UNIVERSITY OF KWAZULU-NATAL

Morphological and molecular characterization of amphistomes from wild ruminants and their snail intermediate hosts in Matebeleland region of Zimbabwe

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As the candidate's supervisor, I have approved this thesis for submission.

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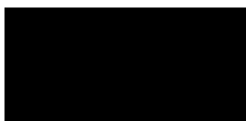

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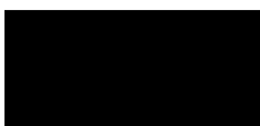

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ABSTRACT

Amphistomosis is a tropically neglected disease that affects both wild and domestic ruminants. The disease is not well understood as to date most amphistome species have no known intermediate hosts. Wild ruminants harbor several amphistome species which they share with domestic ruminants, however, very few studies have focused on amphistomes of these wild ruminants. The lack of molecular information on amphistomes of wild ruminants and their intermediate host snails prompted the need to do a study on the identity of amphistomes species of wild ruminants in Zimbabwe. The main objectives of the study were to (a) conduct a systematic review of amphistomes found in wild ruminants across Africa (b) to conduct molecular and morphological characterization of amphistomes present in the wildlife ruminants, and (c) to detect the presence of DNA of these amphistomes in the intermediate host snails collected from wildlife conservancies and game parks in Matebeleland region of Zimbabwe. The study sites for all this work were areas Matebeleland, located in the southwestern region of Zimbabwe.

A systematic search of literature on the amphistomes of wild ruminants in published between 1900 and 2022 in Africa showed that there are a total of 38 amphistome species belonging to the genera *Calicophoron* (*C.*), *Gastrothylax* (*G.*), *Carmyerius* (*Car.*), *Cotylophoron* (*Cot.*), *Leiperocotyle* (*L.*), *Bilatorchis* (*Bi.*), *Paramphistomum* (*P.*), *Stephanopharynx* (*S.*) and *Gigantocotyle* (*Gi.*) identified and recorded in wild ruminants. It was observed that most studies on amphistomes of wild ruminants were conducted and documented before the year 2000, which not only show the paucity of data in this area but also highlighted the less attention given to these group of parasites by researchers. Studies done post 2000 mostly shifted and focused on amphistomes and other helminth parasites of domestic animals such as schistosomiasis including zoonotic diseases such as fascioliasis which are more common and have a higher impact on public health in Africa. This is understandable as domestic animals are seen as an investment and of economic importance to most countries in Africa. As a result, current amphistome prevalence estimates in Africa may not completely reflect the diversity of host species or geographical areas. Results also revealed that only one study has been done to date on molecular characterization of amphistomes of wild ruminants and future research gaps were identified.

A total of 313 hunter-killed wild ruminants from game parks and wildlife conservancies from Hwange, Nyamandlovu and Beitbridge, were randomly selected and assessed for amphistome infection. The wild ruminant species were identified as *Syncerus caffer* (African buffalo), *Aepyceros melampus* (impala), *Redunca arundinum* (reedbuck), *Kobus ellipsiprymnus* (waterbuck), *Hippotragus niger* (sable), *Tragelaphus strepsiceros* (kudu), *Connochaetes taurinus taurinus* (blue wildebeest), *Sylvicapra*

grimmia (common duiker), *Tragelaphus sylvaticus* (bushbuck) and *Damaliscus lunatus* (tsessebe). From these, amphistome infections were found in African buffalo, sable, waterbuck, kudu, eedbuck and blue wildebeest. The overall prevalence of infection was 10.22% (32/313), with the highest infection rate recorded in 63 % (7/11) in waterbuck. The highest prevalence of amphistomes per locality was in Hwange at 35% (7/20) whilst the lowest prevalence was observed in Nyamandlovu at 5.62% (15/267). Morphology revealed eight amphistome species which included *C. raja*, *C. microbothrium*, *C. phillerouxi*, *C. clavula*, *L. gretillati*, *G. crumenifer*, *Gi. symmeri* and *Orthocoelium (O.) dicranocoelium*. Molecular analysis using ITS-2 region confirmed identity of some of the isolates with a percentage identity of above 97%. This is a first record of *Gastrothylax crumenifer*, *Leiperocotyle gretillati* and *Orthocoelium dicranocoelium* in Zimbabwe.

This was followed by a study on detection of amphistome DNA and identification of snail intermediate hosts of amphistomes from the following six areas in the Matebeleland region of Zimbabwe; Matopos, Inyathi, Beitbridge, Nyamandlovu, Ntabazinduna and Esigodini. A total of 487 freshwater snails were collected from 9 of 19 surveyed water points (from the six mentioned areas) where wild ruminants had been spotted or frequently drink and were morphologically identified as *Biomphalaria (Bio.) pfeifferi, Bulinus (B.) tropicus, B. truncatus, B. globosus*, *Lymnaea (Radix) natalensis, Physa (Phy.) acuta*, *Bellamya* spp. and *Melanoides (M.) tuberculata*. Of these snails, species identifcication was confirmed for *Bio. pfeifferi*, *Bul. tropicus*, *Bul. truncatus*, *Bul. globosus*, *L. (R.) natalensis,* based on the *Cox1* gene. *Bulinus tropicus* and *Phy. acuta* were collected in abundance. Amphistome DNA was detected in 11.9 % (58/487) snails, with the highest infection rate being detected in *B. globosus* (44.4 %). Amphistome DNA from *M. tuberculata* was successfully sequenced and identified as *C. microbothrium*. An additional band was detected in *M. tuberculata*, *B. tropicus* and *B. trancatus* which showed a 96.42% similarity to *Paragonimus* spp. sequence in the GenBank.

Although mapping was done in the review paper, it shows that there still is a lack of current information on amphistomes of wild ruminants. More work therefore needs to be done on wild ruminants as they may be potential reservoirs of amphistomes. In conclusion, this study revealed how wild ruminants harbor diverse amphistome species, some of which have not been documented before in Zimbabwe. This may be attributed to either movement of wild animals across barriers or establishment of new and invasive snails in different parts of the country. Furthermore, the detection of *Calicophoron microbothrium* DNA in snails such as *Melanoides tuberculata* shows that there could be many more snail intermediate hosts of amphistomes. Future work should focus on screening more snails for amphistome infection.

LIST OF ABBREVIATIONS

mL - Microlitre

Cox1 – Cytochrome Oxidase 1

DNA – Deoxyribonucleic acid

IH – Intermediate Host

ITS – Internal transcribed spacer

PCR – Polymerase Chain Reaction

rDNA – Ribosomal Deoxyribonucleic acid

DECLARATION 1 – PLAGIARISM

I, **Madeline S. Sibula** declare that:

1. The research reported in this thesis is my original research, except where otherwise indicated.
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.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. their words have been re-written, but the general information attributed to them have been referenced, and
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section/s.

Signed

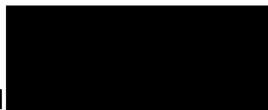
Madeline Sibula

DECLARATION 2-PUBLICATION

Details of publications:

1. Sibula, M.S., Nyagura, I., Malatji, M.P., Mukaratirwa, S. Prevalence and geographical distribution of amphistomes of African wild ruminants: A scoping review. International Journal for Parasitology: Parasites and Wildlife, 2024, 23, 100906. https://doi.org/10.1016/j.ijppaw.2024.100906.
2. Sibula, M.S., Malatji, M.P., Nyahunda, C., Mukaratirwa, S. Species diversity of freshwater snails collected from selected wildlife drinking water sources in Matebeleland region of Zimbabwe and screening of amphistome infections. Veterinary Sciences, 2024, 11, 221. https://doi.org/10.3390/vetsci11050211
3. Sibula, M.S., Ndlovu, P., Jele, T., Malatji, M.P., Mukaratirwa, S. Morphological and molecular identification of amphistomes of wild ruminants from selected game reserves of Zimbabwe-In prepeparation

From all the above publications, my role included carrying out all the experimental work and contributing to the writing of the publications along with my supervisor. Where other authors carried out aspects of the experimental, I either interpreted or consulted with them to understand the data. Co-authors contribution was also that of an editorial nature, checking on the scientific content in their field, and my correct interpretation of the data in their field. Based on their expertise, they may have added minor parts to the manuscripts.

Signed 

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DEDICATION

To my husband Mkhululi and my children Wayne, Zoey and Dawn.

"Bloom where you are planted…."

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Chapter 1

General introduction

1.1 Introduction

Wild animals have often been incriminated as being the source of parasites both in humans and livestock, acting as reservoirs for infection (Thompson et al., 2010). With on-going changes in land-use practices particularly in Zimbabwe, interaction between livestock and wildlife is increasing. This increases the risk of disease incidence, emergence of new diseases and re-emergence of previously diagnosed diseases (Bekker et al., 2012). However, in spite of all these risks, information on parasites remains limited in Zimbabwe particularly in the wildlife community.

Parasites can cause severe population declines and pause as significant threats to wildlife populations (Stringer and Linklater, 2014). Although this is true, parasites also play a positive role in the selection of stronger animals in the wild and natural selection of the ecosystem (Boomker, 2007). This is because weaker animals succumb among many other factors, to heavy parasitic infections and consequently die in the early stages before they can contribute to the gene pool (Malan et al., 1997). However, due to anthropogenic activities and interventions, movement of wildlife are now restricted and mostly confined to game parks with heavy parasitic burdens. Among parasites that have been documented in wild animals, helminths constitute a large group with mostly unidentified species which needs molecular characterisation (Majewska et al., 2021). Helminths that have been recorded in wild ruminants include amphistomes among others (Round, 1968; Boomker, 2007).

Amphistomes are digenetic trematodes that exhibit a heteroxenous life cycle, having both the intermediate and a definitive host (Smyth and Halton, 1983). The intermediate host is usually freshwater snails from the genus *Bulinus* spp., which is mainly found in water bodies and harbours the larval stages (Abrous et al., 2000; Dube et al., 2002; Chingwena et al., 2002). Wild animals and domestic animals may share habitats and as a result, cross infection is likely to occur at interfaces where wild animals and domestic animals interact which include water bodies used as source of drinking water. There is therefore a need to determine prevalence of amphistome infections in wild ruminanst, and further identify and characterise those amphistomes to species level using both morphologically and molecular techniques. Furthermore, identifying freshwater snails present in the habitats used as water points for these animals, sceening for infections, as well as identifying species of infection is also crucial to not only identify the amphistomes species circulating in the wild ruminants, but also identify the the sites with active transmission of dieases, the snail species and wild ruminant species also involved in the transmissions.

Globally, over 70 amphistome species have been reported, with 26 of these being reported in South and East Africa (Pfukenyi and Mukaratirwa, 2018). To date, amphistomes remain poorly understood with characterisation of species being difficult using morphological means as it involves a cumbersome process of sectioning through robust bodies (Lofty et al., 2010). Although this is so, molecular tools have been developed to assist with easy characterization of amphistomes and these include the use of ITS 2 (Dube et al., 2015) and Cox1 (Laidemitt et al., 2017) sequencing. Amongst these tools, the most popularly used tool is the ITS2 sequencing, however, although several studies have been done on molecular characterisation of amphistomes of livestock particularly in Africa, few studies have been done on amphistomes of wild ruminants.

1.2 Problem statement

Zimbabwe is a country whose economy is based on both agricultural and tourism sectors. Because of the diversity of wild animals in the wild, many parasites are yet to be recorded particularly in Zimbabwe, since the checklist of internal parasites of wild animals by Jooste (1984). Amphistome infections, like many other gastro-intestinal parasites result in heavy economic losses in the livestock, through morbidity, mortality, and reduction in wool and milk production in the tropics and subtropics (Taylor *et al*., 2007; Godara *et al*., 2013). So far, there is lack of comprehensive information on the species of amphistomes and their intermediate hosts snails that infect wild ruminants in Zimbabwe. Hence, it is crucial to characterise the amphistome species that are found in wild ruminants, and identify the species linked to severe disease and/or outbreaks and their corresponding intermediate hosts.

According to Eduardo (1987), the warm climate and the vast number of ruminants as possible hosts of parasites makes Africa home to many different amphistomes. Wild ruminants harbor undescribed parasites because of their different feeding habits. As they move during grazing, they pick parasites from various places. According to Gagnon and Chew (2000) there are 78 species of ruminants in Africa belonging to the Bovidae family that are in existence. These range from the small Dik dik weighing 2kg to the large *Syncerus caffer* (African Buffalo) of about 800kg in weight. Despite this background information, studies are only confined to domestic animals which are probably of importance to humans. There have been records of high prevalence of amphistomes in wild ruminants in and out of Africa. A study done by Mahanti *et al*. (2020) in a game reserve in Kerala showed amphistomiasis to be the cause of deaths of several female Sambar deers in the reserve. The main source of this infection was determined to be local cattle slaughtered in abattoirs upstream resulting in contamination of the water canals and consequently the snails transmitting the amphistomes to the Sambar deers. *Calicophoron microbothrium* has been recorded in Roe Deer with a prevalence as high as 53% in Serbia

(Pavlovic et al., 2012) and studies done by (Phiri et al., 2011; Munyeme et al., 2010) reported a prevalence of 100% on Kobus Leche of Zambia. This shows that there is a high prevalence of amphistomes in wild ruminants which go unchecked and may result in outbreaks in the future. Gretillat (1960), Phiri et al. (2011) and Pfukenyi and Mukaratirwa (2018) all agree that wild ruminants may be potential resevoirs of helminth parasites including amphistomes. Despite this, there remains a research gap on amphistomes in the wild of which since the year 2000, very few studies have been done on amphistomes in the wild. Although some adult amphistomes have been collected in wildlife studies, very few scholars characterize them to species level.

Furthermore, snails play a key role in the distribution of amphistomes as they are the only intermediate hosts (Dinnik, 1965). According to Eduardo (1987), the establishment of amphistomes in new environments depends on the presence of suitable intermediate hosts making snails very important in zoogeographic distribution of amphistomes. There are a total of 28 species of amphistomes in African wild ruminants of which only the life cycles of 12 species have been completed. This means there are still so many gaps on amphistome transmission and cycles making it difficult to determine if there is loss of diversity in amphistome species or just a lack of study. According to Lu et al. (2018) there is correlation to a certain extent between snail distribution and parasite diseases and hence mapping the distribution of snails may help clarify interactions with parasitic diseases aiding in the detection and prediction of prevalence of these diseases.

The use of molecular tools in characterizing amphistomes will helpin detection of specific amphistomes in the wild, hence contributing towards understanding the epidemiology of these parasites. Again, from observations being made in phylogenetic analyses, there is a possibility of hybridisation of amphistomes.

1.3 Aims and Objectives

The main aim of this study was to apply PCR-based techniques (ITS 2 and Cox 1 gene sequencing) and morphological techniques (histology) to identify and genetically characterize amphistomes of wild ruminants and their intermediate host snails from Matebeleland region in Zimbabwe.

The specific objectives of this study are to;

1. To do a systematic review of amphistomes found in wild ruminants and intermediate host snails across Africa.

2. To identify and characterise amphistome specimens collected from hunter-killed wild ruminants morphologyically based on histological sectioning and molecularly based on the ITS-2 gene.
3. To determine the diversity of freshwater snails that may harbour amphistomes, and to detect for the presence of amphistome DNA using PCR.
4.

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Chapter 2

Prevalence and geographical distribution of amphistomes of African wild ruminants: A scoping review

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2.1 Abstract

This review summarizes published records on the prevalence, species diversity, geographical distribution, mixed infections, co-infections with other trematodes and intermediate hosts (IHs) of amphistomes (rumen flukes) of wild ruminants in Africa. Literature search was conducted on Google Scholar, PubMed and JSTOR, using a combination of predetermined search terms and Boolean operators. Of the 54 African countries searched, results showed that occurrence of amphistome infections in wild ruminants have only been reported in 23 countries. A total of 38 amphistome species consisting of the following 11 genera were recorded, viz *Bilatorchis, Calicophoron, Carmyerius, Choerecotyloides, Cotylophoron, Explanatum, Gastrothylax, Gigantocotyle, Leiperocotyle, Paramphistomum* and *Stephanopharynx.* These were recorded in 39 wild ruminant species, belonging to the Bovidae family. The genus *Carmyerius* recorded the highest number of species (n = 13) across nine countries Africa. However, *Calicophoron* species (n = 9) were more widely distributed, occurring in 17 countries across all regions of Africa. Species of this genus collectively infected 27 wild ruminant species. However, at a species level, *Cotylophoron cotylophorum* infected the highest number of wild ruminant species. Prevalence of infection based on post-mortem examination ranged from 1.89% in African Buffalo to 100 % in Defassa waterbuck from Egypt and Zambia, respectively. The most common mixed infections recorded were those between amphistomes of the same or different genus. Snail intermediate hosts (IHs) were described for 10/38 amphistome species, and these were predominantly species from Plarnobidae family. Despite the richness in diversity of amphistomes infecting wild ruminants in Africa, there is need to further confirm identity of snail IHs and the amphistome species using both morphological and molecular techniques. Furthermore, more studies are recommended to assess the burden of amphistomosis in commercially reared wildlife/game farming, mixed game and livestock farming systems in Africa.

Keywords: Amphistomes, wild ruminants, snail intermediate hosts, infection, co-infection, distribution, prevalence

2.2 Introduction

Intestinal amphistomosis, variously known as amphistomiasis (Pfukenyi and Mukaratirwa, 2018), paramphistomiasis (Lotfy et al., 2010; Horak, 1971) or paramphistomosis (Huson et al., 2017) is a neglected disease of domestic and wild ruminants (Pfukenyi and Mukaratirwa 2018). The disease is caused by conical flukes, commonly known as amphistomes (Sey, 1988), stomach or rumen flukes (Mitchell et al., 2021) due to their predilection site, and they are characterized by lack of the oral sucker and the positioning of the ventral sucker or the acetabulum at the posterior end of their body (Tandon et al., 2019). They belong to the superfamily Paramphistomoidea Fischoeder, 1901 (Lotfy et al., 2010), which is composed of hundreds of species belonging to 12 families (Jones 2005).

Amphistomes have a wide geographical distribution, with more than 70 species documented in diverse range of domestic and wild ruminant hosts (Ghatani et al., 2012; Ichikawa et al., 2013; Malik et al., 2017; Pfukenyi and Mukaratirwa, 2018; Mitchell et al., 2021). Studies have shown that the prevalence of infection in wild ruminants can be as high as 100 % as recorded by Zieger et al. (1998) in Kobus defassa (Defassa waterbuck) in Zambia. Despite this, the disease remains understudied in wild ruminants which may act as reservoirs for domestic ruminants (Niranjan et al., 2020), and consequently its burden is hugely underestimated particularly in the tropics and subtropics (Phiri et al., 2011; Huson et al., 2017) including Africa where focus on amphistomes infections have been on domestic ruminants (Pfukenyi et al., 2005; Mavenyengwa et al., 2008; Mavenyengwa et al., 2010; Lotfy et al., 2010; Dube et al., 2015; Laidemitt et al., 2017).

Although adult amphistomes have been collected from wild ruminants in recent years, few specimens have been characterized and identified to species level (Munyeme et al., 2010; Munang'andu et al., 2012) and were commonly designated as Paramphistomum spp. despite the vast diversity in amphistome genera in Africa (Pfukenyi and Mukaratirwa, 2018). As a result, losses due to amphistomosis in wildlife in Africa have not been quantified, despite the growing concern of cross-infection of amphistomes with domestic animals (Madzingira et al., 2002; Van Wyk and Boomker, 2011). As the domestic-wildlife interface increases due to scarcity of land for human settlement resulting in diminishing wildlife habitat, it becomes imperative to understand and determine distribution of parasitic fauna, especially amphistomes in wild ruminants (Brown et al., 2022).

Identification of amphistomes is a cumbersome process that involves sectioning through thick robust bodies (Lotfy *et al*., 2010), a process described by earlier taxonomists like (Nasmark, 1937) and (Eduardo, 1982c). Classification and reclassification of amphistomes over the period 1900 to 2000 was done by several taxonomists. Taxonomic groupings of amphistomes are based on different organs

with the different taxonomists focusing on different internal organs and sometimes the teguments, with most taxonomists settling for using the pharynx, acetabulum and the genital atrium. For the sake of this review work, classifications of amphistomes that are considered valid are those done according to (Eduardo, 1980a, Eduardo, 1982a, Eduardo, 1983, Eduardo, 1984, Eduardo, 1982b, Eduardo, 1986, Nasmark, 1937) descriptions and revisions. For the pouched amphistomes we considered classifications according to taxonomists such as (Graber *et al*., 1964, Grétillat, 1964) and revisions by (Sey, 1983) and (Eduardo, 2004). For this review work, we will consider the species that are only present in Africa and are included in the literature we collected.

Amphistomes have two hosts life cycle, with vertebrates serving as the definitive hosts and freshwater snails as intermediate hosts (Tandon et al., 2019). The geographical distribution and prevalence of amphistome infections is influenced by the availability and abundance of susceptible snail intermediate hosts (Eduardo, 1987) and susceptible definitive hosts (Pfukenyi and Mukaratirwa, 2018). Various freshwater gastropods belonging to the genera Bulinus (Müller 1781), Biomphalaria (Preston 1910), Ceratophallus (Brown and Mandahl-Barth 1973) and Galba (Müller 1774) have been implicated as the intermediate hosts of amphistome species in Africa (Dinnik, 1961; Dinnik, 1965; Wright et al., 1979b; Southgate et al., 1989; Pfukenyi et al., 2005). Although a review paper was done by Pfukenyi and Mukaratirwa (2018) on amphistomes of both domestic and wild ruminants, the geographical area was confined to east and southern Africa. There is a need to show distribution of amphistomes in Africa as whole to link the presence of snail IH to establishment of certain amphistome species. However, there are still gaps on the specificity of the incriminated snail IHs in the transmission of the documented amphistome species. Therefore, this review gathered data from peer-reviewed publications on the prevalence, species diversity, geographical distribution, mixed infections, co-infections with other trematodes and intermediate hosts (IHs) of amphistome infections in wild ruminants in Africa and identified research gaps for future studies.

2.3 Methodology

2.3.1 Scoping review

The scoping review aimed to answer the following questions on amphistomes of wild ruminants in Africa: (i) Which amphistome genera/species are currently circulating in wild ruminants in Africa? (ii) What is the geographical distribution of amphistome species of wild ruminants in Africa? (iii) What is the prevalence of infection recorded in Africa? (iv) What are the intermediate snail hosts implicated in the transmission of amphistomes in Africa? (v) Which other trematodes species co-infect with

amphistomes in wild ruminants and their snail IHs in Africa? (vi) What are the predilection sites of the amphistomes in wild ruminants?

The review was guided by the methodological approach described by Arkey and O'Malley (2005) which consisted of the following guidelines: (i) Identification of the research questions, (ii) Searching and identification of relevant articles, (iii) Selection of articles, (iv) Charting of data, collating, summarizing and reporting of the results. To address the questions raised, published peer-reviewed articles and reports reporting on amphistomes of wild ruminants and their snail IHs from Africa were identified and reviewed following guidelines for reporting from PRISMA (Moher et al., 2009).

2.3.2 Search strategy.

The following electronic databases were searched: PubMed, JSTOR, Google scholar. Reference lists of relevant publications were searched as potential leads. Literature searches were carried out from a total of 54 African countries (Figure 2.1). A thorough search was conducted using Boolean operators (AND, OR) and the following terms: Amphistome infection OR Amphistomosis AND wild ruminants OR intermediate hosts OR freshwater snails, Paramphistome infections OR Paramphistomosis AND wild ruminants OR intermediate hosts OR freshwater snails. Relevant articles were selected based on a preliminary screening of the titles and abstracts. All full text articles retrieved were managed in the EndNote reference manager.

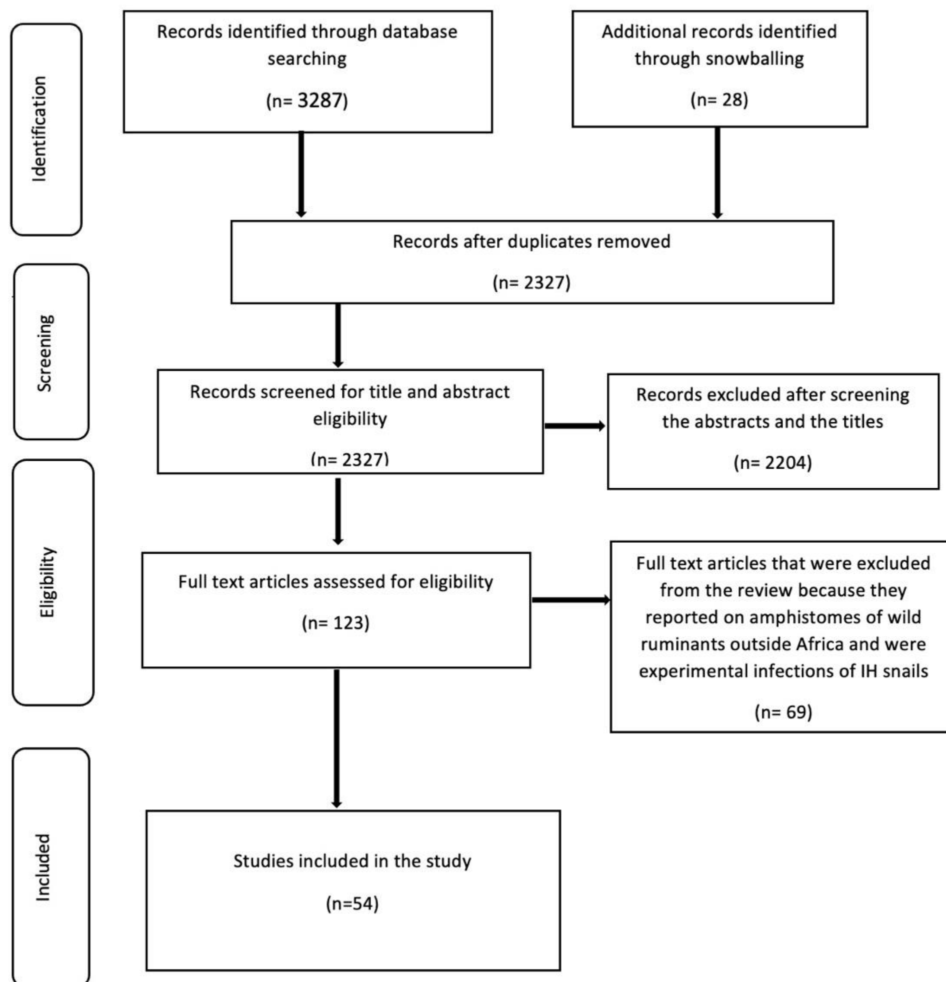

Figure 2.1 PRISMA diagram showing the search and selection process.

2.3.3 Selection criteria

The literature search was not limited to articles in English but included articles in French and Afrikaans published from 1900 to 2022. Articles published in Afrikaans and French were translated using online Google translator. Included in this review were field or case studies explicitly reporting; (i) occurrence of amphistomes in wild ruminants in Africa; (ii) geographical distribution of amphistomes in wild ruminants in Africa; (iii) prevalence of amphistomes in wild ruminants in Africa; (iv) snail IH linked to the transmission of amphistomes in wild ruminants in Africa; (v) co-infection of amphistomes and other trematodes in wild ruminants and snail IHs; (vi) studies reporting post-mortem and coprological examinations outcomes on amphistomes infections in wildlife in Africa. Furthermore, information on predilection site of amphistomes in wild ruminants were also extracted from the selected studies.

Excluded from this review were articles that reported on; (i) amphistome infections in non-ruminant wild animals; (ii) amphistome infections in domestic livestock; (iii) studies conducted outside Africa and, (iv) did not identify the amphistomes (egg/larval/adult stage) to genus level.

2.3.4 Charting, collating, and summarizing the data.

Data was extracted from articles that met the inclusion criteria after appraisal. Information of the authors, region and country, year of publication, intermediate and definitive host species, prevalence, and predilection site of amphistomes were extracted and tabulated.

2.4 Results

2.4.1 PRISMA

A total of 3287 records were obtained from the database search on Google scholar, JSTOR and PubMed, and this included peer reviewed articles, abstracts, reviews, books and duplicated articles (Figure 2.1). Additional 32 articles were obtained through snowballing. Nine-hundred-and-ninety-three duplicated articles were excluded and the titles and abstracts of the remaining 2327 articles were screened of which 2204 articles were deemed ineligible and excluded. One-hundred-and-twenty-three full-text articles were appraised based on the predetermined inclusion criteria, and 69 articles did not meet the criteria and were excluded from the review. Fifty-four articles that met the inclusion criteria and were reviewed, and were distributed across all African regions; Southern Africa (n= 25), East Africa (n=18), Central Africa (n = 11), North Africa (n = 8) and West Africa (n=6) (Figure 2.2).

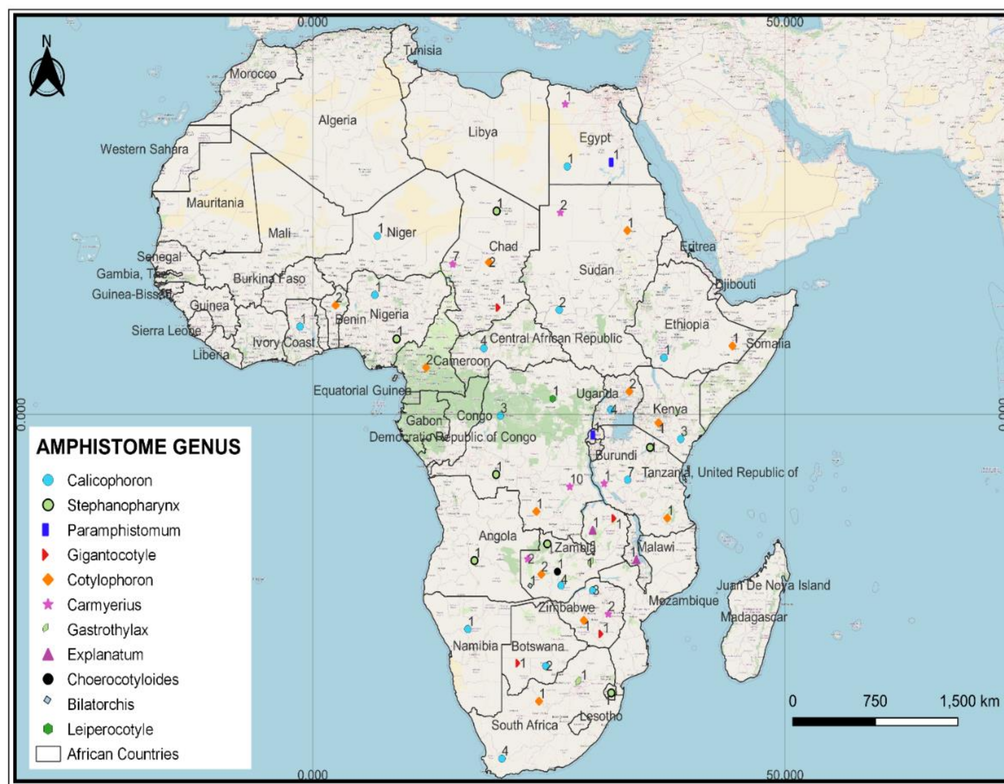

Figure 2.2 Map showing geographical distribution of amphistomes in wild ruminants in Africa (1900-2022)

Of the 54 articles reviewed, 38 articles reported exclusively on amphistome infections in wild ruminants, 15 reported on infections in the snail IHs, and only one article reported on both the IHs and wild ruminant hosts. Thirty-five of the thirty-nine studies identified amphistomes up to species level, whilst 4/39 identify amphistomes up to genus level in the definitive hosts. Four articles reported on co-infection/mixed infection of amphistomes with other trematodes, or between amphistomes of similar or different genera in wild ruminants. Fifteen articles reported on snail infections and identified amphistomes to species level with five records of mixed infections of amphistomes and other trematodes in snail intermediate hosts in Africa.

2.4.2 Checklist of amphistomes species and their wild ruminant host species in Africa (1900-2022)

A total of 38 amphistome records were documented in natural infections in wild ruminants in Africa (Table 2.1, Apendix 2.1) and of these records, 36 records identified amphistomes up to species level. The recorded 39 wild ruminant species from the Bovidae family infected with amphistome species were *viz* impala (*Aepyceros melampus)*, red hartebeest *(Alcephalus busephalus),* bongo *(Alcelaphus boocerus eurycerus),* Lichteinstein's hartebeest *(Alcelaphus lichteinsteini),* lelwel hartebeest *(Alcelaphus lelwel),* hartebeest (*Alcelaphus* spp*.)*, springbok (*Antidorcas marsupialis),* blue wildesteest (*Connochaete taurinus),* black wildebeest *(Connochaete gnou),* African duiker (*Cephalophus* sp.), black-fronted duiker (*Cephalophus nigrifrons),* topi *(Damaliscus korrigum),* blesbuck *(Damaliscus albifrons),* blesbok *(Damaliscus dorcas),* common tsessebe (*Damaliscus lunatus),* Thomson's gazelle (*Gazella thomsoni)*, roan antelope (*Hippotragus equinus),* sable (*Hippotragus niger),* defassa waterbuck *(Kobus defassa),* waterbuck *(Kobus ellipsiprymnus),* kob *(Kobus kob),* red lechwe *(Kobus leche),* nile lechwe *(Kobus megaceros),* puku *(Kobus vardoni),* royal antelope *(Neotragus pygmaeus),* klipspringer *(Oreotragus oreotragus),* oribi *(Ourebia oribi),* gemsbok *(Oryx gazelle),* East African oryx *(Oryx beisa),* common reedbuck *(Redunca arundinum),* bohor reedbuck *(Redunca Redunca),* mountain reedbuck *(Redunca fulvovufula),* African buffalo *(Syncerus caffer),* common duiker *(Sylvicapra grimmia),* common eland *(Taurotragus oryx),* nyala *(Tragelaphus angasi),* sitatunga *(Tragelaphus spekei),* bushbuck *(Tragelaphus scriptus),* greater kudu *(Tragelaphus strepsiceros),* Menelek's bushbuck *(Tragelaphus scriptus meneliki),* mountain nyala *(Tragelaphus buxtoni*).

Carmyerius (*Ca*.) was the genus with the most diverse species, with 13 species documented in in 20 wild ruminants, namely, *Car. bubalis, Car. chabaudi, Car. endopapillatus, Car. exosporus, Car. graberi, Car. gregarius, Car. mancupatus/dollfusi, Car. multivitellarius, Car. papillatus, Car. parvipapillatus, Ca. spatiosus, Car. schoutedeni* and *Car. wenyoni* (Table 2.1, Supplementary table 2.1). The *Calicophoron* (*C.*) genus was the second most diverse genus, with ten species (*Calicophoron* spp., *C. bothriophoron, C. calicophorum, C. clavula, C. daubneyi, C. microbothrium, C. phillerouxi, C. sukari, C. sukumum, C.*

raja). This genus, although second in diversity, infected the highest number (n = 27) of wild ruminant host species when compared to other genera. *Cotylophoron* (*Cot.*) *species* (*Cot. cotylophorum, Cot. fuelleborni, Cot. macrosphinctris, Cot. jacksoni*), and *Paramphistomum* (*P.*) species (*Paramphistomum* spp., *P. cephalophi*, *P. cervi, P. gotoi)* were reported in wild ruminants. However, *Cotylophoron* species utilized more host species (n = 23), followed by *Calicophoron* spp., and *Paramphistomum* spp. were only reported in six wild ruminants. The following were reported as the only species within their genera; *Bilatoschis (Bi.) papillogenitalis*, *Explanatum (E.) explanatum*, *Gigantocotyle (Gi.) symmeri, Gastrothylax (G.) crumenifer, Leiperocotyle (L.) gretillati, Stephanopharynx (S.) compactus* and *Choerocotyloides (Ch.) onotragi*. Of these species, *S. compactus* infected more wild ruminant species (n = 2) comparatively.

Further results showed that the African buffalo (*Syncerus caffer*) was more susceptible to amphistomes infection and recorded the highest number of amphistome species (n = 21). This included seven *Carmyerius* spp. (*Car. mancupatus, Car. gregarius, Car. exoporus, Car. endopapillatus, Car. spatiosus, Car. schoutedeni, Car. graberi*), six *Calicophoron* spp. (*C. calicophorum, C. raja, C. clavula, C. sukari, C. phillerouxi, C. microbothrium*), three *Cotylophoron* spp. (*Cot. macrosphrinctris, Cot. cotylophorum, Cot. fuelleborni*), *G. symmeri*, *S. compactus*, *L. gretillati, P. gotoi and P. cervi. Oryx beisa* infected with *C. daubneyi*, *Tragelaphus scriptus meneliki* with *Cot. cotylophorum*, *Tragelaphus buxtoni* with *Cot. cotylophorum*, *Antidorcas marsupialis* with *C. calicophorum* had the least (n = 1) number of amphistome species infection.

Table 2.1: Checklist of amphistome species of wild ruminants in Africa (1900-2022).

Amphistome spp.	Hosts	Countries	References
Bi. papillogenitalis	Red lechwe	Zambia	Eduardo, 1980
Calicophoron sp.	Tsessebe, red lechwe, defassa waterbuck	Zambia	Zieger *et al*., 1998
C. bothriophoron	Defassa waterbuck	Tanzania, Kenya	Eduardo, 1983
C. calicophorum	African buffalo, blesbuck, black wildebeest, blue wildebeest, impala, lelwel's hartebeest, red hartebeest, springbok	South Africa, Uganda, DRC, Chad and Central African Republic	Mettam, 1932; Prudhoe, 1957; Ortlepp, 1961; Graber et al., 1964
C. clavula	African buffalo, bohor reedbuck, common eland, defassa waterbuck, impala, oribi, puku, roan antelope, red hartebeest, sable	Tanzania, Uganda, DRC, CAR, Republic of Niger, Nigeria, Sudan	Stunkard, 1929; Nasmark, 1937; Prudhoe, 1957; Sey and Graber, 1979a; Eduardo, 1983; Dinnik et al., 1963
C. daubneyi	East African oryx	Ethiopia	Graber et al., 1980
C. microbothrium	African buffalo, blesbok, bohor reedbuck, common eland, defassa waterbuck, impala, kob, lelwel hartebeest, red hartebeest, red lechwe, roan antelope, senegal hartebeest, waterbuck	South Africa, Zambia, Zimbabwe, Tanzania, Kenya, Uganda, Chad, CAR, Ghana, Sudan, Egypt, Botswana	Maplestone, 1923; Baer, 1923; Mettam, 1932; Ortlepp, 1961; Eduardo, 1983; Graber et al., 1964; Myers et al., 1960; Sey, 1977; Halium et al., 2014
C. phillerouxi	African buffalo, bohor reedbuck, deffasa waterbuck, greater kudu, impala, kob, topi, common eland, hartebeest, Thomson's gazelle	Zambia, Uganda, Zimbabwe, Tanzania, DRC, CAR	Eduardo, 1983; Sey and Graber, 1979a; Sach and Sach, 1968; Dinnik et al., 1963
C. raja	African Buffalo, black wildebeest, blue wildebeest, bohor reedbuck, bushbuck, deffasa waterbrck, puku, gemsbok, greater kudu, impala, red lechwe, red hartebeest, Thomson's gazelle	South Africa, Botswana, Zambia, Zimbabwe, Namibia, Tanzania, Kenya	Eduardo, 1983; Ikeuchi et al., 2022, Mijele et al., 2016; Sach and Sach, 1968
C. sukumum	Blue wildebeest, deffasa waterbuck, topi, African buffalo, kob, Thomson's gazelle, impala, puku, hartebeest, common eland	Tanzania	Eduardo, 1983; Sach and Sach, 1968

C. sukari	African buffalo, kob, blue wildebeest, deffasa waterbuck, topi, Thomson's gazelle, impala, puku, hartebeest, common eland	Tanzania	Eduardo, 1983; Sach and Sach, 1968
Car. bubalis	Bongo	Zimbabwe	Sey, 1983
Car. chabaudi	Thomson's gazelle	DRC	Sey, 1983
Car. gregarius	African buffalo, nile lechwe	Sudan, Egypt**,** DRC	Myers et al., 1960; Dollfus, 1963; Sey, 1977; Sey, 1983; Halium et al., 2014
Car. mancupatus/ dollfusi	Red lechwe, roan antelope, common eland, African buffalo, kob, blue wildebeest, deffasa waterbuck, topi, Thomson's gazelle, impala, puku, hartebeest, common eland, bohor reedbuck	Zambia, DRC, Tanzania	Prudhoe, 1957; Wright et al., 1979b; Sach and Sach, 1968
Car. multivitellarius	Thomson gazelle	DRC	Sey, 1983
Ca. schoutedeni	African Buffalo, black-fronted duiker	DRC	Sey, 1983
Ca. wenyoni	Nile lechwe	Sudan	Sey, 1983
Cot. cotylophorum	Impala, common tsessebe, mountain reedbuck, eland, sitatunga, greater kudu, menelik bushbuck, mountain nyala, klipspringer, royal antelope, puku, defassa waterbuck, African buffalo, common eland, bushbuck, lelwel hartebeest, kob, red hartebeest, waterbuck, hartebeest	South Africa, Zambia, Zimbabwe, Ethiopia, DRC, Chad, CAR, Cameroon, Benin, Sudan, Tanzania, Uganda	Maplestone, 1923; Stunkard, 1929; Le Roux, 1932; Le Roux, 1934; Ortlepp, 1961, Nasmark, 1937; Morel, 1959; Prudhoe, 1957; Mettrick, 1962; Graber et al., 1964; Graber et al., 1980; Anderson, 1983; Sach and Sach, 1968; Dinnik et al., 1963
Cot. jacksoni	Red hartebeest, sable	Zambia, Uganda, Tanzania, Kenya	Nasmark, 1937; Eduardo, 1985,
Cot. macrosphinctris	African buffalo	Uganda, CAR	Sey and Graber, 1979b; Eduardo, 1985
G. crumenifer	Red lechwe, sitatunga	South Africa, Zambia	Le Roux, 1932; Ortlepp, 1961; Sey, 1983
Gi. symmeri	African buffalo, red lechwe, greater kudu	Botswana, Zambia, Zimbabwe, CAR	Sey and Graber, 1979a; Eduardo, 1984; Yeh, 1957

L. gretillati	African buffalo	DRC	Eduardo, 1985b
Paramphistomum spp.	Kudu, common reedbuck, tsessebe, grey duiker	South Africa	Boomker et al., 1989; Reinecke et al., 1988; Boomker et al., 1987
P. cephalophi	Black-fronted duiker	Rwanda	Eduardo, 1982
P. gotoi	African buffalo	Egypt	Sey, 1977
S. compactus	African buffalo, blue wildebeest, bohor reedbuck, common eland, defassa waterbuck, impala, Kob, mountain reedbuck, puku, red lechwe, roan antelope, topi	DRC, Chad, CAR, Zambia, Angola, Swaziland, Nigeria, Tanzania	Ortlepp, 1961; Wright et al., 1979b; Eduardo, 1986; Sach and Sach, 1968

2.4.3 Geographic distribution of amphistomes of wild ruminants in Africa (1900-2022)

Results showed that amphistomes have been recorded in 39 wild ruminant species are distributed across 23 countries in Africa (Angola, Benin, Botswana, Cameroon, Central African Republic, Chad, DRC, Egypt, Ethiopia, Ghana, Kenya, Malawi, Namibia, Nigeria, Republic of Niger, Rwanda, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zambia, Zimbabwe) as shown (Table 2.1, Figure 2.2). Calicophoron species were the most distributed, occurring in 17 of 23 reviewed countries, but most commonly reported in the southern and eastern African countries. *Calicophoron microbothrium* was overall the most distributed Amphistome species, recorded in 56.5 % (13/23) of the reviewed countries (Table 2.1). Although majority of *Calicophoron* species reported in multiple countries, 40 % (4/10) of species from this genus were however reported only in one country, i.e., *Calicophoron* spp. in Zambia, *C. daubneyi* in Ethiopia, *C. sukumum* and *C. sukari* in Tanzania. *Carmyerius* species were reported in nine countries across all regions except West Africa. Results showed that seven species were reported in more than two countries, and six species (*Car. bubalis*, *Car. chabaudi*, *Car. graberi*, *Car. multivitellarius*, *Car. schoutedeni* and *Car. wenyoni*) documented in one country each. *Cotylophoron* species have recorded in 13 countries across all regions but shown to occur mostly in the East African countries. *Cotylophoron cotylophorum* is the second most distributed amphistome species following *C. microbothrium*, reported in 12 of 23 reviewed countries. Results showed only Egypt recorded more than one *Paramphistomum* species (*P. cervi* and *P. gotoi*), whereas other *Paramphistomum* spp. and *P. cephalophi* were reported in South Africa and Rwanda respectively. Reviewed results further showed that *Bi. papillogenitalis* and *Ch. onotragi* were documented in Zambia, and *L. gretillati* was recorded in DRC. *Gastrothylax crumenifer* was reported in South Africa and Zambia, and *Gi. Symme*ri was documented in Botswana, Zambia, Zimbabwe and CAR. *Explanatum explanatum* was reported in Malawi and Zambia, whilst the presence of *S*. *compactus* infections in wild ruminants were documented in DRC, Chad and CAR, Zambia, Angola, Swaziland, Nigeria and Tanzania.

2.4.4 Predilection sites for amphistomes of wild ruminants in Africa

The results from reviewed studies showed that the rumen, reticulum, abomasum and omasum are the most common predilection sites for amphistomes. Amphistome species from the *Cotylophoron* genus (*Cot. macrosphinctris*, *Cot. jacksoni*, *Cot. cotylophorum*, *Cot. fuelloborni*), *C. clavula, Bi. papillogenitalis* and *S. compactus* were reported to utilize the rumen as their predilection site (Eduardo, 1983; Eduardo, 1984; Eduardo, 1986). Authors also showed that other species, predominantly those from *Calicophoron* genus (*C. microbothrium*, *C. phillerouxi*, *C. clavula*, *C. daubneyi*, *C. sukumum*, *C. sukari*, *C. calicophorum*, *C. bothriophoron*) were found in the rumen and

reticulum (Eduardo, 1983; Eduardo, 1985, Eduardo, 1986, Sey and Graber, 1979). Results also showed that *Car. spatiosus, Car. mancupatus, C. raja* and *Gi. symmeri* were found in the rumen, reticulum, omasum and/or the abomasum. *Paramphistomum cephalophi* was found in the small intestines (Eduardo, 1982), *L. gretillatti* the intestine (Eduardo, 1985), and *Ch. onatragi* utilizes the caecum (Prudhoe et al., 1964) as their predilection.

2.4.5 Prevalence of amphistome species in wild ruminants in Africa (1900-2022)

Prevalence of amphistomes infections in wild ruminants ranged from 7.1 % - 100 % (Table 2.2). The lowest prevalence was recorded in in *Gazella rufifrons* (7.1 %) in Chad which was infected with *C. microbothrium* (Grabner et al., 1964), followed by grey duiker (8 %) infected with *Paramphistomum* spp. in South Africa (Boomker et al., 1987). The highest prevalence was in Zambian *Kobus defassa* (100%) infected with *Calicophoron* spp. (Zieger et al., 1998), followed by *Cot. cotylophorum* infections in South African *Aeropyceros melampus* (89.1 %) (Anderson, 1983), and *Paramphistomum* spp. also in South African *Damaliscus lunatus* (81 %) (Reinecke et al., 1988).

2.4.6 Mixed Infection of amphistomes species and co-infection with other trematodes in the animal host species (1900-2022)

Co-infection of amphistomes with other trematode species in the same animal host were reported in *Kobus defassa* with *C. calicophorum* + *Fasciola gigantica*, and Kobus lechwe with *Calicophoron* spp. + *F. gigantica* + *Schistosoma* sp. in Zambia (Zieger et al., 1998). In Zimbabwe, *Tragelaphus spekei* was found co-infected with *Car. spatiosus* + *F. tragelaphi* (Pike and Cody, 1966) (Table 2.3). Mixed infections with multiple amphistome species in the same host were common in Chad and Central African Republic (CAR). The lowest prevalence (17/315, 1.89 %) was recorded in *Syncerus caffer* which was infected with *C. microbothrium* + *Car. gregarius* + *P. cervi* in Egypt (Halium et al., 2014) and the highest prevalence of 60 % (3/5) was recorded in *Syncerus caffer* mixed infected with *C. microbothrium* + *Car. endopapillatus* + *Car. spatiosus* + *Cot. cotylophorum* in Chad and RCA (Graber et al., 1964).

Table 2.2 Prevalence of single infections of amphistomes as determined through post-mortem in wild ruminant species in Africa (1900-2022)

Region/Country	Host species	No. examined	No. positive	Prevalence (%)	Amphistome species	Method of identification	References
Southern Africa							
South Africa	*Redunca arundinum* (common reedbuck)	47	19	40.4	*Paramphistomum* spp.	Fluke morphology	Boomker et al., 1989
South Africa	*Damaliscus lunatus lunatus* (Tsessebe)	11	9	81	*Paramphistomum* spp.	Histology	Reinecke et al., 1988
South Africa	*Sylvicapra grimmia* (grey duiker)	13	1	8	*Paramphistomum* spp.	Fluke morphology	Boomker et al., 1987
South Africa	*Aeropyceros melampus* (impala)	46	41	89.1	*Cot. cotylophorum*	N/A	Anderson, 1983
Zambia	*Damaliscus lunatus lunatus* (tsessebe)	3	1	33.3	*Calicophoron* spp.	N/A	Zieger et al., 1998
Zambia	*Kobus leche* (red lechwe)	2	1	50	*Calicophoron* spp.	N/A	Zieger et al., 1998
Zambia	*Kobus defassa* (defassa waterbuck)	6	6	100	*Calicophoron* spp.	N/A	Zieger et al., 1998
East Africa							
Kenya	*Connochaetes taurinus* (Blue Wildebeest)	130	26	20	*C. raja*	Fluke morphology	Mijele et al., 2016
North Africa							
Egypt	*Syncerus caffer* (African buffalo)	-	-	78	*C. microbothrium*	Histology	Sey, 1977
Chad	*Gazella rufifrons* (Thomson gazelle)	14	1	7.1	*C. microbothrium*	Histology	Graber et al., 1964

Table 2.3 Mixed amphistome species infections of species with other trematodes in wild ruminants (1900-2022).

References	Country	Host	Amphistome species	Mixed/ co-infections		Prevalence of infection			Method of identification
				Other amphistomes species	Other trematodes	No. examined	No. infected	Prev. (%)	
Halium et al., 2014	Egypt	*Syncerus caffer*	*C. microbothrium*	*Car. gregarius, P. cervi*	-	315	17	1.89	Fluke morphology
Zieger *et al*., 1998	Zambia	*Kobus defassa*	*C. calicophorum*	-	*F. gigantica*	ND	ND	ND	Fluke morphology
Zieger *et al*., 1998	Zambia	*Kobus lechwe*	*Calicophoron sp.*	-	*F. gigantica, Schistosoma sp.*	ND	ND	ND	Fluke morphology
Wright *et al*., 1979	Zambia	*Kobus lechwe*	*Car. spatiosus*	*Car. mancupatus, S. compactus*	-	ND	ND	ND	Fluke morphology
Pike and Condy, 1966	Zimbabwe	*Tragelaphus spekei*	*Car. spatiosus*	-	*F. tragelaphi*	ND	ND	ND	Fluke morphology
Graber *et al*., 1964	Chad and CAR	*Kobus defassa*	*Car. spatiosus*	*C. microbothrium, Car. parvipapillatus, Car. papillatus, S. compactus*	-	8	3	25	Fluke morphology
Graber *et al.*, 1964	Chad and CAR	*Alcelaphus lelwel*	*C. calicophorum*	*Cot. cotylophorum, C. microbothrium, Car. Spatiosus*	-	11	3	18	Fluke morphology
Graber *et al.*, 1964	Chad and CAR	*Syncerus caffer*	*C. microbothrium*	*Cot. cotylophorum, Car. endopapillatus, Car. spatiosus*	-	5	3	60	Fluke morphology
Graber *et al.*, 1964	Chad and CAR	*Damaliscus korrigum*	*C. microbothrium*	*Car. spatiosus, Car. exoporus, Car. parvipapillatus*	-	8	1	14	Fluke morphology

Graber *et al*., 1964	Chad and CAR	*Kobus kob*	*C. microbothrium*	*S. compactus, Car. spatiosus, Car. papillatus, Car. parvipapillatus*	-	6	3	50	Fluke morphology
Graber *et al*., 1964	Chad and CAR	*Redunca Redunca*	*C. microbothrium*	*S. compactus, Car. spatiosus*	-	2	1	50	Fluke morphology
Graber *et al*., 1964	Chad and CAR	*Hippotragus equinus*	*C. microbothrium*	*S. compactus, Car. spatiosus*	-	9	2	22	Fluke morphology

ND = Not Determined

2.4.7 Checklist of snail intermediate hosts implicated in the transmission of amphistomes species in Africa (1900-2022)

Results showed eleven snail species documented as intermediate hosts of known amphistomes species across eight of 23 reviewed African countries. The species include ten Planorbidae Rafinesque 1815, *viz*, *Bulinus (B.)* (*Physopsis) globosus*, *B*. (*Physopsis) nasutus*, *B. tropicus*, *B. forskalii*, *B. senegalensis*, *B. mairei*, *B. liratus*, *Biomphalaria (Bio.) pfeifferi*, *Anisus (A.) natalensis and Ceratophallus (Ce.) natalensis* and one Lymnaeidae Rafinesque 1815 species, *Galba* (*G.)* (*Lymnaea*) *truncatula* (Table 2.4).

Bulinus tropicus was implicated in the transmission of *C. calicophorum* in South Africa (Porter, 1921; Grobbellar, 1992). *Calicophoron microbothrium* was shown to use a variety of snail species as intermediate hosts, ranging from *B. tropicus* in South Africa (Swart and Reneike, 1921), Zambia (Dannik, 1965) and Kenya (Dinnik, 1962), *B. nasatus* in Kenya (Dinnik, 1965) and *B. globosus* in Zambia (Dannik, 1965). *Calicophoron sukari* has been reported to be transmitted by *Bio. pfeifferii* in Kenya (Dannik, 1965) and Ethiopia (Graber and Daynes, 1974), whereas *C. phillerouxi* infections were documented in *B. forskalii* in Zambia and Tanzania, and *B. senegalensis* in Gambia (Dannik, 1961). *Calicophoron daubneyi* reported in Kenya was the only amphistomes infection recorded in a Lymnaeidae species; *G.* (*L.) truncatula* (Dannik, 1962). *Carmyerius exosporus* was reported solely in *A. natalensis* in Zambia and Kenya (Dannik, 1964, 1965), whereas *Car. mancupatus* was reported in several hosts such as *A. natalensis* (Dannik, 1965), *B. mairei* (Gretillat, 1960), *B. liratus* (Gretillat, 1960) in Zambia and *C. natalensis* (Laidemitt et al., 2017). *Bulinus (Phy.) globosus* was reported to transmit *Ca. parvipapillatus* whilst *B. forskalii* transmitted *S. compactus* in Zambia (Dinnik, 1965).

Occurrence of co-infections between two trematode species in intermediate hosts included co-infections of *C. microbothrium* and *Schistosome bovis* in *B. tropicus* from Kenya (Southgate et al., 1985; Southgate et al., 1989), and *Carmyerius* spp. and *Schistosoma margrebowiei* in *B. forskalii* from Zambia (Wright et al., 1979).

Table 2.4 Checklist of intermediate host snails involved in the transmission of Amphistomes of wild ruminants in Africa (1900-2022).

Amphistome species	IH snail species	Country	References
C. calicophorum	*B. tropicus*	South Africa	Grobbellar, 1922
C. calicophorum	*B. tropicus*	South Africa	Porter, 1921
C. clavula	*B. abyssinicus*	Somalia	Sobrero, 1962
C. microbothrium	*B. tropicus*	South Africa	Swart and Reneicke, 1962
C. microbothrium	*B. tropicus*	South Africa	King and Van As, 2001
C. microbothrium	*B. tropicus*	Zambia	Dinnik, 1965
C. microbothrium	*B. tropicus*	Kenya	Dinnik, 1962
C. microbothrium	*B. (Physopsis) globosus*	Zambia	Dinnik, 1965
C. microbothrium	*B. (Physopsis) nasutus*	Kenya	Dinnik, 1965
C. microbothrium	*B. senegalensis*	Gambia	Wright, et al., 1979a
C. microbothrium	*B. tropicus*	Kenya	Southgate et al., 1985
C. microbothrium	*B. tropicus*	Kenya	Southgate et al., 1989
C. daubneyi	*G. (L.) truncatula*	Kenya	Dinnik, 1962
C. phillerouxi	*B. forskalii*	Tanzania, Zambia	Dinnik, 1961
C. phillerouxi	*B. senegalensis*	Gambia	Dinnik, 1961
C. sukari	*Bio. pfeifferii*	Ethiopia	Graber and Daynes, 1974
C. sukari	*Bio. pfeifferii*	Kenya	Dinnik, 1965
C. exosporus	*A. natalensis*	Kenya	Dinnik, 1964
C. exosporus	*A. natalensis*	Kenya, Zambia	Dinnik, 1965
C. mancupatus	*A. natalensis*	Zambia	Dinnik, 1965
C. mancupatus	*B. mairei*	Zambia	Gretillat, 1960
C. mancupatus	*B. liratus*	Zambia	Gretillat, 1960
C. mancupatus	*C. natalensis*	Kenya	Laidemitt et al., 2017
C. parvipapillatus	*B. (Physopsis) globosus*	Zambia	Dinnik, 1965
S. compactus	*B. forskalii*	Zambia	Dinnik, 1965

2.5 Discussion

Over 70 amphistome species have been previously identified and documented around the world and have been reported to parasitize a diverse spectrum of hosts (Ghatani et al., 2012), mainly domestic and wild ruminants. In this review, we report 38 amphistome species in wild ruminants (36 of which were identified up to species level) distributed in 23 out of the 54 African countries. Although the species were reported across 39 wild ruminant's species, previous reports have shown that majority of the amphistomes reported are shared with domestic ruminants, with exception to *Bi. papillogenitalis*, *Car. bubalis* and *Cot. macrosphinctris* which have only been documented in wild

ruminants in Africa (Pfukenyi and Mukaratirwa, 2018). Munyeme et al. (2010) has suggested that this overlap of species between wild and domestic ruminants may have been attributed to the bi-modal transmission of parasites facilitated by contact between wild and domestic ruminants through shared grazing areas or sources of drinking water.

African buffalo was the most frequently infected wild ruminant across Africa, infected with 21 amphistome species from different genera which include *Calicophon*, *Carmyerius, Cotylophoron, Paramphistomum, Gigantocotyle, Leiperocotyle* and *Stephanopharynx*. A similar trend was observed in Asian countries (Bangladesh, China, India, Indonesia, Iraq, Iran, Japan, Malaysia, Nepal, Pakistan, Phillipines, Sri Lanka, Thailand, Turkey), where over 26 amphistome species belonging to the families Gastrothylacidae and Paramphistomoidea were reported in buffaloes (Tookhy et al., 2022). These infections in African buffalo are not surprising as these ruminants are widely distributed across sub-Saharan Africa and is often considered an important reservoir for livestock diseases (Eygelaar, 2015). Furthermore, Saha et al. (2013) and Nath et al. (2016) have argued that infection of buffaloes with amphistomes may have been due to factors such as their wallowing habit, and bulk ingestion of grasses near the water source (habitats of snail IHs) which in turn increase exposure to ingestion of the metacercariae encysted on the lush grass on the edges of water bodies.

The review showed that at genus level, species from the genus *Calicophoron* were most common and widely distributed across 17 of the 23 reviewed African countries. However, results also showed that at a species level *C. microbothrium* was the most widely distributed species, followed by *Cot. cotylophorum* as compared to the other genera. This was not surprising as *C. microbothrium* is regarded the most common and significant cause of amphistomosis in Africa (Pfukenyi et al., 2005; Pfukenyi and Mukaratirwa, 2018). Furthermore, the wider distribution of both species may have also been attributed to their ability to infect and utilise a wider range of wild ruminant species as their hosts. Amongst least reported amphistomes species were *G. crumenifer*, *C. daubneyi* and *P. cervi*. These three amphistomes have been linked with cases of amphistomosis beyond Africa, in Asia, Europe, and South America, with only *Cal. Daubneyi* having an IH host in Africa (Raza et al., 2009; Gordon et al., 2013; Tehrani et al., 2013; Jones et al., 2015; Jones et al., 2016, Rafiq et al., 2020). The existence of *P. cervi* wasreported for the first time in Africa (Maplestone, 1923, Stunkard 1929) but these claims were ignored due to misdiagnosis (Round, 1968). These presence of *P. cervi* in Halium et al. (2014) could confirm re-emergence of these amphistomes due to several factors including animal trade or introduction of the IH snail vectors.

According to Eduardo (1987), the establishment of an amphistome species in a particular geographical region may be dependent more on the intermediate host as compared to the definitive host. Saito et al. (2023) stated that freshwater snails may be moved from one region to another through migratory

birds, or through aquarium trades (Derraik, 2008; Work & Mills, 2013). Reviewed studies showed that certain amphistome species only utilise Planorbidae species as Intermediate Hosts. Our results showed that the distribution of the widely spread amphistomes genera *Calicophoron* in Africa were linked to the presence and distribution of *B. tropicus, B. globosus*, *B. forskalii*, *B. nasutus, B. senegalensis* and *Bio. pfeifferii* in (Gretillat, 1960; Dinnik, 1961; Swart and Reneicke, 1962; Dinnik; 1962; Dinnik; 1964; Dinnik, 1965; Graber and Daynes, 1974). Several authors indicated that *Bulinus* species are highly susceptible to infection and have a capacity to aestivate during the dry season (Dinnik, 1954; Dinnik, 1965; de Kock et al., 2002), which may explain the dispersion of *C. microbothrium* which was linked with *B. tropicus*, *B. globosus*, and *B. nasutus* (Dinnik, 1962; Swart and Reneicke, 1962 and Dinnik, 1965). *Calicophoron daubneyi* was the only amphistomes species linked with *G. (L.) trancatula* in East Africa (Dinnik, 1962). According to Eduardo (1987), the genus *Calicophoron* appears to have developed in Africa and from there radiated to other areas and its introduction to new environments is limited by the nature of intermediate hosts which show high specificity. Despite the widespread distribution of *Bulinus sp*., a study done by Pederson et al. (2014) in Zimbabwe shows that climate change may cause a reduced spatial distribution of suitable habitat of snails such as *Bulinus globosus.* This may cause a redistribution of amphistomes in the coming years.

The lowest prevalence of amphistomes infection in this review was recorded in *Gazella rufifrons* (7.1 %) and the grey duiker (8 %) infected with *C. microbothrium* from Chad and *Paramphistomum* spp. from South Africa, respectively. These low prevalences may be because browsers generally eat on shrub leaves and buds, limiting their exposure to infective stages of amphistomes (Condy, 1972; VanderWaal et al., 2014). Furthermore, low prevalence of other trematode infection patterns has been noted in browsers such as Sambar deers and Nilgai in Indian (Gupta et al., 2011). It should however be noted that there is most likely to be a bias in the prevalence data as most of the hunted animals are males with fewer female and young animals. Furthermore, the lower number of hunted animals due to restrictions in the hunting quotas may not give a true representation of the prevalence.

The highest prevalence from this review were recorded in Zambian *Kobus defassa* infected with *Calicophoron* spp. (100 %), followed by South African Impala infected with *Cot. cotylophorum*. These Bovidae species are predominantly grazers, and the observed high prevalence may be due to their feeding behavior, which increases exposure to parasites (Phiri et al., 2011). Amoroso et al. (2019) argued that sharing of waterholes may potentially be a source of parasite exposure and subsequent high prevalence especially in the case of amphistomes. However, Zieger et al. (1998) suggested that transmission of parasites in wild ruminants may also be impacted by habitat type and stocking density. The higher the density of animals often lead to contamination of grazing and water source, and consequently lead to infections and re-infection of animals (Condy, 1972; Zieger et al., 1998). This was

later supported by Singh et al. (2009), who mentioned that pasture contamination by parasites may result in a high incidence of parasites.

In general, results showed that Southern African countries recorded some of the highest prevalence rates of amphistomes infections, while East, North, and Central Africa have low to moderate rates. Regional differences in amphistome prevalence are likely due to factors like environmental conditions, ecology, host-parasite interaction, and collection season (Phiri et al., 2011; Tookhy et al., 2022). This variation in prevalence of amphistomes may be due to different climatic conditions, ecological and management systems (Hajipour et al. 2021 Climatic conditions may affect the life cycle of the parasites resulting in regional differences of prevalence amphistomes (Gonzalez-Warleta et al., 2013). In some regions fewer studies were conducted as compared to other regions, this could have attributed to logistical obstacles, insufficient financing, and more studies focusing on areas of interest, such as malaria and schistosomiasis, which are more common and have a higher impact on public health in Africa. As a result, current amphistome prevalence estimates in Africa may not completely reflect the diversity of host species or geographical areas.

Furthermore, all prevalence cases were based on coprological and morphological techniques, which not only failed to identify to species level in most cases but may have underestimated the true prevalence of amphistomes species in Africa. This was supported by several authors who indicated the challenges associated with identifying various species of immature amphistomes based on morphology alone (Chaoudhary et al., 2015; Ikeuchi et al., 2022).

Reviewed studies also documented the presence of mixed infections of amphistomes and other trematodes such as *Fasciola* spp. and *Schistosoma* spp. in both intermediate and definitive hosts. Similar co-infection of amphistomes with other trematodes have been documented outside Africa in Sambar deer, Chital, Neelgai, Gaur and buffalo in India (Gupta et al., 2008; Sing et al., 2009; Gupta et al., 2011; Saha et al., 2013; Swarnarkar et al., 2014) and goats of Pakistan (Ayaz et al., 2013). Furthermore, 60.42 % of Black Bengal goats were found harboring two or more species of amphistomes in India (Uddin et al., 2006), whilst in Pakistan 12.8 % of buffaloes were also reported to harbor multiple species of amphistomes (Nazar et al., 2019). According to Alstedt et al. (2022), co-infection of amphistomes with other trematodes such as *Fasciola* spp. in the intermediate and definitive hosts is possibly due to the similar heteroxenous lifecycle of these trematodes and shared intermediate hosts. Furthermore, exposure to various sources of infection, vast habitats, (Gonzalez-Warleta, 2013; Nazar et al., 2019; Amoroso et al., 2019) and the movement and foraging habits of wild ruminants (Mijele et al., 2016) are all factors that can result in co-infections in ruminants. Moreover, large wild ruminant populations and high stock densities offer opportunities for parasite colonisation and transmission, which can result in the development of mixed or co-infections within a population

(Amoroso et al., 2019; Schmid-Hempel, 2021). According to Munyeme et al. (2012), mixed or co-infections can negatively impact the health and carcass quality of animals. Observations were also made by Pike and Condy (1966) regarding how mixed trematode infections may affect wild ruminants when they encountered animals in poor condition with a heavy mixed parasitic load. Additionally, earlier studies have highlighted the increased susceptibility of infected animals to secondary infections, leading to higher mortality rates (Pfukenyi and Mukaratirwa, 2018). These contemporary investigations continue to emphasize the detrimental effects of co-infections on animal health.

2.6 Conclusions

Results from this review showed natural infection of 39 wild ruminant species with 38 amphistome species occurred across 23 African countries. *Calicophoron microbothrium* and *Cot. cotylophorum* were the most distributed species. High prevalence of amphistome infections were documented in southern Africa compared to all the other regions. The low number of studies showed that there is still paucity of information on amphistome of wild ruminants and their intermediate snail hosts. Future study should focus on determining the geographical expansion and prevalence of amphistomes in their wildlife hosts. Furthermore, future studies should provide more information on the species of intermediate hosts involved in the distribution of amphistomes in Africa. Most importantly, there is need to adopt both morphological characterization and newer molecular tools not only to detect amphistomes in the larval stages for conservation of wildlife, but also study their taxonomy and genetic relationships. The limitations in the studies conducted were that the studies that gave prevalences were not characterizing the amphistomes to species level and sometimes would not mention the worm burden. Worm burden has a bearing on the extent of disease in animals.

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Chapter 3

Morphological and molecular identification of amphistomes of wild ruminants from selected game reserves of Zimbabwe

3.1 Abstract

Amphistomosis is generally a poorly understood trematode disease whose effect has been underestimated in domestic and wild ruminants. Limited work has so far been done on the taxonomy of amphistome species particularly in Africa which is home to diverse species infecting domestic and wild ruminants. This study was conducted in wildlife conservancies and national parks located in Beitbridge, Nyamandlovu, and Hwange located in the Matebeleland region of Zimbabwe during the 2019-2021 game hunting seasons. A total of 313 carcasses of wild ruminants were screened for amphistome infections in the gastrointestinal tracts, and 32/313 (10.22%) animals were infected. Prevalence of amphistomes (number of infected animals) per locality was 5.62% in Nyamandlovu, 35% in Hwange and 33.91% in Beitbridge. A high prevalence of 63% was recorded in *Kobus ellipsiprymnus* (waterbuck) from Beitbridge with a high parasitic burden of more than 10 000 parasites per animal. Parasitic burden was determined by counting a one tenth aliquote of the total amphistomes that were washed and collected from each animal. Higher parasitic burdens show clinical manifestations of amphistomosis compared to those with less. The lowest parasitic burden was observed in *Connochaetes taurinus taurinus* (blue wildebeest) and *Hippotragus niger* (sable) each with less than 10 amphistomes. Eighty-six amphistome specimens were harvested from *Syncerus caffer* (African buffalo) (n=27), *Aepyceros melampus* (impala) (n=17), *Tragelaphus strepsiceros* (kudu) (n=16), waterbuck (n=10), sable (n=7), *Redunca arundinum* (reedbuck) (n=7), and *Connochaetes taurinus taurinus* (blue wildebeest) (n=10) for morphological and molecular identification. Using a combination of histology to study morphological features and ITS-2 rDNA for sequencing molecular techniques, seven amphistome species were identified as follows; *Leiperocotyle gretillati, Calicophoron microbothrium*, *C. raja*, *C. clavula*, *C. phillerouxi, Gigantocotyle* (*G.*) *symmeri*, and *Orthocoelium dicranocoelium*, and *Gastrothylax* (*Ga.*) *crumenifer* (pouched amphistome species). *Orthocoelium dicranocoelium*, *Ga. crumenifer and L. gretillati* in are first records in wild ruminants in Zimbabwe. On performimg a phylogenetic analysis of some isolates with those of domestic animals from Genbank, four distinct clades were formed and did not separate these according to host but according to amphistome species and genera. *Calicophoron* species were separated into two distinct groups whilst the *Leiperococotyle gretillati* isolates from buffalo were separated into their own clade with isolates obtained from snails (Kenya). Although the ITS-2 is a most widely used marker, it may not be useful in discriminating species within the *Calicophoron* genus.

Keywords: Wild ruminants, amphistomes, ITS-2, phylogenetic analysis, morphology

3.2 Introduction

Wild animals have in many instances been implicated as reservoirs of parasites (Kruse et al., 2012). This has led to studies being focused on wild animals on emerging parasitic diseases (Thompson, 2010), whose surveillance has become important. Amphistomes are digenetic trematodes whose life cycle includes an intermediate snail host which harbors the larval stage and a definitive host where the adult stage resides. The definitive hosts include both domestic and wild ruminants, with over 70 species of amphistomes being documented (Jones, 2005). Wild ruminants have been recorded as definitive hosts of several amphistomes species in Africa (Boomker et al., 1987; Eduardo, 1987; Dube et al., 2005; Pfukenyi and Mukaratirwa, 2018). There is to date limited research on amphistomes of wild ruminants (Pfukenyi and Mukaratirwa, 2018), with Ali et al. (2018) confirming that amphistomosis is a poorly understood trematode disease. Outbreaks of amphistomosis have been recorded in wild ruminants such as *Axis axis* (deer) (Balaji *et al*., 2014) and *Rusa unicolo*r (Samba deers) (Mahanti et al., 2021), showing that amphistomes may affect wild ruminants and potentially contributing to population decline. While studying amphistomes in domestic animals is simplified by the easy access to abattoir for collections, this is not the case with wild populations where access to carcasses for specimens collection remain a challenge and this include costs related with travel to conservancies/gameparks and often times the animals are rare to find and/or possibly protected (Perkins et al., 2011).

Distribution of amphistome parasites is worldwide (Tandon *et al*., 2014) with higher incidences occurring in the sub-tropics/tropics (Zintl *et al*., 2014). However, in Africa where they are abundant and diverse wild ruminants, there is still dearth of information on the amphistomes species of ruminants in the wild (Eduardo, 1987). A review by Sibula et al. (2024) reported 36 amphistome species occurring in wild ruminants in Africa, of which Pfukenyi and Mukaratirwa (2018) earlier showed 26 species had been recorded in both domestic and wild ruminants in southern and eastern Africa. These species belonged to six genera, namely, *Calicophoron*, *Carmyerius*, *Gigantocotyle*, *Gastrothylax*, *Orthocoelium* and *Cotylophoron*, with the *Calicophoron* genus being the most dominant (Pfukenyi and Mukaratirwa, 2018). Furthermore, *Calicophoron microbothrium* was the most widely reported and distributed (Eduardo, 1987; Pfukenyi and Mukaratirwa, 2018) and is known to be the cause of amphistomosis.

Zimbabwe's economy relies on agriculture and tourism (Sanderson and Leroux, 2017) and therefore necessary to understand the parasites that may affect both sectors as it is possible for transmission of these parasites from wild animals to domestic animals and vice versa (Mijele *et al*., 2016). To date,

only limited molecular studies have been conducted on amphistomes of wild ruminants in Africa, as seen with *Calicophoron raja* isolates being obtained from black wildebeest and waterbucks (Ikeuchi *et al*., 2022). There is still a need for molecular analysis of a wide range of amphistomes from different host species and geographical areas to improve the understanding of parasite genetic structure, transmission dynamics and gene flow (Ali *et al*., 2018). The ITS-2 rDNA gene has been widely used to distinguish between amphistome species (Lofty et al., 2010; Chamuah et al., 2015; Dube et al., 2015; Laidemitt et al., 2016; Ikeuchi et al., 2022; Khan et al., 2020; Pageum et al., 2023). According to Pfukenyi and Mukaratirwa (2018), 23 out of the 26 amphistome species recorded in southern and eastern Africa have been recorded in wild ruminants. As most of this data was recorded before 2000, it is important to get current information on the amphistome species found in Zimbabwe and to add molecular data of these parasites to enhance knowledge. Therefore, this study aimed at morphologically identifying amphistome species infecting wildlife ruminants from Matebeleand region of Zimbabwe and confirm species identification using the ITS-2 rDNA marker.

3.2 Materials and methods

3.2.1 Study sites

Sample collection was conducted in Hwange, Umguza/Nyamandlovu and Beitbridge localities of the Matebeleland region of Zimbabwe (Figure 3.1). The first location was in Hwange National Park, which is the largest game reserve of Zimbabwe and covers an area of 14600 $km^{2,}$ in North-western region of Matebeleland, Zimbabwe (Dudley *et al*., 2001). The second site was in the Umguza/Nyamandlovu area (Rosslyn safaris) which is adjacent to the Hwange National Park. This is a game ranch that covers about 20 000 hectares of land, lying within region IV in Matebeleland North province. The third site for collection was a wildlife conservancy (Bubiana) that is about 400 000 acres in size and situated in the southern parts of Zimbabwe, that stretches from West Nicholson towards Beitbridge.

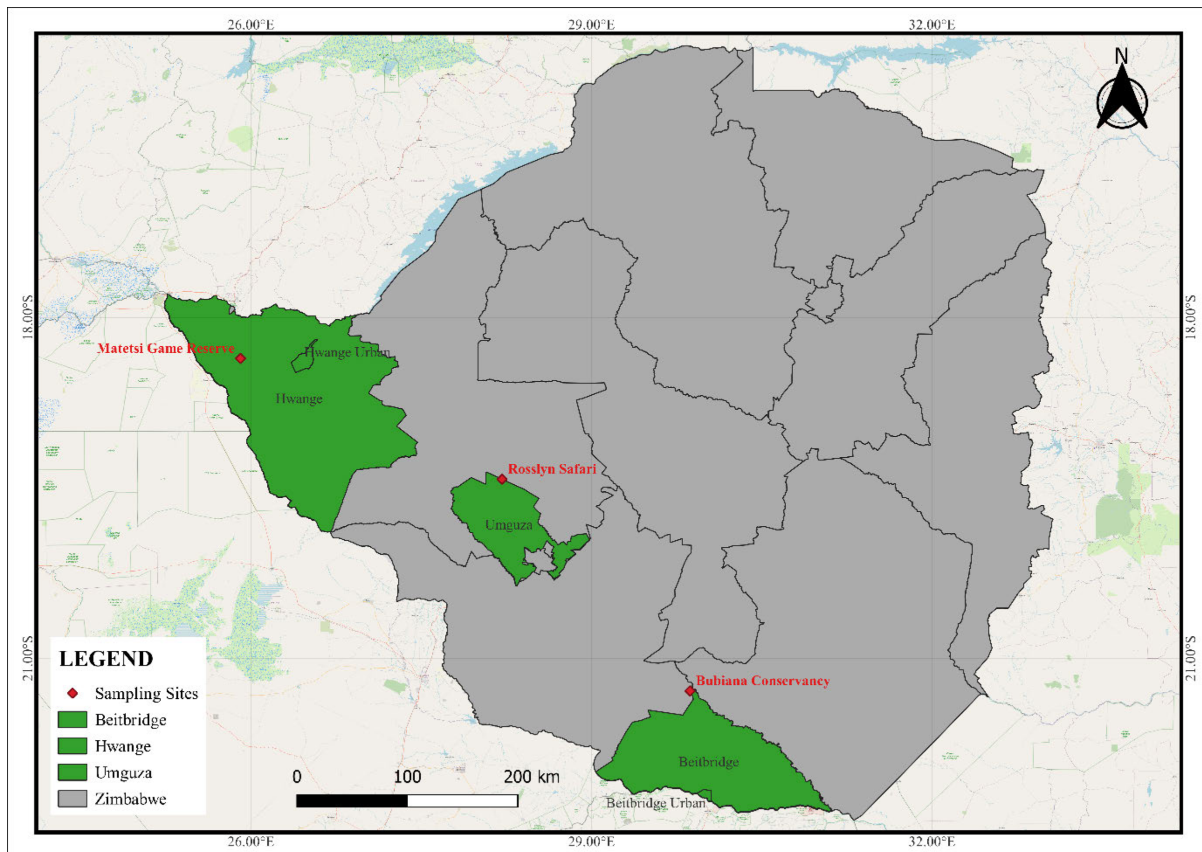

Figure 3.1 Map of Zimbabwe showing areas where amphistomes were collected from wild ruminants

3.2.2 Collection and preservation of amphistomes

A total of 313 carcasses of hunter-killed wild ruminants between were examined between 2019 and 2021 comprising of the following wild ruminants: African buffalo (*Syncerus caffer*) 11, common duiker (*Sylvicapra grimmia*) 5, bushbuck (Tragelaphus scriptus)(11), eland (*Taurotragus oryx*) (7), impala (*Aepyceros melampus*) (127), klipspringer (*Oreotragus oreotragus*) 3, kudu (*Tragelaphus strepsiceros*) 68, mountain reedbuck (*Redunca fulvorufula*) 17, sable (*Hippotragus niger*) 25, tsessebe (*Damaliscus lunatus)14*, waterbuck (*Kobus ellipsiprymnus*) 24, wildebeest (*Connochaetes taurinus*) 20,. Immediately after death the carcasses of all animals except for the African Buffalo were taken to nearby abbatoirs. As done by Zieger et al., the abnominal cavities were opened, examined for amphistomes and eviscerated. All amphistomes present were removed or scooped out washed, put in aliquots of a tenth for enumeration. Once counted and washed, the amphistomes put in containers with 70% ethanol and carried to the lab for further analysis.

Sampling was not random and depended on circumstances as described by Van wyk and Boomker (2011). The total number of wild ruminant species that were sampled and the number of infected animals recorded in each site to determine the prevalence of amphistomosis in wild ruminants hunted are shown in Table 3. 1.

3.2.3 Ethical considerations

The samples in this study were conveniently collected from animals that had been either hunted for reasons other than this study as described by Van Wyk and Boomker (2011).

3.2.4 Morphological characterization of specimens

Amphistomes were selected according to size, morphology and colour as a guide to get different amphistomes. Each selected amphistome was cut into two fragments using a sterile scalpel blade carefully in order not to disturb the internal organs. From each amphistome, the smaller tissue segment was frozen for DNA extraction whilst the remaining larger segments were dehydrated in successive concentrations of ethanol (70% - 100%) for histological sectioning. After dehydration, the samples were cleared using xylene, then sectioned using a microtome and placed on slides for staining with haematoxylin and eosin. Visualization of the sections was done under a stereo microscope (Zeiss) and photomicrographs of each section taken (Appendix 3.2). A device with a rear camera of 50 mega pixels was used for taking the photomicrographs. For identification to species level, the acetabulum, the pharynx, genital atrium, and ventral pouch were used as described by Nasmark (1937), Eduardo (1982, 1983, 1984, 1985) and Sey (1983, 2019).

3.2.5 Molecular analysis

DNA was extracted from a tissue segment of each of the 86 amphistomes specimens using the Zymo Research Quick- DNA ™ Microprep Kit. The ITS-2 rDNA region of the amphistome DNA was then amplified using the primers GA1 (5'-AGAACATCGACATCTTGAAC-3') and BD2 (5'–TATGCTTAAATTCAGCGGGT-3') (Laidemitt et al., 2017). PCR amplification was conducted in a total volume of 10µl, composed of 2 µl of 5X One Taq PCR buffer, 0.1 µl of 10mM dNTPs, 0.2 µl forward primer (10mM), 0.2 µl reverse primer (10 mM), 1.25 µl of 25mM $MgCl_2$, 1 µl DNA template, 0.8µl of One Taq polymerase (NEB, England) and nuclease free water to make up to volume. The cycling conditions were set at initial denaturation at 94℃ for 30 secs, followed by 35 cycles each of 94℃ for 30 secs, annealing at 55℃ for 45 secs and extention at 68℃ for 30 secs, and the final extention was set at 68℃ for 10 minutes. For visualization, the products were run on a 1% agarose gel stained with ethidium bromide and viewed under the UV gel pro and successful fragment were identified at approximately 380 bp. Sanger sequencing was performed at Inqaba Biotechnical Industries (Pretoria, South Africa).

3.2.6 Phylogenetic analyses

Sequences were manually edited and trimmed using Chromas lite with species identification being done on BLAST search against samples from NCBI. The sequence identities and similaries are shown on Appendix 3.3. The sequences were submitted to GenBank and accession numbers obtained

(PP854078-PP854163). A number of isolates were retrieved from NCBI with the following accession numbers from different localities and different hosts: *Orthocoelium dicranocoelium*, Thailand (OM943175); *Calicophoron phillerouxi*, Kenya (KX668977); *Gastrothylax crumenifer*, India (MN371811); *Calicophoron microbothrium*, Kenya (KX668945); *Calicophoron raja*, Kenya (KX668966); , *Calicophoron clavula*, Egypt (MK416145); Paramphistomoidea, Kenya (KX668930) ; Paramphistomoidea, Kenya (KX668934); *Notocotylidae* sp., Vietnam (MT268117); *Ogmocotyle sikae*, China (KF008246); and *Ogmocotyle capricorni*, Japan (AB367788), were used as the outgroups as according to Laidemitt et al. (2017). *Calicophoron raja*, South Africa (LC633276) and *Gigantocotyle gigantocotyle*, South Africa (LC660655) are isolates from wild animals. Alignment was then done using Muscle on Mega 11 with all isolates being trimmed to 373bp. Phylogenetic analyses was inferred using the Neighbour Joining method. The optimal tree is shown with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 404 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021)

3.3 Results

3.3.1 Prevalence of amphistomes

Three-hundred and thirteen (n = 313) wild ruminants consisting of 10 species viz *Syncerus caffer* (African buffalo), *Tragelaphus scriptus* (bushbuck), *Sylvicapra grimmia* (common duiker), *Taurotragus oryx* (eland), *Tragelaphus strepsiceros* (kudu), *Aepyceros melampus* (impala), *Kobus ellipsiprymnus* (waterbuck), *Connochaetes taurinus taurinus* (blue wildebeest), *Hippotragus niger* (sable) and *Redunca arundinum* (reedbuck) were screened for amphistome infection (Table 3.1). Of these, 32/313 (10.54 %) wild ruminants were infected by amphistomes, and these included African buffalo, Kudu, Impala, waterbuck, blue wildebeest, sable and reedbuck. The highest prevalence per location was observed in Hwange (35 %, 7/20) while the lowest prevalence was recorded in Nyamandlovu at (5.62 %, 15/267). The highest prevalence per host species was recorded in waterbucks from Beitbridge (63.63 %, 7/11) followed by African buffaloes from Hwange with a prevalence of 54 % (6/11), and the lowest prevalence was recorded in Impala (3.57 %, 4/112) from Nyamandlovu.

Table 3.1 Prevalence of amphistomes in wild ruminants from Matebeleland region of Zimbabwe.

		Study site/location										
	Total hunted	Hwange			Nyamandlovu/Umguza			West Nicholson/Beitbridge			**Overall positive**	**Overall prevalence**
Animal host	N	N	Positive	% Prevalence	N	Positive	% Prevalence	N	Positive	%P revalence		
African Buffalo	**11**	11	6	54.5	-			-			6	54.5
Common duiker	**5**	-	-	-	5			-				-
Bushbuck	**11**	-	-	-	5	0	0	6	0	0		-
Eland	**7**	-	-	-	4	0	0	2	0	0		-
Impala	**127**	-	-	-	112	4	3.57	15	4	27	8	6.3
Klipspringer	**3**	-	-	-	-							-
Kudu	**68**				63	5	7.93	5	0		5	7.35
Mountain reedbuck	**17**				17	3	17.64				3	17.64
Sable	**25**	9	1	11.11	16	2	12.5				3	12
Tsessebe	**14**				11	0		3				-
Waterbuck	**24**				13	0		11	7	63.63	7	29,2
Wildebeest	**20**				17	1	5.88	3	0		1	5
Eland	**6**				5	0		1	0			
Total	**313**	20	**7**	**35**	267	**15**	**5.62**	46	11	**23.91**	32	**10.22**

3.3.2 Amphistome burden and mixed infection

Results show that animal hosts had an intensity of amphistomes ranging from between 1 to >10 000. The highest intensity of infection was observed in two waterbucks from Beitbridge with more than 10 000 amphistomes specimens collected. The lowest amphistome burden was noted in sables from Hwange and blue wildebeest from Nyamandlovu, with an amphistome burden of less than 10. Mixed infections with two or more amphistomes were recorded in the African buffaloes, sables, Impala and waterbucks. The highest mixed infection of four amphistome species was recorded in waterbuck where one waterbuck has *Cal. clavula*, *Cal. microbothrium*, *Gastrothylax crumenifer* and *Orthocoelium dicranocoelium* and in the African buffalo there was L*eiperocotyle gretillati*, *Cal microbothrium*, *Cal raja* and *Gigantocotyle symmeri*. Most wild ruminants had an intensity range of betwwen 100-1000.

3.3.1 Morphological characterization

Morphologically, eight amphistome species were identified and these were *Calicophoron microbothrium*, *C. clavula*, *C. raja*, *C. phillerouxi*, *Leiperocotyle gretillati*, *Gigantocotyle* (*G.*) *symmeri*, *Gastrothylax* (*Ga.*) *crumenifer* and *O. dicranocoelium* (Appendix 3.2). These species were identified based on diagnostic features indicated in Table 3.2. The most common genus was *Calicophoron,* accounting for 80.2 % (69/86) of the amphistome specimens analyzed (Table 3.4). *Calicophoron microbothrium* was dentified from all wild ruminants which were infected except the blue wildebeest although it had the highest number of specimens collected (39.5 %, 34/86).

This is a first record of *Orthocoelium (O.) dicranocoelium*, *Gastrothylax (G.) crumenifer and Leiperocotyle (L.) gretillati* in wild ruminants of Zimbabwe. Morphologically, *L. gretillati* has an acetabulum that is of the *Cotylophoron* type and a pharynx that is *Calicophoron* type and a genital atrium that is *Cotylophoron* type. The distinguishing feature for this amphistome from *Cot. fuelleborni* is the position of the genital atrium relative to the pharynx and oesophageal bifurcation. It must however be highlighted that the *L. gretillati* specimens in this study had larger mature testes that extended from the middle third of the body to the lower posterior end (Appendix 3.1). Differentiation of most amphistomes species was done morphologically using the acetabulum, the pharynx and the genital atrium (Eduardo 1982a,b; Eduardo, 1983; Eduardo, 1985; Nasmark, 1937). However, one pouched specimen, *Ga. crumenifer* was identified by the acetabulum and the ventral pouch as described by Sey (2019) being triangular with a dorsally directed apex and a testes that is visible just below the pouch. The genital atrium was observed within the ventral pouch.

Table 3.2 Descriptive morphology of amphistomes collected from wild ruminants in Matebeleland region of Zimbabwe.

Species	No. of specimens	Host(s)	Diagnostic features
Calicophoron (C.) microbothrium	35	African buffalo, Kudu, Impala, Reedbuck, Sable, Waterbuck	Acetabulum of the paramphistomum type, pharynx is the Calicophoron type and genitalium of the microbothrium type.
C. clavula	6	African buffalo, Impala, Sable, Waterbuck	Acetabulum is of the paramphistomum type, pharynx is of the Calicophoron type, genitalium is of the clavula type
C. raja	23	African buffalo, Impala, Kudu, Reedbuck, Sable, Wildebeeste	Acetabulum is of the pisum type, pharynx is of the Calicophoron type and genitalium is of the raja type.
C. phillerouxi	4	African buffalo, Impala, Reedbuck	Acetabulum of the Paramphistomum type, pharynx of the Calicophoron type with a deeply lobbed testes and a genital atrium of the microbothrium type. Position of the genitalium is at level with the oesophageal bifurcation
Leiperocotyle gretillati	9	African buffalo	Acetabulum is of the Cotylophoron type, pharynx is of the calicophoron type, genital atrium is Cotylophoron type and is at level with oesophageal bifurcation and has a short oesophagus.
Gigantocotyle symmeri	2	African buffalo, Impala	Acetabulum is enormous and of the symmeri type, pharynx is explanatum type with a deeply lobed testes and genital atrium of the gigantocotyle type.
Gastrothylax crumenifer	5	Waterbuck	Acetabulum is of the Gastrothylax type with a ventral pouch with a dorsally directed apex, pharynx is of the paramphistomum type. Terminal genitalium opens into ventral pouch.
Orthocoelium dicranocoelium	1	Waterbuck	Acetabulum is of the streptocelium type, pharynx is of the dicranocoelium type with a terminal genitalium of the gracile type

3.3.2 Molecular analyses

Molecular analysis of morphologically confirmed *C. microbothrium* had a percentage identity of between 99% to 100% except for a few isolates which had a 99% similarity to *C. clavula* (Table 3.5). However, molecular analysis of *C. raja*, *C. clavula* and *C. phillerouxi* showed that these three species had a 99 to 100% similarity to each other making it hard to distuinguish from each other. Molecular analysis of these isolates gave a 99% identity to isolates recorded as Paramphistomoidea from Kenya.. Molecular analysis yielded a percentage identity of 99% to *Ga. crumenifer* (Accesion number = MN371811) from India and Gastrothylacidae (Accession number = KX668946) from Kenya (Appendix 3.3). The waterbuck was the only host for *Ga. crumenifer* and the African buffalo was the only host for *L. gretillati*. Two amphistome species identified morphologically as *Gi. symmeri* and *O. dicranocoelium* had percentage identities of 98,5 % to *C. microbothrium* (Accession number = KP639635) and 97,86% *Cot cotylophorum* (Accession number = KC503917) respectively.

Table 3.3 Morphological identity of amphistomes collected from wild ruminants in Matebeleland region of Zimbabwe.

Location	**Host species**	**No. of hosts (N)**	**Amphistome species**								**Total**
			C. microbothrium	***C. clavula***	***C. phillerouxi***	***C. raja***	***O. dicranocoelium***	***Ga. crumenifer***	***L. gretillati***	***G. symmeri***	
Beitbridge/ West	Waterbuck	7	3	1			1	5	-	-	10
Nicholson	Impala	4	4	4	1	3	-	-	-	-	12
Hwange	Buffalo	6	13	1	2	1	-	-	9	1	27
	Sable	1	3	-	-	1	-	-	-	-	4
Nyamandlovu/Umguza	Blue wildebeest	1	-	-	-	2	-	-	-	-	2
	Kudu	5	5	-	-	11	-	-	-	-	16
	Sable	2	-	-	-	3	-	-	-	-	3
	Impala	4	3	-	-	1	-	-	-	1	5
	Reedbuck	3	4	-	1	2	-	-	-	-	7
Total		**33**	35	6	4	24	1	5	9	2	86

C. = *Calicophoron*, O. = *Orthocoelium*, Gi. = *Gigantocotyle, Ga.* = *Gastrothylax, L.* = *Leiperocotyle*

Table 3.2 Molecular identity of amphistomes collected from wild ruminants in Matebeleland region of Zimbabwe.

Wildlife species	Amphistome species	No. of specimens identified (N)	Location		
			Beitbridge/West Nicholson	Hwange	Nyamandlovu/Umguza
Waterbuck	*C. microbothrium*	3	3	-	-
	C. phillerouxi	-	-	-	-
	C. clavula	1	1	-	-
	G. crumenifer	5	5	-	-
	O. dicranocoelium	1	1	-	-
Impala	*C. microbothrium*	7	4	-	3
	C. phillerouxi	1	1	-	-
	C. clavula	4	4	-	-
	C. raja	4	3	-	1
	Gi. symmeri	1	-	-	1
Buffalo	*C. microbothrium*	13	-	13	-
	C. phillerouxi	2	-	2	-
	C. clavula	1	-	1	-
	C. raja	1	-	1	-
	Gi. symmeri	1	-	1	-
	L. gretillati	9	-	9	-
Sable	*C. microbothrium*	3	-	3	-
	C. clavula		-	-	-
	C. raja	4	-	1	3
Blue wildebeest	*C. raja*	2	-	-	2
Kudu	*C. microbothrium*	5	-	-	5
	C. clavula		-	-	
	C. raja	11	-	-	11
Reedbuck	*C. microbothrium*	4	-	-	4
	C. phillerouxi	1	-	-	1
	C. clavula		-	-	
	C. raia	2	-	-	2
Total		86	22	31	33

C. = *Calicophoron*, O. = *Orthocoelium*, Gi. = *Gigantocotyle, Ga. = Gastrothylax,* L. = *Leiperocotyle*

3.3.3 Phylogenetic analyses

Phylogenetic analyses of amphistomes obtained in this study with some isolates retrieved from Genbank was able to separate the sequences into 4 clades as shown on figure 3.1. The clades did not separate the species according to host species but were separated according to species. However, the results show separation of the *Calicophoron* genera into two where one has a lot of *Calicophoron raja* isolates and the other *Cal. microbothrium* isolates. *Gastrothylax crumenifer* isolates from this study, grouped with other *Ga. crumenifer* from India where as the *Leiperocotyle gretillati* in this study grouped with Paramphimoidea from Kenya.

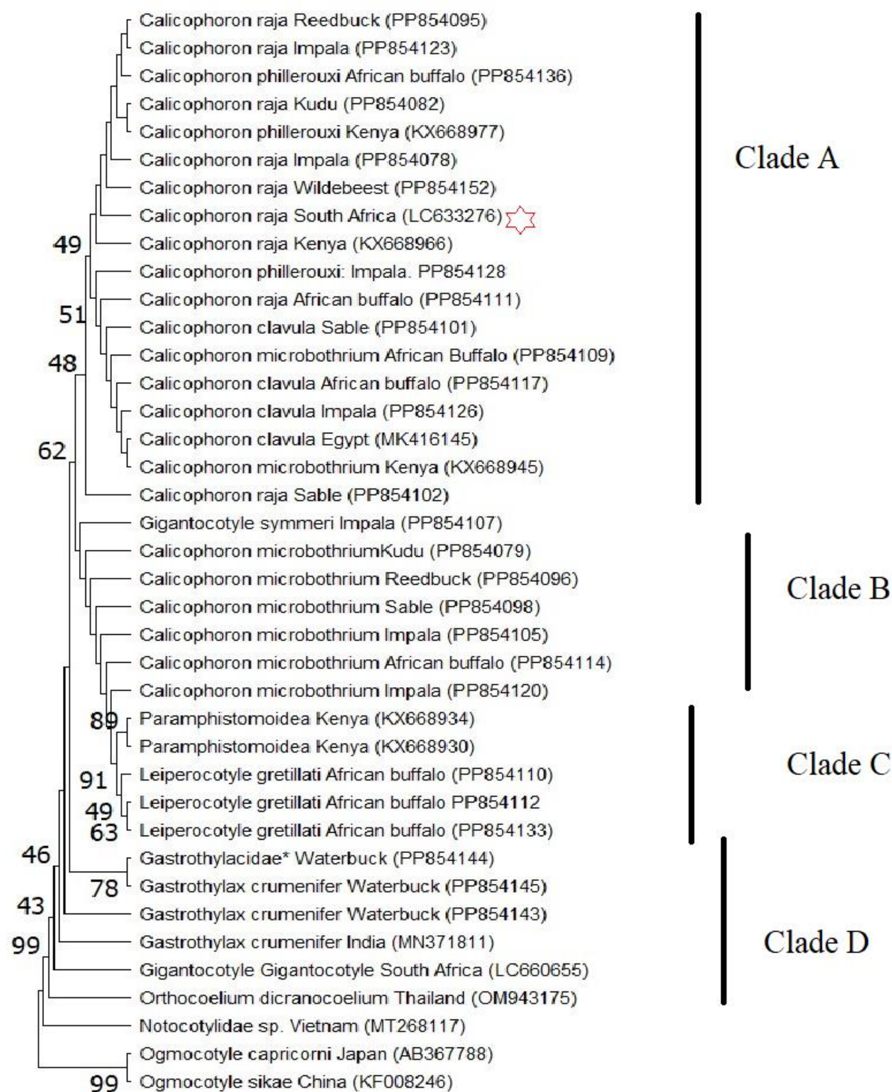

Figure 3.1: Neighbor-joining tree based ITS-2 gene illustrating the relationship between amphistome isolates from wild ruminanta fro Matebeleland in Zimbabwe, and the closest matches from the NCBI GenBank. The nodal support values indicated at 1 000 bootstraps.

3.4 Discussion

More than 26 amphistome species have been documented in Africa, in both wild and domestic ruminants (Pfukenyi and Mukaratirwa, 2018) and more than 70 amphistomes have been recorded globally. The current study revealed eight amphistome species in wild ruminants from matebeleland of Zimbabwe namely, *L. gretillati, C. microbothrium*, *C. phillerouxi, C. raja*, *C. clavula*, *Gi. symmeri*, and *O. dicranocoelium,* and one pouched amphistome species *G. crumenifer*. Previous studies done in this region only reported on amphistomes of domestic animals (Dube et al., 2005; Dube et al., 2010; Dube and Tizauone, 2014; Sibula et al., 2014). The use of both molecular and morphological tools has been done to characterise amphistomes of wild ruminants by Ikeuchi e al. (2022) and these have most been widely used in domestic animal (Dube et al., 2015; Laidemitt et al., 2017) and have revealed amphistome species similar to the ones obtained in this study. Similar techniques were employed in our study to characterize amphistomes such as *Leiperocotyle gretillati*. In this study, *Cal. raja* isolates were collected from several wild ruminants. Phylogenetic analysis of these isolates grouped them with an isolate with a genbank accession number (LC633276) from wild ruminants South Africa and another from domestic animals (KX668966) from Kenya, showing no interspecies variation in amphistomes. In this way, wild ruminants and livestock may share parasites, and wild ruminants may act as reservoirs for these parasites. *Ga. crumenifer* is recorded as the cause of amphistomiasis in India (Swanakar et al., 2021).

The overall prevalence of amphistomes for all the ruminants sampled in this study was 10.22 %. This was lower than the 88 % obtained by Graber and Thal (1980) from 12 different wild ruminant species from Chad, North Cameroon and Central African Republic. The difference in prevalence could be the seasons of collection as observed by Phiri et al. (2006) or due to the differences in the regions where collection was done. In this study, however, seasonal prevalence could not be determined as the hunting season is confined to the drier months. Effective collection of representative samples of amphistomes from wild ruminants is a challenge as it entails movement to specific hunting resort areas (Perkins *et al*. 2011) and collection of specimens requires coordination with the hunters (Reneicke *et al*., 1968).

The highest prevalence of amphistomes in individual wild ruminant hosts in this study was recorded in waterbuck at 63% compared to other species. Similarly, several authors also reported high prevalence of amphistome infections in waterbuck or the Kob species (Zieger et al., 1998; Mun'gandu et al., 2010; Munyeme et al., 2010; Phiri et al., 2011; Graber et al., 1964). However, the lowest prevalence recorded in impala in this study was contrary to the 83 % reported by Anderson (1983). Differences in prevalence in the animal hosts could be due to feeding habits of these ruminants as waterbucks are grazers (Phiri et al., 2011) whilst impalas are mixed feeders that may either browse or graze depending on the foliage present (van Wyk and Boomker, 2011). Furthermore, the waterbuck

generally inhabits areas closer to water, and such environmnets provide easy access to grazing of green grass contaminated with metacercariae from the snail intermediate host.

Prevalence of amphistomes per location of study seemed to be lower in Nyamandlovu despite having the highest number of hunted animals sampled. This could be attributed to the type and suitability of watering holes for the breeding of snails (Titcomb et al., 2021), the type of foliage present, and the ecological habits of the animal. Contrary to van Wyk and Boomker (2011)'s suggestion that smaller ranches may have a higher parasitic burden due to confined space, there seem to be fewer amphistome infections in Nyamandlovu area which was the smallest ranch compared to others. This could be that the water had limited or no vegetation growing that were observed around, thereby limiting the presence of snails in the watering holes. Zieger et al., (1998) also pointed out how stocking densities in these game reserves may directly impact parasitic burden and prevalence. The other reason why there were possibly variations in the prevalences of these particular areas could be attributed to animal hosts present. In Hwange, there were African buffaloes which are not present in Nyamandlovu whilst more waterbucks were also recorded in Beitbridge as well. Saha et al. (2013) suggested that the feeding habits of buffaloes predispose them to infection by a lot of parasites. Waterbucks also have similar feeding behaviours to the Zambian *Kafue lechwe* whose habits also expose them to amphistome infection(Phiri et al., 2011).

Our results also show that some animal hosts like the waterbuck had a high parasitic burden of more than 10 000 amphistomes per animal. Previous studies also correspond with results from our study, where a high amphistome burden was recorded in some wild ruminants (Pike and Condy, 1966; Talbot and Talbot, 1963; Zieger et al., 1998). According to Sey (2019), a massive infection with immature amphistomes is associated with amphistomosis. However, Phiri et al. (2011) observed that wild ruminants harbor a high parasitic burden of adult amphistomes without being severely affected indicating that wild ruminants may have a high tolerance for amphistomes. On the contrary, Pike and Condy (1966) observed that massive amphistome infection of the sitatunga (*Tragelaphus spekii*) in their study resulted in poor health of the animal.

Notably, some wild ruminants were infected by more than one species of amphistomes. Mixed infection was noted in heavily parasitized waterbucks in this study as four amphistome species were found in one animal and three species were also recorded from impala all from the same locality, i.e. Beitbridge. A similar trend of mixed infection was observed by Graber et al. (1964) on seven wild ruminant species with the highest number of five species being recorded in *Kobus defassa* and *Kobus kob*. Wright *et al*. (1979) also isolated 3 amphistomes species from *Kobus lechwe*. This trend of mixed

infections may be attributed to the ecological habits of the *Kobus* species which exposes them infection from grazing contaminated habitats as this family of wild ruminants is semi-aquatic. There has been a record of mixed infection of the Cape buffalo (*Syncerus caffer*) where up to 4 amphistome species were observed by Graber et al. (1964). Similar to our study, prevalences higher than 50% have been observed in Cape buffalo (*Syncerus caffer*) (Sey and Graber, 1979; Graber *et al*., 1964). However, contrary to our study, Halium et al. (2014) observed a low prevalence of amphistomes in Cape buffalo that were slaughtered in an abattoir leading us to believe that these were domesticated. Similar to *Kobus sp*., Cape buffalo have ecological habits that make them reside in semi-aquatic areas and graze on the grass around these areas exposing them to infection. Mixed amphistome species infection has not been recorded before in impala (*Aepyceros melampus*), however, three amphistome species were recorded in these animals for the first time in this study.

In our study, morphological characterisation of the specimens showed the presence of eight species. The most abundant genus was *Calicophoron* and the frequently encountered species was *C.* microbothrium. Similarly, in a study done by Laidemitt et al. (2017), the most abundant genus was *Calicophoron*. According to Pfukenyi and Mukaratirwa (2018) and Sibula et al. (2024), the most abundant genus in Africa is *Calicophoron* with *C. microbothrium* being the most frequently encountered amphistome species. However, using ITS2 sequences in this study, *C. microbothrium* was showed sequences close to other species such as *C. clavula*. In this study, species belonging to the genus *Calicophoron* had high similarity in their ITS2 sequences and these were *C. clavula*, *C. raja* and *C. phillerouxi*. This confirms the confusion earlier taxonomists have encountered concerning the genus *Calicophoron* in particular (Round, 1968) which leads us to believe that the genus is cryptic and advanced molecular tools are needed to fully resolve the differences (Laidemitt et al., 2017).

In this study, *L. gretillati* in Cape buffaloes was recorded for the first time in Zimbabwe. A previous study by Eduardo (1985) recorded this amphistome species in black buffalo in the Democratic Republic of Congo. However, in this study, there were a number of isolates with a 99% similarity to isolates that were recorded by Laidemitt et al. (2017) as Paramphistomoidea. According to the dataset from Laidemitt et al. (2017), this molecular data was obtained from the larval stages found in *Biomphalaria pfeifferi*. Molecular tools used therefore proved useful as molecular data can be used to determine the species of unknown larval forms. This therefore assisted in the completion of the lifecycle of *L. gretillati*, an amphistome confirmed by Eduardo et al. (1987) as predominantly African. Another species being reported for the first time in Zimbabwe and in the host species, *Kobus ellipsiprymnus*, is *G. crumenifer*. Previously, *G. crumenifer* was recorded in *K. lechwe* and *T. spekei* from Zambia (Le Roux, 1932) and in Cape buffalo and *K. lechwe* from South Africa (Ortlepp, 1961).

Phylogenetic analysis in this study revealed sharing of parasite speices among different hosts as there was no segregation of amphistomes according to hosts. There were similarities in the species particularly the *Calicophoron sp*. as suggested by Phiri et al. (2010) and Pfukenyi and Mukaratirwa (2018), and this could be attributed to shared grazing habitats. Although these habitats of wild ruminants have strict barriers to movement of domestic animals, water holes may still be shared causing transmission of these parasites.

Considering that the collection site in Zimbabwe is close to South African border, there is a possibility of movement of either the intermediate hosts or definitive host causing this amphistome species to be established in Zimbabwe or vice versa. Consequently, *G. crumenifer* was the only pouched amphistome that was collected from wild ruminants in this study. It has however been noted that *G. crumenifer* may cause amphistomosis, something that is worth noting for future reference (Sey, 2019). Possible wildlife animal movements between South Africa and Zimbabwe occur on the side of the Limpopo river during the drier months. Another amphistome species that was collected in this study that is a first record in Zimbabwe was *O. dicranocoelium* previously identified by Nasmark (1937) as *Ceylonocotyle dicranocoelium*. The host for this amphistome species was impala and waterbuck from Beitbridge. In Africa, one species from the genus *Orthocoelium* (*O. streptocoelium*) was recorded in *K. ellipsiprymnus*, *K. kob*, *K. thomasi*, and *T. scriptus* (Graber et al., 1964). A report by Dube and Aisien (2010) on *C. dicranocoelium* in domestic animals in Nigeria confirms the presence of *O. dicranocoelium* in Africa.

It was noted that for some isolates the molecular data and morphological data were different. This may sometimes differ when no prior sequences of that particular species were submitted to Genbank as in the case with *Leiperocotyle gretillati*. Another reason why differences are noted or different sequences particularly with Calicophoron species could be because as observed by Ikeuchi et al. (2022), some researchers submit to Genbank without the accompanying morphological data, resulting in a lot of discrepencies on molecular data.

3.5 Conclusions

In this study, a total of 8 species of amphistomes which *L. gretillati, C. microbothrium*, *C. raja*, *C. clavula*, *Gi. symmeri*, *C. phillerouxi* and *O. dicranocoelium* and one pouched amphistome species *G. crumenifer* were identified in 8 species of wild ruminants. The following species were first records in some hosts and in the country and these include *L. gretillati*, *O. dicranocolieum* and *Ga. crumenifer*. Results contributed to filling the knowledge gap of amphistomes species of wild ruminants in Zimbabwe.

3.6 Recommendations

The number of amphistome species reported in this study shows that wild ruminants of Zimbabwe have a rich fauna of amphistome parasites and thus a need for further sudies to map the distribution of amphistome species and their intermediate host snails and cross transmission with domestic ruminants especially in areas where domestic and wildlife interface.

3.7 References

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Chapter 4

Zintl, A., Garcia-Campos, A., Trudgett, A., Chryssafidis, A.L., Talavera-Arce, S., Fu, Y., Egan, S., Lawlor, A., Negredo, C., Brennan, G., Hanna, R.E. Bovine paramphistomes in Ireland. Veterinary parasitology, 2014, 204, 199-208.

Amphistome infection and species diversity of freshwater snails collected from selected wildlife drinking water sources in Matebeleland region of Zimbabwe

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4.1 Abstract

This study aimed at determining the identity of freshwater snails collected from selected water habitats frequented by wildlife as source of drinking water in the Matebeleland region of Zimbabwe, and further screen the identified snails for natural infections with amphistomes using PCR. A total of 487 freshwater snails were collected from six areas in the Matebeleland region of Zimbabwe for identification and screening of amphistome infection. Eight freshwater snail species were morphologically identified and only *Biomphalaria (Bio.) pfeifferi, Bulinus (B.) tropicus, B. truncatus, B. globosus* and *Lymnaea (Radix) natalensis* were confirmed using the *CoxI* gene. *Bulinus tropicus* and *Physa (Phy.) acuta* were the most abundant species at 33.9 % (165/487) and 31.2 % (155/487) respectively. DNA of amphistome was detected in 11.9 % (58/487) of the collected snails. The highest infection rate was detected in *B. globosus* (44.4 %). West Nicholson recorded the highest infection rate (33.9 %), and infection was not detected in *L. (R.) natalensis*, *Phy. acuta* and *Bellamya* spp. Amphistome DNA from *Melanoides (M.) tuberculata* was successfully sequenced and identified as *Calicophoron microbothrium*. An additional band was detected in *M. tuberculata*, *Bul. tropicus* and *Bul. trancatus* which showed a 96.42% similarity to *Paragonimus sp*. sequence in the GenBank.

Keywords: Gastropods, identification, diversity, amphistomes, molecular detection, Zimbabwe

4.2 Introduction

Amphistomes are digenetic trematodes that exhibit a heteroxenous life cycle that includes an intermediate and definitive host (Huson et al., 2017). Although over 70 amphistomes have been recorded globally to date (Ghatani et al., 2012; Malatji et al., 2021), the intermediate host snails have only been described for a few amphistomes species in sub-Saharan Africa (Pfukenyi et al., 2018). Amphistome species use several freshwater snail species as intermediate hosts (IHs) for transmission,

and these include species from the genus *Bulinus* (De Kock et al., 2005; Lofty et al., 2010; Chingwena et al., 2004), *Galba* (Dinnik, 1962; Eduardo, 1987), *Lymnaea* (*Radix*) (Sanabria et al., 2012; Rafiq et al., 2022), *Biomphalaria* (Laidemitt et al., 2012) and *Segmentorbis* (Laidemitt et al., 2012). According to Laidemitt et al. (2012), *Bulinus* species, which are the most widely distributed snails in sub-Saharan Africa, accounts for the wide distribution of *Calicophoron* spp. in Africa. Furthermore, several Bulinus species have been implicated in the transmission of *Calicophoron microbothrium*, an amphistome responsible for most cases of amphistomosis in both wild and domestic ruminants (Pfukenyi et al., 2018).

Like other trematodes, both domestic and wild ruminants get infected with amphistomes by grazing in pasture around drinking water points contaminated with metacercariae (Phiri et al., 2011). Infection in wild ruminants can be confirmed at post-mortem by the presence of immature stages in the duodenum, and adult amphistomes in the fore-stomachs of ruminants, and at ante-mortem through the detection of eggs in faeces (Zintl et al., 2014). However, surveys and monitoring of these infections in free-ranging wild ruminants can be challenging (Ryser-Degiorgis, 2013) due to factors such as accessibility and ethical issues as-sociated with capturing, handling, and releasing wild animals to collect non-invasive faecal samples (Fontoura-Goncalves et al., 2023). Moreover, identification up to species level using morphological characters is a challenge and at ante-mortem the egg morphology is not easily distinguishable with that of other trematodes such as *Fasciola* spp. (Gonzalez-Warleta et al., 2013).

Data from previous research in sub-Saharan Africa have indicated that majority of amphistomes species occurring in wild ruminants are also common in domestic ruminants, with exception to *Bilatorchis (Bi.) papillogenitalis*, *Carmyerius (Car.) bubalis* and *Cotylophoron (Cot.) macrosphinctris* which have only been documented in wild ruminants to date (Pfukenyi and Mukaratirwa, 2018, Sibula et al., 2024). These three species are amongst those with unknown intermediate snail hosts. Amphistomes infection in the intermediate snail hosts, can be detected through shedding of cercariae or squashing the snail soft tissue and visualizing the developmental stages using a light microscope, and subsequent morphological characterization of the cer-cariae/rediae/sporocyst (King and Van-As, 2001; Chingwena et al., 2002; Mereta et al., 2019). However, morphological characterization of these larval stages has limitations as most studies could only discriminate up to the genus level (Nzalawahe et al., 2015; Chimbwana and Nkwengulila, 2017; Mereta et al., 2019) with only a few studies able to distinguish to species level (Wright et al., 1979a; Wright et al., 1979b; Southgate et al., 1985; Southgate et al., 1989). Nonetheless, Kane et al. (2008) indicated that morphological identification of larval trematodes is error-prone and hard or impossible for genus or species level resolution.

Although some recent studies still utilize shedding of cercariae as sole detection method of infection (Mereta et al., 2019), most recent studies couple the use of shedding cercariae with molecular techniques such as Restriction Fragment length Polymorphism (RFLP) of Polymerase Chain Reaction (PCR) products (Molaba et al., 2023) or sequencing of the PCR products for identification up to species level (Lofty et al., 2010; Laidemitt et al., 2016; Molaba et al., 2023). Some studies also amplified the larval DNA directly from the snails, followed by sequencing (Laidemitt et al., 2016; Carolus et al., 2019) or RFLP of the products (Molaba et al., 2023). However, Schols (2019) showed detection of larval infection using PCR was better than cercariae shedding.

According to Carols et al. (2019), knowledge on the prevalence, diversity and ecology of both intermediate host freshwater snails and trematodes is key to understanding disease transmission dynamics and possible control of trematodes of economic and public health significance. Although there are keys to identify freshwater snails and trematode larval stages based on morphological characters, molecular methods are known to provide better resolution on identity of snail and trematodes to species level (Tookhy et al., 2023). Hence, the aim of this study was to determine the identity of freshwater snails collected from selected from water habitats serving as water sources for wildlife in selected game ranches, conservancies and game parks located in the Matebeleland region of Zimbabwe and the amphistome species they may transmit in these habitats.

4.3 Methodology

4.3.1 Study areas and sample collection

Freshwater gastropods were surveyed from water sources frequented by wildlife around game ranches, conservancies and game parks located in Inyathi, Nyamandlovu and Ntabazinduna, located in Matebeleland North, and Esigodini, West Nicholson and Matopos which are in Matebeleland South province of Zimbabwe. The map for the study area were constructed using QGIS (Figure 4.1).. Snail survey conducted in this study was from three different habitat types that include dams, waterholes, and a river. All the habitats had macrophytes (submerged vegetation), with Ntabazinduna sampling site having abundance brown algae macrophytes.

4.3.2 Sampling sites

Sampling sites were pre-selected based on the following criteria: little or no human activity, locality is a drinking water point for wild ruminants, easily accessible and was located in Matebeleland. There were a total of 19 samplling sites from the six study areas. The habitat type and vegetation cover were recorded for each site, and snails were collected using a scooping net and dredge for superficial and deep-water samples respectively by two assistants and spent an hour per site (Carolus et al., 2019). Sediments and aquatic plants were visually inspected for snails. All snails were pooled per site, placed in containers containing water from

the study site and transported to the lab at room temperature. Collection per site was done once during the drier seasons (September- November 2021). Once in the lab, thecollected snails were first washed with distilled water and thereafter preserved in 70% ethanol for morphological and molecular analysis.

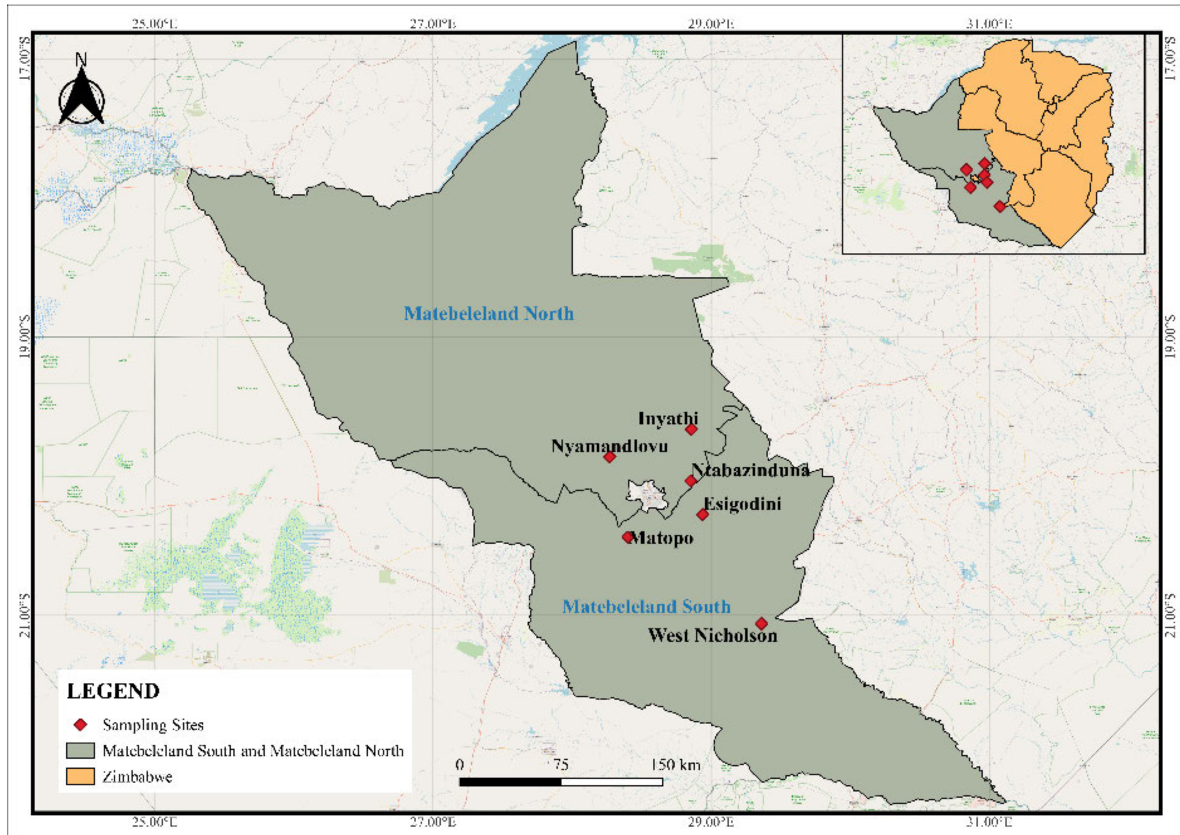

Figure 4.1 Showing all the sampling sites for the study located in Matebeleland region of Zimbabwe from where snail isolates were collected.

4.3.3 Morphological and molecular identification of snails

4.3.3.1 Morphological classification

Morphological identification of snails was based on the classification keys as described by Brown (1994) and this was done before preserving in ethanol for correct identification. Snails of the same species morphologically were grouped, and representative specimens were selected for confirmation using PCR.

4.3.3.2 DNA extraction from gastropods

Selected snail specimens which included 58 *M. tuberculata*, 20 *Bio. pfeifferi*, 165 *B. tropicus*, 61 *B. truncatus*, 18 *B. globosus*, 155 *Phy. acuta*, eight *L. natalensis* and two *Bellamya sp*. were washed with sterile water and excess water was removed with sterile absorbent paper. Tissue from foot of each snail or whole snail tissue was harvested using either a sterile blade or removing the whole snail tissue

and then frozen at -20ºC for two hours before use. DNA was extracted using a modified Quick-DNATM Tissue Miniprep Kit (Zymo Research Corporation, Irvine, CA 92164, USA) protocol.

4.3.3.3 Molecular characterization of gastropods

Extracted DNA were amplified based on the COI region using the primers COI (F) 5'-TAATGTWATTGTTACAGCACATG-3' and COI (R) 5'-GTTGRTATAAAATAGCATCACCW-3' (Carolus et al., 2019). PCR was performed in a total reaction volume of 25µl, composing of 5 µl of 5X One Taq PCR buffer, 0.4 µl of 10mM dNTPs, 0.5 µl forward primer (10mM), 0.5 µl reverse primer (10 mM), 3 µl of 25mM MgCl2, 2 µl DNA template, 0.125 µl of One Taq polymerase (NEB, England) and nuclease free water to make up to the final volume. The cycling protocol for the reaction were as follows: initial denaturation at 94°C for 3 mins, followed by 30 cycles of denaturation at 94°C for 30 secs, annealing at 64°C for 45 secs, extension at 68ºC for 1 min and a final extension of 68ºC for 15 mins. The fragments were separated on 1% agarose stained with ethidium bromide, and successful amplicons were identified by a band at 630 base pairs. Amplicons were sent to Inqaba Biotech for sanger sequencing.

4.3.4 Molecular detection of amphistomes from snail tissue samples

Snails were assessed for amphistome infection using the primers GA1 (5'-AGAACATCGACATCTTGAAC-3') and BD2 (5'-TATGCTTAAATTCAGCGGGT-3') (Laidemitt et al., 2017). *Calicophoron microbothrium* DNA was used as a positive control/signal for amphistome DNA. The PCR mix was composed of 2 µl of 10X PCR buffer (Super Therm), 0.8 µl of 10 mM dNTPs, 0.4 µl forward primer (10µM/µl), 0.4 µl reverse primer (10µM/µl), 1.6 µl 25 mM MgCl2, 1 µl DNA template, 0.2 µl Super Therm polymerase and nuclease free water to make up to a total reaction volume of 20 µl. Amplification was performed under the following thermocycling conditions: 95°C for 3 mins, followed by 30 cycles of 95°C for 30 secs, 55°C for 45 secs, and 72ºC for 1 min, and a final extension of 72ºC for 15 mins. Amplicons were separated on a 1% agarose gels stained with ethidium bromide, and positive isolates were identified by a band at approximately 385bp. Positive amplicons were sent Inqaba Biotechnical Industries (Pretoria, South Africa) for Sanger sequencing.

4.3.5 Phylogenetic relationships of snails and amphistomes from snail tissue samples

Sequences were viewed, assembled and manually edited using BioEdit version 7.2 (Sequence Alignment Editor) (Hall, 1999), and NCBI BLAST (Basic local alignment search tool) was used to identify the closest matches available on GenBank database. Sequences were trimmed to common length of 455 nucleotides for snails and 380 nucleotides for amphistomes. Tamura 3-parameter (T92) was selected as the best model fit for the dataset, and the Neighbour-joining (NJ) and Maximum likelihood trees were generated on MEGA 7 software (Kumar et al., 2016). The phylograms were 50% majority-rule and the nodal support was estimated using 1000 bootstrap pseudo-replicates for both methods.

4.4 Results

4.4.1 Description of snail habitats

From the six localities visited in Matebeleland region (Figure 1), 19 water sites were surveyed and these were predominantly man-made waterholes in Nyamandlovu (n = 13), dams in West Nicholson (n = 2), Matopos National Park (n = 1), Ntabazinduna (n = 1), and Esigodini (n = 1) and lastly, a river in Inyathi (n = 1) (Table 4.1). All habitats had different types and levels of submerged vegetations, and only habitats from West Nicholson, Esigodini and Inyathi had trees on their periphery. The surrounding areas were clear, with decaying matter found in dams in West Nicholson and Ntabazinduna. Results show that although all 13 sites from Nyamandlovu had solely wildlife activity, some sites showed a level of interaction or shared habitat with livestock (n = 4) and to a lesser extent humans (n = 1).

Table 4.1 Description of habitat type and animal/human activity at localities where freshwater snails were collected in the Matebeleland region of Zimbabwe.

Locality	No. of habitats surveyed	No. of habitats with snails	Habitat type	Vegetation cover/ Description	Animals/human activity
Nyamandlovu	13	3	Water-holes made of concrete (typically less than 40m^2)	Sub-merged vegetation in some waterholes but typically some waterholes are clear	Wild ruminants (100%),
West Nicholson	2	2	Dams	A lot of submerged vegetation, trees on the periphery and decaying organic matter	Wild animals (100%)
Esigodini	1	1	Dam	Submerged vegetation and a lot of trees on the periphery	Wild ruminants (50%), livestock (40%) and human activity (10%)
Ntabazinduna	1	1	Dam	Very little submerged vegetation with no trees on the periphery but a lot of decaying organic matter. More brown algae macrophytes observed.	Wild ruminants (30%), livestock (70%)
Matopos	1	1	Dam	A lot of submerged vegetation with no trees on the periphery	Wild ruminants (80%) and 20% livestock
Inyathi	1	1	River	Few submerged vegetation with trees on the periphery	50% wild ruminants and 50% livestock
Total	19	9			

4.4.2 Morphologically identified snail species and their abundance.

Snails were found at nine of 19 sites (47.37 %), while none were found at 10 sites from Nyamandlovu (Table 4.1). A total of 487 gastropods were collected, and these were from Nyamandlovu (n = 212) and Ntabazinduna (n = 174), followed by West Nicholson (n = 59), Esigodini (n = 21), Matopos National Park (n = 18) and to a lower extent Inyathi (n = 3) (Table 4.2). From these collections, eight species were morphologically identified as *M*. *tuberculata*, *B*. *globosus*, *B*. *truncatus*, *B*. *tropicus*, *Bio*. *pfeifferi*, *Phy*. *acuta*, *L*. *natalensis*, and *Bellamya* spp. (Table 4.2).

Table 4.2 Morphological and molecular identity of snail species and number collected in the different localities surveyed in Matebeleland region of Zimbabwe.

Morphology identification	Molecular identification		Number of snails collected per locality						Total	% Overall Prevalence per snail species
	Species	% Similarity	West Nicholson	Esigodini	Inyathi	Matopos	Nyamandlovu	Ntabazinduna		
M. tuberculata	ND	-	58	-	-	-	-	-	58	11.9
Bio. pfeifferi	*Bio. pfeifferi*	100	-	13	-	7	-	-	20	4.1
B. tropicus	*B. tropicus*	99.78	-	-	3	5	58	99	165	33.9
B. truncatus	*B. truncatus*	99.78	-	-	-	-	-	61	61	12.5
B. globosus	ND	-	1	5	-	-	-	12	18	8.2
Phy. acuta	ND	-	-	3	-	-	152	-	155	31.2
L. natalensis	*L. natalensis*	98.68	-	-	-	6	2	-	8	1.6
Bellamya spp.	ND	-	-	-	-	-	-	2	2	0.4
Total			59	21	3	18	212	174	487	
% Prevalence per locality			12.1	4.3	0.6	3.7	43.5	36		

ND = Not done

Bulinus tropicus was the most distributed species across sites and found in four of the six surveyed areas. Furthermore, this species was the most abundant and contributed 33.9 % (165/487) of the collected snail populations. This was followed by *Phy. acuta* which contributed 31.2 % (155/487) and was found in Esigodini and Nyamandlovu*. Bulinus truncatus* and *M. tuberculata*, which were each found in Ntabazinduna and West Nicholson contributed 12.5 % (61/487) and 11.9 % (58/487), respectively. Surprisingly, *Bio*. *pfeifferi*, *B. globosus* and *L. natalensis*, were found in multiple areas, though in low numbers of 20/487 (4.1 %), 18/487 (3.7 %) and 8/487(1.6 %), respectively. The least collected snail species was *Bellamya* spp., which was found in Ntabazinduna and contributed 0.4 % (2/487) of the collected snail population.

4.4.3 Molecular confirmation and phylogenetic relationship of snail species.

Of the eight species morphologically identified, four snail species were confirmed with a BLAST similarity index ranging between 98.24 to 100 % (Table 4.2). Amongst these was 1G, which showed a 100 % homology with *Bio. pfeifferi* from Zimbabwe (DQ084829). However, the phylogenetic tree showed a moderate support between this isolate and other GenBank isolates (Figure 4.2). The *Bio. pfeifferi* isolated formed a well-supported sister clade to *Bulinus* species, which falls under the same family. Our isolate NB showed a 99.78 % identity with *B. truncatus* isolate from Iran (KT365867) and formed a well-supported clade with other *B. truncatus* isolates. These isolates formed a strongly supported monophyletic sister clade with *B. tropicus* isolates, including those from this study (2R and IT) (Figure 4.2) which showed 99.78 % homology with *B. tropicus* from Uganda (MN551550). Lastly, our *L. natalensis* isolates (LN and N3) (Figure 4.2) showed a homology of 98.68 % with *L. natalensis* from Malawi (EU818835). These species formed a well-supported separate clade from the *Bulinus* and *Biomphalaria* clade. However, the relationship between the isolates was moderately to weakly supported. The sequences generated from this study were submitted to NCBI GenBank under the accession numbers PP389543 – PP389548. Sequences for *B. globosus* failed quality control, while sequencing failed for *M. tuberculata*, *Phy. acuta*, and *Bellamya* spp. The time lapse between the PCR and the amplicons reaching South Africa from Zimbabwe, for post clean-up and sequencing might have compromised the quality of the PCR products, and possibly degradation.

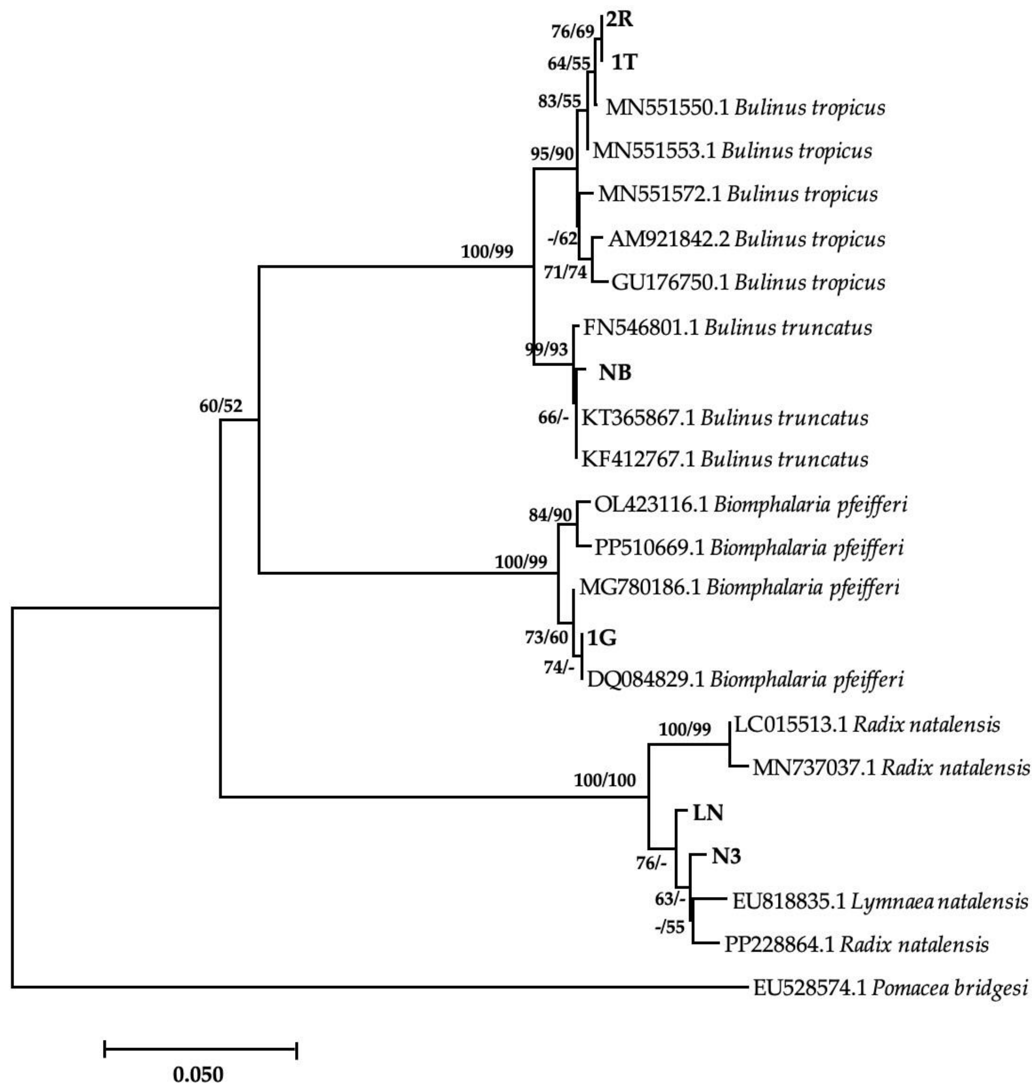

Figure 4.2 Neighbor-joining tree based COI gene illustrating the relationship freshwater snails obtained from game ranches, conservancies and game parks located in the Matebeleland in Zimbabwe, and the closest matches from the NCBI GenBank. The nodal support value indicated in the order: neighbour-joining and maximum likelihood. 2R, IT, NB, 1G, LN, N3 = Isolates from the study.

4.4.4 Molecular detection of amphistome DNA in field-collected snails

All 487 gastropod isolates were individually screened for amphistome DNA based on the ITS-2 gene. A total of 58 out of 487 (11.9 %) snails showed a band at approximately 385bp (Figure 4.3), which was consistent with a *C. microbothrium* isolate used as a control for amphistomes. *Bulinus globosus* recorded the highest prevalence of amphistome infections (44.4 %), followed by *M. tuberculata* (33.9 %) (Table 4.3). No amphistomes DNA was detected in *Phy. acuta*, *L. natalensis* and *Bellamya* spp. The highest prevalence of amphistomes infections in snails per locality was recorded in West Nicholson, with a prevalence of 33.9 % in *M. tuberculata*. In contrast, snails from Inyathi did not harbor any amphistomes, and those from Nyamandlovu had a prevalence of 2.4 % in *B. tropicus* despite the area

recording the highest number of gastropod specimens collected. Sequencing of amphistome amplicons was however only successful for a sample from *M. tuberculata*, which was confirmed as *C. microbothrium* with a percentage similarity of 100 % (Table 4.4) and submitted on GenBank under the accession number PP392962. The remaining samples, which were majority failed quality control.

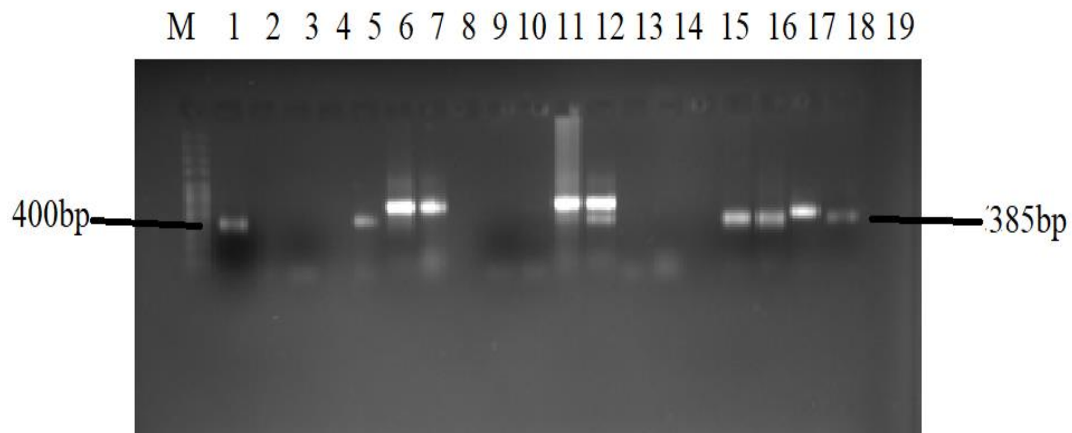

Figure 4.3: Representative gel image of an ITS-2 gene amplification of snail isolates. Isolate 19 is a negative control, isolate 18 is the positive control (*C. microbothrium*) of around 385bp and M is a 100bp marker. Isolate 11 and 12 showing positive mixed infection of snails.

Table 4.3 Prevalence per locality of amphistome infection in freshwater snails collected from Matebeleland region of Zimbabwe as detected by PCR.

Gastropod species	No. of snails screened	Snails positive for amphistome DNA per locality						Total infected	% Prevalence
		West Nicholson	Esigodini	Inyathi	Matopos	Nyamandlovu	Ntabazinduna		
M. tuberculata	58	20	-	-	-	-	-	20	34.5
Bio. pfeifferi	20	-	1	-	0	-	-	1	5
B. tropicus	165	-	-	0	1	5	14	20	12.1
B. truncatus	61	-	-	-	-	-	9	9	14.8
B. globosus	18	0	5	-	-	-	3	8	44.4
Phy. acuta	152	-	-	-	-	0	-	0	0
L. natalensis	12	-	-	-	0	0	-	0	0
Bellamya spp.	2	-	-	-	-	-	0	0	0
Total infected		20	6	0	1	5	26	58	
N	487	59	21	3	18	212	174	487	
% Prevalence	-	33.9	28.6	0	5.6	2.4	14.9	-	-

Additional band was observed at approximately 280-290 base pairs from *M. tuberculata* (n = 9) and *B. tropicus* (n = 1) from Esigodini and *B. truncatus* (n = 1) from Ntabazinduna indicating mixed infection. Sequencing of three of the additional band from *M. tuberculata* and BLAST analysis showed a similarity index of 96.42 % to *Paragonimus kellicotti* (Table 4.4). The sequences were submitted on GenBank as *Paragonimus* sp. under the accession numbers (PP392960, PP392961 and PP392963). Phylogenetic analysis showed that these sequences formed a clade with two *P. kellicotti* isolates from India (KC523868.1) and the United States (JF4177709.1) from *Indoplarnobis exustus* and *Orconectes virilis*, respectively. The genetic distance within this clade was 3 %. This clade also formed a monophyletic sister clade with various *Paragonimus* species, including two more *P. kellicotti* (Figure 4.4). The genetic p-distance between these two clades was 28 %.

Table 4.4 BLAST percentage similarity of trematode DNA based on the ITS-2 gene obtained from snails collected in the Matebeleland region of Zimbabwe.

Sample ID	Fragment size	Species ID based on sequence	% Similarity	IH species (source)
MO1	290bp	*Paragonimus kellicotti*	96.10	*B. truncatus*
MB1	290bp	*Paragonimus kellicotti*	96.42	*M. tuberculata*
MB2	385bp	*C. microbothrium*	100	*M. tuberculata*
NT	290bp	*Paragonimus kellicotti*	96.42	*B. truncatus*

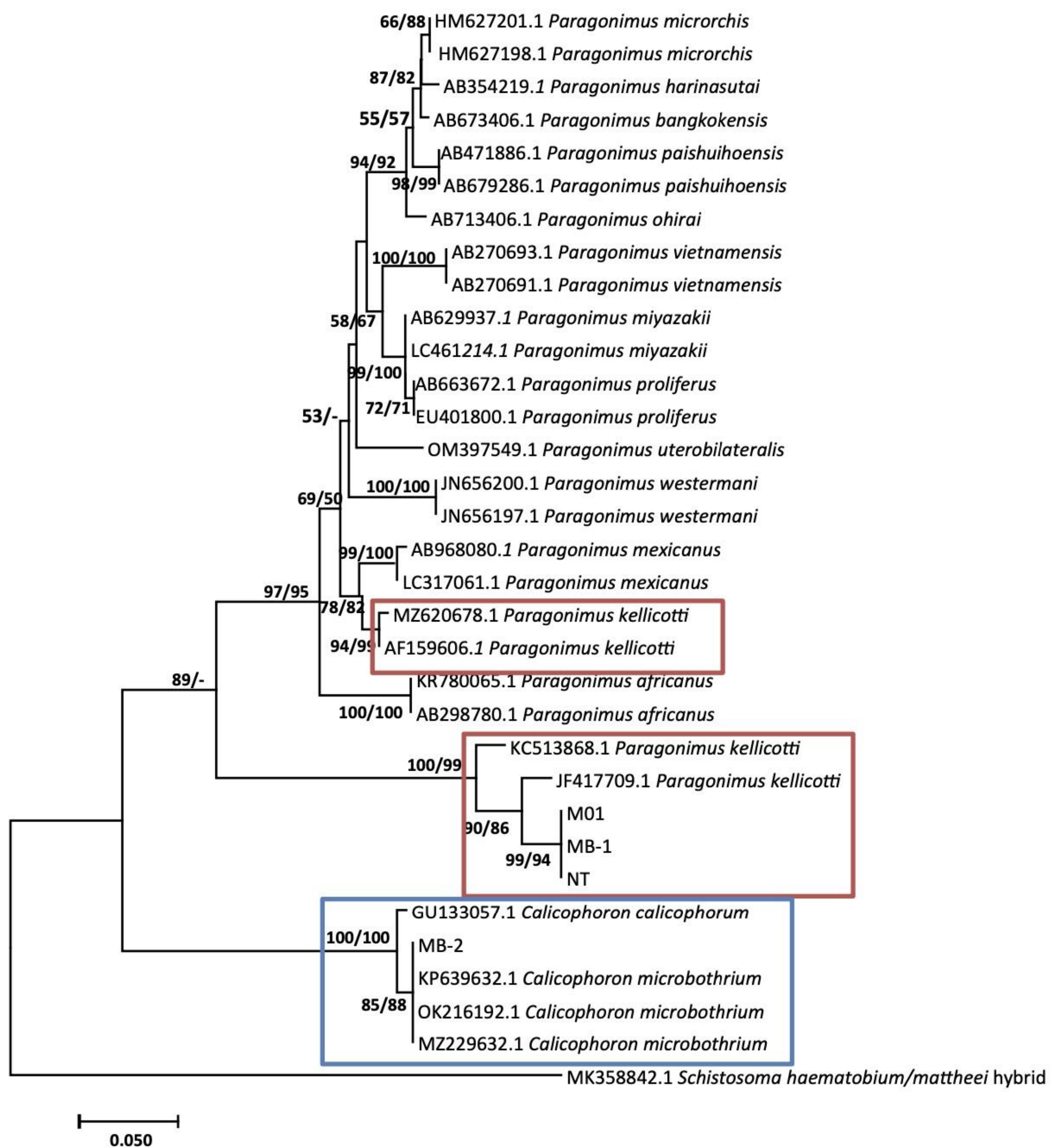

Figure 4.4 Neighbor-joining tree based ITS-2 gene illustrating the relationship trematodes isolates from freshwater snails from the Matebeleland in Zimbabwe, and the closest matches from the NCBI GenBank. The nodal support values indicated in the order: neighbor- joining and maximum likelihood.

4.5 Discussion

According to Min et al. (2022), physico-chemical properties and macrophyte abundance together with other factors influence the diversity of freshwater snails. The most abundant snail species were collected from Ntabazinduna, accounting for 36% of the total number of snails. Middelboe and Markager (1997) suggested that the presence of submerged macrophytes increase periphyton, the food source of *Bulinus* spp. and *Biomphalaria pfeifferi*. In this study, a total of eight snail species

collected from Matebeleland region of Zimbabwe in selected areas included *B. globosus*, *B. tropicus*, *B. truncatus*, *M. tuberculata*, *Bio. pfeifferi*, *L. natalensis* and *Phy. acuta* and these have been implicated in the transmission of amphistome species in wildlife and domestic ruminants (Pfukenyi and Mukaratirwa, 2018) except *Phy. acuta* and *Bellamya* spp.

Results showed that *B. tropicus* was the most widely distributed and abundant species in the Matebeleland region of Zimbabwe. This was not surprising as earlier reports have indicated that this species is the most widely distributed freshwater snail in Zimbabwe (Makura and Kristensen, 1991) and is adapted well to a wide variety of environments (Graber and Daynes, 1974). However, Chingwena et al. (2004) later reported that *B. tropicus* was the most abundant species in the lowveld and the third most abundant snail species following *L. natalensis* and *B. globosus* in the highveld. Although this still explains the abundance of this species in the Matebeleland region which falls within the middle to highveld, *L. natalensis* and *B. globosus* were however surprisingly collected in low numbers in this study. *Physa acuta*, the second abundant species is an invasive snail which was previously reported in Zimbabwe (Appleton, 2003). Although this species showed a limitation in distribution and the one locality where this snail species was collected, had macrophytes in abundance compared to other species. This is not surprising as not only several authors have shown that this species is now widely distributed in Zimbabwe (Schols et al., 2021) and is also invasive (De Kock and Wolmarans, 2007).

Of all the eight snail species screened for amphistome DNA, *M. tuberculata*, *Bio. pfeifferi*, *B. tropicus*, *B. globosus* and *B. truncatus* were positive. Amphistome infections in the *Bulinus* and *Biomphalaria* species were not surprising as a wide range these snail species have been reported to act as intermediate hosts for different amphistome species in sub-Saharan Africa (Dinnik, 1961; Dinnik, 1965, Graber and Daynes, 1974; Wright et al., 1979b; Chingwena et al., 2004). Although *B. tropicus* was reported to act as an intermediate host of *C. microbothrium*, with high prevalence on experimental infections (Chingwena et al., 2004) our study reported a high prevalence of amphistome DNA in *B. globosus*. *Melanoides tuberculata* showed the second highest prevalence of amphistome infection in this study. Previous reports have already confirmed the susceptibility of this species through experimental infections with *C. microbothrium* in Zimbabwe (Chingwena et al., 2004) and South Africa (Malatji et al., 2021), and to another trematode species, *Gastrodiscus aegyptiacus*, in Zimbabwe (Mukaratirwa et al., 2004). Based on the successful identification of *C. microbothrium* from *M. tuberculata* through sequencing, to the best of our knowledge, this is the first study to confirm natural infection of this Thiaridae species with *C. microbothrium* in Africa.

Results showed that amphistome DNA was not detected in three snail species namely *Phy. acuta*, *Bellamya* spp. and *L. natalensis*. Although earlier experimental studies have showed that *L. natalensis*

was refractory to *C. microbothrium* (Chingwena et al., 2004), this species was found infected with *Gastrothylax*/*Paramphistomum* in Zimbabwe (Mukaratirwa et al., 2004). Similar pattern was observed with *Phy. acuta*, which was successfully infected with *G. aegyptiacus* during dissection after experimental exposure (Mukaratirwa et al., 2004), but no amphistomes DNA was detected from this species despite being collected in high numbers, indicating that there might be amphistome species IH specificity and this may be confirmed through experimental studies like those conducted by Chingwena et al. (2004).

In our results, an overall prevalence of 11.9% amphistome infection was noted. However, considering that collection was done in the drier months when parasitemia was high, this could account for the high prevalence observed. It was noted by Pfukenyi and Mukaratirwa (2018) that the larval stages are found in high titers during the drier months. The high prevalence is also of public health importance where habitats are shared with livestock and humans as that could raise disease incidence in livestock resulting in possible amphistomosis outbreaks. Furthermore, although not documented, there is a possibility of amphistomes affecting humans being found in the wild.

Results from this study revealed cases of mixed infections of amphistomes DNA with other with DNA of a trematode species identified as *Paragonimus*-like species. in *B. truncatus* and *M. tuberculata*. *Paragonimus* spp*.* are lung flukes which have been reported worldwide including Africa and mainly in West and Central African region. Species reported in the West and Central Africa include *P. africanus*, *P. gondwanensis*, *P. kerberti* and *P. uterobilateralis* (Cumberlidge et al., 2018). According to Procop (Procop, 2009), *Paragonimus* species usually utilize snail species from families Pleuroceridae and Thiaridae as IHs. While results from this study and previous reports link paragonimiasis with presence of *M. tuberculata* in South Africa (Appleton, 2014), which may explain the infections observed in *M. tuberculata* in this study, the phylogenetic positioning of these isolates and the high genetic distance with other reported *Paragonimus spp.* raises concerns of whether these isolates belong to the genus *Paragonimus*, or the sequences deposited on GenBank are misidentified under the genus *Paragonimus*. Mixed infection of snails with an amphistome and another trematode has been recorded by Wright et al., (1979); Southgate et al. (1985) and Southgate et al. (1989). Some mixed infections may affect the host causing it to be susceptible to infections by other parasites or may adversely affect the snail.

4.6 Conclusions

Eight freshwater snail species common at drinking water sources frequented by wildlife from Matebeleland region of Zimbabwe were identified in this study with *B. tropicus* and *Phy. acuta* as the predominant species and overall prevalence of amphistome DNA (*C. microbothrium*) was 11.9 %.

Prevalence rate was higher in *B. globosus* followed by *M. tuberculata*. The highest number of snails were collected from Nyamandlovu, meanwhile the highest prevalence of amphistome DNA was recorded in Beitbridge. A single sample was confirmed as *C. microbothrium* through DNA sequencing and confirmation of mixed infection with *Paragonimus* species-like trematode. Failure to sequence other amphistome samples shows the need to specifically use the larval stage from the infected snails (sporocysts, rediae or cercariae), to increase the quantity and quality of DNA to be successful in identifying and characterizing the amphistome species found in freshwater snails. There is a need to also develop and apply the use of primers and protocols which do not require sequencing such as the LAMP protocols and PCR-RLFP to discriminate among multiple amphistome species of wildlife in IH snails. We recommend future studies such as collecting amphistome specimens from culled wildlife ruminants from the study locations for morphological and molecular identification.

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Chapter 5

Conclusion

5.1 General discussion and conclusions

The study was set to identify and characterise amphistome species in wild ruminants and freshwater snails in the Matebeleland, Zimbabwe. A systematic literature search on the amphistomes species and their hosts was conducted in Africa between 1900 and 2023. This was conducted to obtain knowledge on the species diversity of amphistomes recorded in wild ruminants, and also determine the distribution and intermediate hosts and wild ruminants involved in the transmission. Literature showed that 36 amphistomes were previously documented in wild ruminants in Africa. Of these, studies also showed that *C. microbothrium* was the most documented species. However, the review mostly highlighted the paucity of studies using molecular techniques to identify the species, despite the wide range of species reported in the continent.

A survey was conducted in wild ruminants killed during professional hunting expeditions from three parks and conservancies to determine the prevalence of amphistome infectionand the snail speciesserving as definitive hosts. This survey was conducted during the hunting season between 2019-2021. Our study showed an overall prevalence of 10.22 %, with high infections recorded in the waterbuck (63%) and intensity of infection of >10 000 parasites per animal.

Morphologically, eight amphistome species were identified across the three sampling sites. Of these, *Gastrothylax crumenifer*, *Leiperocotyle gretillati* and *Orthocoelium dicranocoelium* were first records for Zimbabwe. Our results corroborated with previous studies that the *Calicophoron* genus is common and widely distributed in both wild ruminants and domestic animals in Africa. This genus contributed over 50 % of the specimens collected and showed more species diversity. Likewise, *C. microbothrium* was the most common species. This then led to failure to confirm some isolates to species level. Freshwater snails were collected from water sites which were frequently visited by these animals as source for drinking water to determine the species and to screen for amphistome DNA based using molecular techniques.

Eight snail species were identified from nine habitats and were predominantly Planorbidae (*Bulinus* and *Biomphalaria*) snails, which was not surpising as these snail species are widely distributed. Screening of infections detected amphistome DNA in *Bulinus globosus*, *B. tropicus*, *B. truncatus*, *Bio. pfeifferi*, and *Melanoides tuberculata*. This was not surprising as previous studies also showed that amphistomes species are predominantly transmitted by Planorbidae species, although only 10 out of

36 amphistomes have known or documented intermediate hosts. Prevalence was higher in *B. globosus* from West Nicholson, located in Beitbridge. The study confirmed for the first time a natural infection of *Melanoides tuberculata* with *C. microbothrium* in southern Africa. Additionally, the presence of *Paragonimus* sp. was detected in *M. tuberculata, Bulinus tropicus* and *Bulinus truncatus*.

5.2 Limitations and recommendations

This study hadl two limitations (i) the lack inter species variation observed with the primers designed and primarily used to identify amphistome species. While these primers are predominantly used globally, most studies focus on one species and few isolates, which allows an easy control of data. However, when more than one species is being considered as the case with with this study, the marker was not successful in differentiating between species. (ii) The failure of obtaining sequences for amphistomes species from snails. Despite all isolates amplifying for amphistome infection, species of infection was only determined in one snail specimen. The difficulties in sequencing the amphistome DNA and intra snail DNA highlights the importance of developining species specific primers that can differentiate between species without sequencing, and development of Loop-mediated isothermal amplification (LAMP) protocols, and microsatellites for amphistome species available in Africa. This will assist in identifying the diverse amphistome species of wild ruminants in the African region and linking them to their snail hosts, and also in surveillance and monitoring of infections especially at wildlife-livestck interface, which future studies should focus on.

While sequencing based on the ITS-2 rDNA gene was successful and able to confirm species identification for some species, the primers used showed little to no variation between species, especially within the *Calicophoron* genus. This may have been caused by a number of reasons including submissions that have backing morphological data. A way forward would be to use other regions such as the Cox1 gene together with the ITS

Appendices

Appendix 2.1 Summary of studies reporting on the occurrence of amphistome species and their wild ruminants hosts in Africa between 1900-2022.

Country	Host species	Amphistome species	Diagnostic/ identification technique	Predilection site	Author/ Year
Southern Africa					
South Africa	Impala (*Aepyceros melampus),* Red hartebeest (*Alcephalus busephalus),* Springbok *(Antidorcas marsupialis),* Blue wildebeest *(Connochaete taurinus),* Black wildebeest (*Connochaete gnou),* Blesbuck *(Damaliscus albifrons),* Common Tsessebe (*Damaliscus lunatus),* Red lechwe *(Kobus leche),* Bohor reedbuck (*Redunca redunca),* African Buffalo (*Syncerus caffer)*	*Calicophoron (Cal.) calicophorum, Cal. microbothrium, Cotylophoron (Cot.) cotylophorum, Gastrothylax (Gas.) crumenifer*	Fluke morphology	-	Ortlepp, 1961
South Africa	Impala (*Aepyceros melampus)*	*Cot. cotylophorum*	Fluke morphology	Rumen and reticulum	Anderson, 1983
South Africa	Blesbok *(Damaliscus Dorcas),* Roan Antelope (*Hippotragus equinus),* Impala (*Aepyceros melampus),* Red lechwe (*Kobus leche),* Puku *(Kobus vardoni),* Red hartebeest *(Alcephalus busephalus),* Black wildebeest (*Connochaete gnoue)*	*Cal. microbothrium, Cal. raja*	Fluke morphological	Rumen, reticulum and rarely abomasum	Eduardo, 1983
South Africa	Red lechwe *(Kobus leche)*	*Gas. crumenifer*	Fluke morphology	-	Sey, 1983
South Africa	Grey duiker (*Sylvicapra grimmia*)	*Paramphistomum spp.*	Fluke morphology	-	Boomker et al., 1987
South Africa	Tsessebe (*Damaliscus lunatus lunatus*)	*Paramphistomum spp.*	Fluke morphology	Rumen and reticulum	Reneicke et al., 1988
South Africa	Common reedbuck (*Redunca arundinum*), Kudu (*Tragelaphus strepsiceros*)	*Paramphistomum sp.*	Fluke morphology	-	Boomker et al., 1989

South Africa	Black wildebeest (*Connochaete gnou)*	*Cal. raja*	Fluke morphology and molecular characterization	Stomach	Ikeuchi et al., 2022
Botswana	Red lechwe (*Kobus leche)*	*Gigantocotyle (Gig.) symmeri*	Fluke morphology	Rumen, omasum and abomasum	Eduardo, 1984
Botswana	Red lechwe (*Kobus leche*), Puku (*Kobus vardoni*), Impala (*Aepyceros melampus*)	*Cal. raja, Cal. microbothrium*	Fluke morphology	Rumen, reticulum and rarely abomasum	Eduardo, 1983
Zambia	Eland (*Tragelaphus oryx),* Mountain reedbuck (*Redunca arundinum),* Red lechwe (*Kobus leche),* Sitatunga (*Tragelaphus spekei),* Southern reedbuck (*Redunca arundinum)*	*Cot. cotylophorum, Explanatum (Exp.) explanatum, Gas. crumenifer*	Fluke morphology	-	Le roux, 1932
Zambia	Red lechwe (*Kobus leche*)	*Gig. symmeri*	Fluke morphology	Stomach	Yeh, 1957
Zambia	Lichteinsteini's Hartebeeste (*Alcelaphus lichteinsteini),* Greater Kudu (*Tragelaphus strepsiceros)*	*Carmyerius (Car.) spatiosus, Cot. cotylophorum*	Fluke morphology	Rumen	Le Roux, 1934
Zambia	Mountain reedbuck (*Redunca arundinum),* Puku (*Kobus vardoni),* Red lechwe (*Kobus leche)*	*Choerocotyloides (Cho.) onotragi*	Fluke morphology	Caecum	Prudhoe et al., 1964
Zambia	African buffalo (*Syncerus caffer)*	*Car. spatiosus, Car. mancupatus, Stephanopharynx (Ste.) compactus*	Fluke morphology	Rumen, reticulum and omasum	Wright et al., 1979
Zambia	African buffalo (*Syncerus caffer)*	*Bilatorchis (Bil.) papillogenitalis*	Fluke morphology	Rumen	Eduardo, 1980
Zambia	Bongo (*Alcelaphus boocerus eurycerus),* Red lechwe (*Kobus leche)*	*Car. bubalis, Gastrothylax crumenifer*	Fluke morphology	-	Sey, 1983
Zambia	African Buffalo (*Syncerus caffer),* Bohor reedbuck (*Redunca redunca),* Bushbuck (*Tragelaphus scriptus),* East African oryx (*Taurotragus oryx),* Greater Kudu (*Tragelaphus strepsiceros),* Impala (*Aepyceros melampus)*	*Cal. raja, Cal. phillerouxi, Cal. microbothrium*	Fluke morphology	Rumen, reticulum and rarely abomasum	Eduardo, 1983

Zambia	African buffalo (*Syncerus caffer)*	*Gig. symmeri*	Fluke morphology	Rumen, omasum and abomasum	Eduardo, 1984
Zambia	African buffalo (*Syncerus caffer),* Blue wildebeest (*Connochaete taurinus)*	*Ste. compactus*	Fluke morphology	Reticulum and omasum	Eduardo, 1986
Zambia	Red lechwe (*Kobus leche*), Tsessebe), (*Damaliscus lunatus* lunatus), Defassa waterbuck (*Kobus defassa*)	*Calicophoron sp.*	Fluke morphology	Gastro intestinal tract	Zieger et al., 1998
Zimbabwe	Greater Kudu (*Tragelaphus strepsiceros)*	*Cot. cotylophorum*	Fluke morphology	-	Mettrick, 1962
Zimbabwe	Sitatunga (*Tragelaphus spekei)*	*Car. spatiosus*	Fluke morphology	-	Pike and Condy, 1966
Zimbabwe	African Buffalo (*Syncerus caffer),* Blue wildebeest (*Connochaete taurinus),* Red lechwe (*Kobus leche)*	*Cal. raja, Cal. microbothrium*	Fluke morphology	Rumen, reticulum and rarely abomasum	Eduardo, 1983
Zimbabwe	Sitatunga (*Tragelaphus spekei)*	*Gig. symmeri*	Fluke morphology	Rumen, omasum and obamasum	Eduardo, 1984
Namibia	African Buffalo (*Syncerus caffer),* Black wildebeest (*Connochaete gnou),* Gemsbok (*Oryx gazelle)*	*Cal. raja*	Fluke morphology	Rumen, reticulum and rarely abomasum	Eduardo, 1983
Angola	Mountain reedbuck (*Redunca arundinum)*	*Ste. compactus*	Fluke morphology	Rumen	Eduardo, 1986
Swaziland	Bohor reedbuck (*Redunca redunca*)	*Ste. compactus*	Fluke morphology	-	Ortlepp, 1961
East Africa					
Malawi	Hartebeest (*Alcelaphus sp)*	*Exp. explanatum*	Fluke morphology	Stomach	Maplestone, 1923
Tanzania	African Buffalo (*Syncerus caffer*), Blue wildebeest (*Connochaete taurinus*), Bohor reedbuck (*Redunca redunca*), Impala (*Aepyceros melampus*), Puku (*Kobus vardoni*), Red hartebeest (*Alcephalus busephalus*), Thomson's gazelle (*Gazella thomsoni*), Topi (*Damaliscus korrigum*), Defassa waterbuck (*Kobus deffasa*), Common Eland (*Taurotragus oryx)*	*Cal sukumum, Cal. phillerouxi, Cal. sukari, Cal. raja, Cot. cotylophorum, Ste. compactus, Car. mancupatus*	Fluke morphology	Rumen	Sach and Sach, 1968

Tanzania	African Buffalo (*Syncerus caffer),* Black wildebeest (*Connochaete gnou),* Blue wildebeest (*Connochaete taurinus),* Bohor reedbuck (*Redunca redunca),* Impala (*Aepyceros melampus),* Puku (*Kobus vardoni),* Red hartebeest (*Alcephalus busephalus),* Sable (*Hippotragus niger),* Thomson's gazelle (*Gazella thomsoni),* Topi (*Damaliscus korrigum),* Defassa waterbuck (*Kobus deffasa)*	*Cal. bothriophoron, Cal. raja, Cal. clavula, Cal. phillerouxi, Cal. sukumum, Cal. microbothrium*	Fluke morphology	Rumen, reticulum and rarely abomasum	Eduardo, 1983
Tanzania	Sable (*Hippotragus niger)*	*Cot. jacksoni*	Fluke morphology	Rumen	Eduardo, 1985a
Kenya	Red hartebeest (*Alcephalus busephalus)*	*Cot. jacksoni*	Fluke morphology	-	Nasmark, 1937
Kenya	Common Eland (*Taurotragus oryx),* Defassa waterbuck (*Kobus deffassa)*	*Cal. bothriophoron, Cal. microbothrium*	Fluke morphology	Rumen and reticulum	Eduardo, 1983
Kenya	Blue wildebeest (*Connochaete taurinus)*	*Cal. raja*	Fluke Morphology	Gastro intestinal tract	Mijele et al., 2016
Uganda	African Buffalo (*Syncerus caffer),* Impala (*Aepyceros melampus),* Red hartebeest (*Alcephalus busephalus)*	*Cal. calicophorum, Cal. microbothrium*	Fluke morphology		Mettam, 1932
Uganda	Red hartebeest (*Alcephalus busephalus*)	*Cot. jacksoni*	Fluke morphology	-	Nasmark, 1937
Uganda	African Buffalo (Syncerus caffer)	*Cot. cotylophorum, Cal. clavula, Cal. phillerouxi*	Fluke morphology	-	Dinnik et al., 1963
Uganda	African Buffalo (*Syncerus caffer),* Kob (*Kobus kob)*	*Cal. phillerouxi, Cal. clavula,*	Fluke morphology	Rumen	Eduardo, 1983
Uganda	African Buffalo (Syncerus caffer)	*Cot. macrosphinctris*	Fluke morphology	Rumen	Eduardo, 1985a
Ethiopia	East African oryx (*Oryx beisa),* Klipspringer (*Oreotragus oreotragus),* Menelik bushbuck (*Tragelaphus scriptus meneliki),* Mountain Nyala (*Tragelaphus buxtoni)*	*Cal. daubneyi, Cot. cotylophorum*	Fluke morphology	Gastric chambers	Graber et al., 1980
Rwanda	Black fronted duiker (*Cephalophus nigrifrons)*	*Paramphistomum (P.) cephalophi*	Fluke morphology	Small intestine	Eduardo, 1982
Central Africa					

DRC	Bohor reedbuck (*Redunca redunca),* Kob (*Kobus kob),* Puku (*Kobus vardonii),* Royal antelope (*Neotragus pygmaeus)*	*Cal. clavula, Cot. cotylophorum, Ste. compactus*	Fluke morphology		Stunkard, 1929
DRC	Royal antelope (*Neotragus pygmaeus)*	*Cot. cotylophorum*	Fluke morphology	-	Nasmark, 1937
DRC	African Buffalo (*Syncerus caffer),* Bushbuck (*Tragelaphus scriptus),* Common Eland (*Taurotragus oryx),* Defassa waterbuck (*Kobus defassa),* East African oryx (*Taurotragus oryx),* Oribi (*Ourebia ourebi),* Puku (*Kobus vardonii),* Roan antelope (*Hippotragus equinus)*	*Cal. calicophorum, Cal. clavula, Car. exoporus, Car. mancupatus, Car. endopapillatus, Cot. cotylophorum*	Fluke morphology		Prudhoe, 1957
DRC	Defassa waterbuck (*Kobus defassa)*	*Car. papillatus, Car. parvipapillatus, Car. spatiosus*	Fluke morphology		Gretillat, 1962
DRC	African buffalo (*Syncerus caffer),* Roan Antelope (*Hippotragus equinus)*	*Car. Gregarious, Ste. compactus*	Fluke morphology	-	Dollfus, 1963
DRC	African Buffalo (*Syncerus caffer),* Black fronted duiker (*Gazella thomsoni),* Thomson's gazelle (*Cephalophus nigrifons)*	*Car. chabaudi, Car. multivitellarius, Car. schoutedeni,*	Fluke morphology	-	Sey, 1983
DRC	African Buffalo (*Syncerus caffer),* Bohor reedbuck (*Redunca redunca),* Defassa waterbuck (*Kobus defassa)*	*Cal. clavula, Cal. phillerouxi*	Fluke morphology	Rumen, Stomach	Eduardo, 1983
DRC	African buffalo (*Syncerus caffer)*	*Leiperocotyle (L.) gretillati*	Fluke morphology	Intestines	Eduardo, 1985b
CAR	African Buffalo (*Syncerus caffer),* Bohor reedbuck (*Redunca redunca)*	*Cal. clavula, Cal. phillerouxi, Car. spatiosus, Car. graberi, Car. spatiousus, Gig. symmeri, Ste. compactus*	Fluke morphology	Rumen	Sey and Graber, 1979a
CAR	African Buffalo (*Syncerus caffer*)	*Cot. macrosphynctris*	Fluke	Rumen	Sey and Graber, 1979b
Chad and CAR	African Buffalo (*Syncerus caffer),* Bohor reedbuck (*Redunca redunca),* Roan Antelope (*Hippotragus*	*Cal. calicophorum, Cal. microbothrium, Cot.*	Fluke morphology	Digestive tract	Graber et al., 1964

	equinus), Senegal hartebeest *(Damaliscus korrigum),* Kob (*Kobus kob),* Lelwel hartebeest (*Alcelaphus lelwel)*	*cotylophorum, Car. endopappilatus, Car. spatiosus, Car. exoporus, Car. papillatus, Car. parvipapillatus, Ste. compactus*			
Republic of Niger	African buffalo (*Syncerus caffer)*	*Cal. clavula*	Fluke morphology	Rumen	Sey and Graber, 1979a
West Africa					
Ghana	Waterbuck (*Kobus sp.)*	*Cal. microbothrium*	Fluke morphology	Stomach	Maplestone, 1923
Cameroon	African buffalo (*Syncerus caffer),* African duiker (*Cephalophus* sp.*),* Kob (*Kobus kob)*	*Cot. fuelleborni, Cot. cotylophorum*	Fluke morphology	-	Nasmark, 1937
Benin	Hartebeest (*Alcelaphus busephalus)*	*Cot. cotylophorum*	Fluke morphology	-	Morel, 1959
Nigeria	Roan Antelope (*Hippotragus equinus)*	*Cal. clavula*	Fluke morphology	Rumen	Eduardo, 1983
Nigeria	Kob (*Kobus kob)*	*Ste. compactus*	Fluke morphology	Rumen	Eduardo, 1986
North Africa					
Sudan	Kob (*Kobus kob)*	*Cal. microbothrium*	Fluke morphology	-	Baer, 1923
Sudan	African buffalo (*Syncerus caffer),* Waterbuck (*Kobus sp.)*	*Car. gregarius, Cot. cotylophorum*	Fluke morphology	Stomach	Maplestone, 1923
Sudan	Bohor reedbuck (*Redunca redunca),* Defassa Waterbuck (*Kobus deffasa),* Kob (*Kobus kob)*	*Cal. clavula*	Fluke morphology		Nasmark, 1937
Sudan	Defassa Waterbuck (*Kobus deffassa)*	*Cal. microbothrium*	Fluke morphology	Rumen	Myers et al., 1960
Egypt	African buffalo (*Syncerus caffer)*	*Cal. gotoi, Car. gregarius, Cal. microbothrium*	Fluke morphology	Stomach	Sey, 1977
Sudan	Defassa waterbuck (*Kobus defassa)*	*Cal. clavula*	Fluke morphology	Rumen	Eduardo, 1983
Sudan	Nile lechwe (*Kobus megaceros)*	*Car. wenyoni, Car. gregarius*	Fluke morphology	-	Sey, 1983

Egypt	African buffalo (*Syncerus caffer)*	*Cal. microbothrium*, *Car. gregarius*, *P. cervi*	Fluke morphology and egg morphology	Stomach	Halium et al., 2014

Appendix 3.1: Ethical clearance approval letter

NATIONAL UNIVERSITY OF SCIENCE AND TECHNOLOGY

NUST INSTITUTIONAL REVIEW BOARD

DATE: 29/02/2022

Dear Mrs Sibula

TITLE: **Molecular Characterisation of amphistomes, helminths, ticks and blood parasites of wild ruminants and the snails that carry amphistomes**

Thank you for submitting your Research Proposal for review by the NUST IRB. Please be advised that the IRB reviewed your protocol and it was approved.

The approval by the NUST IRB was based on the following documents you submitted:

(i) Research Proposal Document of the Study,

The approval number for the study is **NUST/IRB/2021/17** and should be used in all correspondence, consent forms and other documents as appropriate.
Approval Date : 29 February 2022
Expiry Date : 28 February 2023

After the expiry date, the project may only continue after renewal. Renewal application process should commence three months before the expiry date.
All problems related to the safety of participants must be reported to the NUST IRB within 3 working days. You should not deviate from the protocol and procedures stated in the proposal. Do not make any adjustments/changes to the protocol and consent forms without prior written approval to the NUST IRB.

Thank you

Dr P. Makoni
Chief Research Officer

PARKS AND WILDLIFE ACT [CHAPTER 20:14] OF 1996

RESEARCH PERMIT

Fee paid: 100.00
Receipt Numbers: 1288992(BYO)
Permit Number: 23(1) (C) (II) 11/2019

ISSUER

PARKS AND WILDLIFE MANAGEMENT AUTHORITY (The Authority)

PERMIT HOLDER

Madeline Sibula ID No. ████ K 03: A Student at NUST (Department of Applied Biology and Biochemistry), Whose registered address for business is P. O Box 939, Ascot Bulawayo and whose contact number: ████

Project Supervisor: Prof S. Mukaratirwa and Prof S. Dube.

The Parks and Wildlife Management Authority is a statutory body established in terms of the **Parks and Wildlife Act [Chapter 20:14] (the Act)** and is the responsible authority for the management and development of National Parks and wildlife resources thereby controlling all related activities and issues this permit in terms of the Act. Permission is hereby granted, on the authority of the Minister of Environment, Tourism and Hospitality Industry in terms of paragraph section 23(1) (c) (ii) of the Parks and Wild Life Act [Chapter 20:14] of 1996.

Purpose of Permit
- To study on parasites of wild animals from Hwange National Park and Matetsi Safari Area with a topic entitled "*morphological and molecular characterization of amphistome isolates from wild animals of the Matebeland Region of Zimbabwe*"

Unless sooner cancelled, amended or modified in terms of section **123(1) (4) (b) of Parks and Wildlife Act Chapter 20:14 of 1996**, this permit is valid from, **1st of January 2019** to **31th of December 2019**, and is issued subject to the following terms and conditions being strictly adhered to:

SWO- Trade and Permits
████
For Director General: Parks and Wildlife Management Authority

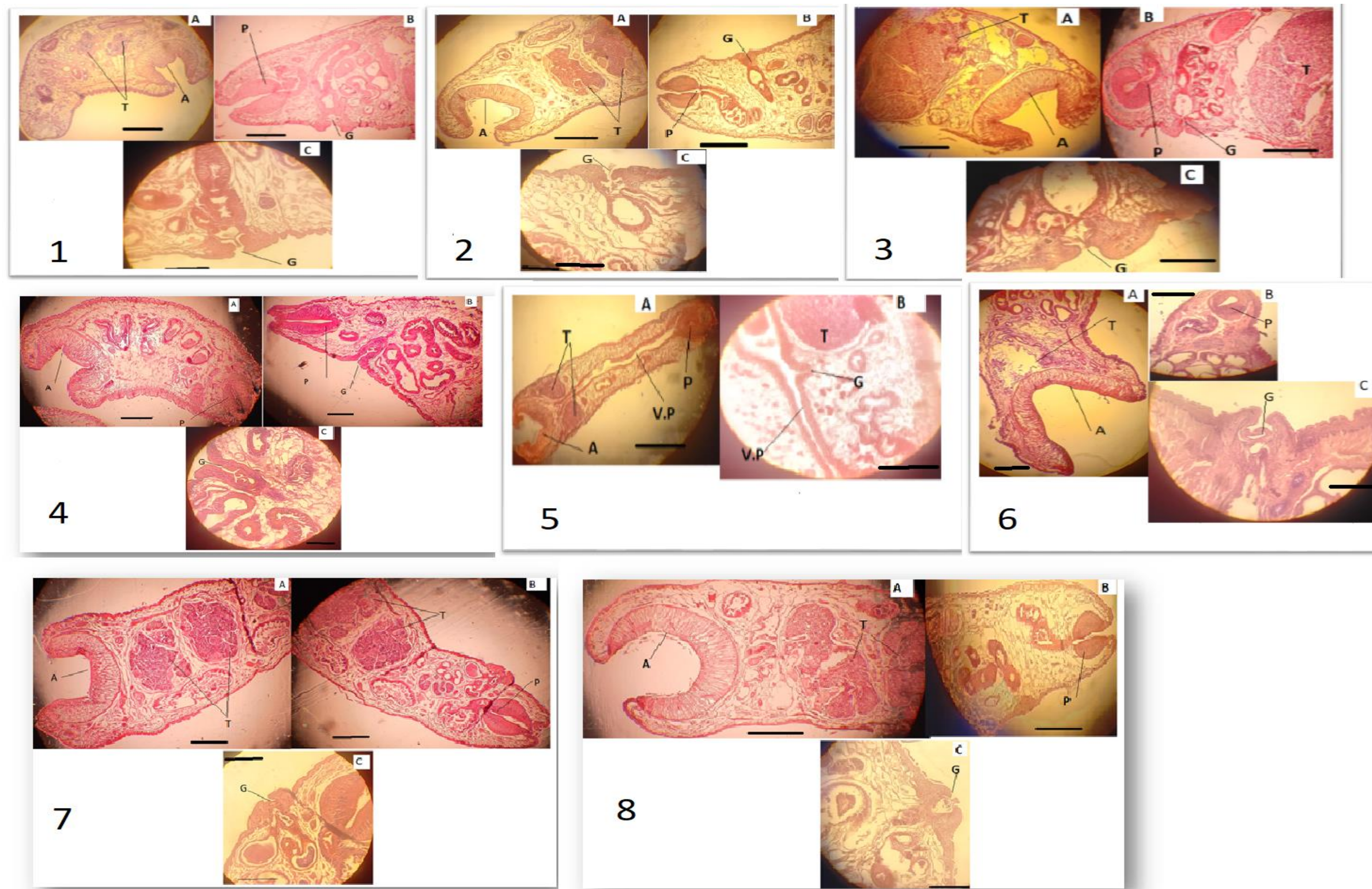
PTO

PARKS AND WILDLIFE MANAGEMENT
PERMITS OFFICE
08 MAR 2019
HEAD OFFICE
P.O. BOX CY 140
CAUSEWAY, HARARE

15077

TERMS AND CONDITIONS

1. The permit holder shall not assign this permit or any interest therein by an operating agreement or otherwise, and shall not transfer any interest therein, except with the approval of the Authority.
2. The permit holder and his bona fide employees only, may carry out research under this permit.
3. This permit shall be issued on an annual basis subject to the terms and conditions herein under. Permit fees are subject to change.
4. Carrying out research with an invalid permit shall be an offense and the Authority has the right to prosecute any offender caught with an invalid permit.
5. This permit shall be renewed annually. The permit holder shall apply for renewal 30 days prior to the expiration of this permit or commencement of the succeeding year.
6. The permit holder shall be deemed to be conversant with and adhere to the provisions of the **Parks and Wildlife Act Chapter 20:14 of 1996 and the laws of Zimbabwe.**
7. This permit is issued without any alteration, any alterations will render in invalid.
8. This permit is not transferable.
9. To report to the Area Manager before and after entering the protected area.
10. To liaise closely with Ecologists of the respective protected area or Ecologist from the nearest Parks office if activity is on private land.
11. To be allowed to do night drives and to travel off road, subject to notifying Area Manager in advance of the specific planned activities.
12. To set up a temporary camp, when necessary with the permission of the Area Manager who specifies the camping terms and conditions.
13. To submit monthly progress reports to the Area Ecologist, Area Manager and Chief Ecologist.
14. **This permit is solely/exclusively issued for Scientific studies/research purposes only.**
15. **No commercial activities/tourism activities in whatever manner are permitted under this permit.**
16. **All recordings video, audio, print or in whatever manner shall not be put to public broadcast without obtaining the corresponding commercial filming permits which shall be charged at commercial filming rates and will be subject Zimpark's intellectual property rights.**
17. Recognition of the participation of the Area Ecologists is emphasized in the publication under co-authorship.
18. To submit a final report to the Director General of Parks and Wildlife Management Authority after the research completion.
19. To submit a bound copy of thesis/dissertation to the Chief Ecologist for the ZPWMA library.
20. Not to interfere with any animals and not to disturb the habitat e.g. destroying trees and no collection of samples without prior authorization.
21. For any wildlife immobilization for purposes of collaring, micro chipping including collection of any biological material such as blood, semen etc. Report each case to the Provincial Veterinary Officer.
22. No samples including genetic material can be exported from Zimbabwe without signing a Biological Transfer Agreement with the Research Council of Zimbabwe.
23. No one is allowed to make Press statement on behalf of the Authority. If there is any need to make any statement, the ZPWMA Public Relations Manager must be informed beforehand.
24. Foreign Researchers to obtain a Research Council of Zimbabwe permit and meet appropriate Immigration requirements.
25. The Director General of Zimbabwe Parks and Wildlife Management Authority reserves the right to cancel the permit at any time if the terms and condition stipulated on the permit are not met.
26. The permit holder agrees to indemnify, protect, release, and hold the Authority from and against all losses, liabilities, damages, costs, investigations, obligations, claims, penalties, causes of action, monitoring, costs, and expenses, incurred by permit holder.



Appendix 3.2 Representative median sections of all amphistome species (1-8) in this study. The amphistome species are: 1. *Calicophoron* (C.) *clavula* 2. *C. microbothrium* 3. *C. phillerouxi* 4. *C. raja* 5. *Gastrothylax crumenifer* 6. *Gigantocotyle symmeri* 7. *Leiperocotyle gretillatti* 8. *Orthocoelium dicranocelium*. For each amphistome species except 5 diagnostic features, Plate A has the acetabulum (a) and testes (t), Plate B has the pharynx and Plate C has the genital atrium (g). Amphistome 5 has a ventral pouch (vp). Scale bars on plates A and B are 1mm whilst that of C is 0.2mm.

Appendix 3.3 GenBank sequence accession numbers of amphistome species identified from the study and similarity index (%) with Genbank sequences from elsewhere

Sample ID	Accession number	Morphological identification	Molecular Species ID on Genbank	Similarity (%)	Genbank Accession number	Country of origin
BW1	PP854142	*C. microbothrium*	*C. clavula*	100	MK416145	Saudi arabia
BW4	PP854143	*Gastrothylax crumenifer*	*Gaastrothylacidae*	99.42	KX668946	Kenya
BW5	PP854144	*Gastrothylax crumenifer*	*Cotylophoron cotylophorum*	97.86	KC503917	India
BWB1	PP854145	*G. crumenifer*	*C. microbothrium*	97.96	KX668988	Kenya
BWB1-2	PP854146	*G. crumenifer*	*Gastrothylax sp.*	97.96	KY786332	India
BWB2-1	PP854147	*G. crumenifer*	*Gastrothylax sp.*	97.94	KY786332	India
BWB2-2	PP854148	*Orthocoelium dicranocoelium*	*C. microbothrium*	97.69	KX668988	Kenya
BWBC3	PP854163	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
C3	PP854149	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
C4	PP854150	*C. clavula*	*C. clavula*	100	KX668956	Kenya
BI-1	PP854119	*C. microbothrium*	*C. microbothrium*	99.71	MZ229631	South Africa
BI-2	PP854120	*C. microbothrium*	*C. microbothrium*	99.71	KP639632	South Africa
BI-3	PP854121	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
BI-4	PP854122	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
BI2-1	PP854123	*C. raja*	*C. raja*	99.05	LC633276	South Africa
BI2-3	PP854124	*C. raja*	*C. raja*	99.70	LC633276	South Africa
BI2-4	PP854125	*C. raja*	*C. raja*	99.66	LC633276	South Africa
BI2-5	PP854128	*C. phillerouxi*	*C. clavula*	99.43	MK416145	Saudi Arabia
BI4-1	PP854129	*C. clavula*	*C. clavula*	100	MK416145	Saudi Arabia

B14-2	PP854130	*C. clavula*	*C. clavula*	99.71	MK416145	Saudi Arabia
BI4- 3	PP854131	*C. clavula*	*C. clavula*	100	MK416145	Saudi Arabia
BI4-4	PP854126	*C. clavula*	*C. clavula*	99.71	MK416145	Saudi Arabia
BD	PP854109	*C. microbothrium*	*C. microbothrium*	100	MZ229632	South Africa
BF1-a	PP854110	*L. gretillati*	*Paramphistomoidea*	99.12	KX668934	Kenya
BF1- b	PP854111	*C. raja*	*C. raja*	99.41	KX668957	Kenya
BF1- c	PP854112	*L. gretillati*	*Paramphistomoidea*	99.41	KX668934	Kenya
BF1 – d	PP854116	*C. microbothrium*	*C. clavula*	100	MK416145	Saudi Arabia
BF1-e	PP854113	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
BF1-f	PP854117	*C. clavula*	*C. clavula*	100	MK416145	Saudi Arabia
BF1-g	PP854114	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
BF1-h	PP854115	*C. microbothrium*	*C. microbothrium*	100	KP639632	South Africa
BF3	PP854118	*L. gretillati*	*Paramphistomoidea*	99.41	KX668934	Kenya
BF4	PP854127	*Gi. symmeri*	*C. microbothrium*	98.5	KP639635	Botswana
BM1-1	PP854134	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
BM1-2	PP854135	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
BM1-a	PP854132	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
BM1-b	PP854139	*C. microbothrium*	*C. microbothrium*	99.42	KP639632.1	South Africa
BM1-c	PP854140	*C. phillerouxi*	*C. phillerouxi*	100	KX668977.1	Kenya
BM1-e	PP854141	*L. gretillati*	*Paramphistomoidea*	99.71	KX668934.1	Kenya
BM1-f	PP854133	*L. gretillati*	*Paramphistomoidea*	98.84	KX668934	Kenya
BM6	PP854137	*L. gretillati*	*Paramphistomoidea*	99.42	KX668934	Kenya
BMC	PP854136	*Cal. phillerouxi*	*Cal. phillerouxi*	100	KX668977.1	Kenya

BME	PP854138	*Leiperocotyle gretillati*	*Paramphistomoidea*	99.71	KX668934	Kenya
HBM	PP854157	*C. microbothrium*	*C. microbothrium*	99.72	KP639632.1	South Africa
HBM1	PP854158	*C. microbothrium*	*C. microbothrium*	99.72	KP639632.1	South Africa
HBM2-1	PP854159	*C. microbothrium*	*C. microbothrium*	99.72	KP639632.1	South Africa
HBM2-3	PP854160	*C. microbothrium*	*C. microbothrium*	99.72	KP639632.1	South Africa
HBM2-4	PP854161	*L. gretillati*	*Paramphistomoidea*	100	KX668934	Kenya
HBM2-5	PP854162	*L. gretillati*	*Paramphistomoidea*	100	KX668934	Kenya
SH1	PP854098	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
SH2-1	PP854099	*C. microbothrium*	*C. microbothrium*	100	MZ229632	South Africa
SH2-2	PP854100	*C. microbothrium*	*C. microbothrium*	100	MZ229632	South Africa
SH3	PP854101	*C. clavula*	*C. clavula*	100	MK416145	Egypt
CC5	PP854151	*C. raja*	*C. raja*	99.69	LC633276.1	South Africa
CC6	PP854152	*C raja*	*C. raja*	99.69	LC633276.1	South Africa
K211	PP854087	*C. raja*	*C. raja*	100	LC633276.1	South Africa
K51	PP854088	*C. raja*	*C. raja*	100	LC633276.1	South Africa
KCI	PP854089	*C. raja*	*C. raja*	99.71	LC633276.1	South Africa
KC21	PP854090	*C. raja*	*C. raja*	100	LC633276.1	South Africa
KC3	PP854091	*C. raja*	*C. raja*	98.86	LC633276.1	South Africa
KC4	PP854092	*C. raja*	*C. raja*	100	LC633276.1	Kenya
KC4-1	PP854093	*C. raja*	*C. raja*	100	LC633276.1	South Africa
KC7	PP854094	*C. raja*	*C. raja*	100	LC633276.1	South Africa
IC1-2	PP854078	*C. raja*	*C. raja*	99.7	LC633276.1	South Africa
IM1	PP854105	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa

IM2	PP854106	*C. microbothrium*	*C. microbothrium*	99.71	KP639632.1	South Africa
IM3	PP854107	*Gi. symmeri*	*C. microbothrium*	98.84	KP639632.1	South Africa
IM4	PP854108	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
IN3-1	PP854080	*C. microbothrium*	*C. microbothrium*	99.72	KP639632.1	South Africa
IN3	PP854079	*C. microbothrium*	*C. microbothrium*	99	KP639632.1	SA
IN201	PP854080	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
IN205	PP854082	*C. raja*	*C. raja*	100	LC633276.1	South Africa
IN208	PP854083	*C. raja*	*C. raja*	100	LC633276.1	South africa
IN209	PP854084	*C. raja*	*C. raja*	99.43	LC633276.1	South africa
IN2010	PP854085	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
IN2010-2	PP854086	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
N5	PP854096	*C. microbothrium*	*C. microbothrium*	100	KP639632.1	South Africa
N51	PP854097	*C. microbothrium*	*C. microbothrium*	99.71	KP639632.1	South Africa
CNY1-1	PP854153	*C. phillerouxi*	*C. clavula*	99.72	MK416145	Saudi Arabia
CNY1-2	PP854154	*C. raja*	*C. raja*	99.71	LC633276	South Africa
CNY3-1	PP854155	*C. microbothrium*	*C. microbothrium*	99.7	KP639632	South Africa
CNY3-2	PP854156	*C. microbothrium*	*C. microbothrium*	99.72	KP639632	South Africa
KM1	PP854095	*C. raja*	*C. raja*	100	LC633276.1	South Africa
SN1	PP854102	*C. raja*	*C. raja*	99.72	LC633276.1	South Africa
SN2	PP854103	*C. raja*	*C. raja*	100	LC633276.1	South Africa
SN3	PP854104	*C. raja*	*C. raja*	100	LC633276.1	South Africa