Molecular characterization of Fasciola isolates collected from sheep, goats and cattle in Kisumu, Baringo and Narok Counties, Kenya

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Abstract

Fasciolosis is a neglected trematode infection of public health and veterinary importance caused by *Fasciola gigantica* and *Fasciola hepatica*. Molecular analysis using the internal transcribed spacers' ITS-1 and ITS-2 of nuclear ribosomal DNA is useful in distinguishing *Fasciola* species. This study aimed to characterize liver flukes from sheep, goats and cattle using these genetic markers. Fifty nine adult *Fasciola* specimens were collected from livers of naturally infected sheep, goats and cattle at selected abattoirs in Kisumu, Baringo and Narok Counties. Sequence comparison of ITS-1 and ITS-2 sequences of *Fasciola* isolates from this study and sequences in Genbank was carried out. A maximum likelihood tree was constructed for phylogenetic analysis. Analysis of ITS-1 and ITS-2 rDNA sequences revealed that *F. hepatica* and *F. gigantica* caused infection in both cattle and sheep and in goats only *F. gigantica* caused infection. The sequenced PCR amplicons showed a close relationship between *Fasciola* species in this study with *Fasciola* isolates from other regions in the world. Phylogenetic analysis showed that sequences of *F. hepatica* are similar to the sequence from Spain, China and Tunisia obtained from GenBank. The sequences of *F. gigantica* in this study have similarity to the sequence from Iran and Burkina Faso. Data from this study provides information that serves as basis for further studies on the distribution of *F. gigantica* and *F. hepatica* in other localities in Kenya, and is also important in designing epidemiological and control programmes for zoonotic fascioliasis.

Introduction

The most widespread liver flukes are of the genus *Fasciola* and include two species: *Fasciola hepatica* and *Fasciola gigantica* causing fasciolosis (Wongkham et al. 2005), but *Fasciola hepatica* is majorly prevalent in temperate regions while *Fasciola gigantica* is commonly distributed in tropical climates (Walker et al. 2008; Yakhchali et al. 2015). *Fasciola* infection is of veterinary importance, especially in regions majorly practicing sheep or cattle farming, resulting in massive economic burdens (Hussein and Khalifa 2010). The World Health Organization has recognized *Fasciola* infection as a Neglected Topical Disease due to its public health significance (Takeuchi-Storm et al. 2017; Sah et al. 2018) and is now considered as an emerging infection in different regions of the world, especially in South America, Africa and Asia (Hussein and Khalifa 2010, Salahi-Moghaddam and Arfaa 2013).

The two *Fasciola* species coexist in many African and Asian countries (Sumruayphol et al. 2020), and can also occur in the same region, although ecological factors influence the transmission of trematodes and the abundance of their freshwater snail intermediate host (Mas-Coma et al. 2005). *Fasciola* parasites can be distinguished using morphological features (Ashrafi et al. 2006), but this can lead to uncertainty in identification of species with intermediate morphological features (Itagaki et al. 2005). Molecular methods have been employed to distinguish between *Fasciola hepatica* and *Fasciola gigantica* (Marcilla et al. 2002), these are the most reliable and sensitive methods for the exploration of genetic variability among flukes (Mas-Coma et al. 2007). They are also very useful tools for epidemiological survey and diagnosis of *Fasciola* species (Mas-Coma et al. 2005).

Molecular analysis was carried out in this study to differentiate adult *Fasciola* species obtained from naturally infected liver of sheep, goats and cattle at selected slaughter houses within the study area. The nucleotide sequences of the ITS-1 and ITS-2 of the nuclear ribosomal DNA (rDNA) of 59 *Fasciola* worms from Kisumu, Baringo and Narok Counties was determined in order to establish the prevalence of *Fasciola* species and identify the domestic animal species most infected by *Fasciola* parasite in the study area.

Materials And Methods

Study area

This study was carried out in Mara river basin in Narok County, Perkera irrigation scheme in Baringo County and Ahero irrigation scheme in Kisumu County, Kenya (Fig. 1). The residents of the study area practice irrigation and livestock farming for both beef and milk production. Perkera irrigation scheme lies in the lowland area with an average altitude of 1100 m above sea level and covers a total area of 2,350 hectares. The rainfall varies from 1000 to 1500 mm in the highlands to 600 mm per annum in the lowlands. The temperatures vary between 25°C to 30°C, however in January the temperatures rises up to 35°C on average. The

Ahero irrigation scheme has an area of 2085.9 Km^2 and annual relief rainfall between 1200 mm and 1300 mm with a mean annual temperature of 23^0C with a range of between 20^0C and 35^0C and the altitude of 1168 m above sea level. The 13,750 km^2 drainage area of the Mara River basin covers the agricultural and forested areas in the upper basin, the open pastureland in the middle portion of the basin and the Masai Mara Game Reserve in Kenya (1718 km^2, all of which is within the Mara River Basin). It lies at an altitude range of 1480-2280 m above sea level. The rainfall is bimodal and the highest annual rainfall amount is received in the high altitude areas with 1100 mm on the average. Livestock accounts for 30% of the agriculture in this region.

Source and collection of adult Fasciola samples

Adult *Fasciola* specimens were collected from livers of naturally infected sheep, goats and cattle at selected abattoirs in three regions, Perkera Irrigation Scheme, Ahero Irrigation Scheme and Mara River Basin. A total of 59 infected livers were collected from three abattoirs from 20 livers (8 sheep, 12 goats) from Perkera, 19 livers (7 sheep, 12cattle) fromAhero, and 20 livers (7 sheep, 4 goats, 9 cattle) from Narok (Table 1). The livers along with gall bladders including the bile duct of all cattle, sheep and goats slaughtered at the abattoir were thoroughly inspected for the presence of liver flukes and the infected ones were removed from the slaughtered animals. The bile ducts were incised longitudinally through the gall bladder and the parasites were removed with the help of fine forceps, taking all necessary precautions to avoid any damage to the parasite. The infected livers were squeezed manually to macerate the parenchyma and the flukes were carefully removed. A total of 354 of individual isolate were obtained (119 form Perkera, 133 form Ahero and 102 from Narok).All samples were thoroughly washed individually 2 to 3 times in physiological saline to remove debris and host cells, and subsequently fixed in 70% ethanol and were carried to the laboratory where they were identified as *Fasciola* species and stored at room temperature for DNA extraction.

Table 1
Location and number of *Fasciola* specimen used for molecular identification

Location	Host	Species	Number of isolates
Perkera	Sheep	*F. hepatica*	4
Perkera	Sheep	*F. gigantica*	4
Perkera	Goat	*F. gigantiga*	12
Ahero	Sheep	*F. gigantica*	7
Ahero	Cattle	*F. gigantica*	12
Narok	Sheep	*F. gigantica*	3
Narok	Sheep	*F. hepatica*	4
Narok	Cattle	*F. gigantica*	7
Narok	Cattle	*F. hepatica*	2
Narok	Goat	*F. gigantica*	4

DNA extraction

One adult fluke from each liver was used for total Genomic DNA was extracted using QIAamp DNA Mini Kits (Qiagen, USA) following the manufacturer's recommendations (Dar et al. 2012). The extracted DNA quality was assessed using 1% agarose gel examined in UV transilluminator and the bands were visualized and photographed.

PCR amplification

PCR amplification was performed according to (Yuan et al. 2016). The internal transcribed spacer 1 (ITS1) and Internal transcribed spacer 2 (ITS2), regions were amplified by PCR using a set of BD1 5'-GTCGTAACAAGGTTTCCGTA-3' and BD2 5'-TATGCTTAAATTCAGCGGGT-3' as forward and reverse primers, respectively. PCR reaction was performed in a total volume of 50

µl containing 2 µl DNA template, 25 µl 2x master mix one taq Quick load, 21 µl of ddH_2O and 1 µl of each primer in a thermocycler (BioRad®) under the following conditions: 94°C for 5 min as initial denaturation, followed by 30 cycles of 94°C for 20s (denaturation), 55°C for 30s (annealing), 72°C for 30s (extension) and final extension of 72°C for 10min. For detection of PCR results, 5µl of the PCR product was examined on 1% agarose gel in TAE buffer at 80V for 45min. The gels stained with ethidium bromide, visualized and photographed using a transilluminator (UVITEC). To estimate the size of the amplicons, a 100bp DNA ladder (Fermentas) was used in gels. The PCR products were sequenced (Sanger dideoxy sequencing, Ingaba biotec) from both directions, using the same primers used in the PCR amplication.

Phylogenetic analysis

The sequences were aligned and compared with those of existing sequences related to *Fasciola* species ITS1 and ITS2, available in the GenBank, using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment was performed with data related to *Fasciola* species from other countries deposited in GenBank, using BioEdit Sequence Alignment Editor Version 7.1.3 software. A maximum likelihood tree was constructed using the MEGA X software and bootstrap analysis using 1,000 replicates (Shafiei et al. 2014; Kumar et al. 2018).

Results

The PCR amplification of ITS-1 and ITS-2 rDNA yielded fragments of approximately 970 bp in length (Fig. 2). The 59 PCR amplicons of ITS-1 and ITS-2 were subjected to direct sequencing which yielded sequences of 946 bp in length. The sequence was composed of the complete ITS-1 sequence of 422 bp, complete 5.8S sequence of 158 bp, and complete ITS-2 sequence of 366 bp. Out of the 59 *Fasciola* samples sequenced, 17% were *F. hepatica* and 83% were *F. gigantica*, there were no intermediate forms of *Fasciola* species. *Fasciola* samples collected from cattle were *F. hepatica* 2 (9.5%), *F. gigantica* 19 (90.5%), sheep *F. hepatica* 8 (36.4%), *F. gigantica* 14 (63.6%) and all samples from goats were *F. gigantica*.

The sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers MZ396875, MZ396876, MZ396877, MZ396878, MZ396885, MZ396925, MZ396926, MZ396928, MZ396929, MZ3969332 for *Fasciola hepatica* and MZ396874, MZ396879- MZ396884, MZ396886- MZ396924, MZ396927, MZ396930, MZ396931 *Fasciola gigantica.* The sequences were aligned with previously published *Fasciola* ITS-1, 5.8S and ITS-2 sequences retrieved from GenBank accession numbers JF432073, MK321604, JF496711, AM900371, MW046875, JF496709,KJ789338, HM746787, HQ197358, KF543340, AJ853848, JF432072, KJ789364, HM746785, MK321597, JF432075, GQ231547, AM709647, AM850107 and AM709621 (Alasaad et al. 2007; Ali et al. 2008; Farjallah et al. 2009; Rokni et al. 2010; Amor et al. 2011; Galavani et al. 2016; Evack et al. 2020; Omar et al. 2021).

The sequences of ITS-1 showed that *F. hepatica* differed form *F. gigantica* in five variable nucleotides, while the ITS-2 sequences of the examined *F. hepatica* was different from *F. gigantica* ITS-2 in seven nucleotides (Table 2). Among the 59 sequenced *Fasciola* isolates there was no nucleotide variation in the ITS-1 sequences. In ITS-2 sequences there was no nucleotide variation among *F. hepatica* but nucleotide variation was observed at two nucleotides of *F. gigantica* at sequence position 842 and 936, two and three *Fasciola* isolates respectively. This indicates the presence of two genotypes of *F. gigantica* isolates that were examines. Sequence variation in position 842 was examined only in Narok among sheep and cattle, while in position 936 it was examined in *Fasciola* samples from the three localities and it occurred in cattle and goat.

Table 2
Comparison of nucleotides at variable sites in ITS-1 and ITS-2 sequences of *Fasciola* from different hosts and geographical locations

Species	Variable sites in ITS-1 and ITS-2 sequences												Accession numbers	Location
	ITS-1 ITS-2													
	18	108	202	280	300	815	842	854	860	911	918	936		
F. gigantica	T	T	T	A	T	C	G	T	T	-	A	G	JF432073	Iran
	T	T	T	A	T	C	G	T	T	-	A	G	MK321604	Chad
	T	T	T	A	T	C	G	T	T	-	A	G	JF496711	China
	T	T	T	A	T	C	G	T	T	-	A	G	AM900371	Niger
	T	T	T	A	T	C	G	T	T	-	A	G	MW046875	Zimbabwe
	T	T	T	A	T	C	G	T	T	-	A	G	MZ396874	This study
	T	T	T	A	T	C	C	T	T	-	A	G	MZ396918	This study
	T	T	T	A	T	C	C	T	T	-	A	G	MZ396931	This study
	T	T	T	A	T	C	G	T	T	-	A	T	MZ396912	This study
	T	T	T	A	T	C	G	T	T	-	A	T	MZ396916	This study
	T	T	T	A	T	C	G	T	T	-	A	T	MZ396919	This study
F. hepatica	C	A	C	T	C	T	G	C	C	T	G	G	AM709647	Spain
	C	A	C	T	C	T	G	C	C	T	G	G	AM850107	Niger
	C	A	C	T	C	T	G	C	C	T	G	G	JF432072	Iran
	C	A	C	T	C	T	G	C	C	T	G	G	JF708027	China
	C	A	C	T	C	T	G	C	C	T	G	G	MZ396875	This study

Phylogenetic tree was constructed for the analysis of phylogenetic diversity of the liver flukes, using ITS1 and ITS2 sequences of *F. gigantica* and *F. hepatica* from this study along with available sequences in GenBank from other regions (Fig. 3). *Fascioloides magna* was used as an outgroup GenBank accession number EF534991 (Khalifa et al. 2016).

Discussion

Fascioliasis is a parasitic disease which is of concern to veterinary and public health sector worldwide (Sy et al. 2021). The genetic markers ITS1 and ITS2 genes of ribosomal DNA have been utilized to discriminate between *Fasciola hepatica* and *Fasciola gigantica* (Amor et al. 2011; Dar et al. 2019). There is limited data on the genetic characteristics of *Fasciola* species in Kenya. Out of the 59 *Fasciola* samples sequenced, 83% and 17% of the isolates were identified as *F. gigantica and F. hepatica* respectively. In the present study, *F. gigantica* caused majority of the infection in cattle compared to infection in sheep. Isolates for *F. hepatica*, 8 out of 10 were found in sheep. This was in agreement with studies from other regions of the world that shows *F. hepatica* are more prevalent in sheep while *F. gigantica* are more prevalent in cattle (Akhlaghi et al. 2017). A study in Tanzania showed that 41 *Fasciola* isolates were *F. hepatica* (Farjallah et al. 2009), in Iran showed that 96.8% of 31 sheep sampled were infected with *F. hepatica* (Rokni et al. 2010). A study in Niger also showed that 66.7% of 12 cattle isolates were *F. gigantica* (Ali et al. 2008). In the past, it was believed that *F. hepatica* was present primarily in the temperate regions, while *F. gigantica* is distributed in some countries in tropical region (Evack et al. 2020). This has been proved not to be the case because several studies have recently documented the presence of *F. hepatica* in Africa and Asia (Amer et al. 2011; Dar et al. 2012; Mucheka et

al. 2015), including this present study that have shown the existence of both *F. hepatica* and *F. gigantica* in the three localities in Kenya. In addition, the present study is the first to demonstrate the presence of *Fasciola hepatica* in the study areas. This could be attributed to environmental and host-related factors that could affect the distribution of *Fasciola* flukes (Amer et al. 2016). Among the two *Fasciola* species, *Fasciola hepatica* has a selective advantage because it has been reported to adapt quickly to external selection pressures such as new hosts, new environments, and medications than *Fasciola gigantica* (Cwiklinski et al. 2015).

Alignment of the sequences of ITS-1 and ITS-2 rDNA with available sequences in GenBank showed ten DNA variable sites in which segregated the Fasciola isolates into two different genotypes, this is consistent with previous studies (Chougar et al. 2019). In this study, the sequences of ITS-1 showed five variable nucleotides that separated between *F. hepatica* from *F. gigantica* and there was no nucleotide variation in the ITS-1 sequences. ITS-2 sequences *F. hepatica* was different from *F. gigantica* in seven nucleotides. Also in ITS-2 sequences there was no nucleotide variation in *F. hepatica* but nucleotide variation was observed at two nucleotides of five *F. gigantica* at sequence position 842 and 936, two and three *Fasciola* isolates respectively. This indicates the presence of two genotypes of *F. gigantica* isolates in the three localities.

Phylogenetic tree constructed using ITS1 and ITS2 sequences of *F. gigantica* and *F. hepatica* from this study along with available sequences in GenBank from other regions showed a close relationship between *Fasciola* species in this study in comparison with *Fasciola* isolates from other regions in the world. All the sequences of *F. hepatica* are in the same group and are similar to the *Fasciola* species of Switzerland, Spain, China and Tunisia. The sequence of *F. gigantica* in this study, fall in the same group and are similar to the *Fasciola* species of Zimbabwe, Iran and Burkina Faso.

Conclusion

This present study has shown the existence of both *F. hepatica* and *F. gigantica* in Kenya. In addition, it is the first to demonstrate the existence of *Fasciola hepatica* in Narok, Kisumu and Baringo Counties in Kenya. Further studies involving more other mitochondrial polymorphic genes, like cytochrome c oxidase gene (CO1), can be carried out to assist understand more on genetic divergence of both *Fasciola gigantica* and *Fasciola hepatica* in the region and other parts of the world. This will also enable the assessment its zoonotic potential and epidemiological surveys especially in areas where there is limited resources making prevention and control strategies difficult to implement and sustain.

Declarations

Funding information

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Conflicts of interests

The authors declare that there are no conflicts of interest.

Availability of data and material

The datasets generated during and/or analysed during the current study are available in the GenBank repository under the following accession numbers [MZ396875-78, MZ396885, MZ396925-26, MZ396928-29, MZ3969332, MZ396874, MZ396897-84, MZ396886-924, MZ396927, MZ396930-31], [https://www.ncbi.nlm.nih.gov/nuccore]

Code availability

Not applicable

Authors' contributions

Cornelius Kibet Kipyegen designed the project, acquired and analysed data and wrote the first draft of the manuscript. Charle I. Muleke and Elick O. Otachi supervised the research, contributed to writing and reviewing of the manuscript. All authors read and approved the final manuscript.

Ethical approval

This study was approved by the Egerton University Research and Ethical Committee (EU/RE/DVC/009) and The Kenya National Commission for Science, Technology and Innovation (NACOSTI/P/15/8095/6943). “The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration 1975, as revised in 2008.”

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Figures

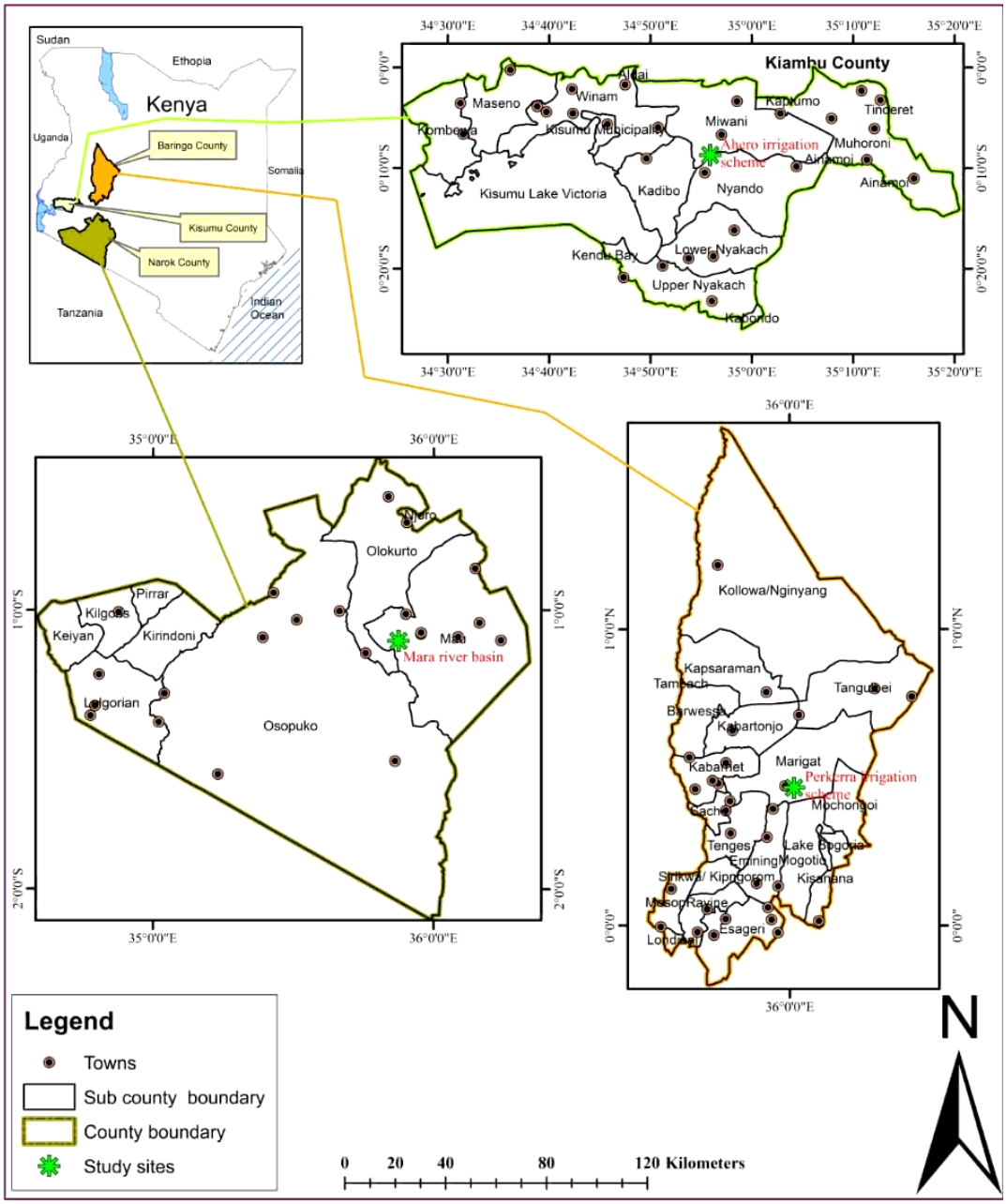

Figure 1

A map of Kenya showing the location of Narok, Ahero and Baringo Counties, where the samples were collected. Source: Kenya: County Fact Sheet (2013). Commission on Revenue Allocation, Second edition

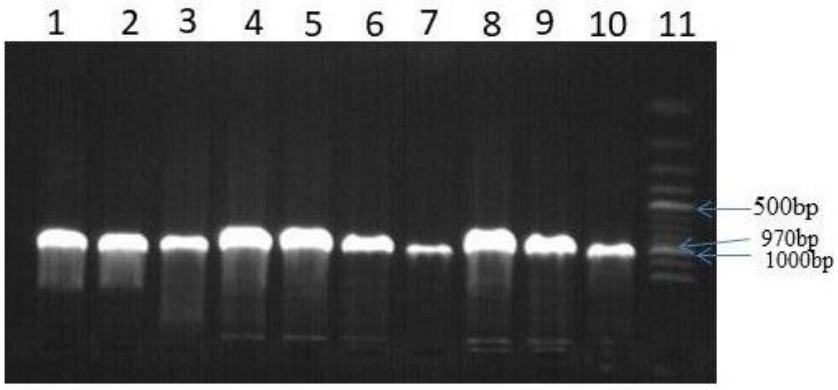

Figure 2

Agarose gel electrophoresis of ITS-1 and ITS-2 rDNA of Fasciola specimen. Well 1- 10 Fasciola species samples, well 11 is DNA ladder of 1kb molecular weight

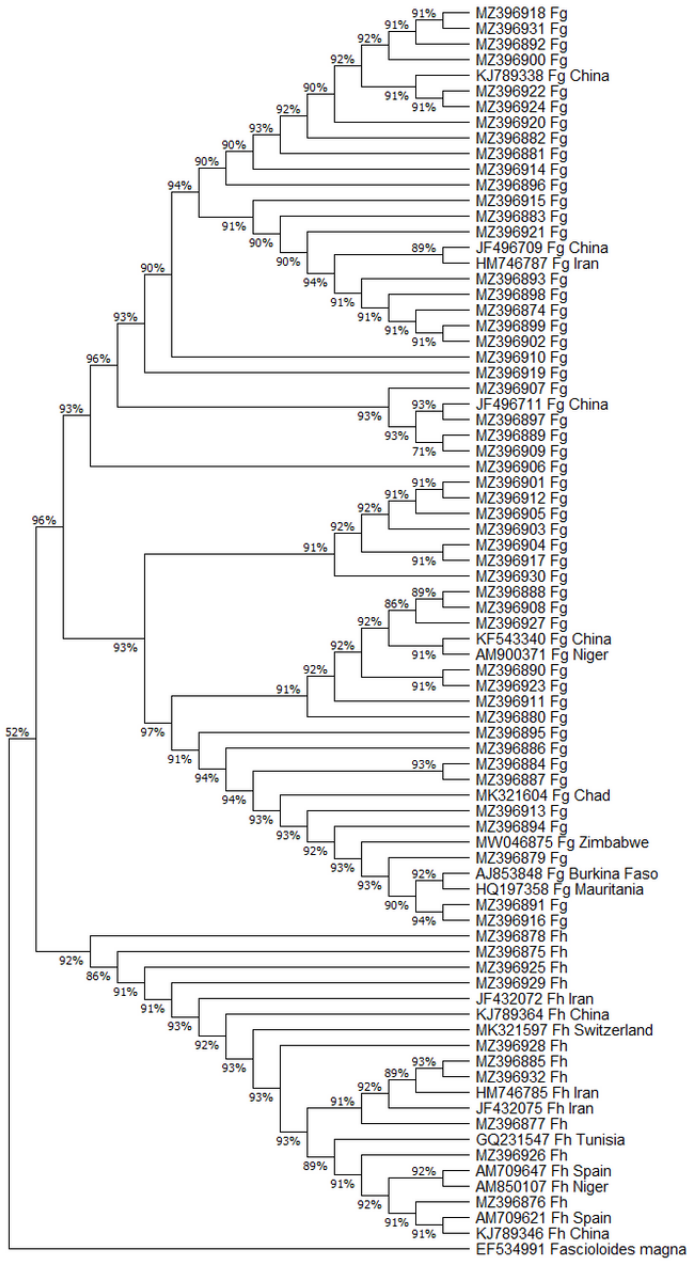

Figure 3

Phylogenetic relationship of ITS-1 and ITS-2 rDNA sequences of isolates of Fasciola hepatica (MZ396875-78, MZ396885, MZ396925-26, MZ396928-29, MZ3969332) and Fasciola gigantica (MZ396874, MZ396879-MZ396884, MZ396886-MZ396924, MZ396927, MZ396930-31) specimens collected from sheep, goat and cattle in Kenya and other representative isolates of F. gigantica and F. hepatica from different regions using Maximum Likelihood method. Phylogenetic tree was obtained using MEGA X [Kumar et al, 2018] with bootstrap values of 1000 replicates set for neighbor-joining.