Food and Waterborne Parasitology 22 (2021) e00114

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Morphological and molecular characterization of *Fasciola hepatica* and *Fasciola gigantica* phenotypes from co-endemic localities in Mpumalanga and KwaZulu-Natal provinces of South Africa

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ARTICLE INFO

Article history:
Received 18 September 2020
Received in revised form 24 January 2021
Accepted 27 January 2021

Keywords:
Fasciola species
Morphometrics
Co-endemic
South Africa
ITS-1/5.8S/ITS-2
CO1

ABSTRACT

Fasciolosis is a food- and water-borne disease caused by digenean trematode species, *Fasciola hepatica* and *F. gigantica*. They are widely distributed and infect a wide range of definitive hosts, causing enormous economic loss due to reduced productivity in domestic ruminants. The two species have been previously reported to be co-endemic in KwaZulu-Natal and Mpumalanga provinces of South Africa. Hybridization between the two species has been reported elsewhere. Despite the overlap of the two species in two provinces, there has been no attempt to determine the presence of the intermediate forms or hybrids. Therefore, this study aimed at morphological and molecular characterization of *Fasciola* spp. collected from cattle slaughtered at abattoirs located in the two provinces of South Africa, where two species are endemic. A total of 71 fluke specimens were collected cattle from abattoirs in Enhlazeni and Nelspruit in Mpumalanga province and Pietermaritzburg in KwaZulu-Natal province of South Africa, and Zimbabwe (Bulawayo abattoir). *Fasciola gigantica* specimen collected from Zimbabwe where it has been confirmed as the only species occurring and this was used as control in the morphological and molecular assessment of the collected specimens. Of the 71 specimens collected, 37 were classified as *F. hepatica*, 12 as *F. gigantica* and 22 as *Fasciola spp* using morphological characters. Of these species, 11 of 37 *F. hepatica* and 6 of 22 *Fasciola sp* were found to be aspermic or having very scanty sperm. Fifteen flukes which were spermatic were all identified morphologically as *F. gigantica* whilst 5 flukes which were aspermic were identified morphologically as *F. hepatica*. Molecular analysis of the same 15 spermatic specimens confirmed the presence of *F. hepatica* ($n = 9$) and *F. gigantica* ($n = 6$) using the CO1 marker and as *F. hepatica* ($n = 4$), *F. gigantica* ($n = 7$) and *Fasciola* sp. ($n = 1$) for the same specimens using the ITS-1/5.8S/ITS-2 marker. The remaining 4 aspermic flukes (one did not resolve) were all identified morphologically as *F. hepatica* and molecular analysis confirmed them as *F. hepatica* ($n = 4$) by both CO1 and ITS-1/5.8S/ITS-2. Phylogenetic analysis based on both CO1 and ITS-1/5.8S/ITS-2 showed that *F. hepatica* species formed a moderately supported monophyletic clade with *F. gigantica*. Their ancestral history was further confirmed by haplotype network, which formed novel haplotypes that corresponded with the structure of the phylogenetic tree. Results from this study showed that morphological characters alone have limitations in identifying *F. hepatica* and *F. gigantica* in areas where the two species occur, although both methods confirmed the presence of *F. gigantica* occurring in Zimbabwe, *F. hepatica* in KwaZulu-Natal, and both species occurring in Mpumalanga province. Therefore,

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https://doi.org/10.1016/j.fawpar.2021.e00114

the use of morphological techniques, complemented by molecular techniques are recommended, especially in endemic areas where the two species are co-endemic.

1. Introduction

Fasciolosis is an important parasitic disease of wild and domestic ruminants including humans, caused by digenean trematodes of the genus *Fasciola* (Mas-Coma et al., 2009; Admassu et al., 2015; Ibrahim, 2017). *Fasciola hepatica* (Linnaeus 1758) and *F. gigantica* (Cobbold 1856) are the most common and widespread flukes implicated in the aetiology of the disease (Le et al., 2007; Admassu et al., 2015). Despite being included in the list of human neglected tropical diseases (NTDs) by the World Organisation Health (WHO) (WHO, 2010; Mas-Coma et al., 2018), fascioliasis has long been well recognised for its impact on livestock productivity (Mas-Coma et al., 2009; Kalu, 2015). Globally, it is estimated that about 17 million people are infected (Mas-Coma et al., 2009) with severe health consequences (Mas-Coma et al., 2014, 2018).

Fasciola species have a wide geographical distribution, ranging from subarctic to tropical regions (Mas-Coma et al., 2009). Lymnaeid snail species act as the intermediate hosts and play a major role in the distribution of these species. *Fasciola hepatica* is widely distributed in Europe (Robinson and Dalton, 1999), temperate regions of Asia, Africa, Oceania and America whilst *F. gigantica* is mainly restricted to Africa and Asia (Mas-Coma et al., 2009; Lotfy and Hillyer, 2003). The two species appear to be sympatric in some subtropical and warm temperate regions, especially in Africa and Asia (Mas-Coma et al., 2009; Kalu, 2015). This overlapping distribution has led to controversies around the taxonomic identification of *Fasciola* species in the Far East countries (Periago et al., 2008) where the presence of *Fasciola* intermediate forms have been reported (Le et al., 2008; Periago et al., 2008) and presumed to be a result of hybridization and introgression processes and these are common in *Fasciola* populations in most Asian countries.

Fasciola hepatica and *F. gigantica* are generally distinguished based on the morphological characteristics of their body length and width (Mas-Coma and Bargues, 1997; Ashrafi et al., 2006; Itagaki et al., 2009). According to Itagaki et al. (2009), these two species have shown to be spermic diploid and able to function meiotically. However, the presence of individuals with intermediate morphological characters of *F. hepatica* and *F. gigantica* has previously caused confusion (Le et al., 2008), which subsequently led to the increase combined use of molecular (Marcilla et al., 2002) and morphometric methods (Ashrafi et al., 2006) to distinguish between the species. The existence of the intermediate forms of *Fasciola* or 'hybrids' were confirmed in Japan and Korea where studies on fluke populations identified individuals which resembled one species with nuclear DNA but have genotype typical of the other species with mitochondrial DNA (Le et al., 2008). However, no substantial work has been done to demonstrate the presence of 'hybrid' or introgressed liver fluke populations outside Asia where the two species overlap (Huang et al., 2004).

Fasciola species in South Africa have been reported in wildlife (Van Wyk and Boomker, 2011; Malatji et al., 2020), cattle (Ndlovu et al., 2009; Mucheka et al., 2015; Chikowore et al., 2019), horse (Alves et al., 1998) and humans (Black et al., 2013). Molecular studies have confirmed the presence of *F. hepatica* in Gauteng (Alves et al., 1998), Mpumalanga (Mucheka et al., 2015; Chikowore et al., 2019) and KwaZulu-Natal (Mucheka et al., 2015) provinces, and *F. gigantica* in Mpumalanga (Mucheka et al., 2015; Chikowore et al., 2019), KwaZulu-Natal (Mucheka et al., 2015) and Eastern Cape (Malatji and Mukaratirwa, 2019) provinces. The two species overlap in Mpumalanga (Chikowore et al., 2019) and KwaZulu-Natal provinces (Mucheka et al., 2015). However, no attempt has been made to determine the presence of intermediate forms or hybrids of *Fasciola* in South Africa in localities where the species are co-endemic. Hence, the objective of this study was to combine morphometric and molecular methods to identify *Fasciola* species collected from cattle slaughtered at abattoirs located in Mpumalanga and KwaZulu-Natal provinces of South Africa where *F. hepatica* and *F. gigantica* are co-endemic.

2. Materials and methods

2.1. Collection of fluke specimen

A total of 71 flukes were collected from the livers of naturally infected cross-breed cattle from abattoirs located in Mpumalanga (Nelspruit = 29 and Enhlanzeni = 14) and KwaZulu-Natal (Pietermaritzburg = 20) provinces of South Africa, and Zimbabwe ($n = 8$) (Fig. 1). Flukes were preserved in 70% ethanol prior transportation to the Parasitology Laboratory, University of KwaZulu-Natal, South Africa.

2.2. Morphological analysis

Liver flukes were removed from the 70% ethanol and stained using the Carmine staining method as described by Gibbons et al. (1996), with minor modifications. The flukes were washed with tap water to remove access ethanol from preservation, followed by rehydration through a graded series of alcohol (70%, 50%, 30%, 10% and distilled water). Flukes were then regressively stained

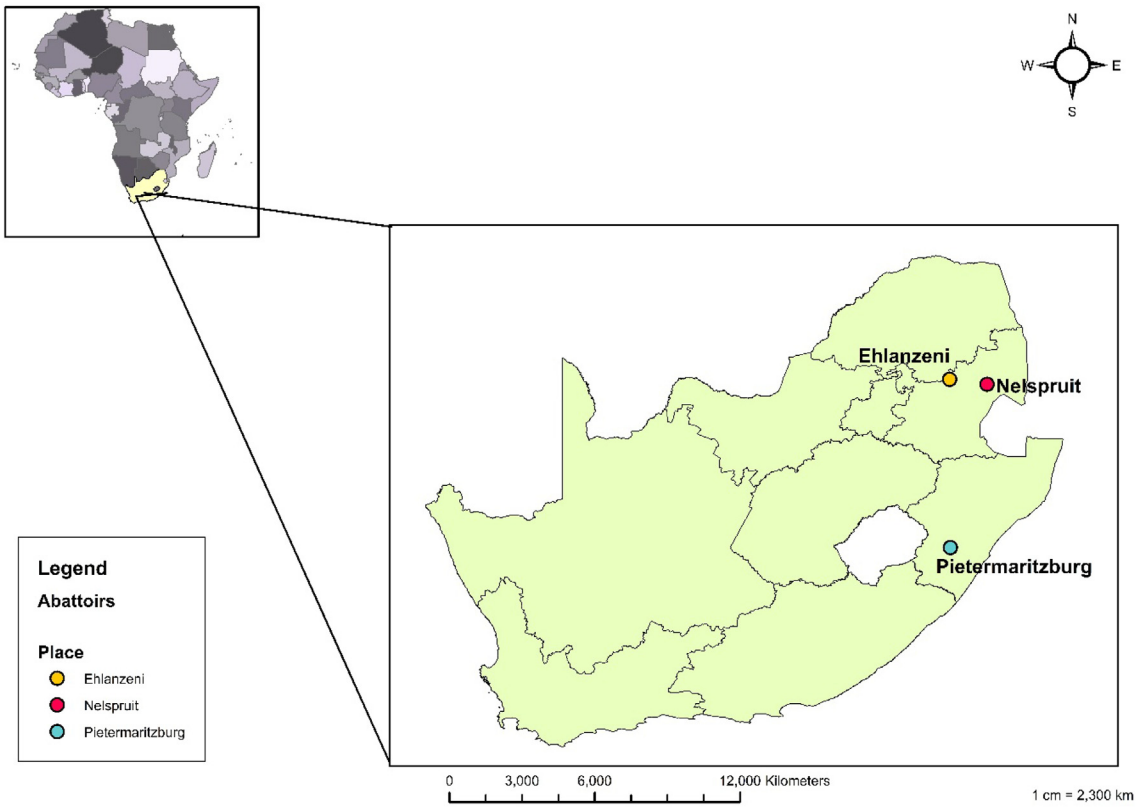

Fig. 1. Sampling collection locations in Mpumalanga and KwaZulu-Natal provinces of South Africa.

by immersing them in Aceto Alum Carmine stain overnight, then washed with distilled water to remove excess stain. This was followed by submerging them in alcoholic acid (2 ml of concentrated HCl in 100 ml of 70% ethanol) for 2–4 h to eliminate excessive staining without losing the pigmentation. Samples were subsequently dehydrated in ascending series of graded alcohols (50% - 70% - 80% - 90% - 100%) and cleared with clove oil for 24 h. The specimens were then mounted using Canada balsam and internal organs examined by light microscope.

The Zeiss Stereomicroscope (V12) integrated with software ZEN and AxioVision was used to visualise and the computer image analysis system (CIAS) was used to measure the length and width of the flukes. The length/width ratio was then manually calculated. The measurements were used to differentiate *Fasciola* species and classified as follows (Mas-Coma and Bargues, 1997): *F. hepatica* were any fluke with a length and width of 20–50 mm and 6–13 mm respectively, and a length/width ratio of 1.88–2.32 mm whilst *F. gigantica* were any isolates 24–27 mm long, 5–13 mm wide with an average length/width ratio of 4.39–5.20 mm (Supplementary Fig. 1). Flukes with intermediate measurements were regarded as an 'intermediate' form of *Fasciola*. Data was tested for normality with Kruskal Wallis test using SPSS 25, One-way analysis of variance (ANOVA) was used to compare the measurements of the 3 categories of specimens. Principal component analysis (PCA) was performed using R-studio (R-studio, 2015).

2.3. Determination of spermic and aspermic flukes

The seminal vesicles of each stained fluke specimen were examined to identify flukes with or without spermatozoa using Zeiss Stereomicroscope (v12) integrated with software ZEN and AxioVision). Spermatic flukes were identified by the presence of a sac-like structure on the anterior end of the body that appear grey in black and white imagery (Fig. 2).

2.4. DNA extractions and amplification

DNA was extracted from small tissue excised from the anterior section of the fluke using the Genomic DNA™ Tissue MiniPrep kit (Zymo Research Corporation) according to the manufacturer's instructions. PCR amplification of the DNA of 20 flukes was performed based on the ITS and CO1 regions using the following primers; S30FE (forward: 5"-GTCGTAACAAGGTTTCCGTA-3″) and S49E6 (reverse: 5"-TATGCTTAAATTCAGCGGGT-3″) (Luton et al., 1992) and FHCO1 (forward: 5"-TTGGTTTTTTGGGCATCCT-3″) and FHCO1 (reverse: 5"-AGGCCACCACCAAATAAAAGA-3″) (Mucheka et al., 2015), respectively. Amplification was performed in a 25 μL reaction volume, each containing 2 μL of genomic DNA, 12.5 μL PCR Master Mix (2×) (Thermo Scientific), 0.5 μL

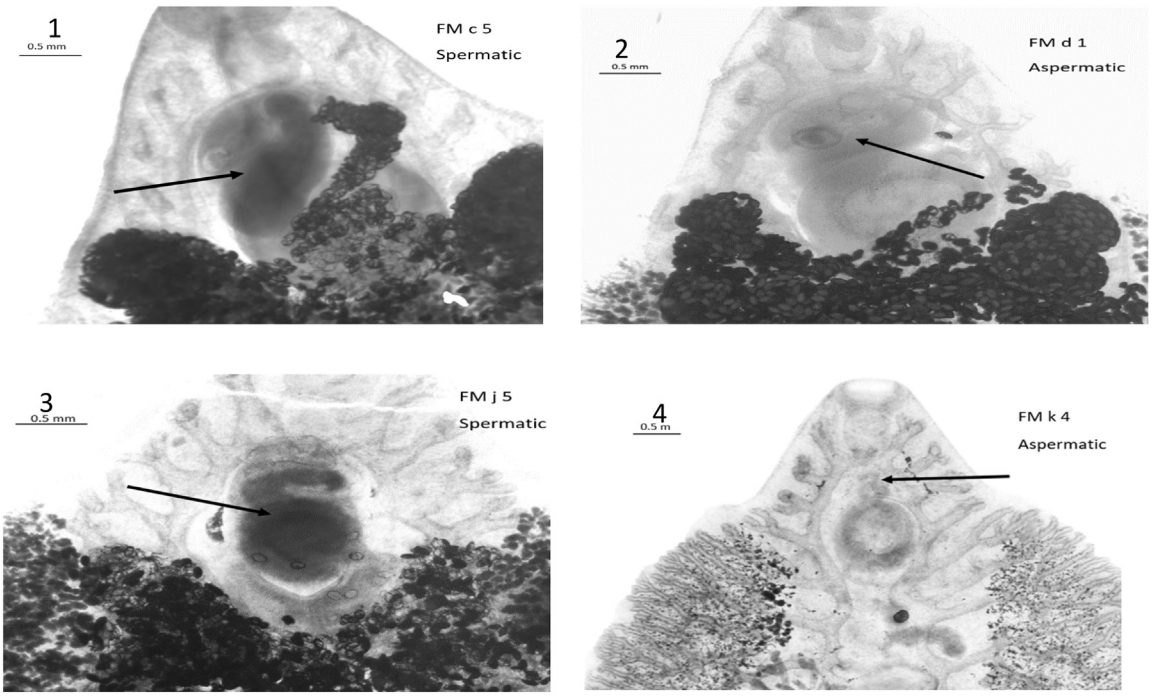

Fig. 2. Seminal vesicles of *Fasciola gigantica* (1) and *F. hepatica* (2–4) stained with Aceto Alum Carmine stain showing the presence (spermatic) or absence (aspermatic) of spermatozoa.

(10 μM) of each primer and 9.5 μL sterile water. PCR amplification was performed under the thermal cycling conditions as described by Mucheka et al. (2015): 94 °C for 1 min, followed by 40 cycles of 95 °C for 1 min, 1 min of annealing temperature at 55 °C for ITS and 59 °C for CO1, 72 °C for 1 min and lastly final extension at 72 °C for 7 min. Fragments were separated by electrophoresis in 1% agarose gel stained with ethidium bromide, at 80 V for 1 h. Amplicons were then sent to Inqaba biotechnical industries (Pty) Ltd. (Pretoria, South Africa) for sequencing.

2.5. Sequencing and phylogenetic analyses

Sequences were assembled, edited manually and aligned with homologus sequences obtained from the GenBank database using the Clustal W (Thompson et al., 1997) option of the BioEdit program (Hall, 1999). Aligned sequences were trimmed to a common length of 450 nucleotides for CO1 and 930 nucleotides for ITS genes. jModeltest (Posada, 2008) was used to select the most appropriate model of nucleotide substitution for use in neighbour-joining, maximum likelihood and Bayesian Inference analyses. The HKY + G and GTR models (Hasegawa et al., 1985) were selected for CO1 and ITS under the AIC information criterion, respectively. Maximum likelihood and neighbour-joining (NJ) trees were generated using PAUP* 4.0 (Swofford, 2002). For both methods, nodal support was estimated using 1000 bootstrap pseudo-replicates. Bayesian analysis was executed in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Four Markov chains were run for 5 million generations, until the standard deviation of the split frequencies was less than 0.01. The first 500,000 trees were discarded as burnin. Phylograms were 50% majority-rule consensus trees with nodal support indicated as posterior probabilities. DnaSP (v 5.10.1) (Rozas et al., 2003) was used to determine the number of haplotypes generated by the dataset and estimate the haplotype diversities. Haplotype networks were constructed using Network 5 (Bandelt et al., 1999) and the median joining rooting method under default (10) weight and with epsilon (ε) set to 0.

3. Results

3.1. Morphological identification

The morphological measurements grouped *F. hepatica, F. gigantica* and presumed intermediate form assigned as *Fasciola sp* specimens based on the body length/width/ratio measurements as indicated in Fig. 3. The average length/width and corresponding standard deviations for *F. hepatica, F. gigantica* and *Fasciola sp* were 21.16 ± 4.29/10.53 ± 1.80 mm, 39.61 ± 1.09/10.44 ± 1.59 mm and 28.87 ± 5.12/9.32 ± 1.72 mm respectively. The mean length/width ratios were 2.02 ± 0.35 mm, 2.79 ± 0.48 mm and 4.41 ± 1.10 mm, respectively. Results also indicated that the difference in the body length of specimens morphologically identified as either *F. hepatica, F. gigantica* or *Fasciola sp* was statistically significant ($P < 0.05$). However, the difference in

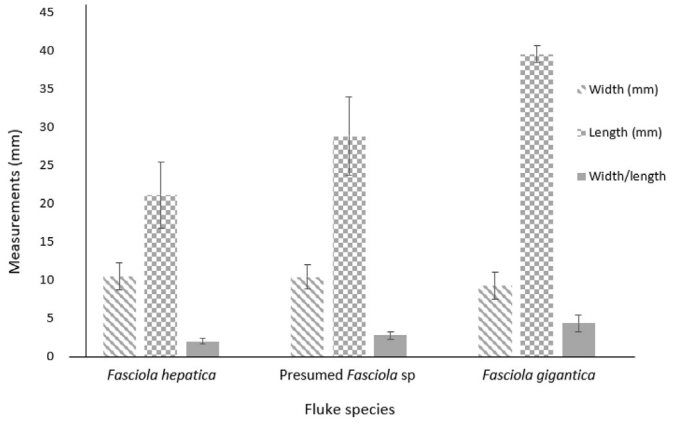

Fig. 3. Summary of combined morphological classification of flukes collected from Mpumalanga, KwaZulu-Natal provinces of South Africa and Zimbabwe based on morphological parameters.

the width was not statistically significant ($P > 0.05$). The length/width ratio between these groups was significantly different ($P < 0.05$) (Fig. 3).

Of the 71 specimens identified morphologically, *F. hepatica* was the only species found in PMB in KwaZulu-Natal, whilst *Fasciola sp*, *F. gigantica* and *F. hepatica* were identified in Enhlazeni and Nelspruit, respectively. Of the 14 specimens from Enhlazeni, 3 were identified as *F. hepatica* and 11 as *Fasciola* sp. In Nelspruit, 14 of 29 specimens were identified as *F. hepatica*, 4 as *F. gigantica* and 11 as *Fasciola* sp. *Fasciola gigantica* was the only species identifies in Zimbabwe (Table 1). All *F. gigantica* specimens identified were found with sperms, whereas 15.5% (11/71) and 8.6% (6/71) *F. hepatica* and *Fasciola sp* were found with little to no sperms (Table 2).

3.2. Molecular identification

Twenty-two specimens morphologically identified as *Fasciola sp*, 10 as *F. hepatica* and 3 as *F. gigantica* were analysed using the CO1 and ITS markers to confirm the species. However, only 20 specimens yielded usable sequences and this includes 10 *F. hepatica* and 3 *F. gigantica* specimens and 7 *Fasciola sp* specimens. The CO1 marker was able to resolve 19 specimens, whilst the ITS marker was able to resolve 15 specimens. Based on the measurements of the morphological characters of twenty (20) liver flukes, the analysis identified and classified 10/20 flukes as *F. hepatica*, 4/20 as *F. gigantica* and 6/20 flukes as *Fasciola sp* (Table 2). Although molecular analysis based on CO1 and ITS markers confirmed the classification of *F. hepatica* and *F. gigantica*, specimens classified as *Fasciola sp* based on their morphological characters were not consistent with that of the CO1 and ITS markers. These markers similarly identified the 6 specimens as *F. gigantica* (5/6) and as *F. hepatica* (1/6) (Table 2).

3.3. Phylogenetic analysis

Phylogenetic analysis based on the 440 nucleotides of CO1 marker showed that the in-groups (clade A) formed a monophyletic clade with respect to the outgroups (Fig. 4). Clade A furthers formed two clades (B and C) which distinguished between *F. hepatica* and *F. gigantica*. Strongly supported Clade B formed two moderate to strong supported subclades representing *F. hepatica* isolates. A well-supported clade (ML and BI) supported clade C represented *F. gigantica* species confirmed identification of nine isolates from this study as *F. gigantica*.

Analysis based on the ITS region formed a strongly supported clade (A) with the outgroups (Fig. 5).This clade included 16 experimental isolates from Mpumalanga province and one from KwaZulu-Natal province, South Africa. This clade shows the difference between *F. gigantica* (clade B) and *F. hepatica* (clade C). Clade B, which is well-supported, included 7 experimental isolates identified as *F. gigantica* and 6 *F. gigantica* isolates from the GenBank. The strongly supported clade C also included 8 isolates identified as *F. hepatica* and 5 *F. hepatica* isolates from the GenBank.

3.4. Haplotype analysis

CO1 sequence alignment of 440 nucleotides yielded 8 haplotypes (Supplementary Table 1), with a relatively high haplotype diversity (0.7892) and the haplotypes presented 23 variable sites between them. (Supplementary Table 2). Haplotypes H_1, H_3 and H_4 were the common haplotypes. *Fasciola gigantica* haplotype H_1, included all *F. gigantica* experimental isolates and one sample from GenBank, with exception to isolate FMC10. This isolate formed a sister clade to the *F. gigantica* isolates (Fig. 3) and a separate haplotype (H_2), which separated from the *F. gigantica* haplotypes H_1, H_6 and H_7 by 5, 9 and 12 mutational steps at sites, respectively (Supplementary Table 3). Typical *F. hepatica* haplotypes H_3 and H_4 included experimental isolates and samples from the GenBank as indicated in Supplementary Table 1. A separate haplotype was formed (H_5), which

Table 1
Presence or absence of sperm in the seminal vesicle in *Fasciola* species from Mpumalanga and KwaZulu-Natal provinces of South Africa and isolates from Zimbabwe.

Province/country	Area	No. of specimens	Absence (−)/Presence (+) of sperm in the seminal vesicles					
			F. hepatica		*F. gigantica*		*Fasciola* sp	
			−	+	−	+	−	+
Mpumalanga	Enhlazeni	14	2	1	0	0	6	5
	Nelspruit	29	4	10	0	4	0	11
KwaZulu-Natal	PMB	20	5	15	0	0	0	0
Zimbabwe		8	0	0	0	8	0	0
Total (%)		71	11 (15 0.5%)	26 (36.6%)	0	12 (16.9%)	6 (8.5%)	16 (22.5%)

PMB – Pietermaritzburg; "+" and "–" represents plenty of and absent to few sperms in the seminal vesicle, respectively.

Table 2
Morphological identification complemented by molecular identification of *Fasciola* species from Mpumalanga and KwaZulu-Natal provinces of South Africa, and Zimbabwe.

Sperm status	*N*	Origin			Morphological ID			Co1			ITS-1/5.8S/ITS-2		
		Mp	Zim	KZN	Fh	Fg	Fsp	Fh	Fg	Fsp	Fh	Fg	Fsp
Presence of sperm	15	14	1	0	5	3	7	6	9	0	4	7	1
Absence of sperm	5	4	0	1	5	0	0	4	0	0	4	0	0
Total	20	18	1	1	10	3	7	10	9	0	8	7	1

Mp = Mpumalanga province; Zim = Zimbabwe; KZn = KwaZulu-Natal province; ID = Identification; Fh = *Fasciola hepatica*; Fg = *Fasciola gigantica*; Fsp = *Fasciola* sp.

represented one *F. hepatica* (FM1) specimen from Mpumalanga province. This haplotype showed the presence of two mutational steps to H_3 at sits 5 and 28 and one mutational steps to H_4 at site 5 (Supplementary Table 3, Supplementary Fig. 2).

Two major haplotypes, each representing *Fasciola* species was formed from the ITS sequence alignment (Supplementary Table 3, Supplementary Fig. 3). These haplotypes showed a moderately high haplotype diversity (0,562), with four variable sites between them (Supplementary Table 4). Haplotype H_1 represents *F. gigantica* and H_2 *F. hepatica*. The haplotype network also showed four mutational steps between *F. gigantica* and between these and *F. hepatica* which occurred at all the sites as indicated in Supplementary Table 4.

4. Discussion

Morphometric analysis of flukes from the present study showed that *F. giganatica* and *F. hepatica* are co-endemic in Mpumalanga province, while only *F. hepatica* was found in KwaZulu-Natal province. *Fasciola gigantica* was initially morphologically distinguished from *F. hepatica* based on its elongated and narrower body, whilst *F. hepatica* was generally shorter, with broad shourders (Mas-Coma and Bargues, 1997; Lotfy and Hillyer, 2003). However, results from this study failed to dictinguish the two, and this in agreement with the study conducted by Peng et al. (2009) and Nguyen et al. (2018), who failed to adequately distinguish between *F. hepatica* and *F. gigantica* using the same characters. However, Sumruayphol et al. (2020) stated that the difference in these body character measurements could lead to misidentification of species, as they could be attributed by factors such as age of the flukes, hosts and season in which the flukes were collected. This was observed in this study, where 7 of the isolates morphologically classified as *Fasciola sp* were identified as either *F. gigantica* or *F. hepatica* by molecular techniques.

Fasciola hepatica and *F. gigantica* are classified as meiototically functional diploids due to their ability to reproduce sexually or through self-fertilisation (Shoriki et al., 2014). According to the author, these individuals produce sperm that is temporarily stored in seminal vesicles, which can be easily observed under a stereomicroscope when the fluke is stained (Shoriki et al., 2014). However, suspected 'hybrids' (*Fasciola* sp) were observed to contain few to no sperm in their seminal vesicles (Itagaki et al., 2009; Peng et al., 2009) and categorized as meiototically dysfunctional (Mohanta et al., 2014). The occurrence of an intermediate form of *Fasciola* was previously reported in Egypt (Periago et al., 2008) and in various Asian countries (Moghaddam et al., 2004; Ashrafi et al., 2006; Le et al., 2008; Periago et al., 2008). There are reports which has also showed that this type of *Fasciola* species showed the presence of different ploidies, all of which are parthenogenetic and do not produce normal sperm (Terasaki et al., 2001; Periago et al., 2008; Mohanta et al., 2014). From our study, 6 of 22 flukes morphologically identified as *Fasciola sp* had little to no sperms in their seminal vesicles, and 16 of the 22 presumed to be *Fasciola sp* had spermatozoa. This phenomenon of 'hybrid' flukes retaining sperm in their seminal vesicle was observed by Hayashi et al. (2018), who suggested that these species should be referred to as hybrid flukes and not aspermic flukes. Furthermore, 11 of 37 *F. hepatica* specimens were also found without sperms, which is in agreement with documented observation of 'pure' *F. hepatica* found to be aspermic triploid in Britain (Fletcher et al., 2004; Le et al., 2008). None of the specimens identified as *F. gigantica* were aspermic in our study.

The distribution of *Fasciola* species is dependent on the availability (Prasad et al., 2008), abundance and distribution of their intermediate host snail (Mucheka et al., 2015; Mahulu et al., 2019). *Fasciola gigantica* was the only species identified in

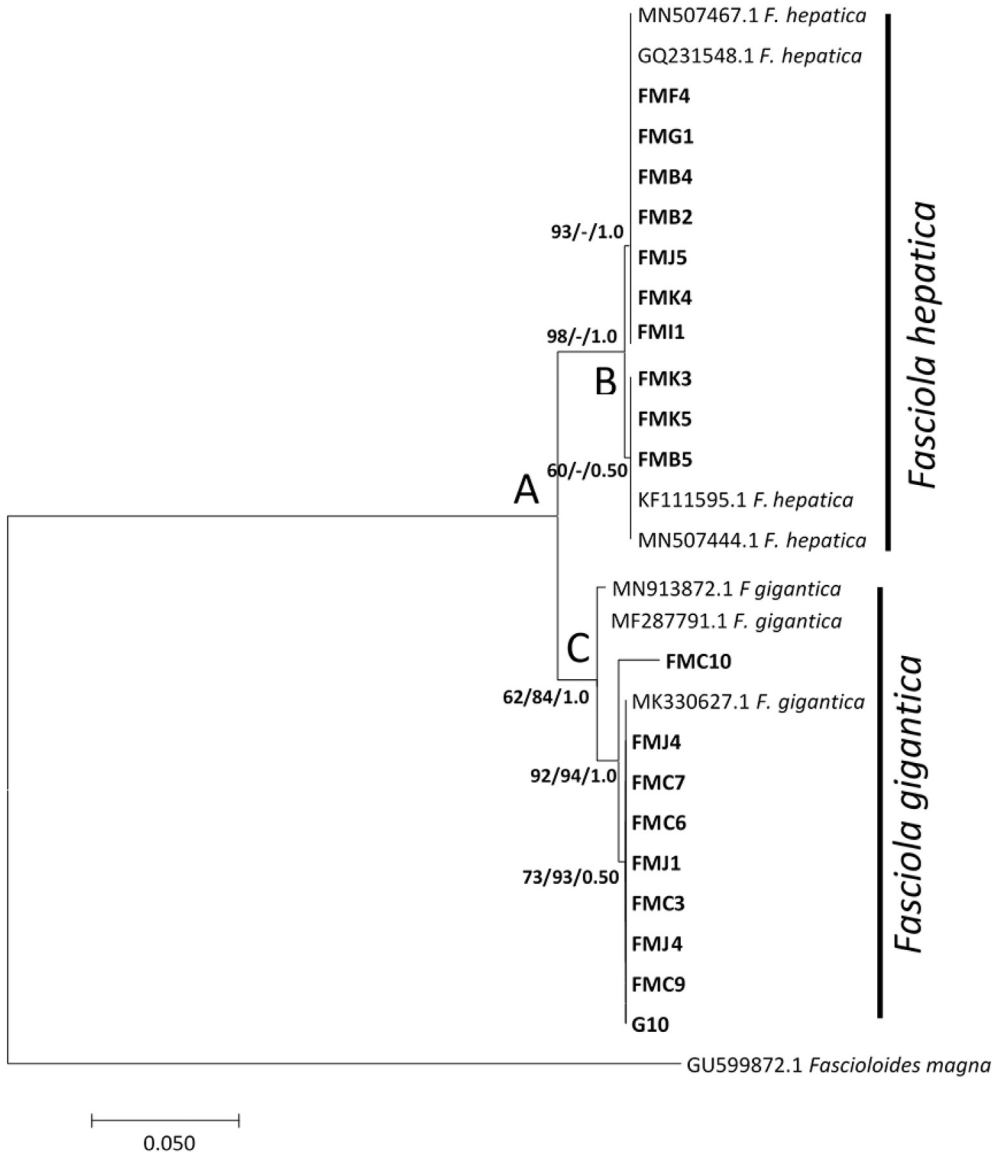

Fig. 4. Neighbour joining tree based on 440 nucleotides of the CO1 region, illustrating the relationships between the experimental samples and the sequences from GenBank (NCBI). Nodal support shown in the order neighbour-joining (NJ), maximum likelihood (ML) and Bayesian inference (BI).

Zimbabwe, and is in agreement with studies that shows that *F. gigantica* is the only *Fasciola* species predominantly occurring in Zimbabwe (Pfukenyi and Mukaratirwa, 2004; Chauke et al., 2014; Mucheka et al., 2015). The distribution and transmission of this species correspond with the distribution of *Radix natalensis*, which act as the intermediate hosts in Zimbabwe (Pfukenyi et al., 2006). Previous studies showed that both *F. hepatica* and *F. gigantica* occur in Mpumalanga and KwaZulu-Natal provinces of South Africa (Mucheka et al., 2015; Chikowore et al., 2019). Furthermore, although phylogenetic analysis by Mucheka et al. (2015) showed that both species overlap in Eshowe in KwaZulu-Natal province and Enhlazeni and Belfast (Chikowore et al., 2019) in Mpumalanga province; studies also showed that *F. hepatica* was predominant in Pietermaritzburg of KwaZulu-Natal and Mpumalanga highveld. Analysis based on the CO1 and ITS markers confirmed the presence of both *Fasciola* species in South Africa, with *F. hepatica* predominantly occurring in Pietermaritzburg of KwaZulu-Natal and Enhlazeni in Mpumalanga provinces, South Africa. Although both *Fasciola* species occurred only in Nelspruit, however, both locations in Mpumalanga province had suspected intermediate form of *Fasciola sp* based on morphological identification. Previous studies have shown that both *R. natalensis* and *Pseudosuccinea columella* occur in Mpumalanga (de Kock and Wolmarans, 1998; Malatji et al., 2019) and KwaZulu-Natal province (Perissinotto et al., 2014; Malatji et al., 2019; Malatji and Mukaratirwa, 2019), which explains the overlap in the distribution of both species in Nelspruit in this study, and previously reported overlap in both Mpumalanga and KwaZulu-Natal provinces.

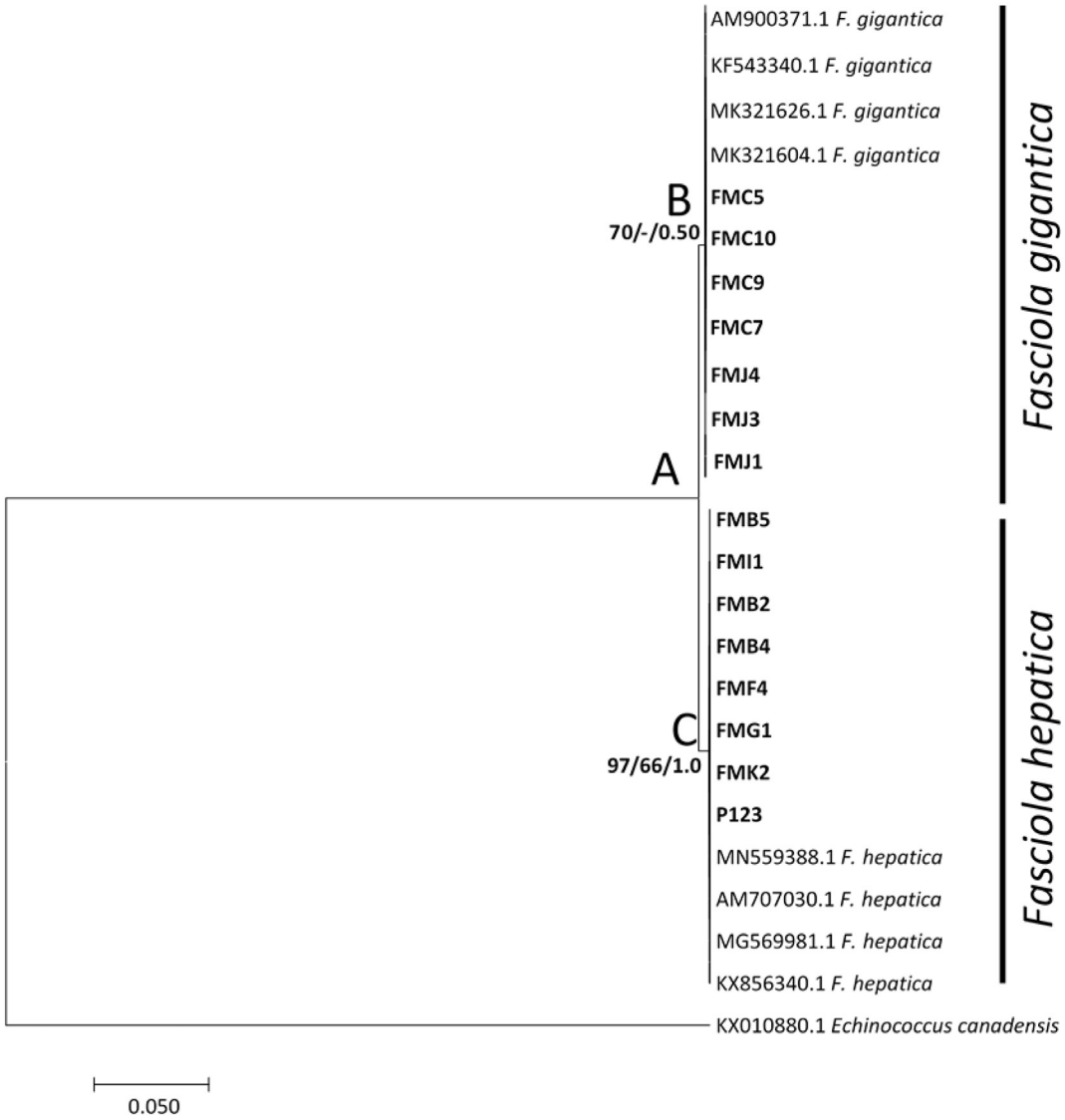

Fig. 5. Neighbour joining tree based on 920 nucleotides of the ITS region, illustrating the relationships between the experimental samples and the sequences from GenBank (NCBI). Nodal support shown in the order neighbour-joining, maximum likelihood and Bayesian inference.

Analysis of the CO1 and ITS regions of 20 specimens, all morphologically classified as *F. hepatica* and *F. gigantica* were confirmed by CO1 and ITS markers. Phylogenetic analysis based on the CO1 marker supported isolates from this study as *F. hepatica* and *F. gigantica*, as they formed well-supported reciprocally monophyletic clades identified by the presence of Genbank-derived isolates. These species formed three novel haplogroups from seven haplotypes, representing *F. hepatica* and *F. gigantica*. The ITS analysis yielded a well-supported *F. hepatica* clade which formed also monophyletic sister clade *for F. gigantica* and showed a clear distinction between the species. Furthermore, the ITS isolates formed two novel haplotypes, each representing the *Fasciola* species and corresponded with the structure of the of the phylogenetic tree, further showing that these species originate from the same ancestors.

5. Conclusion

Results from this study showed that analysis of the morphological characters of the flukes can, to an extent, distinguish between *Fasciola* species. However, factors such as the age of the fluke and hosts, as well as the season in which flukes were sampled are confounders to accurate morphological identification, which may lead to misidentification of flukes due to the presence of flukes with intermediate body measurements. Therefore, a combination of both morphological and molecular techniques to distinguish between *F. hepatica* and *F. gigantica* is crucial especially in areas where both species overlap. Genetic analysis from this study confirmed the occurrence of both *Fasciola* species in Mpumalanga and KwaZulu-Natal province of South Africa, which is

consistent with the presence and distribution of their intermediate hosts. We recommend a wider survey in areas where the two species overlap and the inclusion of Fasciola spp. specimen from sheep and goats in order to ascertain the presence or absence of hybrids or intermediate forms.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank Louis LaGrange for helping in identifying the abattoirs and sampling in Mpumalanga provinces. Our sincere gratitude to the abattoir managers for allowing us to collect the liver flukes.

Ethical consideration

The protocols of this study were reviewed and approved by the Animals Ethics Committee of the University of KwaZulu-Natal (Ref: AREC/044/016D) in accordance with the South African national guidelines on animal care, handling and use for biomedical research.

Funding

Financial support was from SM's research productivity funds from UKZN and National Research Foundation (NRF) of South Africa.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2021.e00114.

References

Admassu, B., Shite, A., Kinfe, G., 2015. A review on bovine fasciolosis. Eur. J. Biol. Sci. 7, 139–146.
Alves, R.M., Van Rensburg, L.J., Van Wyk, J.A., 1998. *Fasciola* in horses in the Republic of South Africa: a single natural case of *Fasciola hepatica* and the failure to infest ten horses either with *F. hepatica* or *F. gigantica*. Onderstepoort J. Vet. Res. 55, 157–163.
Ashrafi, K., Valero, M.A., Panova, M., Periago, M.V., Massoud, J., Mas-Coma, S., 2006. Phenotypic analysis of adults of *Fasciola hepatica, Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. Parasitol. Int. 55, 249–260.
Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036.
Black, J., Ntusi, N., Stead, P., Mayosi, B., Mendelson, M., 2013. Human fascioliasis in South Africa. S. Afr. Med. J. 103, 658–659.
Chauke, E., Dhlamini, Z., Mbanga, J., Dube, S., 2014. Characterization of *Fasciola gigantica* isolates from cattle from South-Western Zimbabwe using RAPD-PCR. IOSR-J Agric. Vet. Sci. 7, 19–25.
Chikowore, T.J., Zishiri, O.T., Mukaratirwa, S., 2019. Phylogenetic analysis of *Fasciola* spp isolated from slaughtered cattle in KwaZulu-Natal and Mpumalanga provinces of South Africa based on the cytochrome C oxidase subunit 1 mitochondrial marker. Onderstepoort J. Vet. Res. 86, 1.
de Kock, K.N., Wolmarans, C.T., 1998. A re-evaluation of the occurrence of freshwater molluscs in the Kruger National Park. Koedoe 41, 1–8.
Fletcher, H.L., Hoey, E.M., Orr, N., Trudgett, A., Fairweather, I., Robinson, M.W., 2004. The occurrence and significance of triploidy in the liver fluke, *Fasciola hepatica*. Parasitology 128, 69–72.
Gibbons, L.M., Jones, A., Khalil, L.F., 1996. Manual for the 8th International Training Course on Identification of Helminth Parasites of Economic Importance. CAB International, Wallingford.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. Information Retrieval Ltd., London, pp. c1979–c2000.
Hasegawa, M., Kishino, H., Yano, T., 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.
Hayashi, K., Ichikawa-Seki, M., Mohanta, U.K., Shoriki, T., Chaichanasak, P., Itagaki, T., 2018. Hybrid origin of 273 Asian aspermic Fasciola flukes is confirmed by analyzing two single-copy genes, pepck and pold. J. Vet. Med. Sci. 18 17-0406.
Huang, W.Y., He, B., Wang, C.R., Zhu, X.Q., 2004. Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. Vet. Parasitol. 120, 75–83.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Ibrahim, N., 2017. Fascioliasis: systematic review. Adv. Biol. Res. 11 278–28.
Itagaki, T., Sakaguchi, K., Terasaki, K., Sasaki, O., Yoshihara, S., Van Dung, T., 2009. Occurrence of spermic diploid and aspermic triploid forms of *Fasciola* in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA. Parasitol. Int. 58, 81–85.
Kalu, E., 2015. Bovine fascioliasis: a review. IOSR-J Agric. Vet. Sci. 8, 23–26.
Le, T.H., De, N.V., Agatsuma, T., Blair, D., Vercruysse, J., Dorny, P., Nguyen, T.G.T., McManus, D.P., 2007. Molecular confirmation that *Fasciola gigantica* can undertake aberrant migrations in human hosts. J. Clin. Microbiol. 45, 648–650.
Le, T.H., Van De, N., Agatsuma, T., Nguyen, T.G.T., Nguyen, Q.D., McManus, D.P., Blair, D., 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. Int. J. Parasitol. 38, 725–730.
Lotfy, W.M., Hillyer, G.V., 2003. *Fasciola* species in Egypt. Exp. Pathol. Parasitol. 6, 9–22.
Luton, K., Walker, D., Blair, D., 1992. Comparisons of ribosomal internal transcribed spacer from two congeric species of flukes (Platyhelminthes: Trematoda: Digenea). Mol. Biochem. Parasitol. 56, 323–328.
Mahulu, A., Clewing, C., Stelbrink, B., Chibwana, F.D., Tumwebaze, I., Stothard, J.R., Albrecht, C., 2019. Cryptic intermediate snail host of the liver fluke *Fasciola hepatica* in Africa. Parasite Vector 12, 573.
Malatji, M.P., Mukaratirwa, S., 2019. Molecular detection of natural infection of *Lymnaea (Pseudosuccinea) columella* (Gastropoda: Lymnaeidae) with *Fasciola gigantica* (Digenea: Fasciolidae) from two provinces of South Africa. J. Helminthol. 1–6. https://doi.org/10.1017/S0022149X19000129.

Malatji, M.P., Lamb, J., Mukaratirwa, S., 2019. Molecular and morphological characterization of liver fluke intermediate host lymnaeids (Gastropoda: Lymnaeidae) snails from selected regions of Okavango Delta of Botswana, KwaZulu-Natal and Mpumalanga provinces of South Africa. Vet. Parasitol. Reg. Stud. Rep. 17, 100318. https://doi.org/10.1016/j.vprsr.
Malatji, M.P., Pfukenyi, D.M., Mukaratirwa, S., 2020. *Fasciola* species and their vertebrate and snail intermediate hosts in East and Southern Africa: a review. J. Helminthol. 94.
Marcilla, A., Barguess, M.D., Mas-Coma, S., 2002. A PCR-RFLP assay for the distribution between *Fasciola hepatica* and *Fasciola gigantica*. Mol. Cell. Probes 16, 27–333.
Mas-Coma, S., Bargues, M.D., 1997. Human liver flukes: a review. Res. Rev. Parasitol. 57, 145–218.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.
Mas-Coma, S., Bargues, M.D., Valero, M.A., 2014. Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario. Parasitology 141, 1918–1946.
Mas-Coma, S., Bargues, M.D., Valero, M.A., 2018. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. Parasitology 145, 1665–1699.
Moghaddam, A.S., Massoud, J., Mohmoodi, M., Mahvi, A.H., Periago, M.V., Artigus, P., Fuentes, M.V., Bargues, M.D., Mas-Coma, S., 2004. Human and animal fascioliasis in Mazandaran province, northern Iran. Parasitol. Res. 94, 61–69.
Mohanta, U.K., Ichikawa-Seki, M., Shoriki, T., Katakura, K., Itagaki, T., 2014. Characteristics and molecular phylogeny of *Fasciola* flukes from Bangladesh, determined based on spermatogenesis and nuclear and mitochondrial DNA analyses. Parasitol. Res. 113, 2493–2501.
Mucheka, V.T., Lamb, J.M., Pfukenyi, D.M., Mukaratirwa, S., 2015. DNA sequence analyses reveal co-occurrence of novel haplotypes of *Fasciola gigantica* with *F. hepatica* in South Africa and Zimbabwe. Vet. Parasitol. 214, 144–151.
Ndlovu, T., Chimonyo, M., Muchenje, V., 2009. Monthly changes in body condition scores and internal parasite prevalence in Nguni, Bonsmara and Angus steers raised on sweetveld. Trop. Anim. Health Prod. 41, 1169–1177.
Nguyen, T.B.N., Van De, N., Nguyen, T.K.L., Quang, H.H., Doan, H.T.T., Agatsuma, T., Le, T.H., 2018. Distribution status of hybrid types in large liver flukes, *Fasciola* species (Digenea: Fasciolidae), from ruminants and humans in Vietnam. Korean J. Parasitol. 56, 453.
Peng, M., Ichinomiya, M., Ohtori, M., Ichikawa, M., Shibahara, T., Itagaki, T., 2009. Molecular characterization of *Fasciola hepatica, Fasciola gigantica*, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. Parasitol. Res. 105 809.
Periago, M.V., Valero, M.A., El Sayed, M., Ashrafi, K., El Wakeel, A., Mohamed, M.Y., Desquesnes, M., Curtale, F., Mas-Coma, S., 2008. First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta. Egypt. Infect. Genet. Evol. 8, 51–58.
Perissinotto, R., Miranda, N.A.F., Raw, J.L., Peer, N., 2014. Biodiversity census of lake St Lucia, iSimangaliso wetland park (South Africa): gastropod molluscs. ZooKeys 1.
Pfukenyi, D.M., Mukaratirwa, S., 2004. A retrospective study of the prevalence and seasonal variation of *Fasciola gigantica* in cattle slaughtered in the major abattoirs of Zimbabwe between 1990 and 1999. Onderstepoort J. Vet. Res. 71, 181–187.
Pfukenyi, D., Mukaratirwa, S., Willingham, A.L., Monrad, J., 2006. Epidemiological studies of *Fasciola gigantica* infections in cattle in the highveld and lowveld communal grazing areas of Zimbabwe. Onderstepoort J. Vet. Res. 73, 37–51.
Posada, D., 2008. jModelTest: Phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Prasad, P.K., Tandon, V., Biswal, D.K., Goswami, L.M., Chatterjee, A., 2008. Molecular identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae) based on the ribosomal internal transcribed spacer regions. Parasitol. Res. 103, 1247–1255.
R studio Team, 2015. R Studio Integrated Development for R. R Studio Inc, Boston, MA http://www.rstudio.com.
Robinson, M.W., Dalton, J.P., 1999. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos. Trans. R. Soc. B Biol. Sci. 364, 2763–2776.
Rozas, J., Sámchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.
Shoriki, T., Ichikawa-Seki, M., Devkota, B., Rana, H.B., Devkota, S.P., Humagain, S.K., Itagaki, T., 2014. Molecular phylogenetic identification of *Fasciola* flukes in Nepal. Parasitol. Int. 63, 758–762.
Sumruayphol, S., Siribat, P., Dujardin, J.P., Dujardin, S., Komalamisra, C., Thaenkham, U., 2020. *Fasciola gigantica, F. hepatica* and *Fasciola* intermediate forms: geometric morphometrics and an artificial neural network to help morphological identification. Peer J. 8 e8597.
Swofford, D., 2002. Phylogenetic Analysis Using Parsimony (Paup), Version 4. Sinauer Associates, Sunderland, MA.
Terasaki, K., Itagaki, T., Shibahara, T., Noda, Y., Moriyama-Gonda, N., 2001. Comparative study of the reproductive organs of *Fasciola* groups by optical microscope. J. Vet. Med. Sci. 63, 735–742.
Thompson, J.D., Gibson, F., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.
Van Wyk, I.C., Boomker, J., 2011. Parasites of south African wildlife: XIX. The prevalence of helminths in some common antelopes, warthogs and a bushpig in the Limpopo province, South Africa. Onderstepoort J. Vet. Res. 78, 1–11.
World Health Organization, 2010. Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases (No. WHO/HTM/NTD/2010.1). WHO.