-H4 and Fh-H5) (table 2). The seven intermediate sequences were split, with six belonging to *F. hepatica* haplotype Fh-H2 and one forming a unique haplotype of Fh-H11. From the 46 rDNA ITS-2 region sequences from Pakistan in the present study, there were just three haplotypes present (Fg-H1, Fg-H3 and Fg-H6) (accession nos: KM259915, KM259916 and KM259917) (table 1). The haplotype Fg-H1 was the most common and was represented by 44 sequences from 14 populations (F6C, F11G, F12G, F13G, F14C, F16G, F17B, F15B, F18G, F19G, F21S, F22S, F23S, F20G) of *Fasciola* sampled from Sahiwal, Rawalpindi, Multan, RY Khan and DG Khan abattoirs of Punjab province and Quetta and Mastoung abattoirs of Baluchistan province. This haplotype has been previously reported from *F. gigantica* from Indonesia (accession no.: AB010977). The haplotype Fg-H3 was represented by one sequence from a single population (F12G) sampled from the Rawalpindi abattoir of Punjab province. This haplotype has previously been reported from *F. gigantica* from Burkina Faso, Egypt and Kenya (accession nos.: AJ853848, EF612482, EF612484). The haplotype Fg-H6 was represented by two sequences from two populations (F16G, F23S) sampled from Multan and Quetta abattoirs of Punjab and Baluchistan provinces (table 2). This haplotype has not been previously reported in the literature.

A maximum likelihood (ML) tree was constructed to examine the phylogenetic relationship between rDNA ITS-2 haplotypes. The six and three different haplotypes of *F. gigantica* and *F. hepatica*, respectively, fell into two distinct phylogenetic clades corresponding to the species of origin (fig. 1). Exceptions were Fg-H9 which was identified as *F. gigantica* but may also be a hybrid and grouped with Fh-H11, the other hybrid, in the *F. hepatica* clade (fig. 1). The unique Fg-H6 haplotype, identified for the first time in this study, clustered with the *F. gigantica* clade.

Discussion

Fasciola is very cosmopolitan in distribution, being found throughout all regions of the world, including temperate, tropical and subtropical regions. *Fasciola hepatica* infection is found in temperate and tropical areas where sheep and cattle are raised and in humans, typically where they consume raw watercress (Mas-Coma *et al.*, 2009). Infection with *F. gigantica*, on the other hand, is found more commonly in tropical and subtropical regions of the world (Mas-Coma *et al.*, 2014). The presence of both *F. gigantica* and *F. hepatica*, and the existence of intermediate forms, has been reported in livestock from Iran (Rokni *et al.*, 2010; Amor *et al.*, 2011b), Egypt (Marcilla *et al.*, 2002; Dar *et al.*, 2012; El-Rahimy *et al.*, 2012), Niger (Ali *et al.*, 2008), Japan (Itagaki *et al.*, 1998, 2005; Ichikawa & Itagaki, 2010), Korea (Agatsuma *et al.*, 2000), China (Huang *et al.*, 2004; Liu *et al.*, 2014) and Vietnam (Le *et al.*, 2008). A number of studies revealed single-species infections of *F. hepatica*, reported in Tunisia, Algeria and Italy (Garippa, 2009; Farjallah *et al.*, 2013) and *F. gigantica* has been reported in India (Velusamy *et al.*, 2006; Prasad *et al.*, 2008; Raina *et al.*, 2013) and Mauritania (Amor *et al.*, 2011a).

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Table 1. The rDNA ITS-2 alignment of *F. hepatica* and *F. gigantica* sequences from GenBank along with the three haplotypes from the present study, showing variable positions and insertions.

Species	Country	Variable positions in the ITS-2											Accession numbers
		207	218	231	270	276	284	298	324	334	341	342	
F. hepatica	Australia	T	T	T	C	C	C	T	T	G	T	A	AB207148
	China	T	T	T	C	C	C	T	T	G	A	T	AJ557568
	Egypt	T	T	T	C	C	C	T	T	G	T	A	EF612479
	France	T	T	T	C	C	C	T	T	G	A	T	AJ557567
	Japan	T	T	T	C	C	C	T	T	G	T	A	AB207150
	Niger	T	T	T	C	C	C	T	T	G	T	A	AM900370
	Spain	T	T	T	C	C	T	T	T	G	T	A	AM707030
	Spain	T	T	T	C	C	C	T	T	G	T	A	AM709498
	Tunisia	T	T	T	C	C	T	T	T	G	T	A	GQ231546
	Tunisia	T	T	T	C	C	C	T	T	G	T	A	GQ231547
	Turkey	T	T	T	C	C	C	T	T	G	T	A	FJ467927
	Turkey	T	T	T	C	C	C	T	T	G	T	A	FJ593632
	Uruguay	T	T	T	C	C	T	T	T	G	T	A	AB010974
F. gigantica	Burkina Faso	T	T	C	T	T	C	T	//	A	T	A	AJ853848
	China	C	T	C	T	T	C	T	//	A	A	T	AJ557569
	Egypt	T	T	C	T	T	C	T	//	A	T	A	EF612482
	Indonesia	C	T	C	T	T	C	T	//	A	T	A	AB010977
	Japan	C	C	C	T	T	C	T	//	A	T	A	AB207151
	Kenya	T	T	C	T	T	C	T	//	A	T	A	EF612484
	Niger	C	T	C	T	T	C	T	//	A	A	T	AM900371
	Vietnam	C	C	C	T	T	C	T	//	A	T	A	EU260063
	Zambia	T	T	A	C	C	C	C	//	G	T	A	AB010975
	Zambia	T	T	C	T	T	C	C	//	A	T	A	AB010976
Fasciola spp. (intermediates)	Japan	T	T	T	C	C	C	T	T	G	T	A	AB207150
	Vietnam	T	T	T	C	C	C	T	T	G	T	A	EU260064
	Vietnam	T	T	T	C	C	C	T	T	G	T	A	EU260066
	Vietnam	T	T	T	C	C	C	T	T	G	T	A	EU260067
	Vietnam	T	T	T	C	C	C	T	T	G	T	A	EU260068
	Vietnam	T	T	T	C	C	C	T	T	A	T	A	EU260069
	Vietnam	T	T	T	C	C	C	T	T	G	T	A	EU260071
Fasciola spp. collected in this study	Pakistan	C	T	C	T	T	C	T	//	A	T	A	Haplotype 1
	Pakistan	T	T	C	T	T	C	C	//	A	C	A	Haplotype 6
	Pakistan	T	T	C	T	T	C	T	//	A	T	A	Haplotype 3

Table 2. Haplotypes of rDNA ITS-2 from *F. hepatica* and *F. gigantica* showing the number of sequences (in brackets) representing unique ITS-2 alleles, relative to country of origin.

Haplotype	Species	Countries
Fg-H1 (44)	*F. gigantica*	Pakistan, Indonesia
Fg-H3 (4)	*F. gigantica*	Pakistan, Burkina Faso, Egypt, Kenya
Fg-H6 (2)	*F. gigantica*	Pakistan
Fg-H7 (2)	*F. gigantica*	Japan, Vietnam
Fg-H8 (2)	*F. gigantica*	China, Niger
Fg-H9 (1)	*F. gigantica/hybrid*	Zambia
Fg-H10 (1)	*F. gigantica*	Zambia
Fh-H2 (6)	*F. hepatica*	Australia, Egypt, Japan, Niger, Spain, Tunisia, Turkey, Vietnam
Fh-H4 (3)	*F. hepatica*	Spain, Tunisia, Uruguay
Fh-H5 (2)	*F. hepatica*	China, France
Fh-H11 (1)	*F. hepatica/hybrid*	Vietnam

In Pakistan, the surveillance record of fascioliasis showed an estimated prevalence of 17.68% in Bahawalpur, 23.97% in Multan and 10.48% in Lahore from Punjab province (Khan *et al.*, 2009), 4% prevalence in Hyderabad from Sindh province, 7.7–16.2% prevalence in Quetta from Baluchistan province and 5.9% prevalence in the northern Khadagzai area, Dir district and the Hindu Kush Range (Afshan *et al.*, 2014).

In the present study, adult specimens of *F. gigantica* infecting small and large ruminants from seven localities of two provinces were characterized by sequencing rDNA ITS-2 regions. Previous studies have shown that these sequences provide reliable genetic markers for the accurate differentiation and identification of *Fasciola* spp. (Farjallah *et al.*, 2013). We found three distinct haplotypes from rDNA ITS-2 sequences recovered from all individuals sequenced from Pakistan, Fg-H1, Fg-H3 and Fg-H6. The former two are identical to previously reported *F. gigantica* sequences and the latter was unique but clustered with *F. gigantica* haplotypes on the phylogenetic tree. The Fg-H1 haplotype has been found as the predominant form across widespread geographical areas of Pakistan and is shared with Indonesia with very low frequency (Itagaki & Tsutsumi, 1998). The Fg-H3 haplotype was identified in Pakistan and shared with the most widespread geographical regions of the world, including Burkina Faso (Mas-Coma *et al.*, 2005), Egypt and Kenya (Lotfy *et al.*, 2008). In fact, the unique Fg-H6 haplotype was identified in Pakistan and is not shared with any other geographical regions of the world (table 2). The occurrence of the shared haplotypes (Fg-H1, Fg-H3) in a wide geographical area of Pakistan could be linked to on-going as well as historical activities related to animal migrations (Mas-Coma *et al.*, 2009; Amor *et al.*, 2011a). Considering the proven usefulness of the rDNA ITS-2-based sequence analysis, both for the unequivocal differentiation between *F. hepatica* and *F. gigantica* and the demonstration of the existence of an intermediate genotype (Huang *et al.*, 2004; Amor *et al.*, 2011a), of particular interest is the fact that none of the study isolates were found to be either *F. hepatica* or mixed infection of both or intermediate species. We found all the isolates to

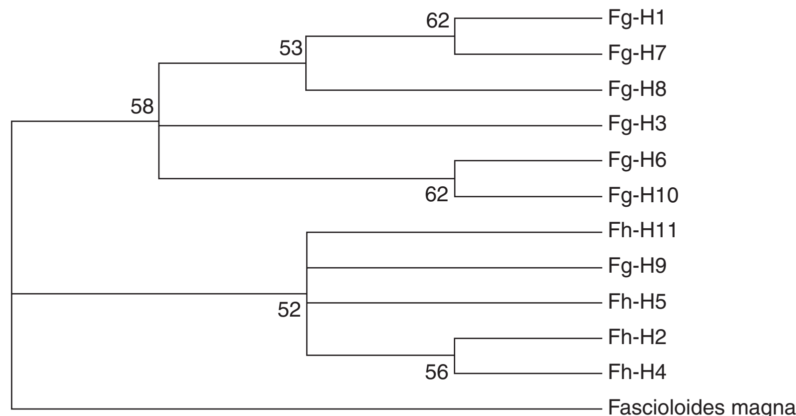

Fig. 1. Phylogenetic analysis of six haplotypes obtained from *F. gigantica* rDNA ITS-2 sequences, and five haplotypes obtained from *F. hepatica* and *Fasciola* hybrid rDNA ITS-2 sequences, from different countries, including our region of study (Fg-H1, Fg-H3 and Fg-H6). *Fasciola gigantica* and *F. hepatica* haplotypes are identified with Fg or Fh respectively. The sequences were aligned by Geneious software and the tree obtained by maximum likelihood (ML) analysis using a Kimura 2 model (K2 + G) of substitution. Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50, respectively, are represented at the base of the nodes. The phylogeny is rooted with the rDNA ITS-2 sequence of *Fascioloides magna* (GenBank accession number EF534994).

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be *F. gigantica*, when matched with the other, previously reported, ITS-2 rDNA sequences of *F. gigantica.*

We have used previously published *F. hepatica* and *F. gigantica* isolates, along with the *F. gigantica* of the present study, to provide more information on the genetic variation of the ITS-2 rDNA locus. The SNPs at positions 231, 270, 324 and 334 all showed invariant fixed differences between the two species, even when the isolates were from diverse geographical origins. We also identified a number of intraspecific variable positions previously reported at position 207, 218, 284, 298, 341 and 342 in *F. hepatica* and *F. gigantica* (table 1). The fixed interspecific variation and distinct pattern of intraspecific variations in ITS-2 rDNA sequence between the two species provided strong evidence for the presence of a single species, *F. gigantica,* in the Pakistani region (table 1).

The results reported here contrast with previous publications, which have reported a predominantly single-species infection with *F. hepatica* or low-level mixed infections of both *F. hepatica* and *F. gigantica* (Ahmed *et al.*, 2005; Ijaz *et al.*, 2007; Iqbal *et al.*, 2007; Gadahi *et al.*, 2009; Kakar *et al.*, 2011; Akhtar *et al.*, 2012; Shahzad *et al.*, 2012; Afshan *et al.*, 2013; Ashraf *et al.*, 2014). Further, in a more recent morphometric analysis of *Fasciola* isolates from buffalo originating from the districts of Punjab province, similar to some of those included in this study, Afshan *et al.* (2013) reported *F. hepatica* and *F. gigantica*, along with intermediates of both species. The difference between the results here and those of previous studies may be due to the fact that the previous identifications were based purely on morphological analysis. There are limitations associated with morphometric analysis, especially in terms of varying parameters with varying diagnostic value used in discriminating the two species and intermediates (Lotfy *et al.*, 2008). Together with inconsistency in morphological features attributed to both species and poorly characterized intermediate *Fasciola* forms, it is difficult to discern accurately between isolates of the two species, either *F. hepatica* or *F. gigantica* (Itagaki *et al.*, 2009; Ichikawa & Itagaki, 2010).

In conclusion, the molecular identification and the phylogenetic analysis of *Fasciola* from Pakistan confirm, for the first time, that all the specimens of liver flukes from small and large ruminants from different localities belong to the species *F. gigantica*. The present genetic analysis of *F. gigantica* has implications for the diagnosis and control of fascioliasis in the region. Showing the potential for mis-identification of *Fasciola* species, this article highlights the need for accurate species identification in order to understand parasite distributions and hybrid zones. Without reliable identification, it will be impossible to determine the differential disease outcomes and epidemiology of different species, or to assess the extent and impacts of hybridization. Therefore more studies using more polymorphic genetic markers are needed for further molecular analysis of a wide range of isolates from different host species and geographical areas, in order to better understand the genetic variability and population structure within *Fasciola* spp., and their transmission dynamics in Pakistan.

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Conflict of interest

None.

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