Population genetic studies of *Fasciola* species from cattle and selected wildlife species in Zimbabwe and localities of KwaZulu-Natal and Mpumalanga provinces of South Africa, based on nuclear ribosomal and mitochondrial DNA sequences

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ABSTRACT

The objective of the study was to confirm the species and determine the genetic diversity of the confirmed *Fasciola* species from cattle and selected wildlife hosts from Zimbabwe and KwaZulu-Natal and Mpumalanga provinces of South Africa. This was based on analysis of DNA sequences of the nuclear ribosomal internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase 1 (CO1) regions. Flukes were collected from livers of 57 cattle at four abattoirs in Zimbabwe and 47 cattle at four abattoirs in South Africa. DNA was extracted from each fluke and 3 wildlife, alcohol preserved, duiker, antelope and eland samples from Zimbabwe. The ITS and CO1 regions of individual flukes were amplified by the polymerase chain reaction (PCR) and sequenced. Aligned sequences (ITS 506 base pairs and CO1 381 base pairs) were analyzed by neighbour-joining, maximum parsimony and bayesian inference methods. The phylogenetic trees revealed the presence of *Fasciola gigantica* in cattle from Zimbabwe and *F. gigantica* and *Fasciola hepatica* in the samples from South Africa. *Fasciola hepatica* was more prevalent (64%) in South Africa than *F. gigantica*. *Fasciola gigantica* was the only species found in Zimbabwe save one sample and an antelope and a duiker which were found to be *F. hepatica*. This is the first molecular confirmation of *Fasciola* species in Zimbabwe and South Africa. Knowledge on the identity and distribution of these liver flukes at molecular level will allow disease surveillance and control in the studied areas.

PREFACE

The experimental work described in this thesis was carried out at the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban. This was carried out from April 2013 to November 2014 under the supervision of Professor Sam Mukaratirwa and co-supervision of Professor Jenny Lamb and Professor Davies Pfukenyi.

This study represents original work by the author and has not otherwise been submitted in any form for a degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly noted in the text.

Signed: _____ **Name:** Vimbai Mucheka **Date:**

DECLARATION 1 – PLAGARISM

I, Vimbai Mucheka declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other University.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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CHAPTER 1

1.0 INTRODUCTION

Fasciolosis is a parasitic disease affecting a wide range of mammalian species especially wild and domestic ruminants including humans. The disease is either caused by the liver fluke *Fasciola gigantica* or *F. hepatica* (WHO 1995). Global economic losses associated with fasciolosis due to weight loss, reduced productivity, draught capacity, fertility and milk production are estimated to be at least US$3.2 billion annually (Spithill *et al*., 1999). Recently, fasciolosis has gained public health importance due to its zoonotic aspect resulting in a number of human cases due to the two *Fasciola* species as well as the hybrid form being reported (Schweizer *et al*., 2005).

Fasciola life cycle is dependent on the presence of a snail as an intermediate host and hence, the distribution of the parasite follows that of the intermediate snail host (Mas-Coma *et al*., 2005). *Lymnaea truncatula*, the intermediate host snail for *F. hepatica* is mainly found in cold and mild climatic regions hence it is common in temperate regions of Europe, America and Australia (Mas-Coma and Bargues 1997). In contrast, *F. gigantica* is found in tropical and sub-tropical regions of Africa and Asia, where *Lymnaea* species have been reported as the main intermediate host (Thanh 2012).

In Zimbabwe, fasciolosis of domestic animals assumed to be caused by *F. gigantica* is endemic in most parts of the country (Vassilev 1999). The intermediate host of *F. gigantica* in Zimbabwe is *Lymnaea natalensis* and it tolerates various climatic conditions and can be found in different reservoirs such as rivers, streams, ornamental ponds and cattle drinking troughs (Davies 1982). However, *L. natalensis* is less prevalent in the drier districts of the country compared to higher rainfall areas (Pfukenyi and Mukaratirwa 2004). It has also been noted that as the numbers of the intermediate host increases the prevalence of *F. gigantica* also increases (Pfukenyi *et al*., 2006).

Diagnosis of *Fasciola* species infection in livestock has been based on parasitological methods such as coprological examination as well as identification of morphological characteristics of the fluke (Thanh 2012). Although coprological examination has a high specificity the sensitivity is low and species identification is not possible using the egg morphology (Adedokun *et al*., 2008). Advanced techniques for identification and determining the phylogeny of parasites have been

developed and include several molecular methods that are DNA based (Thanh 2012). Molecular methods include karyotyping, microsatellites and the analysis of ribosomal and mitochondrial DNA sequence markers (Thanh 2012). Markers have been used in separating *Fasciola gigantica* from F. *hepatica* and have led to the discovery of "intermediate variants of *Fasciola*" in various hosts using the nuclear ribosomal internal transcribed spacers (ITS) 1 and 2 (Itagaki *et al*., 2009). The nuclear rDNA markers are particularly useful for species confirmation (Hills and Dixon 1991) whereas mtDNA markers such as cytochrome oxidase 1 (CO1) and the nicotinamide dinucleotide dehydrogenase subunit-1 (ND1) are more variable and can be used to distinguish closely related species and populations of *Fasciola* (Semyenova *et al*., 2005).

Reports on the presence of *F. gigantica* in Zimbabwe and South Africa are based on morphology of eggs from coprological studies (Alves *et al*., 1988) and adult flukes collected from livestock slaughtered at abattoirs (Vassilev and Jooste 1991). *Fasciola hepatica* (Williams 1914), which was most likely a case of misidentification (Jooste 1989) has been reported once in Zimbabwe. Other *Fasciola* species reported to date in Zimbabwe include *F. tragelaphi* from a sitatunga (*Tragelaphus spekei*) (Pike and Condy 1965) and a cow (Mukaratirwa and Brand 1999) and *F. nyanzae* from a hippopotamus (*Hippopotamus amphibius capensis*) (Jooste 1989). Hence, this study seeks to provide information on the *Fasciola* spp from cattle using molecular techniques and to determine the population genetic structure of the identified species in Zimbabwe and selected sites in KwaZulu-Natal and Mpumalanga provinces of South Africa.

1.1 Main Objective

To determine the distribution of *Fasciola* spp from different geographical locations in Zimbabwe and selected sites in KwaZulu-Natal and Mpumalanga provinces of South Africa

1.2 Specific objectives

1. To determine the *Fasciola* species present in Zimbabwe and selected sites in KwaZulu-Natal and Mpumalanga provinces of South Africa using DNA sequences of the nuclear ribosomal ITS1-5.8S-ITS2 region, referred to hereafter as the ITS region.

2. To determine the genetic diversity within and between the different populations in Zimbabwe and selected locations of KwaZulu-Natal using mitochondrial cytochrome oxidase 1 (CO1) DNA sequences.

CHAPTER 2

2.0 LITERATURE REVIEW

Fasciolosis in ruminants is mainly caused by *F. gigantica* and/or *F. hepatica*, though, two other species; *F. nyanzae* (Jooste 1989) *and F. tragelaphi* have been reported in wild life and cattle by Pike and Condy (1965) and Mukaratirwa and Brand (1999) respectively.

2.1 Morphology of *Fasciola gigantica* and *F. hepatica*

Fasciola hepatica is leaf shaped, flattened and is approximately 35mm long and 15mm wide on its widest part. The anterior and posterior ends of the fluke are narrow with the former end being conical with distinct shoulders (Stemmermann 1953). *Fasciola gigantica* is elongate, with a conical anterior end but however, bigger than *F. hepatica*, and is 75mm long and 15mm wide with less pronounced shoulders (Stemmermann 1953). On electron microscope, the surface of both flukes is covered with spines except the oral and ventral suckers. The spines are fewer on the ventral surface, small at the posterior end and they gradually increase in size from the middle to the anterior end (Dangprasert *et al*., 2001). *Fasciola* spp possess sensory papillae on their surfaces with the dorsal surface having more papillae and spines (Dangprasert *et al*., 2001). In countries where both *Fasciola* species are present it is difficult to differentiate them based on morphology due to the presence of intermediate forms of the parasite with varying morphological characteristics (Marcilla *et al*., 2002).

2.2 Life cycle

Adult flukes are hermaphrodites and produce approximately 20 000 eggs/day which are passed out in faeces (Durbin 1952). In wet environments with adequate light and temperatures above 10^{o}C, eggs embryonate within 2 weeks (Graczyk and Fried 1999). Miracidia are released once the eggs hatch and these penetrate the intermediate snail host of the genus *Lymnaea.* In the intermediate host there is development and multiplication of the miracidia into sporocysts followed by rediae, daughter rediae and finally cercariae (Graczyk and Fried 1999). Shedding cercariae then find plants on which they encyst as metacercariae. Animals and humans are infected by ingesting metacercariae on water plants and grass respectively or drinking water

contaminated with the metacercariae (Graczyk and Fried 1999). Once inside the host, the metacercaria excysts in the stomach in humans and duodenum in animals and then penetrates the gut wall, peritoneal cavity and reaches the liver (Cheesbrough 2005). The immature liver flukes migrate in the liver parenchyma for a period of 8 weeks and then enter the bile duct where they mature to adults (Graczyk and Fried 1999).

Adult flukes reproduce by cross and self-fertilisation in the bile ducts of the host whereas the immature stages are involved in asexual reproduction (Cheesbrough 2005). The flukes survive for years in the liver and produce thousands of eggs that are passed out in faeces via the bile duct into intestinal tract and the cycle continues (Boray 2007). It may take approximately 3 to 4 months for a fluke to develop into an adult and start producing eggs and Lymnaeid snails act as the intermediate hosts (Miliotis and Bier 2003).

2.3 Intermediate hosts

Lymnaeid snails are known to be the intermediate hosts of the main two *Fasciola* species (Thanh 2012). *Lymnaea truncatula* the intermediate host of *F. hepatica* is common in mild climatic regions of America, Australia and especially Europe (Mascoma and Bargues 1997; Kock *et al*., 2003). The distribution of *L. truncatula* in sub Saharan Africa is limited to regions in South Africa with lower temperatures and widely distributed in South Africa (Kock *et al*., 2003). *Lymnaea columella* is more widely distributed in South Africa compared to *L. truncatula* but the importance of *L. columella* in the transmission of *Fasciola* species has not been established in South Africa (Kock *et al*., 2003). In Egypt *F. gigantica* and *F. hepatica* cause fasciolosis and *L. cailliaudi* transmits *F. gigantica* whilst *L. columella* transmits both *F. gigantica* and *F. hepatica*. *Lymnae natalensis* the intermediate host of *F.gigantica* is common in the tropical and subtropical regions of Africa (Thanh 2012). The snail is more prevalent in South Africa than both *L. trancatula* and *L. columella* (Kock and Wolmarans 2008). In Zimbabwe, *L. natalensis* is the intermediate host of *F. gigantica* and is found in a variety of water habitats; streams, dams, rivers and ponds (Davies 1982; Pfukenyi *et al*., 2005).

2.4 Distribution of *Fasciola* spp

The distribution of *F. gigantica* and *F. hepatica* follows that of the snail intermediate hosts found in both the tropical and temperate regions (Prasad *et al*., 2008). *Lymnaea truncatula*, the

intermediate snail host for *F. hepatica* is mainly found in cold and mild climatic zones and hence, *F. hepatica* is mainly common in the temperate regions of Europe, Australia and America (Mas-Coma and Bargues 1997). Other *Lymnaeid* species are mainly found in the tropical and sub-tropical regions of Africa and Asia making *F. gigantica* the most common species in these regions (Dinnik and Dinnik 1964).

Overlaps in distribution of *F. gigantica* and *F. hepatica* occur in some African and Asian countries resulting in hybridization of species and presence of intermediate forms of the parasite in countries such as Japan, Iran and Egypt (Periago *et al*., 2008*)*. *Fasciola hepatica* has been introduced to new environments a result of the introduction of its intermediate host, *L. truncatula*, from European countries to other continents (Mas-Coma *et al*., 2005). In Egypt where *F. gigantica* and *F. hepatica* are present, intermediate forms of the two species have been identified morphologically (Periago *et al*., 2008). In countries such as Japan, Taiwan, Korea it has been difficult to identify the varieties of the hybrid species (Marcilla *et al*., 2002). Morphological types resembling *F. hepatica* and *F. gigantica* and intermediate forms have been reported in these Asian countries (Marcilla *et al*., 2002). This overlap in distribution causes diagnostic problems due to variations in morphology, causing controversy in taxonomic identification of *Fasciola* species in these countries (Periago *et al*., 2008).

In Zimbabwe *F. gigantica* is widespread and occurs all year round, with a high prevalence in high rainfall compared to drier areas (Vassilev1999; Pfukenyi 2003). *Lymnaea natalensis*, the intermediate snail host is widespread in Zimbabwe and is known to thrive in a variety of conditions (Davies 1982). The fresh water snail has been collected in both the highveld and lowveld regions of Zimbabwe with more snails being found in the highveld (Chingwena *et al*., 2002; Pfukenyi *et al*., 2006)). The highveld is characterized by a mean annual rainfall of 800-1200mm and has plenty of water reservoirs such as dams and streams whilst the lowveld has less rainfall (400-650mm), flat land and prone to droughts (Chingwena *et al*., 2002). The disease is endemic in domestic ruminants in areas with mean annual rainfall of 1016mm (Dinnik and Dinnik 1964). High prevalence of *F. gigantica* in Zimbabwe is also attributed to the presence of numerous man-made water bodies such as dams as well as perennial streams in the highveld (Pfukenyi and Mukaratirwa 2004). The epidemiology of *F. gigantica* in Zimbabwe has been extensively studied by Pfukenyi and Mukaratirwa (2004) and high prevalence of the mature

flukes have been reported during the wet season (November to April) compared to the dry season (May to October). *Lymnaea natalensis* was observed to increase during the dry season and declining in the cold months (June and July) and then increasing in October (Pfukenyi 2003). Animals are normally infected during grazing especially when they ingest metacercariae on herbage closer to water bodies and this is common in drier months when grazing pastures are reduced (Pfukenyi 2003). Besides domestic ruminants, *F. gigantica* has been reported in wildlife such as impala (*Aepyceros melampus*), blue wildebeest (*Connochaetes taurinus*), tsessebe (*Damaliscus lunatus*), giraffe (*Giraffa camelopardalis*), sable antelope (*Hippotragus niger*), common duiker (*Sylvicapra grimmia*), buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*) and kudu (*Tragelaphus strepsiceros*) (Christe 1966; von Roth and Dalchow1967; Jooste 1987, 1989) in Zimbabwe. *Fasciola hepatica* (Williams 1914), which was most likely a case of misidentification (Jooste 1989) has been reported once in Zimbabwe. Other *Fasciola* species reported to date in Zimbabwe include *F. tragelaphi* from a sitatunga (*Tragelaphus spekei*) (Pike and Condy 1965) and a cow (Mukaratirwa and Brand 1999) and *F. nyanzae* from a hippopotamus (*Hippopotamus amphibius capensis*) (Jooste 1989). In South Africa, *Fasciola gigantica* has been reported in an impala (Horak 1978) and *F. hepatica* in a kudu (*Tragelaphus strepsiceros*) (Alves *et al*., 1988).

2.5 Pathogenesis in domestic ruminants

The impact caused by the parasites depends on the number of metacercariae that are ingested at a given time and most lesions take place in the liver parenchyma and bile ducts (Soulsby 1986).

2.5.1 ***Acute fasciolosis***

This condition is due to consumption of copious amounts of metacercariae in a short space of time (Behm and Sangter 1999) for example animals feeding on pastures that are highly concentrated with metacercariae due to drought or overstocking (Boray 2007). Huge numbers of immature flukes migrate in the liver parenchyma resulting in traumatic hepatitis and severe damage of the liver parenchyma causing hemorrhaging into the peritoneal cavity (Boray 2007). Acute fasciolosis is rare in cattle but occurs more often in sheep (Müller 2007). The bacteria *Clostridium novyi* which usually resides in sheep liver multiplies due to tissue necrosis causing

"Black disease" (Boray 2007) and this condition is common in sheep rearing continents such as Europe, America and Australia (Soulsby 1986).

2.5.2 *Chronic fasciolosis*

Chronic fasciolosis is due to gradual consumption of low numbers of metacercariae at a given time over a long period of time by ruminants and even in humans (Soulsby 1986). The eventual slow accumulation of adult flukes in the liver causes obstruction of bile flow resulting in stunted growth, weight loss, reduced reproduction, anemia, abdominal pain and diarrhea (Radostits *et al*., 2007). Constant irritation of the bile ducts by adult flukes results in calcification of the bile ducts and liver fibrosis (Soulsby 1986). The adult flukes obtain nutrients by sucking blood resulting in anemia and chronic cholangitis (Boray 2007). Hepatocytes and other tissue components in the tracks are extensively destroyed and hepatocytes are filled with eosinophils, lymphocytes and macrophages and coagulative necrosis occurs in tissues in proximity to the tracks (Rahko 1969). Thrombosis of hepatic blood vessels, arteritis and phlebitis occur in migratory regions and eventually necrosis of vessel walls (Soulsby 1986). Macrophages in larger tracks actively participate in the healing process of the tissue. Fibroblasts, mesenchymal tissue rich in blood capillaries and bile ductuli continuously replace the absorbed tissue in the tracks resulting in liver cirrhosis in the areas of migration (Rahko 1969).

2.5.3 Gross pathology

The colour of the migratory tracks in the liver changes from brown red to grey with a hyperemia (Rahko 1969). Bigger tracks resemble red plaques due to hemorrhage caused by the mature flukes (Rahko 1969). In chronic infections there is dilatation of the bile ducts, and the lumen of the ducts will contain numerous flukes and a brownish mucous exudate (Gajewska *et al*., 2005). Fibrosis of the liver and atrophy of the central and left lobes with enlarged hepatic lymph nodes occurs (Rahko 1969).

2.6 Economic losses

Fasciola species cause significant losses in the livestock sector due to reduced productivity, weight gain and decreased milk production and fertility (Charlier *et al*., 2007). In sheep, reduced production and wool quality, poor growth rates of lambs and increased replacement of lambs has

been observed (Boray 2007). Losses have been estimated to be US$3.2 billion annually (Mas-Coma *et al*., 2005). In Zimbabwe, in addition to what was noted by Charlier *et al*. (2007), losses due to mortalities, poor carcass quality and reduced performance in draught animals as well as liver condemnations have been reported (Vassilev and Jooste 1991; Pfukenyi and Mukaratirwa 2004). Most losses are as a result of liver trimming and condemnations with 46% of cattle livers being condemned due to fluke infection in 1986 (Chambers 1987), whilst a maximum of 43.2% livers were condemned between 1988 and early 1990 (Vassilev and Jooste 1991) and 37.1% were condemned between 1990 to 1999 (Pfukenyi and Mukaratirwa 2004). Fasciolosis has also gained public health importance due to its zoonotic aspect (Thanh 2012). No reports of Fasciolosis in humans in Sub-saharan Africa have been made (Kock and Wolmarans 2008).

2.7 Treatment and Control

Several antihelmintics are used to control fasciolosis, however, some drugs are only effective against mature stages of the parasite (Spithill *et al*., 1999). Triclabandazole is an antihelmintic effective for both mature and immature stages of *F. hepatica* (Wolff *et al*., 1983) and *F. gigantica* (Waruiru *et al*., 1994). Deworming regime recommended by Pfukenyi and Mukaratirwa (2004) for Zimbabwe for control of fasciolosis in domestic ruminants is to treat animals three times a year i.e, December/ January to control chronic fasciolosis, beginning of the cold season April/May to reduce contamination of pastures by the parasite and another treatment at the end of the dry season in August. Control of the Intermediate snail host may be useful in reducing fasciolosis in ruminants in Zimbabwe (Pfukenyi and Mukaratirwa 2004). Molluscicides have been strategically used in Malawi to reduce the infected snail population (Mzembe and Chaudhry 1981). However, molluscicides have been recommended for use in water reservoirs such as dams and rivers due to the persistence of the compound in the environment resulting in the killing of non-targeted animals and plants in the habitat (Spithill *et al*., 1999). In Zimbabwe the use of molluscicides in the highveld maybe expensive due to the vast number of snail habitats in the region (Pfukenyi and Mukaratirwa 2004).

2.7 Human fasciolosis

Human fasciolosis is caused by both *F. gigantica* and *F. hepatica* (Mas-Coma *et al*., 2005). Transmission is due to environmental contamination and thus, infections are more prevalent in

sheep and cattle rearing areas (Thanh 2012). Domestic and wild animals play a role in the transmission and these include cattle, sheep, goats, donkeys, pigs and wild animals such as the deer, buffalo and eland (WHO 2007).

The disease is a problem in young children especially in Andean countries such as Peru, Chile, Equador and Bolivia (Thanh 2012). Prevalence rates in these countries are high (68.2%) and as low as 3% in Northern Africa (Esteban *et al*., 1997). Symptoms of fasciolosis in humans include abdominal pain, weight loss, indigestion and diarrhea (Mas-Coma *et al*., 1999). Diagnosis of human fasciolosis has been through parasitological methods such as the Kato-Katz method for examination of eggs in faeces (Esteban *et al*., 2002).

2.8 Identification of *Fasciola* species

2.8.1 Phenotypic methods

Accurate identification of *Fasciola* species is necessary for effective clinical management of infection and for epidemiological surveys (Thanh 2012). Morphology of eggs has been used to distinguish *F. hepatica* from *F. gigantica*, they are bigger in *F. gigantica* (150±196/90±100 mm) than in *F. hepatica* (130±150/63±90 mm) (Marcilla *et al.*, 2002)

Using morphology in distinguishing adult flukes and eggs has limitations (Rokni *et al*., 2009). Furthermore, there is no egg laying during migration of the immature parasite in the liver and a low, inconsistent number of eggs are laid when there is a low parasite burden (Hillyer 1999).

Morphometric methods, based on measurements of distances between organs of flukes, have helped in distinguishing *F. gigantica* and F. *hepatica* from different countries (Periago *et al*., 2006; Ashrafi *et al*., 2006). Adult fluke measurements such as body length, width and perimeter, cone length and width, diameter of oral and ventral suckers can be measured and compared to determine the differences between the species of the parasite. These morphometric methods of identification rely on a computer image analysis system (CIAS) (Thanh 2012). This technique resulted in a variety of morphological types of *Fasciola* being identified from countries such as Japan, Korea, China and Vietnam (Periago *et al*., 2008; Itagaki *et al*., 2009). Intermediate forms of *Fasciola* that have been identified are probably due to hybridization of the two species (Mas-Coma *et al.*, 2009). This complicates morphological identification and differentiation of the

Fasciola species due to the variety of phenotypic characters resulting from hybridization, especially in places where there is species overlap (Marcilla *et al*., 2002).

Morphology alone cannot give precise identifications because some species may look similar but are genetically different (Knowlton 1993). This method of identification also requires great expertise for precision; however there has been a reduction in the number of taxonomists, hence the need for molecular approach (Hebert *et al*., 2003). Combining molecular and other diagnostic methods such as morphometrics can help in making a more precise species identification of parasites.

2.8.2 Molecular techniques for identification of Fasciola spp

Molecular methods are ideal for species identification, strain confirmation and population genetic studies (Thanh 2012). Most of these molecular methods are polymerase chain reaction (PCR)-dependent targeting a common gene which is adequately divergent between taxa (Hills and Dixon 1991). The targeted region must be long enough to provide adequate variable characters to appreciate differences and similarities for analysis (Huang *et al*., 2004). However, this is determined by the function of the genome. Functional constraints generally result in fewer mutations as some nucleotide positions remain constant whilst diversity exists on others (Shaw *et al*., 2005). Coding regions have more functional constraints than non-coding regions which are said to offer more information for phylogenetic studies (Shaw *et al*., 2005).

The use of mitochondrial and/or nuclear DNA sequencing to identify or diagnose species and determine their boundaries falls under the phylogenetic or genetic species concepts (Maddison 1997; Baker and Bradley 2006). There are a number of molecular techniques which are PCR based and these include DNA fingerprinting, hybridization methods, sequencing of selected genes, DNA barcoding and microsatellites (Thanh 2012).

2.8.4 Markers for Fasciola species

Evolutionary phylogenetic and population genetic studies of a variety of organisms, including *Fasciola,* have been carried out based on ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) markers (Le *et al*., 2000, Huang *et al*., 2004, Zarowiecki *et al*., 2007).

2.8.5 Ribosomal DNA (rDNA) markers

The nuclear rDNA markers (ITS-1 and ITS-2) are located between the 18S, 5.8S and 28S ribosomal RNA genes (Fig. 2.1); these non-coding regions have been used in a number of phylogenetic studies to differentiate *Fasciola* spp from different hosts and locations, resulting in the discovery of the intermediate forms of *Fasciola* (Itagaki *et al*., 2009) They are also useful in the identification and confirmation of *Fasciola* species (Itagaki and Tsutsumi 1998; Prasad *et al*., 2008). The ITS-1 marker has been useful in the differentiation of *F. hepatica* and *F. gigantica* from 3 provinces in Iran, where the presence of both species was confirmed both molecularly and morphologically (Mohammad *et al*., 2010). These markers were also used to confirm the presence of *F. gigantica* and intermediate forms of *F. gigantica* and *F. hepatica* in Vietnam (Le *et al*., 2008). Molecular characterization of *F. gigantica*, *F. hepatica* and intermediate forms from China was carried out using the ITS-2 marker (Huang *et al*., 2004). Flukes of Indian origin were also identified using ITS-1 and 2 markers and it was proven that these flukes are similar to isolates from China, Japan, Indonesia and Zambia (Prasad *et al*., 2008). Several researchers have indicated that ITS-1 and ITS-2 are good markers for interspecific variations and phylogenetic studies of parasites (Itagaki and Tsutsumi 1998; Le *et al*., 2000; Zarowiecki *et al*., 2007). Nuclear rDNA is useful because it has numerous variable regions flanked by conserved regions which make it ideal for molecular studies (Hills and Dixon 1991).

2.8.6 Mitochondrial DNA markers

Certain mitochondrial DNA markers are useful in both phylogenetic and population genetic studies of liver flukes (Walker *et al*., 2006). Mitochondrial CO1 has been used in genotypic and species identification of *Fasciola* (Zarowiecki *et al*., 2007). The mitochondrial nicotiamide dinucleotide dehydrogenase subunit-1 (ND1) has been used to determine the relationship between *Fasciola* spp from Thailand and those from other Asian countries (Pannigan *et al*., 2012). Mitochondrial ND1 and CO1 genes were also used to determine the lineages of *F. hepatica* from 20 different locations in China, Turkey, Bulgaria, Russia and Turkmenistan. Haplotypes were identified; 10 with CO1 and 13 with ND1 (Semyenova *et al*., 2005).

Mitochondrial DNA is useful in determining genetic diversity of flukes; many mitochondrial genes evolve more rapidly than coding regions of the nuclear genome, making them suitable for separating closely related organisms at species and subspecies level (Ai *et al*., 2011).

There is inadequate information on the molecular characterization of *Fasciola* species in Africa and the public health importance of the species (Ai *et al*., 2011). Most studies on *Fasciola* in southern Africa were based on morphology and currently there are no reports on the molecular characterization of the fluke in South Africa and Zimbabwe based on ITS-1 and 2 and mitochondrial markers (CO1 and ND1). Hence, the aim of this study was to identify *Fasciola* species and determine their phylogenetic relationships for samples from Zimbabwe and selected locations in South Africa.

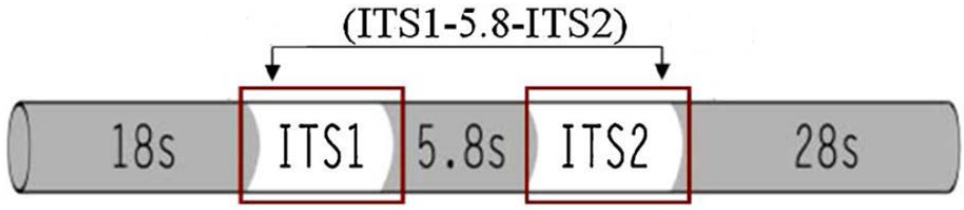

Figure 2.1: Schematic diagram of ITS-1 and ITS-2 markers (Adapted from Le *et al*., 2000)

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sampling

Liver flukes were collected from 57 cattle from four main abattoirs in Zimbabwe (Koala in Harare and the Cold storage Commission abattoirs in Chinhoyi, Mutare and Bulawayo) that have a large slaughter capacity and a wide catchment area of slaughter animals (Fig. 3.1). Alcohol preserved wildlife fluke samples from an antelope, eland and duiker of which the geographical origin is unknown were provided by the Department of Veterinary Services (DVS), Ministry of Agriculture, Zimbabwe. In addition, flukes were collected from cattle livers in abattoirs located in Pietermaritzburg, Glencoe and Eshowe of KwaZulu-Natal as well as Ehlanzeni, Gert Sibande and Newcastle of Mpumalanga provinces of South Africa (Table 3.1).

3.2 DNA extraction

Approximately 25mg of each fluke was used for DNA extraction using a DNeasy® DNA Blood and Tissue Kit (QIAGEN Inc.) according to the manufacturer's instructions.

3.2.1 Spectrophotometry

Spectrophotometry was used to quantify the fluke DNA samples. One µl of double-deionised water was used to zero a Nanodrop Spectrophotometer and 1µl of AE buffer from Qiagen was then used to calibrate the instrument. One µl DNA solution was added and the concentration determined.

3.2.2 Agarose gel electrophoresis

A 1% (w/v) suspension of agarose in 0.5X TBE buffer was heated to dissolve the agarose; 100µl ethidium bromide (0.05 mg ml^{-1}) was added to the solution prior to casting of the gel, to allow visualization of the DNA bands by transillumination with UV light. Eight µl of each DNA sample was mixed with 2µL loading dye (Fermentas 6X Orange) prior to loading into the well.

Five µl molecular weight marker III (Roche, Germany) or O'Gene Ladder (100 bp) (Fermentas) were co-electrophoresed with the samples. Samples were electrophoresed at 100 V for 30 minutes in 0.5X TBE running buffer. A Uvitec UV transilluminator was used to visualize the DNA bands and the image was captured using a Uvitec digital camera. This was done in order to determine the integrity of the DNA (the presence of a sharp high-molecular weight band is indicative of intact DNA, whereas a lack of such a band, combined with the presence of a smear in the lane, is indicative of the presence of degraded DNA).

3.3 Polymerase chain reaction (PCR) and sequencing

The polymerase chain reaction was used to amplify the genomic DNA region comprising ITS-1/5.8S rDNA/ITS-2 region using primers S30FE (forward: 5′-GTCGTAACAAGGTTTCCGTA -3′) and S49E6 (reverse: 5′-TATGCTTAAATTCAGCGGGT-3′), which were designed based on conserved sequences in the 5.8S and 28S genes of *Fasciola* species. The ITS region of all cattle liver fluke samples was amplified with the ITS primer. None of the wildlife samples were amplified with this genetic marker.

The mitochondrial cytochrome oxidase (CO1) region was amplified for half of the Zimbabwean and most KwaZulu-Natal cattle liver flukes. In addition, Zimbabwe wildlife liver flukes from an antelope, duiker and eland were also amplified with the same marker. Cytochrome oxidase 1 primer regions FHCO1 (forward: 5′-TTGGTTTTTTGGGCATCCT-3′) and FHCO1 (reverse: 5′-AGGCCACCACCAAATAAAAGA3′) were used to amplify the flukes.

The PCR amplifications for both markers were performed in 25µL volumes. Each reaction contained 4µL of DNA, 11µL of sterile water, 8µL of TopTaq master mix (QIAGEN Inc.) and 1µL of each primer (100µM) (forward and reverse) per reaction. PCR was performed in a thermocycler (BIORAD) under the following conditions: 94°C for 5 min (initial denaturation), followed by 40 cycles at 95°C, 1 min (denaturation), Ta, 1 min (annealing), 72°C, 1 min (extension), and a final extension of 72°C for 7 min, Ta, the annealing temperature was 55°C for the ITS region and 59°C for the CO1 region.

An aliquot (8µl) of the reaction products was electrophoresed through a 1% agarose gel (as previously described) in order to allow separation and isolation of the amplification products. The amplified ITS and CO1 bands were selected by their positions relative to the co-electrophoresed molecular weight marker and excised from the gel. The excised bands were stored in a 1.5ml microfuge tube at -20°C ready for sequencing.

3.4 DNA sequencing

Unpurified PCR products of 104 1TS-1-2 and 52 CO1 *Fasciola* samples were sent to the Central Analytical Unit of the University of Stellenbosch for DNA sequencing by the Sanger dideoxy method. DNA fragments were sequenced in the forward and reverse directions using the primers used in the initial amplification.

3.5 Data analysis

ITS1-2 and CO1 sequences were edited with Bioedit Sequence Alignment Editor, version 5.0.9 for windows 95/98 NT (Hall 1999). Sequences were determined by comparison with previously published *Fasciola* sequences in the Genbank for both markers (Table 3.1). Multiple alignment of sequences was done using the Clustal W function of Bioedit (version 5.0.9) (Hall 1999) and were further edited by visual inspection. Sequences were trimmed to a uniform length and files were imported into Clustal X, version 1.81 (Thompson *et al.,* 1997), and re-saved as nexus (.nxs) files.

3.5.1 Molecular phylogenetic analysis

3.5.1.1 Neighbour-joining, Maximum parsimony and Bayesian Inference

PAUP4.0b10 for Macintosh (Swofford 2002) was used to create maximum parsimony and neighbour-joining trees for both ITS and CO1 sequences. To determine the most appropriate evolutionary model (GTR + G) to use in neighbour-joining and Bayesian inference analysis, jModelTest 0.1.1 (Posada 2008) was used to apply Akaike's information criterion. Starting trees were obtained via stepwise addition. The addition sequence was random, with 10 replicates and

one tree held at each step during the stepwise addition. The heuristic search option was used to search for the shortest tree using the tree bisection-reconnection (TBR) branch swapping option. Nodal support was assessed using bootstrap resampling analysis (100 replicates) (Felsenstein 1985). Bayesian inference was carried out in MrBayes version 3.2.1 (Ronquist and Huelsenbeck, 2001). Four Markov chains were run for 5 million generations to ensure that the standard deviation of the split frequencies was less than 0.01. The print frequency was 1000 and the sample frequency was 100. The burnin value of 50 000 was determined empirically in several initial runs.

3.5.1.2 Haplotype and population genetic analyses

(DNA Sequence Polymorphism) DnaSP version 4.90.1 was used to determine the number of haplotypes in each data set (Rozas *et al.*, 2003). TCS version 1.13 was used to create a statistical parsimony haplotype network in order to determine the relationships between haplotypes (Clement *et al.*, 2000). Analysis of the dataset for population genetics was carried out in DnaSP to determine haplotype (*h*) and nucleotide (π) diversity values.

3.5.1.3 Pairwise genetic distance analyses

Individual pairwise genetic distance between experimental haplotypes and mean p-distances between *F.hepatica* and *F.gigantica* sample groups were calculated in Mega Version (Hutchison and Templeton, 1999).

Table 3.1: Summary of sample types where liver flukes were collected from and geographical locations in Zimbabwe and South Africa.

Country	Sample type	Abattoir	Location	Number of samples	Latitude	Longitude
Zimbabwe	*Cattle*	Bulawayo	Bulawayo	2	-17.39	30.39
			Matebeleland	2	-20.86	28.42
			Matebeleland North	4	-19.79	28.23
			Matebeleland South	2	-20.46	29.49
		Chinhoyi	Guruve	4	-16.66	30.70
			Hurungwe	4	-16.45	29.36
			Makonde	1	-17.11	30.17
			Mazowe	3	-17.51	30.97
			Mhondoro	2	-18.34	30.62
			Mutoko	2	-17.38	32.21
			Mzarabani	5	-16.36	31.13
			Nyabira	3	-17.48	30.83
			Zvimba	9	-17.48	30.46
			Chiredzi	2	-20.79	31.41
		Harare	Mazowe	1	-17.51	30.97
			Masvingo	3	-20.27	31.06
		Mutare	Chipinge	2	-20.37	32.53
			Manicaland	4	-18.92	32.17

			Marange	2	-19.48	32.40
	Antelope	-	DVS	1	ND	ND
	Duiker	-	DVS	1	ND	ND
	Eland	-	DVS	1	ND	ND
South Africa	*Cattle*					
		Eshowe	Eshowe	7	-28.890	31.471
		Glencoe	Glencoe	10	-28.183	30.150
		Pietermaritzburg	Pietermaritzburg	15	-29.601	30.379
		Gertsibande	Gertsibande	2	-32.546	30.268
		Ehlanzeni	Ehlanzeni	13	-45.819	31.015

DVS - Department of Veterinary Services Zimbabwe; ND – Not Determined

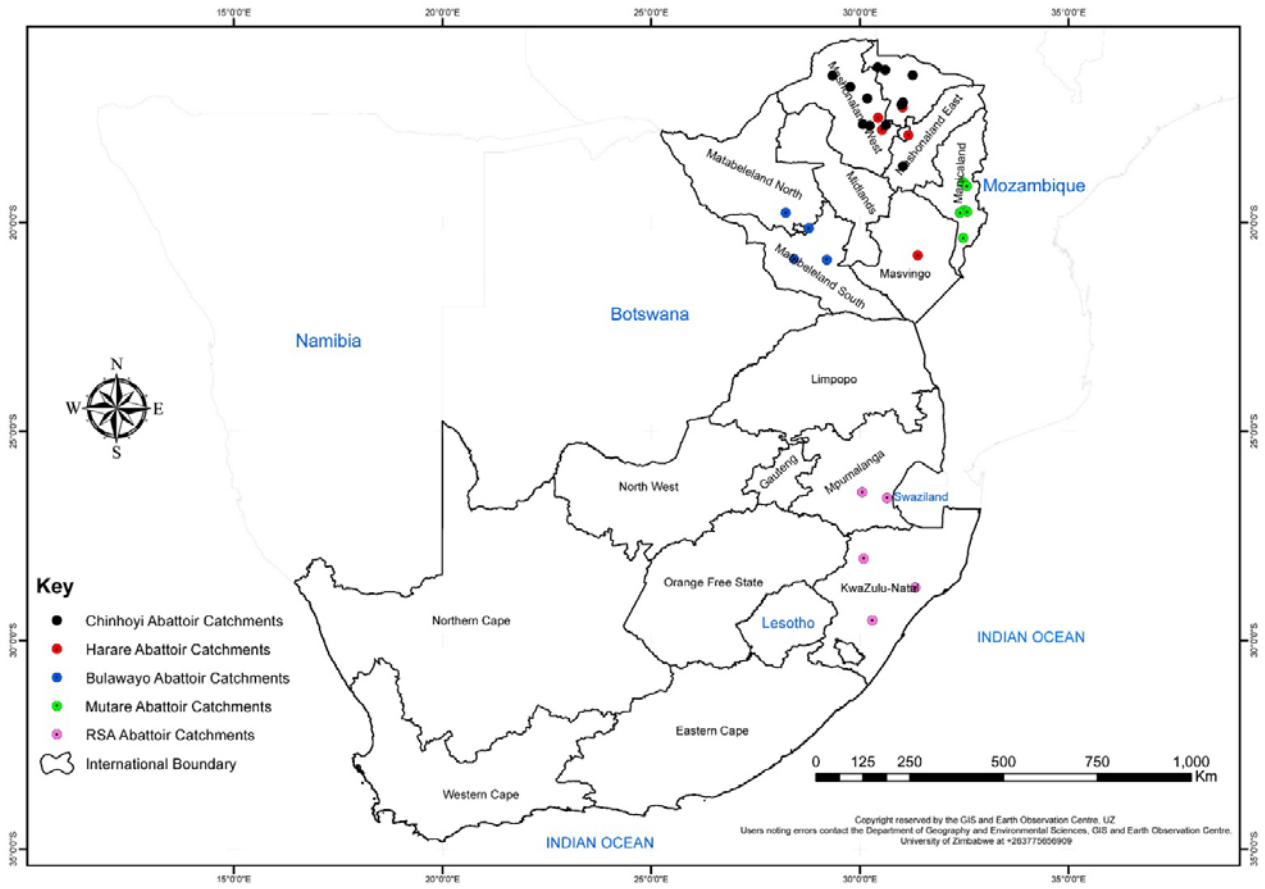

Figure 3.1: Abattoir catchment areas for cattle flukes from Zimbabwe and South Africa

CHAPTER 4

4.0 RESULTS

4.1 DNA Purity and molecular weight

The DNA extracted from cattle flukes yielded discrete high-molecular weight bands with no smearing, indicating that it was not degraded. The ITS and CO1 regions amplified strongly and produced clear bands when separated by agarose gel electrophoresis (Fig 4.1). Fragment length of the trimmed ITS1-2 alignment was 506 base pairs (bp) whilst that for CO1 was 381 bp. Both ITS and CO1 experimental sequences were BLAST searched against the NCBI Genbank to obtain closest matches for inclusion in the analyses (to allow identification of experimental clades) and to obtain outgroups for use in rooting the phylogenetic analyses.

4.2 ITS phylogenetic trees

Neighbor-joining, maximum parsimony and Bayesian analyses produced ITS trees with similar topologies. In Fig 4.2, the neighbour-joining tree is presented, with nodal support values from the maximum parsimony and Bayesian inference analyses indicated at the nodes. *Fasciola* formed a well-supported monophyletic clade (1.00/93/99 – Bayesian posterior probability, neighbour joining bootstrap %, maximum parsimony bootstrap %) with respect to the out-group *Fascioloides magna*. The *Fasciola* clade formed two reciprocally monophyletic sister clades, namely *F. gigantica* and *F. hepatica* respectively. The *F. gigantica* clade is so named as it includes all Genbank-derived samples of *F. gigantica*. All flukes isolated from Zimbabwe cattle are part of the *F. gigantica* clade, which is well-supported (0.93/90/94). According to the phylogenetic species concept (Maddison 1997) they can therefore be identified as *F. gigantica*. However, within the same clade, Guruve 7 and Chimanimani 69 isolates showed a very shallow divergence

from the rest of the Zimbabwe *F. gigantica* isolates. Phylogenetic analyses also revealed that some of the South African *Fasciola* isolates from both Mpumalanga and KwaZulu-Natal provinces (Gert Sibande 16, 18; Ehlanzeni 23-27 & 30-31); all Eshowe isolates except Eshowe 2) were also *F. gigantica* as they were in the same clade as the Zimbabwe isolates.

The *Fasciola hepatica* clade was moderately supported (0.89/73/76), and so named because it contained all of the Genbank samples of *F. hepatica*. Also present within this clade and therefore identified as *F. hepatica* were South African *Fasciola* isolates from KwaZulu-Natal province (Eshowe 2, all Glencoe and all Pietermaritzburg) and Mpumalanga province (Ehlanzeni 17, 19-22, 28-29). The *F. hepatica* clade contained a weakly-supported subclade (0.79/60/62) comprising all samples except JF496716, which was basal. In the analyses, *F. hepatica* and F. *gigantica* were distinguished clearly as separate species according to the phylogenetic species concept, as they form reciprocally-monophyletic clades.

4.3 CO1 phylogenetic tree

The length of the trimmed aligned CO1 sequence was 381 base pairs. Phylogenetic analysis of the CO1 sequences revealed a strongly supported *Fasciola* clade (0.99/98/98) with respect to the outgroup *Fasciolopsis buski* (Fig 4.3). The *Fasciola* clade was divided into three major subclades; (A) a strongly supported *F. hepatica* clade (0.99/98/99, (B) a strongly-supported clade comprising Genbank-derived *F. hepatica* and *Fasciola* spp. (0.90/93/98) and (C) a moderately-supported *F. gigantica* clade (-/52/73).

The *F. gigantica* clade (C) comprised *Fasciola* isolates from all Zimbabwe cattle, eland 12 and some South African cattle (Eshowe 1, 3 & 5, KwaZulu-Natal). All experimental samples (clade C2) were sister to a strongly supported (0.92/80/91) Genbank *F. gigantica* clade (C1).

The strongly supported *F. hepatica* clade (A) was divided into two shallow sister clades, A1 and A2. Clade A1 was moderately supported and contained isolates from KwaZulu-Natal, South Africa [Glencoe & all Pietermaritzburg (PMB) isolates (except PMB1)]

and one Zimbabwe isolate (Matebeleland North 59). Unsupported sister clade A2 comprised isolates from KwaZulu-Natal, South Africa (Eshowe 2 & PMB1) and from Zimbabwe (antelope 1 & duiker 8). *Fasciola hepatica* is present in both clades A and B.

4.4 Haplotype networks

4.4.1 ITS haplotypes

Haplotype analysis of *Fasciola* samples based on 506 nucleotides of the ITS1-2 gene yielded 11 haplotypes (5 for *F. gigantica*, 5 for *F. hepatica* and 1 for *Fasciola* spp) excluding sites with gaps (Table 4.1). The haplotype gene diversity (hd) was 0.6388.

ITS1-2 Haplotype Network

There were 11 haplotypes. Except for haplotype 5 which was separated from haplotype 1 by 3 mutational steps; neighbouring haplotypes were separated by one mutational step (Fig 4.4). Haplotype 1, identified as *F. gigantica*, included most of the Zimbabwe isolates (except Guruve 7 – haplotype 3 and Chimanimani 69 – haplotype 4), some South African isolates (from Mpumalanga and KZN) and Genbank-derived *F. gigantica* haplotypes. Haplotype 2, identified as *F. hepatica* contained most of the South African isolates (KZN and Mpumalanga samples) as well as Genbank-derived *F. hepatica* samples. None of the Zimbabwe and South African isolates belonged to the other remaining haplotypes. Haplotypes 5, 6, 7, 8, 9, 10 and 11 comprise Genbank samples exclusively.

4.4.2 CO1 haplotypes

Haplotype analysis of *Fasciola* samples based on 381 nucleotides of the CO1 gene yielded 32 haplotypes based on 381 sites excluding sites with gaps (Table 4.2). The haplotype diversity (hd) was 0.8943.

CO1 Haplotype Network

A statistical parsimony analysis of the CO1 region of *Fasciola* species showing mutational relationships of haplotypes is presented in Figure 4.5. When set at a 95% parsimony criterion, TCS (Clement *et al.,* 2007) yielded three separate networks, corresponding to *F. hepatica*, *F. gigantica* and a *Fasciola* species (comprising Genbank-derived samples only). The *F. gigantica* and *F. hepatica* networks are separated by 20 mutational steps. *F. gigantica* is separated from the unidentified *Fasciola* species by 10 mutational steps

Fasciola gigantica isolates

All the Zimbabwe isolates identified as *F. gigantica* were found to belong to haplotypes 2, 3, 4, 6, 8 and 9, with haplotype 2 being the most common. All the KZN isolates identified as *F. gigantica* also belonged to haplotype 2. None of the Genbank *F. gigantica* isolates were found to belong to these haplotypes. Haplotype 2, which comprised most isolates, was separated from haplotypes 8 (Muzarabani 17 isolate) and 4 (eland 12) by 1 mutational step. Haplotype 6 (Matebeleland 1 isolate) was separated from haplotype 8 by 2 mutational steps. Haplotype 3 (Banket 40, Bulawayo 65, Makonde 45 and 47, Muzarabani 13 and 14) was separated from haplotypes 4 and 8 by 2 mutational steps. One mutational step separated haplotype 9 (Nyabira 53) from haplotype 3. Experimental haplotypes 3 and 8 were each separated from the closest Genbank haplotype (10) by four mutational steps.

Fasciola hepatica samples

The Zimbabwe cattle isolate (Matebeleland North 59) and the wildlife isolates (antelope 1 & duiker 8) which were identified as *F. hepatica* belonged to haplotypes 7 and 1, respectively. Except for Eshowe 2 and PMB 1, which were found to belong to haplotype 1, the rest of the South African isolates which were identified as *F. hepatica* were found to belong to haplotype 5. Haplotype 5 was separated from haplotype 7 (Matebeleland North 59) by 1 mutation and from haplotype 1 (antelope 1, duiker 8, Eshowe and PMB1 samples) by 2 mutational steps.

4. 5 Genetic Distances

4.5.1 ITS Genetic distance analysis

Genetic p-distances between haplotypes based on 506 nucleotides of the nuclear ribosomal ITS region of experimental samples of *Fasciola hepatica* and *Fasciola gigantica*. Genetic distances between *F. hepatica* samples range from 0.21 to 1.27% and between *F. gigantica* samples range from 0 to 0.42%. Mean genetic p-distance between *F. hepatica* and *F. gigantica* samples based on the ITS region (506 nt) is 1.0% (Table 4.2).

4.5.2 CO1 Genetic distance analysis

Genetic p-distances between haplotypes based on 381 nucleotides of the mitochondrial cytochrome oxidase I region of experimental samples of *Fasciola hepatica* and *Fasciola gigantica*. Genetic distances between *F. hepatica* samples range from 0.26 to 0.79% and between *F. gigantica* samples range from 0.26 to 5.77%. Mean genetic p-distance between *F. hepatica* and *F. gigantica* samples based on the COI gene (381 nt) is 5.5% (Table 4.3)

Table 4.1: Summary of ITS haplotypes for the studied *Fasciola* and Genbank isolates

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola gigantica	1	Manicaland(1-4), Mazowe (1 -6), Zvimba (1-7, 19-20), Muzarabani (13- 14), Mhondoro (29-30), Mutoko (37-38), Makonde(41- 42, 49), Nyabira(50-51), Mazowe (1-5), Chiredzi (52-53), Masvingo (54, 59), Matebeland (60,63, 64, 65), Bulawayo(66-67), Marange, Eshowe (1-5), Guruve (7-10), Marange 68,Chimanimani (70-74), Mpumalanga 1 (23-27,30-32),	JN82895, HM74678, AJ85384, JF43207, JF43207, JF49670, JF49671, AM90037, AM85010, JF49671	91
	3	Guruve 7	Nil	1
	4	Chimanimani	Nil	1
	10	Nil	JF49670	1
	11	Nil	JF49671 F, JF49671	2

Table 4.1: *(cont.)*

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola hepatica	2	PMB(1- 5, 10, 1T-9T,), Eshowe 2, Glencoe(1- 5), Mpumalanga 1 (28-29), Mpumalanga 2 (17, 19-20, 21-22, Glencoe (11-14), Newcastle15	AM707030, AM709498-AM709500, AM709609-AM709622, AM709643-AM709649, AM850107, AM900370, GQ231546, GQ231547, HM746785, HM746786, JF432071, JF432072, JF432075-JF432078, JF708029, JN828954, JN828959, JN828960.	74
	5	Nil	JF496716	1
	6	Nil	JF708026	1
	7	Nil	JF708034	1
	8	Nil	JF708036	1
Fasciola spp	9	Nil	JF708041, JF708042, JF708043	3
Out-group	12	*Fascioloides magna*		

Table 4.2: Summary of CO1 haplotypes for the studied *Fasciola* and Genbank isolates

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola gigantica	2	Banket 39, Chimanimani (69-72, 75), Chipinge (71-72), Eshowe (1, 3, 5), Guruve 6, Hurungwe 34, Manicaland (2-3), Marange (67-68), Masvingo 55, Matebeleland 2, Matebeleland North (61-62), Matebeleland South (63-64), Mazowe (1-3), Muzarabani(16,18), Nyanyadzi 74	Nil	27
	3	Banket 40, Bulawayo 65, Makonde (45,47), Muzarabani (13-14)	Nil	6
	4	Eland	Nil	1
	6	Matebeleland 1	Nil	1
	8	Muzarabani 17	Nil	1
	9	Nyabira 53	Nil	1
	11	Nil	GU11246	1

Table 4.2: *(cont.)*

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola gigantica	12	Nil	GU11247	1
Fasciola hepatica	1	Antelope1, Duiker 8, Eshowe 2, PMB 1	AF216697, GQ231550, KF111595, KF111623	8
	5	Glencoe 1-Glencoe 5, PMB 2-PMB 5	GQ121276, GQ231548-9, KF111585 KF111587, KF111590, KF111602, KF111615, KF111618, KF111626, KF111629, M93388, X15613	22
	7	Matebeleland north 59	Nil	1
	21	Nil	KF111576, KF11158-4, KF111586, KF111588, K111589, KF111591-K111594, KF111597-K111599, KF111601, KF111603-4, KF111606-K116013, KF111619-k111620, KF111628	29
	22	Nil	KF111577, KF111579, KF111581,KF111596	4
	15	Nil	GU112457	1
	23	Nil	KF111600	1

Table 4.2: *(cont.)*

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola spp	14	Nil	GU112454-GU112456, GU112469-GU112470	4
	25	Nil	KF111614, KF111616, KF111617	3
	16	Nil	GU112482	1
	17	Nil	GU112483	1
	18	Nil	GU112484, GU112845, GU112486	3
	19	Nil	KF111574, KF111578	2
	20	Nil	KF111575	1
	24	Nil	KF111605	1
	26	Nil	KF111621	1
	27	Nil	KF111622	1
	28	Nil	KF111624	1
	29	Nil	KF111625	1
	30	Nil	KF111627	1

Table 4.2: *(cont.)*

Taxon	Haplotype	Studied *Fasciola* isolates	Genbank isolates	No. isolates in haplotype
Fasciola spp	31	Nil	GU11247-GU11248	2
	32	Nil	GU11249	1

PCR Amplification

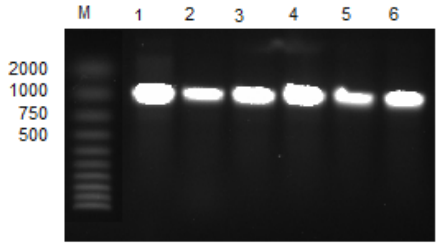

Figure 4.1: Gel electrophoretic separation (2%) of PCR amplicons of the ITS1-2 region of representative *Fasciola* samples from Eshowe, KwaZulu-Natal, South Africa. Lanes 1-6 show the amplified DNA. Lane M contains a molecular ladder.

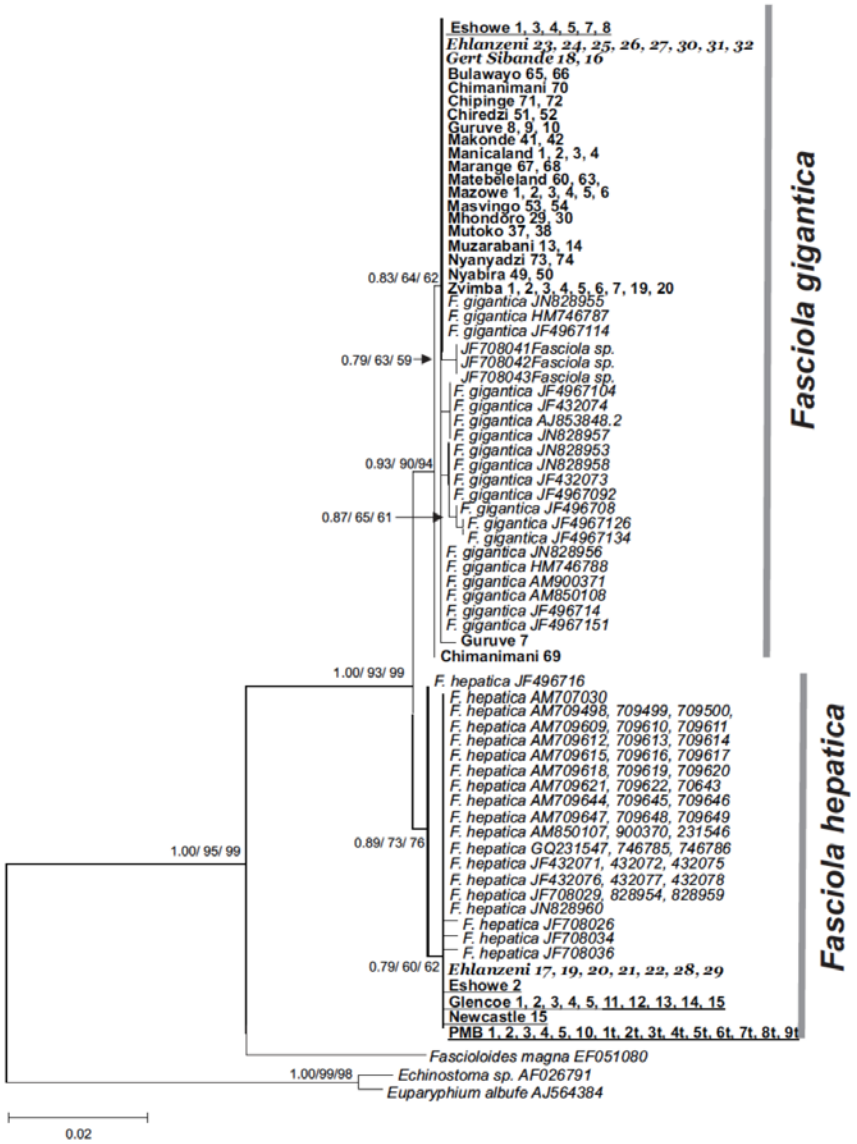

Figure 4.2: Phylogenetic tree based on 506 nucleotides of the nuclear ribosomal ITS region showing relationships between *Fasciola* samples and outgroups. Nodal support is indicated as Bayesian posterior probability/ neighbour-joining bootstrap %/ maximum parsimony bootstrap %.

Normal font + bold = Zimbabwe, **<u>normal + bold + underlined</u>** = KwaZulu-Natal, RSA, ***italics + bold*** = wildlife *normal + italics* = Genbank sample.

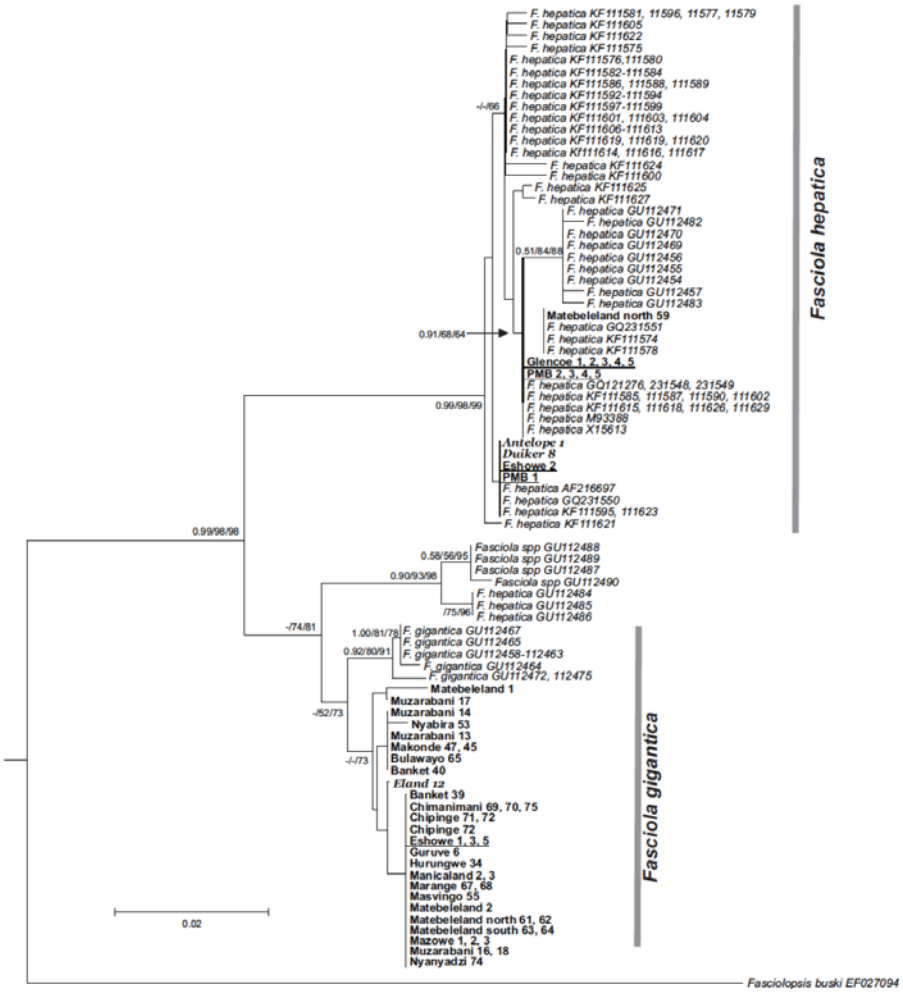

Figure 4.3: Phylogenetic tree based on 381 nucleotides of the mitochondrial cytochrome oxidase I gene showing relationships between *Fasciola* samples and outgroups. Nodal support is indicated as Bayesian posterior probability/ neighbour-joining bootstrap %/ maximum parsimony bootstrap %. **Normal font + bold = Zimbabwe**, **<u>normal + bold + underlined</u>** = KwaZulu-Natal, RSA, ***italics + bold*** = wildlife *normal + italics* = Genbank sample.

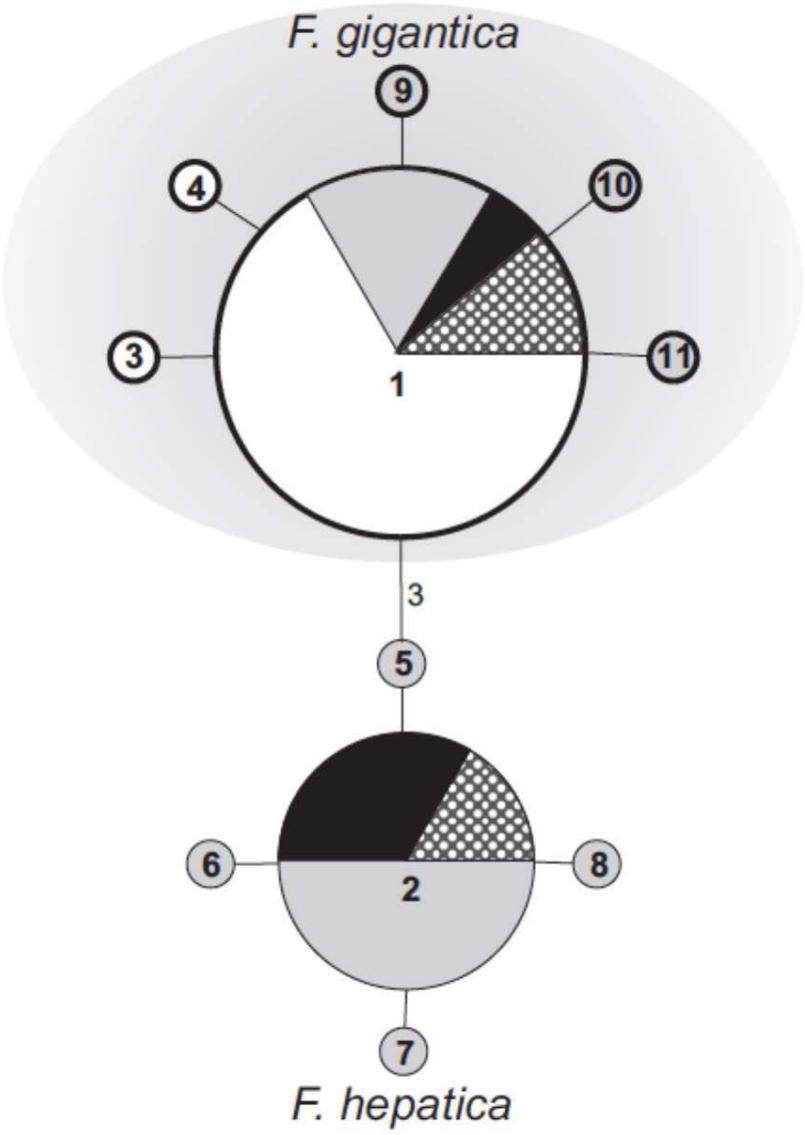

Figure 4.4: Statistical parsimony haplotype network based on 506 nucleotides of the nuclear ribosomal ITS region of *Fasciola* species. White fill=Zimbabwe; black=KwaZulu-Natal; light

grey=Genbank sample; spotted=Mpumalanga. Numbers adjacent to lines connecting haplotypes represent number of mutational steps, where greater than one.

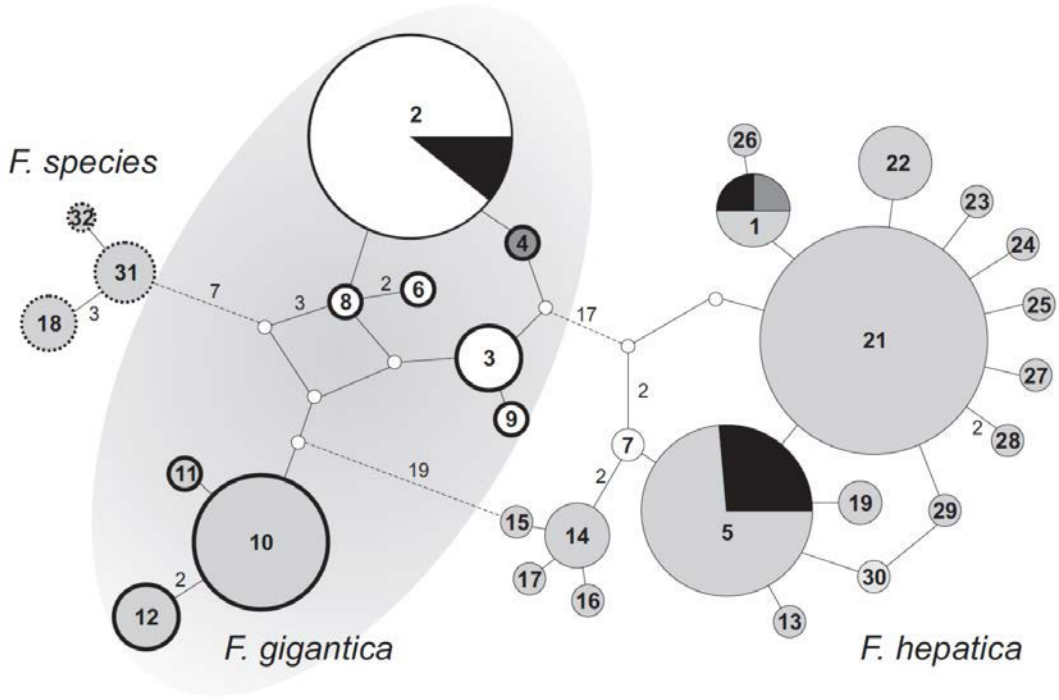

Figure 4.5: Statistical parsimony haplotype network based on 381 nucleotides of the mitochondrial cytochrome oxidase 1 gene of *Fasciola* species. Solid lines represent 95% parsimony connections. Dotted lines represent <95% parsimony connections. Key: white fill=Zimbabwe; black=KwaZulu-Natal; dark grey= wild life; light grey=Genbank sample. Numbers adjacent to lines connecting haplotypes represent number of mutational steps, where greater than one.

Table 4.2: Genetic p-distances between haplotypes based on 506 nucleotides of the nuclear ribosomal ITS region of experimental samples of *Fasciola hepatica* and *Fasciola gigantica*. Hap=haplotype.

		1	**4**	**10**	**3**	**9**	**11**	**5**	**2**	**6**	**7**
F. gigantica											
F. gigantica	**Hap1**										
F. gigantica	**Hap4**	0.00									
F. gigantica	**Hap10**	0.00	0.00								
F. gigantica	**Hap3**	0.21	0.21	0.21							
F. gigantica	**Hap9**	0.21	0.21	0.21	0.42						
F. gigantica	**Hap11**	0.21	0.21	0.00	0.42	0.42					
F. hepatica	**Hap5**	0.63	0.42	0.63	0.84	0.84	0.84				
F. hepatica	**Hap2**	0.84	0.63	0.85	1.05	1.05	1.05	0.21			
F. hepatica	**Hap6**	1.05	0.85	1.06	1.27	1.27	1.27	0.42	0.21		
F. hepatica	**Hap7**	1.05	0.85	1.06	1.27	1.27	1.27	0.42	0.21	0.42	
F. hepatica	**Hap8**	1.05	0.85	1.06	1.27	1.27	1.27	0.42	0.21	0.42	0.42

Mean genetic p-distance between *F. hepatica* and *F. gigantica* samples based on the ITS region (506 nt) = 1.0%

Table 4.3: Genetic p-distances between haplotypes based on 381 nucleotides of the mitochondrial cytochrome oxidase I region of experimental samples of *Fasciola hepatica* and *Fasciola gigantica*. Hap=haplotype.

		1	**5**	**7**	**3**	**2**	**4**	**6**	**8**
F. hepatica	**Hap1**								
F. hepatica	**Hap5**	0.52							
F. hepatica	**Hap7**	0.79	0.26						
F. gigantica	**Hap3**	5.25	5.51	5.25					
F. gigantica	**Hap2**	5.51	5.77	5.51	0.26				
F. gigantica	**Hap4**	5.25	5.51	5.25	0.52	0.26			
F. gigantica	**Hap6**	5.77	6.04	5.77	1.05	0.79	1.05		
F. gigantica	**Hap8**	5.25	5.51	5.25	0.52	0.26	0.52	0.52	
F. gigantica	**Hap9**	5.51	5.77	5.51	0.26	0.52	0.79	1.31	0.79

Mean genetic p-distance between *F. hepatica* and *F. gigantica* samples based on the COI gene (381 nt) = 5.5%

CHAPTER 5

5.0 Discussion

Classification of trematodes such as *Fasciola* species has been based on morphological features (Prasad *et al*., 2008). However, reports have shown that it is difficult to distinguish adult *Fasciola* spp using morphological features (Moghaddam *et al*., 2004). In countries where the two species overlap, it is a challenge due to morphological variations between and within species (Prasad *et al*., 2008; Marcilla *et al*., 2002). Combining diagnostic methods such as molecular and morphometrics can help in the accurate species identification of parasites (Hebert *et al*., 2003). Molecular analyses of nuclear ribosomal ITS (Marcilla *et al*., 2002; Itagaki *et al*., 2005) and mitochondrial ND1and CO1 (Itagaki *et al*., 2005) sequences have been successfully used to discriminate between *F. hepatica* and *F. gigantica* in many regions of the world but not southern Africa.

In our study, ITS and CO1 sequences were used to confirm and determine the genetic variation of *Fasciola* isolates from Zimbabwe and selected locations of South Africa. Phylogenetic analysis revealed that *F. gigantica* is the main species present in Zimbabwe. However, *F. hepatica* was found in one cattle sample from Matebeleland north and in one antelope and one duiker based on CO1 sequences. Both *F. hepatica* and *F. gigantica* were found to be present in KwaZulu-Natal (KZN) and Mpumalanga provinces of South Africa. However, *F. hepatica* was more prevalent as it constituted 64% of the South African samples.

Comment [u1]: This staetement fits more in the Results section than Discussion section.

The distribution of *Fasciola* species is determined by the availability of the snail intermediate hosts (Prasad *et al*., 2008). *Lymnaea* species are known to be the intermediate hosts of the *F. gigantica* and *F. hepatica* (Thanh 2012). *Fasciola hepatica* is common in Europe, America and Asia where *Lymnaea truncatula* act as the main intermediate host (Walker *et al*., 2008). *Lymnaea natalensis* is found in the tropical and sub-tropical regions of Africa and is the main intermediate host of *F. gigantica* in the continent (Dinnik and Dinnik 1964, Walker *et al*., 2008). Based on slaughterhouse studies and coprological examinations, Vassilev (1999), Pfukenyi (2003) and Pfukenyi *et al*. (2006) showed that *F. gigantica* is the common species in Zimbabwe and results of the present study confirmed this finding. An eland sample was also positive for *F. gigantica* and this confirms previous findings of this species in eland as well as other several

wildlife species in Zimbabwe (Christe 1966; von Roth and Dalchow1967; Jooste 1987, 1989). *Fasciola gigantica* is prevalent in all provinces of Zimbabwe and this is attributed to the wide distribution of *L. natalensis* which thrives in a variety of conditions (Davies 1982, Chingwena *et al*., 2002; Pfukenyi *et al*., 2006). The finding of one *F. hepatica* from one animal from Matabeleland North province of Zimbabwe could be attributed to imports of infected cattle, particularly bulls from South Africa which is quite common in Zimbabwe. In addition to the cattle sample, antelope and duiker *Fasciola* isolates were also confirmed to be *F. hepatica*. Previously, *F. hepatica* was documented once in cattle in Zimbabwe (Williams 1914) but the finding was reported as a misidentification (Jooste 1989). Hence, further molecular-based studies are required to determine the extent of *F. hepatica* infection in the country.

The presence of *L. natalensis*, *L. truncatula* and *L. columella* has been reported in South Africa (Kock *et al*., 2003; Kock and Wolmarans 2008). *Lymnaea natalensis* is the most prevalent intermediate host of *F. gigantica* in South Africa (Kock and Wolmarans 2008). In the present study, *F. gigantica* was found in cattle samples from KwaZulu-Natal (Eshowe) and Mpumalanga (Ehlanzeni and Gert Sibande) provinces. In South Africa and Lesotho, *L. truncatula* is common in low temperature regions (Kock *et al*., 2003). However, *L. columella* is more widely spread in South Africa compared to *L. truncatula* (Kock *et al*., 2003). Even though *L. truncatula* is the intermediate host of *F. hepatica* in Europe, its role in transmission in South Africa is unknown (Kock *et al*., 2003). Similarly, the role of *L. columella* in transmission of *F. gigantica* and *F. hepatica* has not been established in South Africa and Zimbabwe (Kock *et al*., 2003). *Fasciola hepatica* was the most prevalent species in KwaZulu-Natal (KZN), whilst both species were equally present in Mpumalanga province. KwaZulu-Natal borders with Lesotho and *L. trancatula* the known intermediate host for *F. hepatica* is abundant in the swampy areas of Lesotho (Kock *et al*., 2003). Cool temperatures, wet and humid conditions of KwaZulu-Natal may enable rapid reproduction, survival and spread of the snail (Kock *et al.*, 2003) from Lesotho once introduced to KwaZulu-Natal. However, Mpumalanga Province is further away from Lesotho compared to KwaZulu-Natal. *Lymnaea columella* which is widespread in South Africa (Kock *et al*., 2003) and resistant to extreme temperatures (Brown 1994), maybe the likely intermediate host for *F. hepatica*, whilst the most prevalent snail *L. natalensis* is responsible for *F. gigantica*, in Mpumalanga province. Reports of fasciolosis in humans in southern Africa are scanty with only one report of infection with *F. hepatica* in school children of KwaZulu-Natal

(Schutte *et al.*, 1981). It is likely that both *L. truncatula* and *L. columella* are transmitting *F. hepatica* in South Africa (Kock and Wolmarans, 2008). *Lymnaea truncatula* is also reported to be spreading from European countries to other continents and thus, *F. hepatica* has adapted to new environments (Mas-Coma *et al*., 2005). However, lack of data on the presence and confirmation of *F. hepatica* in *L. truncatula* and *L. columella* in the present study makes it difficult to conclude on the role of these snails in the transmission of *F. hepatica*. Hence, further studies are required to determine the host-parasite relationship of these snails and *F. hepatica* in South Africa.

The present study revealed the presence of both *F. gigantica* and *F. hepatica* in Eshowe of KZN and Ehlanzeni of Mpumalanga, provinces of South Africa. In Africa, *Fasciola* species overlap has been reported in Ethiopia (Walker *et al*., 2008) and Niger (Ali *et al*., 2008). This poses the risk of hybridization and co-infection where both species co-exist. Both species have also been reported in Egypt and phenotypic variations of the adult fluke have been identified suggesting hybridization of the two species (Periago *et al*., 2008).

Phylogenetic analysis revealed more genetic variability with the CO1 sequences than in ITS sequences, mean genetic distance between *F. hepatica* and *F. gigantica* was 5.5% with CO1 and 1% with ITS, this is in agreement with previous studies (Le *et al*., 2007). *Fasciola gigantica* and *F. hepatica* were distinguished as separate species with ITS according to the phylogenetic species concept as they formed monophyletic clades. However, only *F. gigantica* was monophyletic with CO1 whilst *F. hepatica* was not, as it was present in clades A (*F. hepatica* only) and B (*F. hepatica* and *Fasciola* species). *Fasciola hepatica* in clade B, are probably misnamed and may be hybrid species. The less variable ITS sequences identified one main haplotype (haplotype 1) for *F. gigantica* samples in both Zimbabwe and South Africa. Haplotype 1 is also represented among the Genbank samples from Iran, Burkina Faso and Niger. However, there was slight species variation of *F. gigantica* amongst the Zimbabwe samples. Guruve 7 and Chimanimani 69 isolates differed by one mutation from the rest of the Zimbabwean samples, and represent two novel *F. gigantica* ITS haplotypes. All *F. hepatica* samples from South Africa were identical based on ITS sequences (haplotype 2). Haploytpe 2 is also represented among the Genbank samples from Niger and Spain.

Analysis of the more variable COI region, revealed a greater number of experimental haplotypes of both *F. gigantica* and *F. hepatica.* In the case of *F. gigantica*, all experimental haplotypes (2, 3, 4, 6, 8, and 9) were novel, and not represented on the NCBI Genbank. Haplotype 2, which comprised most isolates, was separated from haplotypes 8 (Muzarabani 17 isolate) and 4 (eland 12) by 1 mutational step. *Fasciola gigantica* from Eshowe (KZN) formed part of the most common haplotype (haplotype 2), along with most *F. gigantica* samples from Zimbabwe, to which it is genetically identical. It is possible that this genetic form of *F. gigantica* shares the same intermediate host in both South Africa and Zimbabwe, and that it is the widely-distributed *Lymnaea (Radix) natalensis* (Walker *et al*., 2008). The experimental haplotypes in the more divergent COI dataset differed from each other by between one and 5 mutational steps, and were four mutational steps distant from the closest Genbank haplotype (haplotype 10) represented among the Genbank samples from Niger.

The COI marker showed one mutational difference between *F. hepatica* haplotype 5 from KZN, South Africa and haplotype 7 from Matebeleland north, Zimbabwe. *Fasciola hepatica* found in antelope 1 and duiker 8 from Zimbabwe is genetically identical to the Eshowe 2 and PMB 1 samples from South Africa. This similarity between the Zimbabwe wildlife and South Africa cattle *F. hepatica* isolates is difficult to explain.

The present study is the first to confirm the presence of *F. gigantica* in cattle in Zimbabwe and both *F. gigantica* and *F. hepatica* in KZN and Mpumalanga provinces of South Africa using a genetic approach. It has also demonstrated *F. hepatica* as the most prevalent species in the localities sampled in KwaZulu-Natal. In addition, it is the first to confirm *F. gigantica* and *F. hepatica* in wildlife samples (eland, antelope and duiker) from Zimbabwe.

The genetic characterization and population genetic variability of *Fasciola* species in South Africa and Zimbabwe is important as it aids in disease surveillance and control of the parasite in the studied areas (Farjallah *et al.,* 2009). Currently there are no reports on the economic losses due to fasciolosis in South Africa (Kock *et al.,* 2003). In Zimbabwe losses due to mortalities, poor carcass quality, liver trimming and condemnations have been reported (Chambers 1987; Vassilev and Jooste 1991; Pfukenyi and Mukaratirwa 2004). Knowledge of losses due to fasciolosis creates an awareness of the importance of the parasites let alone its public health importance as zoonoses in African countries (Kock *et al*., 2003; Pfukenyi *et al*., 2006). The high

prevalence of *Fasciola* species in cattle in the studied areas also poses risk of human infections as these ruminants are reservoirs of infection. Zoonotic fasciolosis is known to be rare in Africa (Kock and Wolmarans, 2008) however; this could be as a result of underreporting which has been noted in countries such as Egypt and Ethiopia (Kock and Wolmarans, 2008).

Further studies are necessary to determine the prevalence of *Fasciola* species in other provinces of South Africa and to determine the actual snail intermediate host involved in the transmission *F. hepatica* in KZN and Mpumalanga provinces of South Africa. There is also need to explore the economic losses associated with the parasite in South Africa as well as to determine whether genetic variation in the species affects control of the parasite.

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