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

by

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ABSTRACT

Fascioliasis is a food- and waterborne disease. It is one of the most common helminthic infections in domesticated ruminants. The disease is caused by liver flukes, *Fasciola hepatica* and *Fasciola gigantica.* Increased attention has been geared toward studying these flukes due to their ever-expanding geographical distribution, enormous economic impact, increased human infections, increased resistance to treatment and the existence of hybrid forms. Both these species are co-endemic in the Mpumalanga and KwaZulu-Natal provinces of South Africa and even though, hybrids have been reported in other areas where both species exist it has not been attempted in South Africa. Therefore, this study was conducted to determine the existence of *Fasciola* hybrids in South Africa using morphological and molecular characterization. A total of 71 flukes were collected from naturally infected cattle slaughtered at abattoirs located in Enhlazeni and Nelspruit in Mpumalanga province and Pietermaritzburg in KwaZulu-Natal province of South Africa as well as control samples from Zimbabwe (Bulawayo abattoir) where only *F. gigantica* exist. The samples were categorized morphologically as either *F.hepatica*, *F.gigantica,* or *Fasciola* sp. The morphometrics (body length, body width, and length/width) were analyzed through a PCA and produced three distinct groups. A one-way ANOVA indicated that the length and length/width could be used to differentiate the species ($P < 0.05$) and the width was not useful in differentiating the species ($P > 0.05$). Molecular analysis based on ITS-1/5.8S/ITS2 marker showed that specimens morphologically identified as *Fasciola* sp were *F.gigantica,* with one sample morphologically identified as *F.gigantica* was molecularly identified as *Fasciola* sp. Similar results were observed with the CO1 marker, however, one sample came up as unknown, this sample however, formed a well-supported sister clade to *F. gigantica.* . It was also observed that aspermatic specimens are not only limited to hybrids, as some individuals that were molecularly identified as *F. hepatica* lacked sperm in their seminal vesicles. This study confirms species identification of *F. hepatica* and *F.gigantica* cannot be solely based on morphological characters where both these species are co-endemic. This was also the first study to report the existence of hybrid *Fasciola* spp. in South Africa.

PREFACE

The research contained in this dissertation was completed by the candidate, while based in the Discipline of Parasitology, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, supervised by Professor S. Mukaratirwa. The research was financially supported by Prof. Mukaratirwa, through his research productivity incentive funds from the University of KwaZulu-Natal and a bursary awarded to the candidate by the National Research Foundation (NRF) of South Africa. The contents within this study represent original work by the author and have not been submitted in any form to another tertiary institution.

DECLARATION

I Sayurika Haridwal, declare that:

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

1. This thesis has not been submitted for any degree or examination at any other university.
2. This dissertation does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.
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Date: 21/09/2020

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LIST OF ABBREVIATIONS

µl – Microliter

H_2O – Water

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic Acid

ITS – Internal Transcribed Dpacer

CO1 – Mitochondrial Cytochrome c Oxidase

PCR – Polymerase Chain Reaction

Taq - *Thermus aqueous*

TBE – Tris/ Borate/ EDTA

UV – Ultraviolet

MEGA – Molecular Evolutionary Genetics Analysis

DnaSP - DNA Sequence Polymorphism

TCS – Transitive Consistency Score

ANOVA – One-way Analysis of Variance

Fh – *Fasciola hepatica*

Fg – *Fasciola gigantica*

*F.*sp – *Fasciola* species

BW – Body Width

BL – Body Length

NTDs – Neglected Tropical Diseases

CIAS – Computer Image Analysis

PCA – Principal Component Analysis

RFLP - Restriction Fragment Length Polymorphism

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mutational step. Source of isolates and sequence is represented by colour or pattern: White fill – Mpumalanga, Black fill – KwaZulu-Natal, Dark grey – GenBank sequences, crosses – outgroup.

CHAPTER 1: INTRODUCTION

1.1 Background

Globally, parasitic foodborne zoonotic diseases have become more recognized, due to the increasing number of cases being reported and/or the availability of better diagnostic tools (Dorny et al., 2009). Communication has also played a key role in identifying those parasites that are responsible for such diseases and their prevalence over the years (EFSA, 2009). One such disease that is quickly gaining recognition is fasciolosis, and it is classified as a re-emerging and emerging disease in many countries (Mas-Coma, 2004).

It is estimated that around 10% of the human population residing in industrialized countries are susceptible to foodborne zoonotic diseases including parasitic diseases such as fasciolosis (Shao et al., 2011). Fasciolosis is a foodborne parasitic zoonoses transmitted via the consumption of food and water contaminated with metacercariae of *Fasciola* spp (Keiser and Utzinger, 2009; Shao et al., 2011).

The disease is highly prevalent amongst wild and domesticated ruminants (Chikowore, 2017; Itagaki *et al*., 2005) and has also been observed in humans across five continents (Jaja *et al*., 2017). A global economic loss of approximately ZAR 46.6 billion (USD 3.2 billion) per annum has been reported in livestock through liver condemnation and loss of productivity (Spithill et al., 1999; Mas-Coma *et al*., 2005; Ai et al., 2011).

The main causative agents for fasciolosis in livestock are *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 (Narva *et al*., 2011).

Both *F. hepatica* and *F. gigantica* have a di-heteroxenous life cycle, with only the spectra intermediate host being different (Mas-Coma *et al*., 2005) (Figure 1). *Fasciola gigantica* is primarily prevalent in the tropical regions of Asia and Africa, with limited distribution of populations in Southern Europe, Uzbekistan, Turkmenistan, Iran, Iraq, India and Pakistan (Mas-Coma, 2004; Ashrafi *et al*., 2006; Yakhchali *et al*., 2015).*Lymnaea natalensis* snail species are endemic to these tropical areas, which is the main intermediate host of *F. gigantica* (Thanh 2012; Mucheka, 2014). *Fasciola hepatica* is far more successful in its geographical distribution than *F. gigantica*, as it is found on five continents: Europe, Africa, Asia, the Americas and Oceania (Itagaki *et al*., 2005). The main intermediate snail host for *F. hepatica* is *Galba trancatula* (Mas-Coma and Bargues 1997). Fasciolosis has one of the widest known longitudinal, latitudinal and altitudinal distribution among water-borne parasitic diseases (Itagaki *et al*., 2005; Ashrafi *et al.,* 2006).

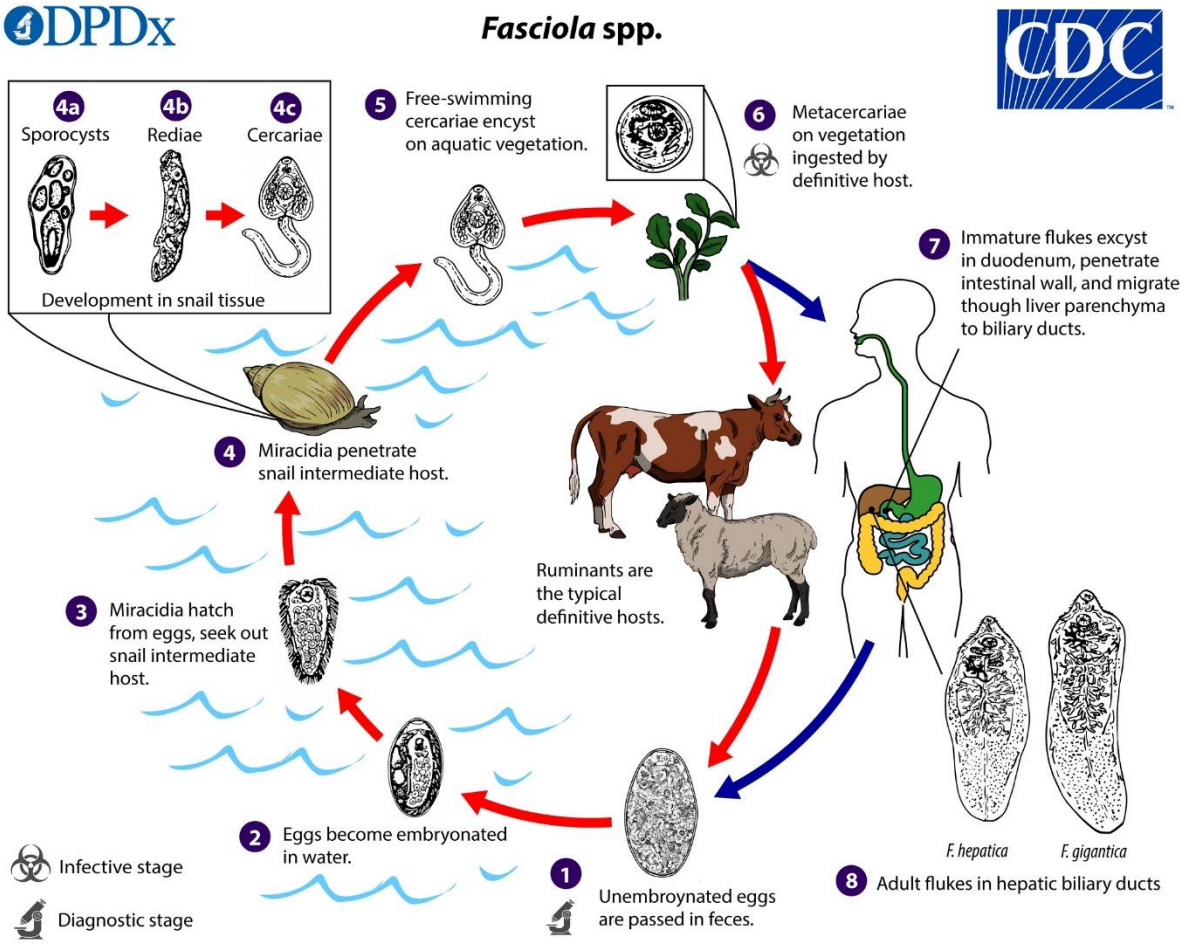

Figure 1: Life cycle of *Fasciola* species. Unembryonated eggs are passed into the external environment via faeces (1). Eggs are embryonated in water (2), miracidia hatch from the egg (3) and actively search and infect a suitable snail host (4). The miracidia undergo three developmental stages within the snail host, viz, (sporocysts (4a), rediae (4b), and cercariae (4c). The cercariae are shed from the snail (5) and encyst on surrounding aquatic plants and then develop into metacercariae. The metacercariae infect the definitive mammalian host when the definitive host consumes the infected aquatic plants (6). Once ingested the metacercariae excyst in juvenile flukes and penetrate the small intestine and migrate to the abdominal cavity eventually reaching the liver (7). Then it moves into the bile duct and develops into an adult and produces eggs (8) (Source:CDC.gov,2018).

The two species of flukes follow a cosmopolitan distribution, which means it can be found almost all over the world (Dorny *et al.,* 2009; Mas-Coma *et al*, 2005). *Fasciola gigantica* is present in warmer tropical and subtropical areas, whilst *F. hepatica* is more prevalent in cooler temperate regions, however, the geographical distribution of the two different species tend to overlap in some areas (Mas-Coma and Bargues, 1997; Thanh, 2012). The overlap of the two species has brought about reports on the existence of hybrids or intermediate forms in areas where the two species co-exist (Ashrafi et al., 2006; Periago *et al.,* 2008). This phenomenon of intermediate forms has been intensively studied in Japan and revealed that the flukes could be diploid, triploid or mixoploid

(Terasaki *et al*., 2000). It was also discovered that they were parthenogenic allowing for asexual reproduction (Terasaki *et al*., 2000).

Fasciola spp populations studied in Japan and Korea displayed individual flukes that contained nuclear and mitochondrial sequences from both species and some individual's nuclear DNA which represented one species. These individuals also had a mitochondrial genotype representing another species (Agatsuma *et al*., 2000; Le *et al*., 2008). Dowling and Secor (1997) concluded that there was evidence of hybridization and introgression between *F. gigantica* and *F. hepatica* species, producing individuals that were polyploid and parthenogenic. The hybrid Individuals expressing these characteristics were found to be frequently aspermic (Dowling and Secor, 1997; Hayashi *et al*., 2017).

The major concern of fascioliasis is its public health impact as a foodborne zoonosis (Mas-Coma *et al*., 2009). Studies are unclear as to how many humans are infected globally, it was estimated in the mid 90's approximately 830 000 people were infected and 27 million at risk (Periago *et al*., 2008). In 2011 both Shahbazi *et al* (2011) and Tolan (2011) indicated that there were 17 million infections with at least 91.1 million people at risk (Tolan, 2011). In 2015 there were approximately 35 to 72 million infections with a further 180 million at risk (Nyindo and Lukamgaire, 2015).

Most human cases are found in low income, and resource-poor livestock farming communities as a result of being in close contact with their livestock and depending on potentially contaminated natural water sources for their livelihood (Nyindo and Lukambagire, 2015). A few decades ago, human cases were either low or undocumented, however, in recent years, the number of cases has increased, making fascioliasis an important zoonotic disease (Marcilla *et al*., 2002; Mas-Coma, 2004; Mas-Coma *et al*., 2009; Nyindo and Lukambagire 2015; Chikowore, 2017). The increase in infections can be attributed to the geographical expanding distribution of the snail intermediate hosts (Chikowore, 2017).

The distribution of the intermediate host directly influences the distribution of *Fasciola* spp. (Prasad *et al*., 2008). Both *F. gigantica* and *F. hepatica* utilize snails belonging to the Lymnaeid species as their intermediate host (Thanh 2012; Mucheka *et al*., 2015). *Galba truncatula* Muller, 1774 is a European snail species that is used by *F. hepatica* as an intermediate host in some parts of Africa (Brown, 1994; Walker *et al*., 2008; Malatji and Mukaratirwa., 2019). According to Mas-Coma (2009), the geographical distribution of *F. hepatica* and *G. truncatula* in sub-Saharan Africa has three categories, namely: (i) the north-western African region; (ii) the large western and central African region; and (iii) the Eastern Africa region from the northern Mediterranean shore to southern Africa. This distribution is also described by Brown (1994), where *G. truncatula* is found across South Africa and Lesotho, other

isolated areas include Ethiopia, Kenya, and Tanzania (Malatji and Mukaratirwa, 2019). Meanwhile, *Fasciola gigantica* distribution range is far more limited than that of *F. hepatica,* as it is only restricted to tropical and subtropical regions of Africa and a few Asian countries (Ashrafi *et al*., 2004; Mas-Coma *et al*., 2005). This limitation in distribution is attributed to the restricted dispersion ability of the intermediate snail host (Malatji and Mukaratirwa, 2019). *F. gigantica* preferred intermediate host is *Lymnaea (R) natalensis* Krauss, 1848 in Africa (Brown, 1994; Mas-Coma, 2005; Walker *et al*., 2008).

Optimal environmental conditions influence the distribution of both *G. truncatula* and *L. (R.) natalensis* (Jaja *et al*., 2012). With the influence of climate change, areas that were previously unfavourable have now become favourable, therefore the geographical distribution of these snails have extended (Jaja *et al*., 2012). The expansion of geographical distribution allows for the co-existence of both *G. (L.) truncatula* and *L. (R.) natalensis* and hence the overlap of *F. gigantica* and *F. hepatica* (Ashrafi et al., 2006 and Periago et al., 2008). The geographical overlap of *F. gigantica* and *F. hepatica* has been observed in Africa, and more specifically in South Africa (Mas-Coma *et al*., 2009; Mucheka *et* al., 2015; Chikowore *et al.,* 2017). The overlaps are categorised as either local, when the climate in an area is favourable all year round to both species of snail, and zonal when highlands with mild and cool temperature favours *F. hepatica* and *G. (L.) truncatula* system whereas the low-lying areas with warm/hot temperature favours to *F. hepatica* and *Lymnaea* species (Mas-Coma *et al.,* 2009; Ashrafi *et al*., 2015; Malatji and Mukaratirwa, 2019). The phenomenon of zonal overlap has been confirmed in 108 African countries (Ashrafi *et al*., 2015).

There are three Lymnaeid species recorded in South Africa; *L. (R.) natalensis, L. (G.) truncatula L. (R.) auricularia* and *L. (P.) columella* as well as the presence of both *Fasciola* species (Mucheka *et al*., 2015; Malatji and Mukaratirwa, 2019). *Lymnaea (P.) columella* has been reported to cohabit with *L. (R.) natalensis* in the Mpumalanga province and with *L. (G.) truncatula* in KwaZulu-Natal province, with confirmed cases of *L. (P.) columella* infected with *F. gigantica* infections in both provinces (Mucheka *et al*., 2015; Malatji and Mukaratirwa, 2019). *Fasciola hepatica* has a high prevalence in cattle in KwaZulu-Natal, while Mpumalanga had both *Fasciola* species are present in equal numbers (Mucheka *et al*., 2015). This shows that *L. (P.) columella* is possibly acting as an intermediate host for both *Fasciola* species (Bargues and Mas-Coma 2005), as the climate in Mpumalanga does not favour the survival of *L. (G.) truncatula* (Mucheka *et al*., 2015).

With both *Fasciola* species present in South Africa and cohabiting in some areas (Mucheka *et al.,* 2015), there is a possibility of hybridization (Chikowore *et al*., 2017). Hence this study seeks to determine the existence of hybrids/intermediate forms between *F. hepatica* and *F. gigantica* in South

Africa using morphometric analyses supported by molecular analyses as well as to determine if those hybrid/intermediate forms are aspermic.

1.2 General Objectives

To determine the existence of hybrids between *F. gigantica* and *F. hepatica* in locations where the two species cohabit in South Africa

1.2.1 Specific objectives

1. To describe the morphological characters of *Fasciola* spp populations collected from a location where *F. gigantica* and *F. hepatica* cohabit.

2. To determine the presence of hybrids between *Fasciola gigantica* and *F. hepatica* in in areas where the two species cohabit using a combination of morphological descriptions and DNA sequences of the nuclear ribosomal ITS1-5.8S-ITS2 region.

3. To determine the presence of aspermic flukes in localities where *F. gigantica* and *F. hepatica* cohabit.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Neglected tropical diseases (NTDs) are diseases of humans that are prevalent in low income and developing countries and are often ignored by the developed world (Tolan, 2011). Little or no attention is paid towards these diseases and hence very limited information is known about their prevalence, geographical distribution and burden in resource-poor communities to warrant funding for their control and prevention (Tolan, 2011). According to Hotez and Kamath (2009), these diseases are preventable, however, there are still around 1.5 billion people in sub-Saharan Africa that are affected. One of these NTDs that is fast re-emerging is fascioliasis (Mas-Coma *et al*, 2009). Fasciolosis is a disease caused by the two species of liver flukes, viz. *F. hepatica* and *F. gigantica* (Young *et al*., 2011). The disease was previously considered as a parasitic disease of high veterinary importance only, but as of recent times have become an important zoonotic disease with a high number of the human population at risk (Marcilla *et al.,* 2002).

2.2 *Fasciola* spp

The genus *Fasciola* consists of the following common species; *F. jacksoni, F. buski, F. hepatica* (Linnaeus, 1758) and *F. gigantica* (Cobbold, 1856) with the two latter species having a greater occurrence and economic and public health importance (Caple *et al*., 1978; Mas-coma *et al*., 2005; Yakhchali *et al.,* 2015). *F. hepatica* and *F. gigantica* mainly infect wild and domestic ruminants with human infections on the rise (Shahbazi *et al.,*2011). Though these two species morphologically appear similar, the main aspect that usually differentiates them is in the shape and size where *F. gigantica* has a slender shape, the length of an adult ranges between 26-76 mm and the width from 5-13 mm (Marcilla *et al*., 2002) whilst *F. hepatica* is substantially shorter than *F. gigantica,* with the length of the adult ranging between 18-32mm and width from 7-14mm (Mas-Coma, 1997). *F. hepatica* has apparent broad shoulders as compared to *F. gigantica* with the cephalic cone of *F. hepatica* being longer (Marcilla *et al*., 2002; Narva *et al.,* 2011). Furthermore, *F. gigantica* has centripetal branches, and most of the intestinal branching are more toward the midline of the fluke. The ovaries have more branches compared to that of *F. hepatica*. The distance between the posterior border of the body and the testis vary in both these species with *F. hepatica* having a range of 3-13mm and 6-19mm for *F. gigantica*. *F. gigantica* also contains larger eggs (≥ 150) whereas, *F. hepatica* has smaller eggs (≤ 140) (Sahba *et al.,* 1972; Marcilla *et al*., 2002; Hussein and Khalifa, 2010; Narva *et al.,* 2011;).

2.3 Life cycle

F. hepatica and *F. gigantica* has a similar life cycle (Figure 1) (Graczyk and Fried, 1999). The life cycle is di-heteroxenous (Rojas *et al*., 2014) and was first described by Thomas (1883) and occurs in four distinct stages (Figure 1).

2.3.1 *Fasciola* egg development

Hermaphroditic adults of both fluke species are present in the bile ducts of the mammalian hosts, where adults can produce approximately 10 000 eggs per day (Taira and Saitoh, 2010; Mucheka, 2014). The eggs are passed onto the external environment via faeces of the definitive host (Beesley, 2016). According to Chappell (2013), most digenea typically have eggs that are opaque and brown in appearance. The brown colouration is a result of quinone tanning and this results in an exterior that is more resistant to the external environment. However, *Fasciola* eggs differed from most digeneans, as the proteins were not linked by quinones and rather cross-linked by disulphide and dityrosine bridges. Chappell (2013), further explained that *Fasciola* does contain the enzyme phenolases, which allows for the conversion of o-phenol to o-quinone during quinone tanning, and this causes some confusion in the integrity of the eggs. The viability of the eggs is directly related to the type of

environment it is liberated into, and these are sometimes only viable when they are released into aquatic environments, because these eggs are vulnerable to desiccation despite the quinone tanning (Chappell, 2013). A miracidia develops and hatch from the egg when all suitable conditions are met, such as fresh water environment with temperatures ranging between 15-25°C and adequate sunlight (Mucheka *et al*., 2015; Beesley, 2016).

2.3.2 Life cycle within the intermediate host and definitive host

The miracidia actively swim in water seeking for an appropriate freshwater snail belonging to the family Lymnaeid and penetrate the snail tissue (Mucheka, 2014; Malatji and Mukaratirwa, 2019). The miracidia can detect specific snail species through attraction by the chemicals excreted by snails and penetrations occurs through the mantle, tentacles or foot of the snail (Beesley, 2016).

Within the intermediate host, the miracidia multiply and develop into a sporocyst, which remain in the foot of the snail and within, the sporocyst develop multiple redia stages which will then mature into cercariae. The emergence of cercariae from the snail is dependent on the availability of light and appropriate temperatures ranging between 9 and 26°C (Mas-Coma and Bargues, 1997).

Rediae then develop into cercariae within 6-7 weeks at 20-25°C and the cercariae are then shed into water sources (Mas-Coma and Bargues, 1997; Valero et al., 2009). Once these are released/shed from the intermediate host, the cercariae shed their tails and encyst on the surrounding herbage and/or aquatic vegetation as metacercaria (Mas-Coma and Bargues, 1997; Achiorno and Martorelli, 2016; Beesley, 2016; Smith, 2016;).

The metacercaria is relatively resilient to extreme temperatures, however, it is highly susceptible to desiccation and therefore they are mainly found on the underside of leaves of aquatic plants/vegetation as an adaptation to protect themselves from direct light (Beesley, 2016). They are ingested by susceptible animals during grazing. Within the gastrointestinal tract of the definitive host, the metacercariae excyst into juvenile flukes which penetrate the small intestinal wall and migrates towards abdominal cavity within two hours of ingestion (Chikowore, 2017) to reach the liver within six days. Within the liver, they migrate for approximately five to 8 weeks, and during this time they also feed on liver tissue (Chikowore, 2017). Eventually they enter the bile duct where they mature into adult flukes (Mucheka, 2014; Malatji and Mukaratirwa, 2019). In the bile ducts, the flukes reproduce both sexually and asexually producing eggs which are expelled through the bile duct into the faeces of the definitive and the life cycle continues (Mucheka, 2014).

Metacercariae can also be accidentally ingested by humans and this normally occurs when humans ingest aquatic plants that contain the metacercariae, as well as drinking water contaminated by free-floating metacercaria (Mas-Coma *et al*., 2005; Tolan, 2011).

2.4 Pathogenesis of fasciolosis

The severity of the disease is influenced by the number of metacercariae ingested by the definitive host at a given time (Mucheka, 2014) which results in either acute or chronic fascioliasis.

2.4.1 Acute fasciolosis

Acute fasciolosis is caused by the ingestion of large amounts of metacercaria (Mucheka, 2014; Ruiz-Campillo *et al*., 2017) all at once. This phenomenon is commonly observed in sheep or cattle grazing in areas with a high concentration of metacercariae and this usually during the dry season when grazing is confined to edges of dams or streams with contaminated vegetation (Boray, 2017; Ruiz-Campillo *et al*., 2017). According to the NADIS (2017), infected sheep experience sudden death due to liver damage and haemorrhaging. Internal bleeding occurs when the large number of immature flukes migrate from the intestinal epithelium and through the liver, and while migrating they cause severe damage to the liver parenchyma (Boray, 2017).

2.4.2 Chronic fasciolosis

The chronic phase of fascioliasis is also known as the obstructive phase (Mas-Coma *et al.,* 2014). This results from trickle infection and the immature flukes enter the body of the definitive host gradually over a long period of time. Once inside they migrate into the liver and mature into adult flukes, therefore causing an obstruction in bile ducts (Mucheka, 2014). At this stage the bile ducts appear to be dilated as they contain numerous worms and a brownish mucus may ooze through the bile duct at post-mortem (Gajewska *et al*., 2015).

The consequences of the obstruction of bile duct ranges from stunted growth, weight loss, anaemia, diarrhoea, and severe abdominal pain (Radostits *et al*., 2007). The blocked bile ducts of the definitive host become calcified and liver fibrosis develops (Mucheka, 2014). The flukes feed on blood of the definitive host, therefore causing anaemia and chronic cholangitis which is a consequence of bacteria that settles on the damaged tissue (Mucheka, 2014; Ruiz-Campillo *et al*., 2017).

There is also extensive damage to the hepatocytes and surrounding tissue causing necrosis (Ruiz-Campillo *et al*., 2017). The surrounding blood vessels also succumb to necrosis as the infection causes

the formation of blood clots and damage caused during migration may lead to the development of scar tissues (Radostits *et al.,* 2007).

2.4.3 Gross pathology

When infected with fascioliasis, the liver appears to be grey in colour rather than a normal red-brown colour with hyperaemia occurring (Mucheka, 2014). The mature flukes are large and cause haemorrhaging, thus making the larger tracks to appear as red plaques (Mucheka, 2014).

2.5 Human fasciolosis

Humans are normally infected by the ingestion of infective *Fasciola* metacercariae encysted on aquatic vegetables such as watercress (Mas-Coma *et al*., 2018). The rate of transmission is dependent on the area, the presence of livestock in the area and season. Prevalence of the disease is higher in children between the ages of 5 and 15 years (Thanh, 2012; Mucheka, 2014; Mas-Coma *et al*., 2018). Urban dwellers are also at risk of contracting fascioliasis through the ingestion of wild plants sold in markets (Mas-Coma *et al*., 2018).

Symptoms of infection in humans range from abdominal pain, weight loss, indigestion and diarrhoea (Mucheka *et al.,* 2014). It is estimated that the global burden of fasciolosis is 2.4 million people across 5 continents and the disease has been reported in at least 51 countries (Black *et al*., 2013; Jaja *et al*., 2017). In South Africa, three cases were reported in 1964 with two in the city of Cape Town and one in city of Johannesburg, but in recent times there were only two reported cases from the Western Cape (Black *et al*., 2013).

2.6 Economic losses due to fasciolosis

Fasciola species infect a wide range of animals and more especially ruminants (cattle, buffalo, sheep, and goats), as well as humans (WHO 1995; Ashrafi and Mas-Coma, 2014). This disease is prevalent in livestock in developing countries and therefore, the true burden of disease is unknown due to the lack of documentation (Tolan, 2011; Jaja *et* al., 2017). However, the prevalence of this disease in tropical regions is roughly estimated to be 25 to 100% (Toet *et al*., 2014; Jaja *et al*., 2017).

Effects of infection of animals include reduced weight, productivity, fertility and milk production. Animals also experience complications during pregnancy, and often experience late abortions (Spithill *et al*., 1999; Charlier *et al*., 2007; Jaja *et al*., 2017). In dairy cows, fasciolosis is responsible for 3.8% to a 15.2% decrease in milk production globally, which contributes to the ZAR 46.6 billion (USD 3.2 billion)

annual global production loss (Spithill *et al*., 1999; Mas-coma *et al*., 2005; Ai *et al.,* 2011). Sheep that are infected produce poor quality wool and the growth of infected lambs is decreased (Boray, 2017).

In South Africa, economic loss due to liver condemnation at three abattoirs was estimated between 2010 and 2012 to be ZAR 44, 930 (USD 3456.2) due to *Fasciola* infections. A study conducted in Zambia in 2013 showed that at least 164,600 kg of liver was infected and condemned, and this amounted to USD 592, 560 (Nyirenda *et al*., 2019). Other economic loses include the treatment of the infected animals, and it costs approximately ZAR 15-20 (~USD 1.5) per animal in South Africa (Jaja *et al*., 2017).

2.7 Epidemiology

Fasciolosis is prevalent in domestic animals world-wide (Yakhchali *et al*., 2015) and human cases have been observed in 51 countries over 5 continents, with an estimated 17 million people infected and approximately 91.1 million people at risk of contracting the disease (Tolan, 2011; Shahbazi *et al*., 2011; Yakhchali *et al*., 2015).

Mas-Coma *et al*. (2009) noted a change in the epidemiology of *Fasciola* species over the last few years. The prevalence of infection has decreased in some areas and increased in others (Keiser and Utzinger, 2009; Ezatpour *et al*., 2015). The decline in the number of infections is mainly attributed to social and economic development, education, stricter food regulations, urbanization and use of chemical fertilizers (Keiser and Utzinger, 2009; Broglia and Kapel, 2011).

While some areas experience a decrease in infections, other areas are seeing an increase, and according to Keiser and Utzinger (2009), this can be attributed to man-made dams, irrigation systems and aquaculture, as Lymnaeid snail distribution expands as these systems expand. Aquaculture is rapidly expanding, with an annual growth of 5.8% between 2001-2016 (Obiero *et al*., 2019). Lymnaeid snails successfully adapt and to these man-made systems and these have been observed in Peru and Egypt (Mas-Coma *et al.,* 2007). Other factors which have influenced the increase in low income countries, is the lack of basic knowledge on these flukes and their mode(s) of transmission and regulations on food safety (Keiser and Utzinger, 2009; Tolan, 2011).

2.7.1 Geographical distribution

The geographical distribution of *Fasciola* species is highly dependent on the intermediate host (Ibrahim, 2017). The most common intermediate snails are lymnaeids species from the genus *Lymnaea*, *Radix*, *Galba* and *Pseudosuccinea* (Ibrahim, 2017). According to Ashrafi (2006), the occurrence of *F. hepatica* was originally restricted to parts of Europe, but since then the fluke has

successfully expanded to all continents except Antarctica and has high prevalence in temperate regions (Valero *et al.*, 2005). *F. hepatica* is transmitted mainly by *G. truncatula* Muller, 1774, even though *G. truncatula* is of European origin, it is present in some parts of Africa acting as an intermediate host of *F. hepatica* (Brown, 1994; Walker *et al*., 2008; Malatji and Mukaratirwa, 2019). The distribution of *F. hepatica* and *G. truncatula* in sub-Saharan Africa can be grouped into three categories, such as: (i) the north-western African region; (ii) the large western and central African region; and (iii) the Eastern Africa region from the northern Mediterranean shore to southern Africa (Mas-Coma, 2009). This is supported by Brown (1994) who has observed *G. truncatula* across South Africa and Lesotho, and other African countries including Ethiopia, Kenya, Tanzania and Zambia (Malatji Mukaratirwa 2019; Nyirenda *et al.,* 2019). *F. giganica* has a distribution that is typically confined to tropical and subtropical regions of Africa and a few Asian countries (Ashrafi *et al*., 2004; Mas-Coma *et al*., 2005). The limitation in dispersion of *F. gigantica* is directly correlated to the dispersion of the snail intermediate host, *R. natalensis* (Krauss, 1848) in Africa (Brown, 1994; Walker *et al*., 2008; Malatji and Mukaratirwa, 2019).

2.8 Identification of *Fasciola* species

2.8.1 Phenotypic analysis

Fasciola species have been commonly identified and differentiated by morphological characteristics at the egg stage or adult stage (Marcilla *et al*., 2002). The adult and the egg of *F. gigantica* is larger in size (width 90-100 µm x length 150-196µm) than that of *F. hepatica* (width 63-90µm x length 130-150µm) (Mas-Coma and Burges, 1997). However, this is not reliable as populations may have a variety of morphological characteristics that overlap between the two species (Taydayon *et al*., 2015). Due to the limitations of the morphological approach, molecular methods have been used to differentiate *Fasciola* species (Ai *et al.,* 2011). With the use of these molecular approaches, differentiating the intermediate form between the two species is now possible (Marcilla *et al*., 2002; Tadayon *et al*., 2015). There are a variety of molecular approaches that are now used to discriminate among species and detect if hybridization is occurring in populations where cohabiting has been reported (Ai *et al*., 2011).

2.8.2 Molecular analysis

Most phylogenetic studies on *Fasciola* species has made use of mitochondrial (mtDNA) and ribosomal DNA (rDNA) markers (Mucheka, 2014; Chikowore, 2017). These genes are normally used in these

studies as they are single-copy genes as they tend to be far more informative than multi-copy genes (Chokowore, 2017).

2.8.2.1 Ribosomal DNA (rDNA) markers

Nuclear ribosomal DNA (rDNA) contain internal transcribed spacers (ITS-1 and ITS-2), and these spaces are present between the 18S, 5.8S and 28S ribosomal genes (Itagaki and Tsutsumi, 1998). They are normally used in species identification and determining if intermediate forms of *Fasciola* exist in certain locations (Itagaki and Tsutsumi, 1998; Itagaki *et al*., 2009; Ai *et al*., 2011).

The ITS-1 marker has been used successfully in many studies, indicating the presence of both species of flukes in many areas, as well as the presence of intermediate forms in some areas (Mucheka, 2014).

2.8.2.2 Mitochondrial DNA (mtDNA) marker

CO1 and mitochondrial nicotiamide dinucleotide dehydrogenase subunit-1 (ND1) are the two commonly used mitochondrial DNA markers (Mucheka, 2014). The CO1 gene is primarily used alongside the ITS-1 and ITS-2 region in species identification (Zarowiecki *et al.,* 2007; Mucheka, 2014) whilst ND1 is not as widely used; however, ND1 becomes useful when determining the relationship between *Fasciola* species from different geographical regions (Mucheka, 2014).

Mitochondrial DNA markers are slightly more sensitive than nuclear ribosomal DNA since mitochondrial DNA experiences a far more rapid evolution to that of nuclear ribosomal DNA. This rapid evolution allows for a better identification of closely related species (Ai *et al., 2011;* Teofanova *et al.,* 2012*)*.

There is clear evidence from published reports that intermediate forms between *F. hepatica* and *F. gigantica* exist in many regions where the two species cohabit (Peng *et al.,* 2009; Afshan *et al.,* 2014). However, there are no studies done in sub-Saharan Africa, especially in South Africa, where the two species have been found to cohabit (Chikowore, 2017).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling

Flukes were collected from fresh livers of naturally infected cattle from abattoirs located in Nelspruit and Ehlanzeni located in Mpumalanga province and Pietermaritzburg in Kwa-Zulu Natal province of South Africa. Mpumalanga is an inland province that ranges from >1500m above sea level, it has a maximum temperature of 27.4 °C and a minimum of 19 °C with an average rainfall of 683 mm per year. KwaZulu-Natal experiences a maximum temperature of 27.6 °C and a minimum of 4.2 °C, and an annual rainfall 897mm. Collected flukes were immediately washed in a physiological saline solution (0.9) and placed in universal bottles containing 70% ethanol and stored until they were sent back to the laboratory. Flukes were then grouped based on their morphological appearance. There were 3 groups established (Figure 5): *F.gigantica*, *F.hepatica* and *Fasciola sp.* These were then stored again in universal bottles containing 70% ethanol until further analysis.

3.2 Fixation, staining and examination

Before fixation and staining a sample of the fluke commenced, a small portion tissues was snipped on the lateral side of each fluke for molecular analysis (Figure 4).

The protocol as described by Gibbons *et al.* (1996) was followed;

I. Collected flukes preserved in 70% ethanol were rehydrated.
II. Rehydration was done using a solution of graded series of ethanol (70%, 50%, 30%, 10% and distilled water). The flukes in their subjected groupings were placed in the ethanol solution, starting at 70% and descending toward distilled water. The flukes remained in each solution for ±24 hours.
III. After rehydration the specimens were regressively stained with aceto-alum carmine. Specimens were left in the stain for approximately 24 hours and later destained in distilled water.
IV. After destaining, the specimens were subjected to differentiation by submerging the specimens in 1% HCl for 2-4 hours with constant monitoring and checking visibility of internal organs of the flukes. After differentiation they were then rinsed in distilled water.
V. The next step followed was to dehydrate the stained specimen by graded ethanol. To prevent curling during this process each specimen was lodge between two glass slides and tied with a rubber band and submerged in an ascending series of ethanol (10%, 30%, 50%, 70%, 90%, 100%). The specimens were submerged for 24-48 hours in each solution.

VI. The specimens were then cleared in clove oil for approximately 12-24 hours which gave the specimens a translucent appearance. The clove oil was then drained away from the specimens and each specimen was fixed in Canada Balsam.

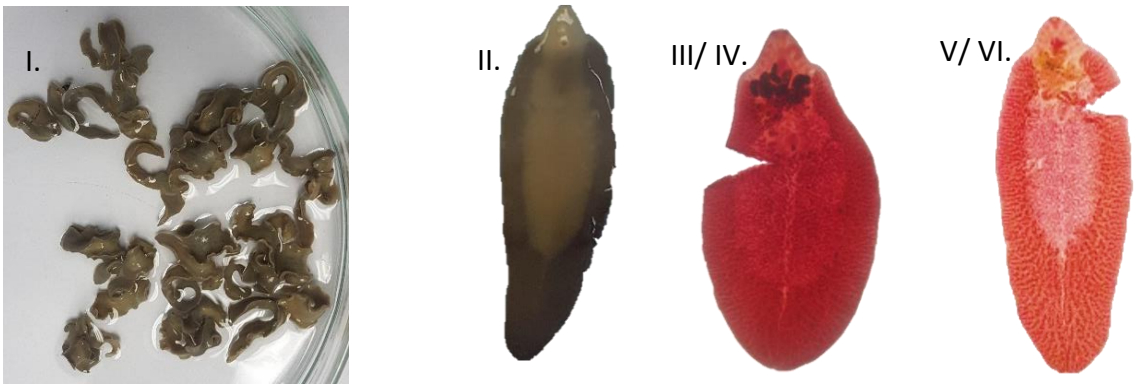

Figure 2: Visual representation of the fluke through the staining and fixation stages (I. Preserved specimen in 70% ethanol; II. Rehydrated specimen; III/IV. Destained specimen; V/VI. Cleared specimen ready for fixing).

3.3 Morphological analysis

3.3.1 Measurement of specimens

Morphometric parameters of each fluke were measured with a computer image analysis system (CIAS), this was based on standardised measurements which are useful for the differentiation between the two *Fasciola* species (Shafiei *et al*., 2014). SteREO Discovery.V12 (Zeiss) with a digital camera (Nikon DS-Fi3) was used to capture zoomed in images which were analysed through integrated software ZEN and AxioVision. The same hardware and software were used to capture the seminal vesicle of each fluke. The parameters measured and the position of the seminal vesicle are depicted in Figure 3.

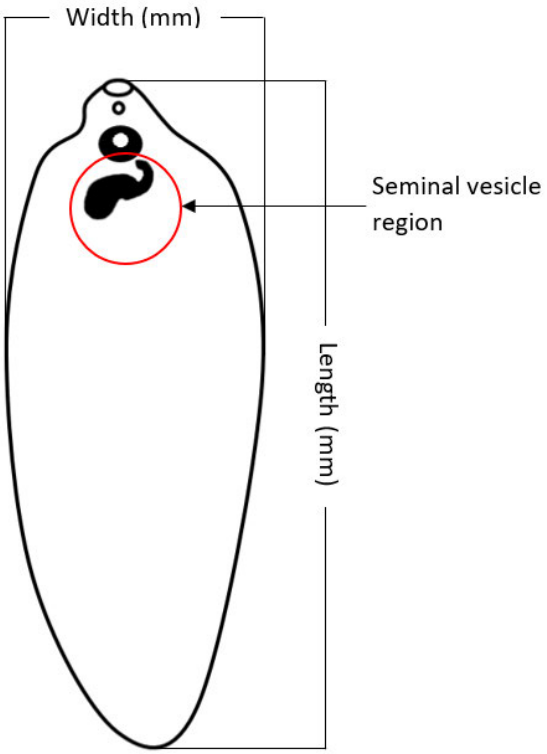

Figure 3: Visual representation of morphometric parameters used and location of the seminal vesicle.

3.3.2 Data analysis

One-way analysis of variance (ANOVA) was used to compare the measurements of the 3 categories of specimens (*F. gigantica*, *F. hepatica* and *Fasciola sp.* (intermediates)).

3.4 DNA Extraction

Prior to the flukes being placed in carmine solution for staining, a small piece of the fluke was excised (Figure 4) anterior to the ventral sucker (Beesley et al., 2017). The extraction of DNA was done using a DNeasy® DNA Blood and Tissue Kit (QIAGEN Inc.), extraction done according to the manufacturer's instructions. A nanodrop was used to determine the quantity and quality of the DNA.

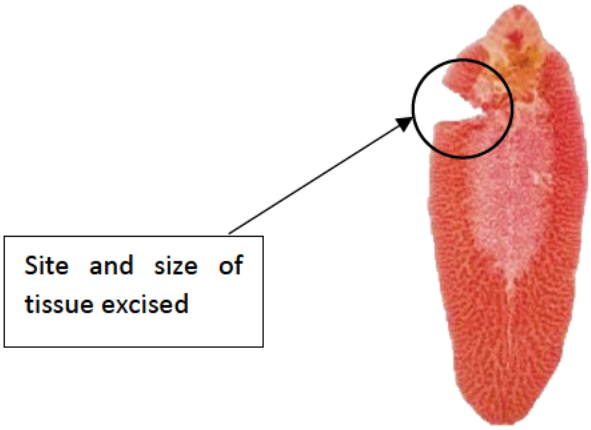

Figure 4: Visual indication of the area where the tissue portion was excised from the fluke specimen for DNA extraction for PCR.

3.5 PCR and electrophoresis

The ITS-1/5.8S rDNA/ITS-2 gene and Mitochondrial cytochrome oxidase (CO1) was amplified by polymerase chain reaction (PCR) from the extracted DNA. The ITS-region was amplified with the S30FE (forward: 5′-GTCGTAACAAGGTTTCCGTA 3′) and S49E6 (reverse: 5′-TATGCTTAAATTCAGCGGGT-3′) primers. These primers were designed using the conserved sequences in the 5.8S and 28S genes of the *Fasciola* species (Mucheka, 2014). CO1-region was amplified with the FHCO1 (forward: 5′-TTGGTTTTTTGGGCATCCT-3′) and FHCO1 (reverse: 5′AGGCCACCACCAAATAAAAGA3′) primers (Mucheka, 2014). These were used on flukes that appeared mainly appeared to be the hybrids.

The PCR reaction for both CO1 and ITS primer contained 2µl DNA, 12.5µl TopTaq Master mix, 0.5µl of each primer (forward and reverse), 10µl of distilled water, making up a reaction volume of 25µl. The PCR was performed in a thermocycler (BIORAD) under the following conditions for ITS primer: 5 minutes initial denaturation step at 95°C, followed by 40 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 55°C and 1 minute polymerization at 72°C, and the program ended with a final polymerization step for 7 minutes at 72°C. The steps were the same for the CO1 reaction, but the annealing temperature was at 59°C.

Fragments were separated by electrophoresis in 2% agarose gels (in 0.5× TBE buffer). The gel was made by adding 2g of agarose powder to 100ml 1x TBE buffer. This was then heated to dissolve the powder. Once the agarose powder was dissolved and the solution was slightly cooled, 100µl of Ethidium bromide (0.5 mg/ml) was added. This was then placed in a mould and left to harden for an

hour. Within each well, 5µl of PCR product was thoroughly combined 1µl loading dye and placed into a well. This was done for all samples. Three microlitres of 100bp gene ladder was placed in the last well. The samples were then run at 80V for 60 minutes in 0.5x running buffer. A Uvitec UV transilluminator fitted with a Uvitec digital camera was used both visualize and capture an image of the DNA bands.

3.6 Sequencing

Sanger sequencing was done at Inqaba biotechnical industries (Pty) Ltd. (Pretoria, South Africa) with ABI 3500XL sequencer using the Big Dye technology. DNA fragments were sequenced in the forward and reverse directions using the primers used in the initial amplification.

3.7 Molecular Analysis

3.7.1 Data analysis

Sequenced samples were returned as ABI files. Sequences were corrected, edited and aligned alongside homologue sequences retrieved from the GenBank database. Alignment was done using Clustal W (Thompson *et al*., 1997) option on BioEdit program (Hall, 1999). Aligned sequences for the ITS gene were trimmed to common length of 930 nucleotides, and 450 nucleotides for the CO1 gene. jModelTest tool (Posada, 2008) was used to statistically select best-fit models of nucleotide substitution to use in neighbour-joining, maximum likelihood and Bayesian Inference analyses. The Hasegawa-Kishino-Yano (HKY-G) model (Hasegawa et al., 1985) was the most suitable model to be used under the AIC information criterion. PAUP* 4.0 (Swofford, 1998) was used to generate neighbour-joining (NJ) and Maximum likelihood trees.

Nodal support for both methods was based on an estimation of 1000 bootstrap pseudo-replicates. The Bayesian posterior probabilities were calculated on Mr Bayes v3.2 (Ronquist and Huelsenbeck, 2003). Four Markov chains were run for 5 million generations, this was run until the split frequencies had a standard deviation less than 0.01 and a burn in value of 500,000 trees. Phylograms were based on 50% majority-rule consensus trees with nodal support indicated as posterior probabilities. DNAsp v 5.10.1 (Librado & Rozas, 2009) was used to generate the haplotype data file and determine the number of haplotypes. A statistical parsimony haplotype network was created in TCS v1.2.1 (Clement et al., 2000).

CHAPTER 4: RESULTS

4.1 Morphological analysis

Morphometric measurements (mean ± standard deviation, maximum and minimum body length and width, and body length and body width ratio) of the examined liver flukes are indicated in Table 1. These measurements coupled visual observations with the naked eye (Figure 5) was used to group flukes. The range between within each experimental group is as follows: *F. hepatica* (BL: 13-31mm; BW: 6-14mm), *F. gigantica* (BL: 30-47mm; BW: 7-12mm) and the presumed hybrid form assigned as *Fasciola sp* (BL: 18-35mm; BW: 6,5-13mm). The mean ratio of BL to BW, was able to be inferred for flukes, as: 1.47-2.75/1; 1.88-3.77/1, and 3.13-5.77/1 respectively.

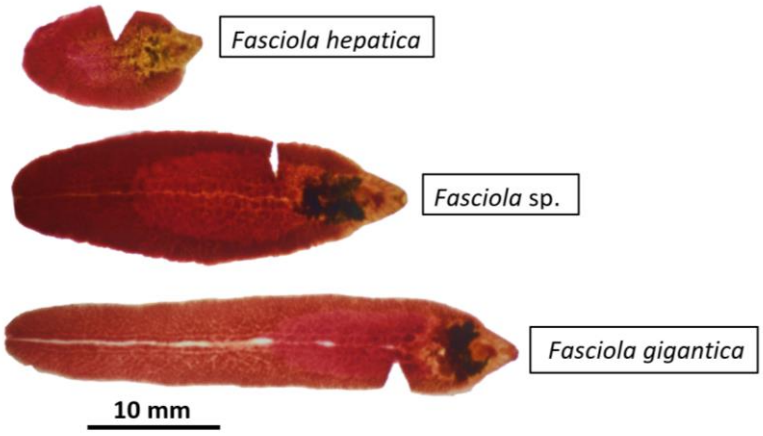

Figure 5: Representative adult *Fasciola* liver fluke individuals sorted out for groups of *Fasciola hepatica* (BL: 13-31mm; BW: 6-14mm), *Fasciola* sp. (BL: 18-35mm; BW: 6,5-13mm). and *Fasciola gigantica* (BL: 30-47mm; BW: 7-12mm). Size indicated by a scale bar (10mm).

The average length with their corresponding standard deviations for *F. hepatica*¸ *Fasciola* sp. and *F.gigantica* were 21.16±4.29, 28.87±5.12 and 39.61±1.09 mm respectively. The widths were 10.53±1.80, 10.44±1.59, and 9.32±1.72 mm respectively. The mean for length/width were 2.02±0.35, 2.79±0.48 and 4.41±1.10 mm respectively (Table 1). Results from the ANOVA test indicated those grouped that were morphologically identified as either *F. hepatica, Fasciola* sp. or *F. gigantica* had significantly different lengths ($P < 0.05$). There was no significant difference between their widths ($P > 0.05$). The length/width ratio between these groups was significantly different ($P < 0.05$) (Table 1).

Table 1: Summary of the mean length, width and length/width with corresponding standard deviations and the minimum and maximum measurements for the flukes in each group.

		Fasciola hepatica (36)	Presumed *Fasciola* sp (24)	*Fasciola gigantica* (11)	P-value
Width (mm)	Min-max	6.93 – 14.46	6.91 – 13.74	7.10 – 12.08	
	Mean ± sd	10.53 ± 1.80	10.44 ± 1.59	9.32 ± 1.72	P = 0.09
Length (mm)	Min-max	13.29 – 31.39	18.29 – 35.99	30.93 – 47.37	
	Mean ± sd	21.16 ± 4.29	28.87 ± 5.12	39.61 ± 1.09	$P = 4.98^{-16}$
Width/length	Min-max	1.47 – 2.75	1.88 – 3.77	3.13 – 5.77	
	Mean ± sd	2.02 ± 0.35	2.79 ± 0.48	4.41 ± 1.10	$P = 2^{-16}$

(sd=standard deviation)

Results from the principal component analysis (PCA) (Table 2), show that PC1 and PC2 accounted for 99.5% of all variability between the 2 species and the suspected hybrid. PC1 accounted for 64.8% of variability alone. PC2 contributed 3.47% to the variation between the selected parameters, suggesting that morphometrics using these parameters are not enough to differentiate the 3 species.

Table 2: Factor loadings from principle component analysis of liver flukes from Mpumalanga and KwaZulu-Natal provinces of South Africa, and Zimbabwe.

Variables	PC1	PC2	PC3
Length	-0.6386	-0.4395	-0.6317
Width	0.2868	-0.8977	0.3345
Length/width	-0.7141	0.0324	0.6993
% Variance	0.648	0.347	0.005
% Cum. Variance	0.6483	0.995	1.000

PC – Principal component, Cum. – Cumulative

Table 3 shows the prevalence of aspermatic flukes in specimens morphologically identified as *F. gigantica*, *F. hepatica* and *Fasciola* sp. The prevalence of aspermatic flukes was highest in flukes collected from Ehlanzeni (MP) (57%; 8/14), followed by Pietermaritzburg (KZN) (25%; 5/20) and Nelspruit (MP) (13.8%; 4/29) (Table 3). To note is the absence of aspermatic *F. gigantica* flukes from the 3 study localities in South Africa and Zimbabwe. Both Enhlazeni and Nelspruit of the Mpumalanga contained spermatic *Fasciola sp.* Only hybrid specimens are said to be aspermatic, however there are some *F. hepatica* specimens that appear to be aspermatic.

Table 3: Morphological identification of *Fasciola* species with or without spermatozoa in the seminal vesicle

Province/ country	Area	No. of specimens	Absence (-)/Presence (+) of sperm in the seminal vesicle					
			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 (15.5%)**	**26 (36.6%)**	**0**	**12 (16.9%)**	**6 (8.5%)**	**16 (22.5 %)**

PMB – Pietermaritzburg; "+" and "–" represent a high amount of and no/few sperms in the seminal vesicle, respectively

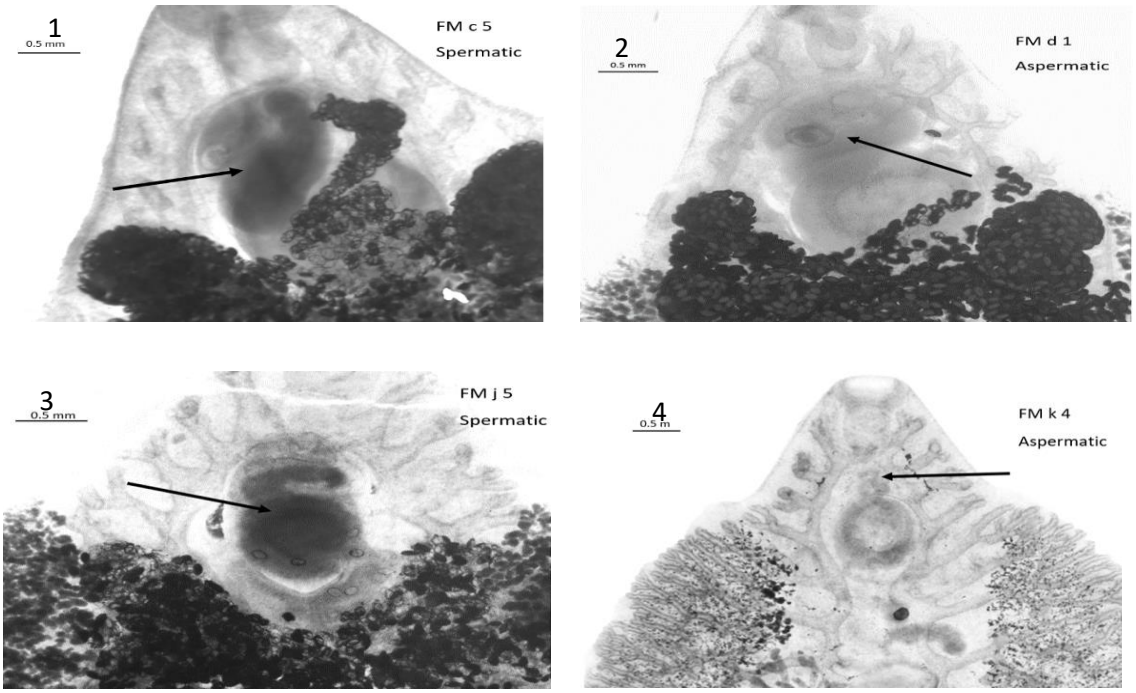

Figure 6: Seminal vesicles of stained *Fasciola gigantica* (1) and *F. hepatica* (2 – 4) showing the presence or absence of spermatozoa.

Figure 6 is a visual representation of the anterior portion of liver flukes that show presence of spermatozoa in the seminal vesicle (1 and 3) and the absence of spermatozoa in the seminal vesicle (2 and 4). Flukes that contain spermatozoa have a sack-like structure on the anterior end of the body that appear grey in black and white imagery.

4.2 Molecular analysis

Flukes that were morphologically classified as either *F. hepatica*, *F.gigantica* or *Fasciola* sp. were analyzed using molecular methods to confirm identity of flukes.

4.2.1 Molecular phylogenetic analysis

Cytochrome c oxidase I (CO1) analysis

From the 71 samples, only 19 (9 *F.hepatica*, 3 *F.gigantica*, 7 *Fasciola* sp. according to morphological analysis) were resolved for CO1 and a phylogenetic tree was constructed by using CO1 gene sequences of the *Fasciola* species (Figure 7). Clade A formed two sister clades (Clade B and C), where clade B consisted of *Fasciola hepatica* (clade D) and *Fasciola gigantica* (clade E) and clade C (*Fasciola* sp.). Clade B formed a highly supported (bootstrap 100%) monophyletic group to clade C. Clade D diverges into 2 highly supported external nodes. Clade E also diverges into two external nodes and one experimental sample diverges into a weakly supported group. The genetic p-distance (Appendix 1) among the experimental *F. hepatica* isolates ranged from 0 to 7%. The genetic p-distance between all the *F. gigantica* experimental isolates within the same clade was 0% and this included the one experimental isolate (G10) obtained from Zimbabwe. The mean genetic p-distance between the divergent *F. gigantica* (FMC10) isolate and the other *F. gigantica* isolates was ~2.6%.

Internal transcribed spacer (ITS-1/5.8S/ITS-2) analysis

Sixteen (8 *F.hepatica*, 2 *F.gigantica*, 6 *Fasciola* sp. according to morphological analysis) samples produced strong usable bands and analysis based on the ITS region, they formed a strongly supported clade (A) (bootstrap 100%) with the outgroup (Figure 7). This clade differentiates *Fasciola* sp., *F. hepatica* and *F. gigantica*. *Fasciola sp*. Isolates from the GenBank and one experimental isolate (FMC6), formed a weakly supported clade (clade B) (bootstrap 59%) to *F. hepatica* and *F. gigantica*. This clade includes 15 experimental isolates from Mpumalanga province and one from KwaZulu-Natal province, South Africa. Within clade B, a well-supported subclade C (bootstrap 79%) was formed, which include 7 experimental isolates identified as *F. gigantica*, 8 isolates as *F. hepatica* and 6 *F. gigantica* and 5 *F. hepatica* isolates from the GenBank. The ITS marker also identified isolate FMC10, which could not be identified by CO1, as *F. gigantica*. Furthermore, isolate FMC6 identified as *F. gigantica* by CO1 by morphological characters, was identified as a *Fasciola sp.* by ITS marker. The mean genetic p-distance between *F.gigantica* and *F.hepatica* isolates is 1%, (Appendix 2). The genetic p-distance is 0% between GenBank sequences for *Fasciola* sp. and experimental isolate FMC6.

4.2.2 Haplotype analysis

CO1 sequence alignment yielded 7 haplotypes from 450 nucleotides sequences, of which 166 sites were variable (Table 4). When set on 95% parsimony criterion, transitive consistency score (TCS) yielded a neighbour-joining network consisting of three major groups and an outgroup. Haplotypes 1, 2 and 5 were made up of *Fasciola hepatica*. Haplotype 1 consisted of 3 experimental isolates and 2 GenBank *F. hepatica* samples (MN006833.1 and KF111595.1). Haplotype 2 separated from haplotype 1, consisted of 6 experimental isolates and 3 GenBank *F. hepatica* (MN006843.1, GQ231548.1 and KT182304.1). Haplotype 5 just consisted of one experimental sample. Haplotype 4, a typical *F. gigantica* haplotype, included all *F. gigantica* experimental isolates and samples from GenBank samples (MK330627.1, KT182295.1 and KT182288.1). These are all isolates presented in clade E of Figure 7, with exception to isolate FMC10 which formed a sister clade to the *F. gigantica* isolates (Figure 7). This isolate further formed a separate haplotype, which is separated from the *F. gigantica* isolates by 6 mutational steps (Figure 9). This haplotype (Haplotype 3) differs at four variable sites from haplotype 4 (contains all other *F.gigantica* samples) (Table 6).

The alignment of the ITS sequence showed 67 variable sites, generating 5 haplotypes (Table 4 and 7). Each species formed its own haplotypes, which was congruent with the phylogenetic analysis (Figure 10). Haplotype 1, typical *F. gigantica*, included all experimental *F. gigantica* isolates and samples from the GenBank (KF543340.1, JF496711.1, JN828953.1, AJ853848.2, KX856339.1 and MN608169). Haplotype 2, typical *F. hepatica* which included all *F. hepatica* experimental isolates and samples from the GenBank (AM707030.1, MN559388.1, MG56998.1, KJ789346.1 and KX856339.1). Haplotype 3 included *Fasciola sp.* samples from the GenBank (MN821535.1, MH790325.1) and one experimental isolate.

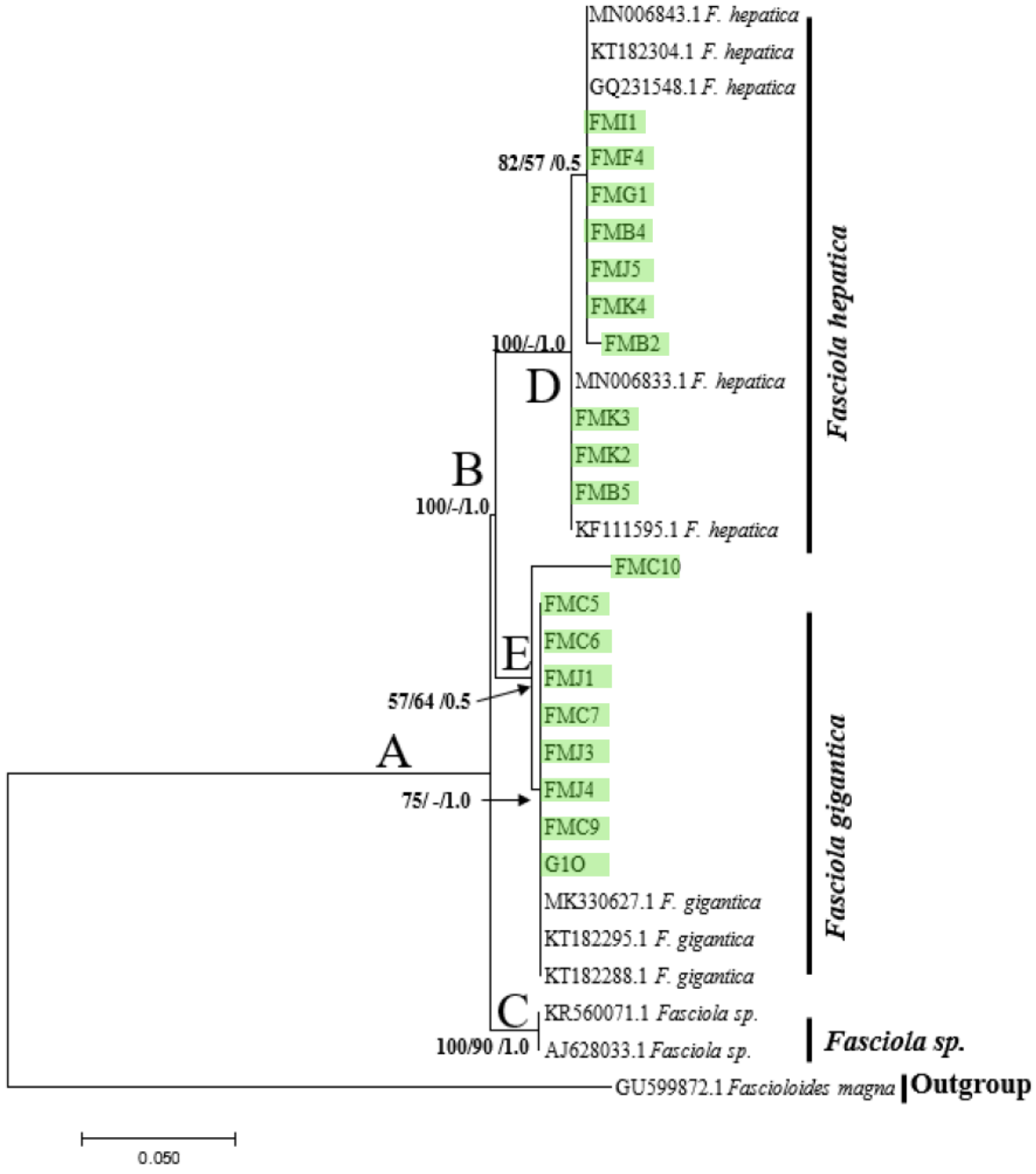

Figure 7: Neighbour-joining tree based on 450 nucleotides of the CO1 region, illustrating the relationships between the experimental samples (shaded in green). GenBank sequence have accession numbers adjacent to species names and have been downloaded from NCBI Genbank. Nodal support shown in the order neighbour-joining, maximum likelihood and Bayesian inference.

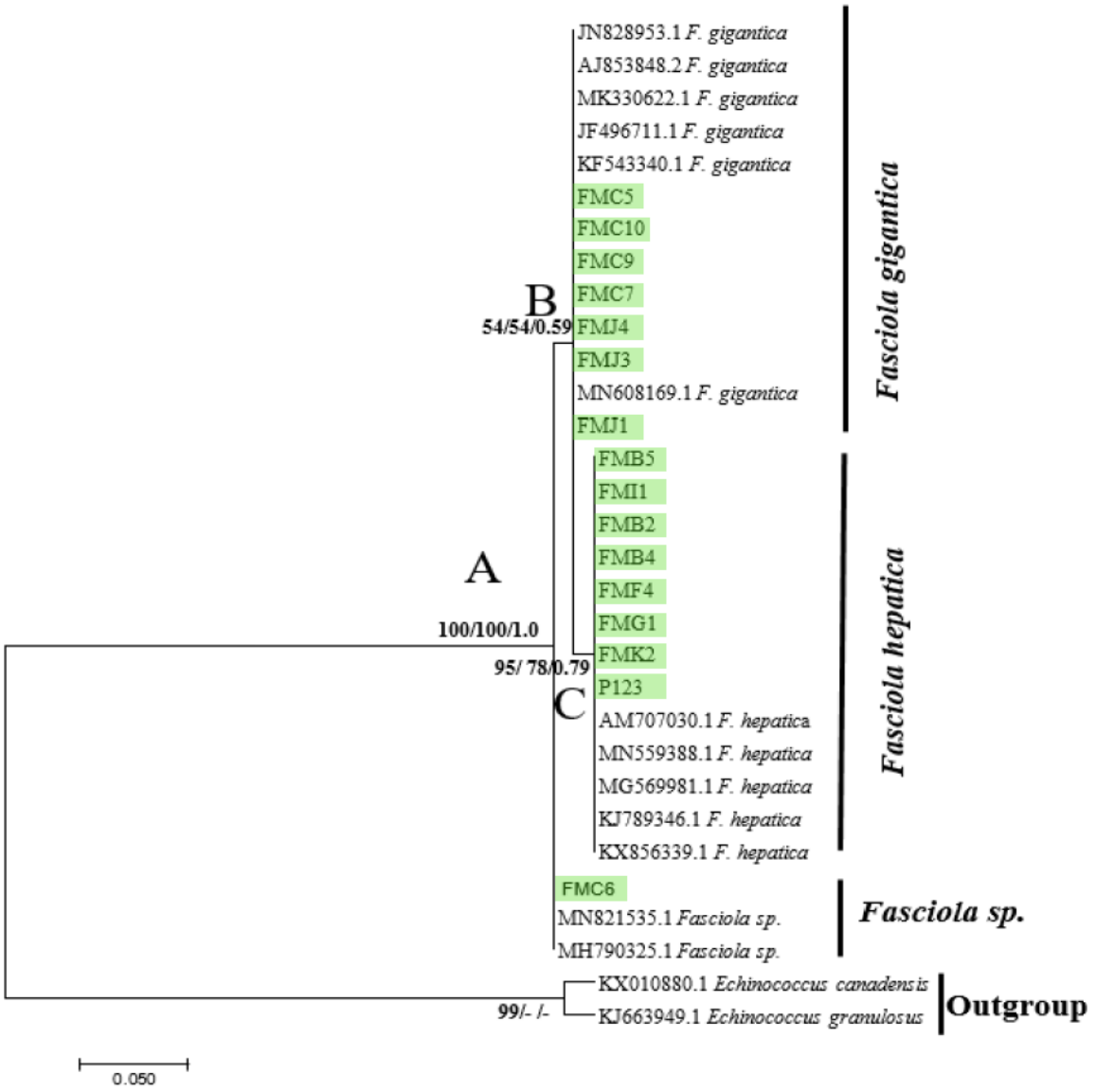

Figure 8: Neighbour joining tree based on 930 nucleotides of the ITS region, illustrating the relationships between the experimental samples (shaded in green) GenBank sequence have accession numbers adjacent to species names and have been downloaded from NCBI Gnebank. Nodal support shown in the order neighbour-joining, maximum likelihood and Bayesian inference.

Table 4: Sequence diversity of CO1 and ITS genes in *Fasciola* isolates from the experimental sequences and GenBank sequences.

	CO1	ITS
G+C content	0.341	0.478
Variable sites	166	67
Parsimony informative sites	34	64
Nucleotide diversity	0.09406	0.07233
Nucleotide variance	0.00199	0.00120
Haplotypes generated	7	5
Haplotype diversity	0.7839	0.658
Haplotype variance	0.00231	0.00257
Tajima's D	-2.4636	-1.8508

Table 5: Haplotype data and haplotype status of the GenBank and experimental isolates based on CO1. Experimental isolates are depicted in bold.

Haplotypes	No. of isolates	Samples	Species ID
H_1	5	**FMB5**, **FMK2**, **FMK3**, MN006833.1, KF111595.1	*F hepatica*
H_2	9	**FMB2, FMB4, FMF4, FMI1, FMG1, FMK4**, MN006843.1, GQ231548.1, KT182304.1	*F. hepatica*
H_3	1	**FMC10**	*F. gigantica*
H_4	11	**FMC5, FMC6, FMC7, FMC9, FMJI, FMJ3, FMJ4, G10,** MK330627.1, KT182295.1, KT182288.1	*F. gigantica*
H_5	1	**FMJ5**	*F. hepatica*
H_6	2	KR560071.1, AJ628033.1	*Fasciola sp.*
H_7	1	GU599872.1	*Facioloides magna*

Table 6: Nucleotide variable sites in the CO1 region of *Fasciola* haplotypes of isolates from GenBank and experimental data.

Species	Haplotype	Nucleotide variable sites																	
		2	4	5	6	9	15	24	36	51	64	66	67	69	70	71	75	77	84	86
F. hepatica	H_1	T	T	T	A	T	G	A	A	G	T	G	C	C	T	T	T	G	G	T
	H_2	T	T	C	A	T	G	A	A	G	T	G	C	C	T	T	T	G	G	T
	H_5	T	A	C	A	T	G	A	A	G	T	G	C	C	T	T	T	G	G	T
F. gigantica	H_3	C	T	T	A	C	A	G	A	G	T	C	T	T	C	A	C	C	C	C
	H_4	C	T	T	A	C	A	G	A	G	T	G	T	T	T	A	T	T	G	T
Fasciola spp.	H_6	T	T	T	G	T	A	G	G	A	C	G	T	T	T	A	T	T	G	T

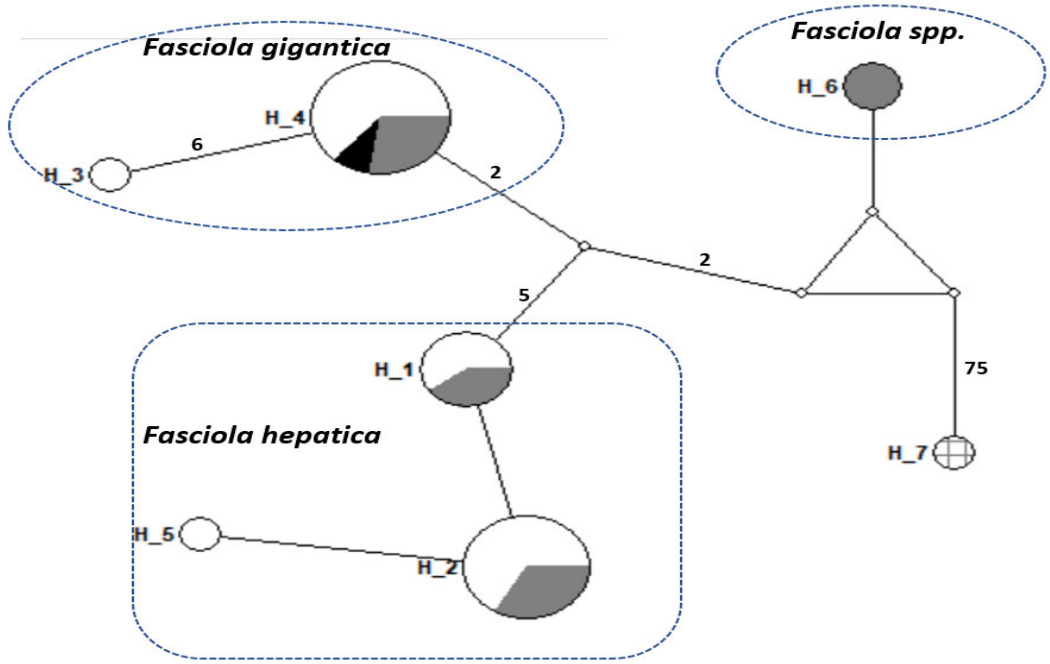

Figure 9: Median joining haplotype network representing mutational relationships between study samples and sequences retrieved from the GenBank based on the CO1 marker. Circle size is proportional to number of samples within the haplotype. Mutational steps are depicted by the numbers adjacent to lines, the lines separate adjacent haplotypes; lines without numbers indicate one mutational step. Source of isolates and sequence is represented by colour or pattern: White fill – Mpumalanga, Black fill – KwaZulu-Natal, Dark grey – GenBank sequences, crosses – outgroup.

Table 7: Haplotype data table showing the haplotype status of the GenBank and studies isolates from Mpumalanga province, South Africa and Zimbabwe, based on ITS-1/5.8S/ITS-2. experimental isolates are depicted in bold.

Haplotypes	No. of isolates	Samples	Species ID
H_1	13	**FMC5, FMC7, FMC9, FMC10, FMJ1, FMJ3, FMJ4**, KF543340.1, JF496711.1, JN828953.1, AJ853848.2, KX856339.1, MN608169.1	*F. gigantica*
H_2	13	**FMB2, FMB4, FMB5, FMF4, FMG1, FMI1, FMK2, P123,** AM707030.1, MN559388.1, MG56998.1, KJ789346.1, KX856339.1	*F. hepatica*
H_3	3	**FMC6**, MN821535.1, MH790325.1	*Fasciola sp.*
H_4	1	KX010880.1	*Echinococcus Canadensis*
H_5	1	KJ663949.1	*E. granulosus*

Table 8: Nucleotide variable sites in the ITS region of *Fasciola* haplotypes of isolates indicated in table 7.

Species	Haplotype	Nucleotide variable sites			
		43	94	148	161
F. hepatica	H_2	T	T	A	T
F. gigantica	H_1	A	C	T	C
Fasciola spp.	H_3	A	C	G	C

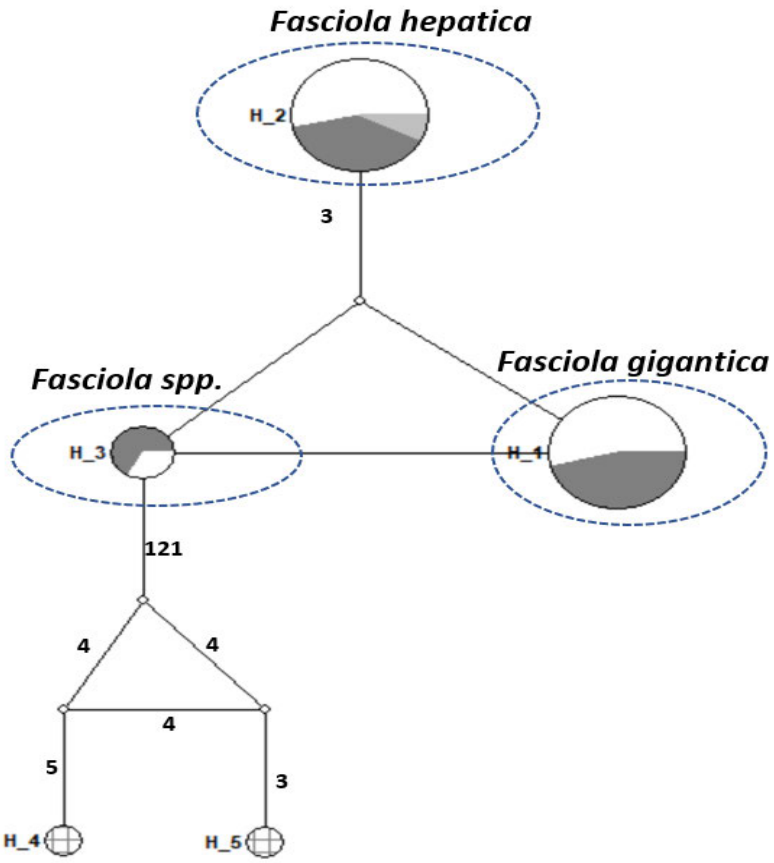

Figure 10: Median joining haplotype network representing mutational relationships between study samples and sequences retrieved from the NCBI GenBank based on the ITS marker. Circle size is proportional to number of samples within the haplotype. Mutational steps are depicted by the numbers adjacent to lines, the lines separate adjacent haplotypes; lines without numbers indicate one mutational step. Source of isolates and sequence is represented by colour or pattern: White fill – Mpumalanga, Black fill – KwaZulu-Natal, Dark grey – GenBank sequences, crosses – outgroup.

Morphological results supported by molecular results is shown in Table 9. All samples categorised as either *F. hepatica* or *F. gigantica* morphologically, were supported by the molecular results for CO1 and ITS analysis, except for one sample (FMC6). This sample was morphologically categorised as *F. gigantica*, while CO1 analysis confirms this, the results from the ITS analysis identifies this sample as *Fasciola* sp. Individuals morphologically identified as *Fasciola* sp. (FMC9, FMJ1, FMJ3, FMJ4) were all identified as *F. gigantica* with both CO1 and ITS analysis. FMK3 was identified as *F. hepatica* with CO1 analysis. The sample from Zimbabwe did not show any variation in haplotype and can be considered a pure species and used as a control.

Table 9: Morphological identification supported by molecular identification of *Fasciola* spp. from Mpumalanga and KwaZulu-Natal provinces, South Africa and Zimbabwe as well as an indication of the presence or absence of spermatozoa in individuals. Samples highlighted in yellow and red indicated those that were morphologically identified as *Fasciola sp,* but molecularly identified as either *F.gigantica* or *F.hepatica.* Bright-yellow indicates a morphologically identified *F.gigantica* as a molecularly identified *Fasciola sp.* using the ITS primer.

Sample ID	Area of collection	Sperm in the seminal vesicle	Morphological identification	Molecular identification	
				CO1	ITS-1/5.8S/ITS-2
FMB2	Mpumalanga	+	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMB4	Mpumalanga	-	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMB5	Mpumalanga	-	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMC5	Mpumalanga	+	*F. gigantica*	*F. gigantica*	*F. gigantica*
FMC6	Mpumalanga	+	*F. gigantica*	*F. gigantica*	*Fasciola sp.*
FMC7	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMC9	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMC10	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMF4	Mpumalanga	_	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMG1	Mpumalanga	+	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMI1	Mpumalanga	+	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMJ1	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMJ3	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMJ4	Mpumalanga	+	*Fasciola sp.*	*F. gigantica*	*F. gigantica*
FMJ5	Mpumalanga	+	*F. hepatica*	*F. hepatica*	-
FMK2	Mpumalanga	+	*F. hepatica*	*F. hepatica*	*F. hepatica*
FMK3	Mpumalanga	+	*Fasciola sp.*	*F. hepatica*	-
FMK4	Mpumalanga	-	*F. hepatica*	*F. hepatica*	-
GT10	Zimbabwe	+	*F. gigantica*	*F. gigantica*	-
P123	KwaZulu-Natal	-	*F. hepatica*	-	*F. hepatica*

CHAPTER 5: DISCUSSION

In this study, a combination of morphological and molecular techniques was used to identify the *Fasciola* species from localities where *F. gigantica* and *F. hepatica* overlap. The study closely focused on the specimens that could not be morphologically identified as either species and considered to be hybrid forms between the two species.

The distribution of *Fasciola* species is dependent on their intermediate host (Mucheka *et al*., 2015; Mahulu *et al*.,2019). Studies conducted by Malatji and Mukaratirwa (2019), showed that the intermediate snail hosts for *F. hepatica* and *F*. *gigantica* are both present in South Africa (Mucheka *et al*., 2015; Chikowore, 2017; Malatji and Mukaratirwa, 2019). Both species of *Fasciola* were present in Mpumalanga and this is supported by a study conducted by Mucheka *et al* (2015). The overlap is attributed to the presence of *L. (P.) columella* snail, as this species of snail is an intermediate host to both species of *Fasciola* (Bargues and Mas-Comas, 2005). A plausible explanation for this could be attributed to *L. (P.) columella's* ability to adapt to a wide range of climatic conditions in South Africa (De Kock, 1989; Kemp *et al*.,2016; Malatji and Mukaratirwa, 2019). According to a study conducted by Malatji and Mukaratirwa (2019), *L. (P.) columella* has been confirmed to transmit *F. gigantica*

Results obtained from this study showed an apparent co-existence of both *Fasciola* species in the province of Mpumalanga, and this is in agreement with the study conducted by Mucheka *et al*. (2015). This co-existence is primarily due to the presence of *L. (R.) natalensis* (IH of *F. gigantica*) in the Mpumalanga province, as well as the existence of *L. (P.) columella* (de Kock, 1989; Mucheka *et al*., 2015; Chikowore *et al*., 2017) which can transmit both *F. hepatica* and *F. gigantica*. Mucheka *et al*. (2015) reported cattle co-infected with both *Fasciola* species, therefore increasing the chances of hybridization. By observation, the isolates from Mpumalanga were either categorised as *F. hepatica*, *F. gigantica* or *Fasciola sp* (hybrids). Those that were classified as hybrids did not have distinct *F. hepatica* (broad and short) or *F. gigantica* (narrow and long) features (Narva *et al*., 2011). Body width and body length were the two main aspects taken into consideration. According to Peng *et al*. (2009), these measurements and the ratio of these two measurements were adequate in species discrimination (Periago *et al*., 2007).

Although Peng *et al.* (2009) and Nguyen *et al* (2018) described body morphometrics such as length, width and length/width as inadequate for species identification and the morphometric results obtained from this study could not distinguish between *F. gigantica* and *F. hepatica*. The suspected hybrid species that were identified as either species overlapped and this is in agreement with a report by Itagaki *et al.* (2009) were *F. hepatica* could not be morphologically distinguished from *Fasciola sp.*

Results from this study also showed that individual flukes that were morphologically categorised as hybrids were molecularly distinguished as either *F. gigantica* or *F. hepatica.* This morphological misidentification could be attributed to factors such as the age of the flukes, the hosts and seasons in which the flukes were retrieved (Sumruaypol *et al*., 2020).

Due to the ability of *F. hepatica* and *F. gigantica* to reproduce sexually or by self-fertilization, they are classified as meiotically functional diploids (2n = 20) (Terasaki *et* al., 2000; Shoriki *et al*., 2014). Individuals produce sperm that is temporarily stored in a male reproductive organ called the seminal vesicle, which can be easily observed under a stereomicroscope when the fluke is stained (Shoriki *et al*., 2014). Whereas the suspected hybrids (*Fasciola* sp) are said to contain few to no spermatozoa in the seminal vesicle (Peng *et al*., 2009; Itagaki *et al*., 2009), and such individuals have been reported in many Asian countries such as Japan (Watanabe and Iwata 1954; Itagaki and Akane 1959; Oshima *et al*. 1968), India (Varma 1953), Korea (Chu and Kim 1967), the Philippines (Kimura et al 1984), and Iran (Ashrafi et al. 2006) (Peng *et al*., 2009). They are classified as meiotically dysfunctional diploids, triploids, and occasionally mixoploids and display abnormal spermatogenetic ability and reproduce asexually by parthenogenesis (Mohanta *et al*., 2014). Some flukes which were classified as *Fasciola* sp. from Japan have been reported to have 20 chromosomes (2n = 20) which are diploids, it was also found that some specimens were triploids, and these contained 30 chromosomes (3n = 30). There were also *Fasciola* sp. specimens that demonstrated mixoploidy as these specimens contained both 20 (2n = 20) and 30 (3n =30) chromosomes within one specimen (Terasaki *et al*., 1998; Terasaki *et al*., 2000; Mohanta *et al*., 2014).

The aspermatic specimens were also reported to contain three types of DNA in nuclear ITS1 type (Peng *et al*., 2009). One type is the same to the sequence of *F. hepatica* (Fh), the other type is identical to that of *F. gigantica* (Fg) and the third type is a combination of the other two types (Fh/Fg) (Mohanta *et al*., 2014). These sequences are termed Fh, Fg and Fh/Fg respectively (Ichikawa and Itagaki, 2010; Mohanta *et al*., 2014; Hayashi *et al*., 2015). Studies suggest that the Fh/Fg type in the nuclear DNA is a consequence of hybridization between *F. hepatica* and *F. gigantica* (Itagaki *et al.,*2005; Peng *et al*., 2009; Mohanta *et al.,* 2014).

However, in this study samples that were identified as *F. hepatica* by both ITS and COI marker did not have sperm present in their seminal vesicle. Whereas, the sample (FMC6) that was identified as *Fasciola sp.* using ITS marker did contain sperm. Another sample (FMC10), which could not be identified using the CO1 marker was also categorised as spermatic. The phenomenon of hybrid flukes retaining sperm in their seminal vesicle was also observed by Hayashi *et al* (2017) and suggested that these isolates should be referred to as hybrid flukes and not aspermatic flukes (Mohanta *et al*., 2014).

However, when subjected to a molecular analysis based on CO1 marker, the majority of the samples that were morphologically categorised as *Fasciola sp* were identified as *F. gigantica* and just one sample was *F. hepatica*. The same outcome was observed using the ITS marker. One sample in particular (FMC10) from this study, could not be identified using the CO1, however, it formed a well-supported sister clade with *F. gigantica* with a small mean genetic difference of ~2.6%. The FMC10 haplotype (H_3) differed from the rest of the *F. gigantica* samples on two variable sites. This low diversity could indicate that they have recently expanded their distribution (Hayashi *et al*., 2015). The ITS analysis further supported this and identified this sample as *F. gigantica.* This sample formed a haplotype (Haplotype 1), with other *F. gigantica* samples from the study and Genbank-derived samples. A sample (FMC6) from the ITS analysis identified as *Fasciola sp*, formed a clade with *Fasciola sp* GenBank-derived samples that were weakly-supported by *F. hepatica* and *F. gigantica* sister clades. This specific sample was identified as *F. gigantica* in the CO1 analysis. Flukes that were molecularly identified as either *F. hepatica* or *F. gigantica* and did not present any spermatozoa could have been be due to ageing (Hayashi *et al*., 2016).

The formation of a subclade in the ITS analysis could suggest the existence of a hybrid between the two species (Malatji and Mukaratirwa, 2019). There was only one mutational step between the *F. gigantica* haplotype and the *Fasciola sp* haplotype and which had a mean genetic difference of 1% and 2% respectively.

The present study indicated that the use of morphological characters alone is inadequate in the identification of *Fasciola* species from areas where *F. hepatica* and *F. gigantica* are endemic. Morphological techniques coupled with molecular techniques are recommended in *Fasciola* species identification, especially in areas where both species are co-endemic. Only one isolate pointed to the possible existence of hybrids in the study area, however, this was not conclusive as the sample size for molecular identification was small. It was also observed that aspermatic specimens are not only limited to hybrids, as some *F. hepatica* specimens lacked sperm in their seminal vesicles

In conclusion, discrimination between *Fasciola* species is of high importance due to the economic losses associated with it, as well as the risk of human infection. The fecundity of hybrid individuals is said to be far greater to that of *F. gigantica* (Hayashi *et al*., 2017). The hybrids are also suspected to have a greater dispersal rate and host expansion (Walker *et al*., 2008; Cwiklinski *et al*., 2015) therefore monitoring their dispersal and distinguishing a preferred intermediate host for these hybrids is highly important. This is the first study to indicate the presence of intermediate *Fasciola* forms in South Africa in a location where *F. hepatica* and *F. gigantica* are endemic. Further studies are required to give

substantial evidence for the existence of these hybrids in South Africa. A PCR-RFLP (restriction fragment length polymorphism) method could be adapted to aid in the discrimination of species and the detection of hybrids, as this method can identify Fh, Fg or Fh/Fg sequence (Hayashi *et al*., 2015). Traditional methods such as using ITS markers used by Ali *et al*. (2008); Itagaki *et al*. (2009); Liu *et al*. (2014); Chaudhry *et al*. (2016) and may be inefficient to detect small genetic changes associated with hybridization. Furthermore, larger sample sizes are required from cattle from a variety of locations in the provinces where the main intermediate host exist along with *L. (P.) columella*. The main shortcoming for this study was that DNA extraction failed for many samples and there was only a limited amount of tissue that could be utilized and thus could not be repeated.

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APPENDIX

Appendix 1: Genetic p-distance matrix between *Fasciola* specimens from Mpumalanga (FM..), KwaZulu-Natal (P123), GenBank samples and outgroups for ITS analysis

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	FMK3																														
2	FMK4	0,00																													
3	FMJ5	0,00	0,00																												
4	FMC10	0,06	0,07	0,07																											
5	FMC5	0,04	0,04	0,04	0,03																										
6	FMB2	0,01	0,00	0,00	0,07	0,05																									
7	FMK2	0,00	0,00	0,00	0,06	0,04	0,01																								
8	FMC6	0,04	0,04	0,04	0,03	0,00	0,05	0,04																							
9	FMJ1	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00																						
10	FMC7	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00																					
11	FMB4	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04																				
12	FMG1	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00																			
13	FMF4	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00	0,00																		
14	FMJ3	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04																	
15	FMJ4	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00																
16	FMI1	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04															

17	FMC9	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,04																							
18	FMB5	0,00	0,00	0,00	0,06	0,04	0,01	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04																						
19	G1O	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,04	0,00	0,04																					
20	MN006833.1 F. hepatica	0,00	0,00	0,00	0,06	0,04	0,01	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04	0,00	0,04	0,04																			
21	KF111595.1 F. hepatica	0,00	0,00	0,00	0,06	0,04	0,01	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04	0,00	0,04	0,04	0,00																		
22	GQ231548.1 F. hepatica	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04	0,00	0,04	0,04	0,00	0,00																	
23	MN006843.1 F. hepatica	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04	0,00	0,04	0,04	0,00	0,00	0,00																
24	MK330627.1 F. gigantica	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,04	0,00	0,04	0,00	0,00	0,04	0,04	0,04	0,04															
25	KT182295.1 F. gigantica	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,04	0,00	0,04	0,00	0,00	0,04	0,04	0,04	0,04	0,00														
26	KT182288.1 F. gigantica	0,04	0,04	0,04	0,03	0,00	0,05	0,04	0,00	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,04	0,00	0,04	0,00	0,00	0,04	0,04	0,04	0,04	0,00	0,00													
27	KT182304.1 F. hepatica	0,00	0,00	0,00	0,07	0,04	0,00	0,00	0,04	0,04	0,04	0,00	0,00	0,00	0,04	0,04	0,00	0,04	0,00	0,04	0,04	0,00	0,00	0,00	0,00	0,04	0,04	0,04												
28	KR560071.1 Fasciola sp.	0,05	0,05	0,05	0,06	0,03	0,06	0,05	0,03	0,03	0,03	0,05	0,05	0,05	0,03	0,03	0,05	0,03	0,05	0,03	0,03	0,05	0,05	0,05	0,05	0,03	0,03	0,03	0,05											
29	AJ628033.1 Fasciola sp.	0,05	0,05	0,05	0,06	0,03	0,06	0,05	0,03	0,03	0,03	0,05	0,05	0,05	0,03	0,03	0,05	0,03	0,05	0,03	0,03	0,05	0,05	0,05	0,05	0,03	0,03	0,03	0,05	0,00										
30	GU599872.1 F. magna	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,38	0,37	0,37									

Appendix 2: Genetic p-distance matrix between *Fasciola* specimens from Mpumalanga (FM..), Zimbabwe (G10), GenBank samples and outgroups for CO1 analysis

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	MN608169.1 F. gigantica																														
2	FMJ1	0,00																													
3	FMB5	0,01	0,01																												
4	FMJ3	0,00	0,00	0,01																											
5	FMI1	0,01	0,01	0,00	0,01																										
6	FMB2	0,01	0,01	0,00	0,01	0,00																									
7	FMB4	0,01	0,01	0,00	0,01	0,00	0,00																								
8	FMF4	0,01	0,01	0,00	0,01	0,00	0,00	0,00																							
9	FMG1	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00																						
10	FMJ4	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01																					
11	FMC7	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00																				
12	FMC9	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00																			
13	FMK2	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01																		

14	FMC10	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01																										
15	FMC6	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,01	0,01	0,01	0,02	0,01																									
16	FMC5	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01																								
17	P123	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01																					
18	KF543340.1 F. gigantica	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01																				
19	JF496711.1 F. gigantica	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01	0,00																			
20	MK330622.1 F. gigantica	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,00																		
21	JN828953.1 F. gigantica	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,00	0,00																	
22	AJ853848.2 F. gigantica	0,00	0,00	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,00																
23	AM707030.1 F. hepatica	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,01	0,01	0,01	0,01															
24	MN559388.1 F. hepatica	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00														
25	MG569981.1 F. hepatica	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00													
26	KJ789346.1 F. hepatica	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00												
27	KX856339.1 F. hepatica	0,01	0,01	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,00	0,01	0,02	0,01	0,00	0,01	0,01	0,01	0,01	0,01	0,00	0,00	0,00	0,00											
28	MN821535.1 Fasciola sp.	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,01	0,01	0,01	0,02	0,01	0,00	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,02	0,02										
29	MH790325.1 Fasciola sp.	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,01	0,01	0,01	0,02	0,01	0,00	0,01	0,02	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,02	0,02	0,00									
30	KX010880.1 E. canadensis	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,53	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,53	0,53								
31	KJ663949.1 E. granulosus	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,53	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,54	0,53	0,53	0,02							