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MOLECULAR CONFIRMATION OF A *FASCIOLA GIGANTICA* × *FASCIOLA HEPATICA* HYBRID IN A CHADIAN BOVINE

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ABSTRACT

Fascioliasis is a zoonotic infection of humans and, more commonly, ruminants. It is caused by 2 liver fluke species, *Fasciola hepatica* and *Fasciola gigantica*, which differ in size. The traditional morphological methods used to distinguish the 2 species can be unreliable, particularly in the presence of hybrids between the 2 species. The development of advanced molecular methods has allowed for more definitive identification of *Fasciola* species, including their hybrids. Hybrids are of concern, as it is thought that they could acquire advantageous traits such as increased pathogenicity and host range. In 2013, we collected flukes from *Fasciola*-positive cattle, sheep, and goats slaughtered in 4 Chadian abattoirs. DNA from 27 flukes was extracted, amplified, and analyzed to identify species using the *ITS1+2* locus. Twenty-six of the 27 flukes were identified as *F. gigantica*, while the remaining fluke showed heterozygosity at all variable sites that distinguish *F. hepatica* and *F. gigantica*. Cloning and sequencing of both alleles confirmed the presence of 1 *F. hepatica* and 1 *F. gigantica* allele. To our knowledge, this is the first unambiguous, molecular demonstration of the presence of such a hybrid in a bovine in sub-Saharan Africa.

Fascioliasis is a liver fluke infection affecting ruminants and occasionally humans that, while treatable, is difficult to diagnose. It is acquired by ingesting infective metacercariae on vegetation grown in contaminated water or ingesting the contaminated water itself (Andrews, 1999). Animal fascioliasis leads to economic loss because of reduced herd productivity resulting from diminished fertility, reduced production of wool and milk, and increased mortality (Bernardo et al., 2011). Spillover infections from animals to humans were, until recently, thought to occur only occasionally (Mas-Coma et al., 2009). However, studies suggest that the frequency of human cases is on the rise (WHO, 2015). Human infections, while seldom fatal, cause pain, liver and gallbladder damage, and injury to other organs in the case of ectopic migration (Lotfy and Hillyer, 2003; Fürst et al., 2012).

Fascioliasis is caused by 2 species of the genus *Fasciola*, namely, *Fasciola hepatica* and *Fasciola gigantica* (Mas-Coma et al., 2007). Distinguishing the 2 *Fasciola* species is often accomplished by morphometry or inferred from geography, but this is not reliable as there can be overlaps in the size of the 2 species and in their geographic range (Moazeni et al., 2012; Afshan et al., 2014). Advances in molecular methods now allow species identification and provide a powerful tool to identify potential hybrids (Agatsuma et al., 2000; Huang et al., 2004; Itagaki et al., 2005; Amor et al., 2011b; Shalaby et al., 2013).

Traditionally, it has been thought that *F. hepatica* exists in the temperate regions of the Americas, Europe, and Oceania, while *F. gigantica* is present across Africa and Asia. However, recent reports of *F. hepatica* in Africa and Asia have challenged these long-held beliefs (Agatsuma et al., 2000; Huang et al., 2004; Ali et al., 2008; Walker et al., 2008; Amer et al., 2011; Dar et al., 2012; Mucheka et al., 2015).

In Africa, *F. hepatica* has been confirmed by molecular analysis in Algeria (Farjallah et al., 2009), Ethiopia (Le et al., 2012), and Tunisia (Farjallah et al., 2009). Geographic co-occurrence (but not necessarily co-infection) of the 2 species has been genetically proven in Egypt (Amer et al., 2011; Dar et al., 2012), Niger (Ali et al., 2008), South Africa (Mucheka et al., 2015), Tanzania (Walker et al., 2008), and Zimbabwe (Mucheka et al., 2015). Further reports of *F. hepatica* from Africa based on morphology have been published from Côte d'Ivoire (Utzinger et al., 2010), Ethiopia (Abebe, 2010), Kenya (Ogambo-Ongoma, 1969), and Morocco (Khallaayoune et al., 1991). *Fasciola gigantica* has been documented at Lake Chad (Jean-Richard et al., 2014), but, to date, neither *F. hepatica* nor hybrids have been detected in Chad.

Given that *F. hepatica* is present in a few African countries and that it is difficult to distinguish from *F. gigantica* by clinical, coprological, or immunological methods (Mas-Coma et al., 2005), it is conceivable that *F. hepatica* has merely been overlooked across Africa, including Chad. Furthermore, morphology as a method for species determination has not been rigorously tested in Africa, and hence, *Fasciola* species may have been misidentified. The presence of hybrids complicates matters further (Itagaki et al., 1998; Valero et al., 2005; Inoue et al., 2007; Afshan et al., 2014).

There have been repeated reports of hybrids (or 'intermediate forms') of *F. gigantica* and *F. hepatica* in regions where both species are present (Agatsuma et al., 2000; Huang et al., 2004; Itagaki et al., 2005; Amor et al., 2011b; Shalaby et al., 2013). Hybrids have been reported by molecular means in Egypt (Amer et al., 2011), Islamic Republic of Iran (Amor et al., 2011b), Japan (Itagaki et al., 2005), Korea (Agatsuma et al., 2000), Pakistan (Mufti et al., 2014), People's Republic of China (Huang et al., 2004; Peng et al., 2009), Saudi Arabia (Shalaby et al., 2013), Thailand (Wannasan et al., 2014), and Vietnam (Nguyen et al., 2012). Hybrid forms, as determined by morphology, have been reported in Bangladesh (Ahasan et al., 2016), Egypt (Periago et al., 2008), Islamic Republic of Iran (Ashrafi et al., 2006), and Pakistan (Afshan et al., 2014). These reports are of interest because *F. hepatica* flukes have shown considerable adaptability to new hosts, both intermediate and definitive, environmental conditions, and pharmaceutical treatments (most importantly triclabendazole) raising concerns that hybrid forms may have greater propensity for geographic and host expansion (Seehausen, 2004; Walker et al., 2008; Cwiklinski et al., 2015).

Species-specific single-nucleotide polymorphisms (SNPs) in the nuclear ribosomal DNA are commonly used to differentiate *Fasciola* species. Previous investigations have identified up to 16 polymorphic positions in the internal transcribed spacer 1 (*ITS1*) and *ITS2* (*ITS1+2*) marker; 11 of these are commonly used to distinguish *F. hepatica* from *F. gigantica* (Ali et al., 2008; Itagaki et al., 2009; Amor et al., 2011a, 2011b; Liu et al., 2014; Chaudhry et al., 2016).

In the current investigation, 27 adult *Fasciola* flukes, collected from 19 slaughtered livestock in Chad, were molecularly characterized using the *ITS1+2* nuclear marker to confirm species and any hybrid forms (Itagaki et al., 2009; Liu et al., 2014). This analysis of Chadian *Fasciola* was part of a larger phylogenetic investigation that included Ivorian and Swiss *Fasciola* specimens.

Liver flukes were collected from the carcasses of 24 cattle, 2 goats, and 1 sheep during post-mortem inspection at 4 abattoirs located on the shores of Lake Chad (Bol, Gredaya, and Sidjé) and at 1 abattoir in N'Djamena, the capital of Chad, in 2013. All work was done in accordance with Chadian ethical regulations.

After collection, the flukes were rinsed with sterile water, put in 96% ethanol, and stored at room temperature. In June 2015, the samples were re-preserved for shipping to Switzerland. Twenty-three of the 27 samples were shipped in 96% ethanol, however, 3 (TCDF050, TCDF062.02, and TCDF082) were shipped in neutral buffered 10% formalin (stored for 29 days in formalin). Upon arrival, all flukes were rinsed with double distilled water and fully immersed in 96% ethanol.

Approximately one-quarter of each fluke was cut with a new or bleached scalpel blade and deposited into a lysis solution of 50 mM Tris, 1% SDS and proteinase K, then incubated at 56 C for 12–14 hr with agitation. DNeasy column kits (Qiagen, Hilden, Germany) were used for DNA extraction, adhering to the manufacturer's protocol with the exception of adjusting the amount of lysis solution and proteinase K. Chadian flukes did not necessarily lyse within 24 hr; therefore, more lysis solution was added (up to 380 µl lysis solution and 20 µl proteinase K) and the flukes were left at 56 C until they were fully lysed (up to 25 hr).

The combined *ITS1+2* region of 27 Chadian flukes was amplified in 10 µl polymerase chain reactions (PCRs) containing 1 µl of Buffer BD 10×, 1.6 µl $MgCl_2$ 25 mM, 0.2 µl 10 mM dNTP mix (2.5 mM of each dNTP), 0.4 µl of each 10 mM primer (BD1, forward: 5′-GTCGTAACAAGGTTTCCGTA-3′, and BD2, reverse: 5′-TATGCTTAAATTCAGCGGGT-3′) (Luton et al., 1992), 0.2 µl of FIREPol 5 U/µl (Solis BioDyne, Tartu, Estonia), 1 µl of DNA, and 5.2 µl of sterile water. The PCR protocol was initiated by 3 min at 94 C; followed by 45 cycles of 40 sec at 94 C, 45 sec at 60 C, and 1.5 min at 72 C; followed by a final extension step of 5 min at 72 C.

The PCR products were loaded onto 0.8% agarose (Promega, Madison, Wisconsin) gels with 1× TAE (Thermo Scientific, Waltham, Massachusetts) and run at 50 V for 20 min. Multiple bands were produced, and the band of the correct length, 1,000 base pairs (bp), was excised from the gel for sequencing. The Wizard® SV gel and PCR cleanup system (Promega) was used for gel cleanup. Finally, PCR products were sent to Microsynth (Balgach, Switzerland) for uni-directional Sanger sequencing using the BD1 primer. The sequence was compared to sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information) and matched *F. gigantica ITS1+2* sequences.

The PCR of the *ITS1+2* loci produced sequences of 970 bp. The sequences from the 27 specimens were aligned and analyzed using CodonCode Aligner version 6.0.2 (CodonCode Corporation, Centerville, Massachusetts). Thirteen SNPs were observed, 11 of those were from known species determining variable sites, plus another 2 sites at positions 107 (3 samples) and 413 (2 samples). These 2 SNPs were heterozygous (R and W, respectively) and did not differ between *Fasciola* species.

Twenty-six of the 27 specimens (96%) conformed to the pattern associated with *F. gigantica* at 10 of the 11 *ITS* species-specific variable sites (GenBank accession nos. MK321604-07, MK321610, MK321614-20, MK321623-26, MK321629-32, MK321634, MK321636, MK321639, and MK321641-43). The exception, specimen TCDF062.02 from a bovine, exhibited heterozygosity at 9 of the 11 sites and a sudden breakdown of the clean sequence after the insertion/deletion (indel) at position 911. This breakdown is the hallmark of a heterozygous variable site, which instead of having a nucleotide mutation has an indel in 1 of the 2 alleles. The Sanger sequence chromatogram for sample TCDF062.02 (Fig. 1) illustrates the sudden breakdown of a clean sequence at a specific bp (position 911, as indicated by the vertical line through the chromatogram). This observation and the heterozygous variable sites indicated that the specimen could be a hybrid. To confirm that there was an *F. gigantica* and an *F. hepatica* allele, the DNA of specimen TCDF062.02 was cloned using a Gibson assembly, and the clones were sequenced (see Suppl. Data for the methods) (Gibson et al., 2009).

Minor SNPs in individual sequences from the clones were due to the Taq polymerase used, and hence, a consensus sequence was built and it revealed heterozygosity at 10 of the 11 variable sites.

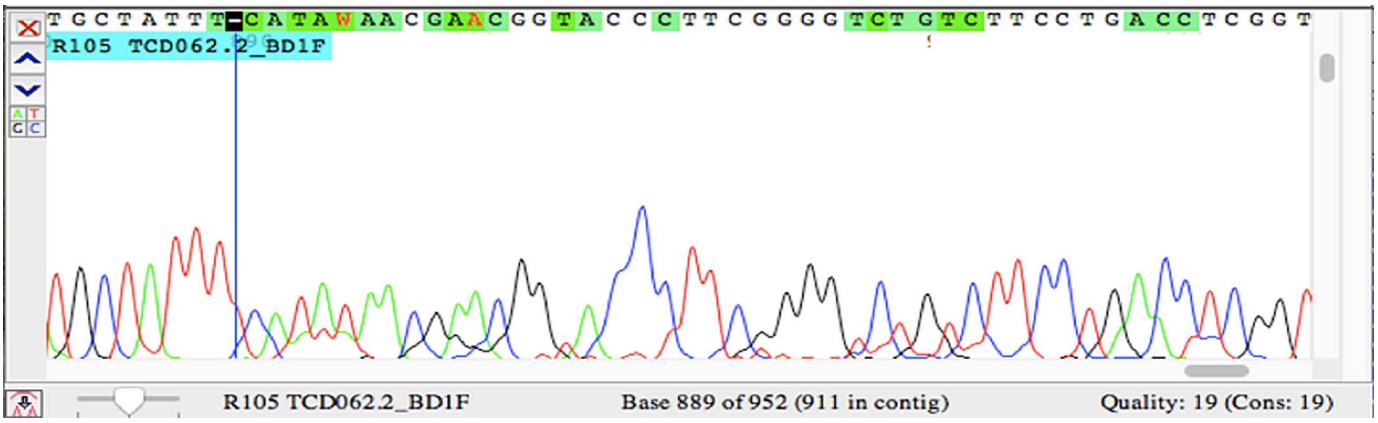

Figure 1. A section of the *ITS1+2* Sanger sequence chromatogram from Chadian *Fasciola hepatica* × *Fasciola gigantica* hybrid sample TCDF062.02. The vertical line indicates the breakdown of the clean sequence at position 911 due to a deletion in 1 of the alleles. Color version available online.

Thirteen clones were sequenced, 1 group (6 clones) matched the expected pattern for *F. hepatica* (GenBank accession no. MK321644) and the other group (6 clones) matched the pattern for *F. gigantica* (GenBank accession no. MK321645), confirming that this specimen is a hybrid between the 2 species.

A second larger analysis of the 11 species determining variable sites was performed using the sequences from all 27 Chadian specimens, in addition to sequences from Ivorian and Swiss specimens and sequences retrieved from GenBank (Table I). It revealed that 1 of the species determining variable sites, position 791, proved problematic for species determination in *F. gigantica*. While this site seems to be "T" consistently in *F. hepatica* specimens, it appears to vary geographically in *F. gigantica* flukes.

Twenty-six of 27 *Fasciola* flukes collected from cattle, goats, and sheep in Chad were found to be *F. gigantica* when analyzed using the 11 species determining variables sites. One specimen, from a bovine, was heterozygous at these sites, and cloning revealed alleles from the 2 *Fasciola* species. It was the only specimen from this bovine included in this analysis. To our knowledge, this is the first unambiguous proof of the occurrence of *Fasciola* hybrids in sub-Saharan Africa and only the second report from Africa. Hybrids have been detected in Egypt (in a buffalo) and in Saudi Arabia (in a goat); however, the goat was imported from Sudan, and it is not known if the hybrid came from Saudi Arabia or Sudan (Amer et al., 2011; Shalaby et al., 2013). There are only a few publications that used advanced molecular methods to determine species of African *Fasciola*, and only 5 sequences are currently available in GenBank. Therefore, the absence of reports of sub-Saharan African hybrids could be due to a paucity of prior investigations.

The hybrid fluke was taken from a cow that belonged to the semi-nomadic ethnic community of Arabs, who, at the end of the dry season, when the pastures around their villages are depleted, bring their livestock to the shores of Lake Chad for grazing (Weibel et al., 2008; Jean-Richard et al., 2014; Greter et al., 2017). At this time, Lake Chad is home to a large population of mobile pastoralists who travel hundreds of kilometers throughout the Sahelian region each year in search of forage. Jean-Richard et al. (2014) and Greter et al. (2017) found the prevalence of fascioliasis in cattle around Lake Chad is high (68% and 31% in slaughtered and live cattle, respectively). Presumably, these mobile livestock are picking-up *Fasciola* parasites and depositing their eggs along the migration route, including at Lake Chad. As the Arab cattle are exposed to Lake Chad, and the mobile livestock there have likely contaminated the area with *Fasciola* from throughout the Sahel, it is difficult to know with any certainty where this hybrid originated.

Site 791 of the *ITS1+2* marker has been included in a panel of 11 variable sites for distinguishing species of *Fasciola* in prior publications. The consensus in the literature is that *F. gigantica* should present a "C" at this site; however, this appears to be true only for *F. gigantica* from Asia and Niger. All *F. gigantica* samples and the hybrid from our investigation, as well as 3 out of 5 sequences from African *F. gigantica* (GenBank accession nos. EF612484, AB010976, and AJ853848) showed a homozygous "T" at this position (although sequence AJ853848 was incorrectly reported as "C" in Liu et al. 2014). The 2 sequences from Niger (GenBank accession nos. AM900371, AM850108) showed "C." *Fasciola gigantica* from the Middle East and Northern Africa also consistently show a "T" at this site, indicating that site 791 is not a reliable marker for distinguishing the 2 species.

There are several limitations to our investigation, which are offered for discussion. While there are a number of publications reporting the species of *Fasciola* found in Africa, many of them do not use the same method to determine species (morphology, molecular methods, or different molecular markers). Consequently, there are only a few *ITS1+2* sequences currently available from African *Fasciola*. Furthermore, among those publications that use *ITS1+2*, there are differences in the approach to the variable sites. Some studies use only one of the *ITS1+2* markers (only *ITS1* or *ITS2*), while others include more variable sites than those mentioned here (Ali et al., 2008; Itagaki et al., 2009; Amer et al., 2011; Choe et al., 2011). The reason for these differences is not clear, as there appear to be only 10 sites that reliably distinguish *F. gigantica* from *F. hepatica*; the 11 reported here minus site 791.

Despite our efforts to obtain a mitochondrial sequence for the hybrid, the sequence was of very poor quality. This may be due to the fact that the specimen was exposed to formalin. Formalin degrades DNA by causing cross-linking between nucleic acids and proteins, which can affect the success of PCR amplification (Vitošević et al., 2018). However, the 3 samples in this investigation that were exposed to formalin amplified in the *ITS1+2* PCR well. Furthermore, only 1 hybrid was found in this relatively small sample. Larger sample size may reveal more

Table I. Comparison of nucleotides at 11 variable positions of the *ITS1/2* marker of *Fasciola* samples collected from Chad, Côte d'Ivoire, and Switzerland and sequences deposited in NCBI GenBank. Abbreviations: n/a, no sequence for this part. Y and W indicate heterozygous positions with nucleotides C/T and A/T, respectively.

			Variable positions in *ITS1/2* sequences											
Species	Origin*	n†	18	108	202	280	300	791	815	854	860	911‡	918	Accession no.
Fasciola hepatica	Switzerland§	1	C	A	C	T	C	T	T	C	C	T	G	MK321597-602
	France	1	C	A	C	T	C	T	T	C	C	T	G	JF708034
	Spain	1	C	A	C	T	C	T	T	C	C	T	G	JF708036
	Niger	5	C	A	C	T	C	T	T	C	C	T	G	AM850107 AM900370
	Tunisia	1	C	A	C	T	C	T	T	C	C	T	G	GQ231546
	Tunisia	1	C	A	C	T	C	T	T	C	C	T	G	GQ231547
	Iran	1	C	A	C	T	C	T	T	C	C	T	G	JF432072
	Egypt	17	n/a	n/a	n/a	n/a	n/a	T	T	C	C	T	G	AB553720-36
	Egypt	67	C	A	C	T	C	n/a	n/a	n/a	n/a	n/a	n/a	LC076128-48 LC076150-52 LC076154-96
	Saudi Arabia	1	C	A	C	T	C	n/a	n/a	n/a	n/a	n/a	n/a	HE972273
	China	1	C	A	C	T	C	T	T	C	C	T	G	JF708026
	Japan	1‖	C	A	C	T	C	n/a	n/a	n/a	n/a	n/a	n/a	AB207145
Fasciola gigantica	Niger	13	T	T	T	A	T	C	C	T	T	—	A	AM900371 AM850108
	Chad§	26	T	T	T	A	T	T	C	T	T	—	A	MK321603-643
	Ivory Coast§	15	T	T	T	A	T	T	C	T	T	—	A	MK321603-643
	Burkina Faso#	1	T	T	T	A	T	T	C	T	T	—	A	AJ853848
	Kenya	1	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	EF612472
	Kenya	1	n/a	n/a	n/a	n/a	n/a	T	C	T	T	—	A	EF612484
	Mauritania	2	T	T	T	A	T	T	C	T	T	—	A	HQ197358, HQ197359
	Zambia	6‖	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	AB207142
	Zambia	6‖	n/a	n/a	n/a	n/a	n/a	T	C	T	T	—	A	AB010976
	Iran	1	T	T	T	A	T	T	C	T	T	—	A	JN828953
	Egypt	1	T	T	T	A	T	T	C	T	T	—	n/a	KF425321
	Egypt	9	n/a	n/a	n/a	n/a	n/a	T	C	T	T	—	A	AB553694-702
	Saudi Arabia	1	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	HE972274
	Indonesia	1‖	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	AB207143
	Thailand	1‖	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	AB207144
	Indonesia: Thailand	1‖	n/a	n/a	n/a	n/a	n/a	C	C	T	T	—	A	AB207149
	China	3	T	T	T	A	T	C	C	T	T	—	A	JF496709, JF496714 KF543340
	Japan	1‖	T	T	T	A	T	n/a	n/a	n/a	n/a	n/a	n/a	AB207146
	Japan	2‖	n/a	n/a	n/a	n/a	n/a	C	C	T	T	—	A	AB207151, AB207152
	Vietnam	1	n/a	n/a	n/a	n/a	n/a	C	C	T	T	—	A	EU260063
	South Korea	1	n/a	n/a	n/a	n/a	n/a	C	C	T	T	—	A	HQ821455
	India	1	n/a	n/a	n/a	n/a	n/a	C	C	T	T	G	A	KJ720004
Fasciola hybrids	China	1	n/a	n/a	n/a	n/a	n/a	T	T	C	C	T	G	AJ557570 clone I
	China	1	n/a	n/a	n/a	n/a	n/a	T	C	T	T	—	A	AJ557571 clone II
	China	1	Y	W	Y	W	Y	n/a	n/a	n/a	n/a	n/a	n/a	AJ628428
	Egypt	2	C	A	C	T	C	n/a	n/a	n/a	n/a	n/a	n/a	LC076149, LC076153
	Egypt	2	Y	W	Y	W	Y	n/a	n/a	n/a	n/a	n/a	n/a	AB553691, AB553692
	Egypt	1	n/a	n/a	n/a	n/a	n/a	T	Y	Y	Y	T	R	AB553737
	Egypt	1	n/a	n/a	n/a	n/a	n/a	T	Y	Y	Y	—	R	AB553738
	Saudi Arabia	1	C	A	T	A	A	n/a	n/a	n/a	n/a	n/a	n/a	HE972275
	Japan	1‖	Y	W	Y	W	Y	n/a	n/a	n/a	n/a	n/a	n/a	AB207147
	Japan	1‖	n/a	n/a	n/a	n/a	n/a	Y	Y	Y	Y	T	R	AB207153
	South Korea	7	Y	W	Y	W	Y	n/a	n/a	n/a	n/a	n/a	n/a	AB211237
	Chad§	1	Y	W	Y	W	Y	T	Y	Y	Y	—/T	R	MK321644-645

* Origin as reported in GenBank.
† Number of samples investigated.
‡ Dashes (—) = Deletion.
§ Specimens from this study.
‖ One entry in GenBank, but appears to be consensus sequence from numerous specimens from the publication.
Site 791 for this specimen is reported as "C" in Liu et al. (2014), but as "T" in Amor et al. (2011a,b), it is reported as "T" in GenBank.

hybrids and strengthen the case for hybrids in Chad and perhaps elsewhere in sub-Saharan Africa.

Table I lists *ITS1+2* sequences deposited in GenBank, although it is not a comprehensive list of all *Fasciola ITS1+2* sequences available in GenBank. Some samples were not included due to unclear descriptive information. For example, a number of sequences were labeled in GenBank as *Fasciola* spp., although the meaning of this is neither clear nor consistent among samples. In addition, not all studies have used the same method or loci to determine species and, as mentioned previously, some analyses have reported sequences incorrectly. These issues limit our ability to compare specimens from other investigations and underscore the meticulous nature of sequence analysis work. Further investigations from different parts of sub-Saharan Africa with larger sample sizes than reported here are warranted to provide better resolution concerning which species are present in Africa and where exactly they are located.

Fasciola hepatica has been found to adapt quickly to external selection pressures such as new hosts, new environments, and medications leading to a selective advantage (Walker et al., 2008; Cwiklinski et al., 2015). Hybrids of *F. gigantica* and *F. hepatica* are of considerable concern as they may have increased adaptability, allowing them to expand their geographic and host range as well as acquire increased resistance to medications. This poses a threat to animal and human health as well as animal production industries in areas that are endemic for fascioliasis, particularly in resource-constrained settings where prevention and control strategies may be difficult to implement and sustain.

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