University of New Mexico
UNM Digital Repository

Biology ETDs Electronic Theses and Dissertations

Spring 5-9-2018

REVEALING BIOTIC DIVERSITY: HOW DO COMPLEX ENVIRONMENTS INFLUENCE HUMAN SCHISTOSOMIASIS IN A HYPERENDEMIC AREA

Martina R. Laidemitt

Follow this and additional works at: https://digitalrepository.unm.edu/biol_etds

Recommended Citation
Laidemitt, Martina R.. "REVEALING BIOTIC DIVERSITY: HOW DO COMPLEX ENVIRONMENTS INFLUENCE HUMAN SCHISTOSOMIASIS IN A HYPERENDEMIC AREA." (2018). https://digitalrepository.unm.edu/biol_etds/279

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biology ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.

Martina Rose Laidemitt

Candidate

Department of Biology

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Eric S. Loker, Chairperson

Dr. Jennifer A. Rudgers

Dr. Stephen A. Stricker

Dr. Michelle L. Steinauer

Dr. William E. Secor

REVEALING BIOTIC DIVERSITY: HOW DO COMPLEX ENVIRONMENTS INFLUENCE HUMAN SCHISTOSOMIASIS IN A HYPERENDEMIC AREA

By

Martina R. Laidemitt

B.S. Biology, University of Wisconsin- La Crosse, 2011

DISSERT ATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

July 2018

ACKNOWLEDGEMENTS

I thank my major advisor, Dr. Eric Samuel Loker who has provided me unlimited support over the past six years. His knowledge and pursuit of parasitology is something I will always admire. I would like to thank my coauthors for all their support and hard work, particularly Dr. Sara Brant, Mr. Martin Mutuku, Ms. Eva Zawadzki, Dr. Gerald Mkoji, Ms. Larissa Anderson and Dr. Helen Wearing. I also thank my committee members, Drs. Jennifer Rudgers, Stephen Stricker, William Evan Secor, and Michelle Steinauer for their expertise, time, insight, and critiques of this work.

I thank my lab mates and members the parasitology group at UNM: Dr. Erika Gendron, Ms. Melissa Sanchez, Ms. Janeth Pena, Mr. Jon Schultz, Dr. Sarah Buddenborg, Ms. Lijun Lu, Dr. Bishoy Hanna, Dr. Ben Hanelt, Dr. Bruce Hofkin, Ms. Caitlin Babbitt, Ms. Heather Mercer, Dr. Ramesh Devkota and Dr. Si-Ming Zhang thank you for your discussions, encouragement and friendship. Also, to my collaborators, Dr. Eric Lelo, Mr. Ibrahim Mwangi, Mr. Geoffery Mania, and Joesph Kinuthia at the Kenyan Research Medical Institute for your assistance in the field and help with this work.

Lastly, but most notably I would like to thank my family for their love and support over the years. I thank my childhood friends, Ms. Lacey Squier, Ms. Beth Jerabek, and Ms. Kortney Hamilton for their encouragement. They knew long before I did that I would be writing this dissertation. I particularly thank Sam Hodder for his endless support throughout this process even when I would be away from home for long periods of time in the field. Finally, I thank our dogs, Mr.

Sheebs and River for putting up with my shenanigans and for their companionship. They may have missed a few walks over the years, but they always greeted me with a wagging tail.

Revealing biotic diversity: How do complex environments influence human schistosomiasis in a hyperendemic area

By

Martina R. Laidemitt

B.S. Biology, University of Wisconsin- La Crosse, 2011

Ph.D. Biology, University of New Mexico, 2018

ABSTRACT

Human schistosomiasis is one of the great neglected tropical diseases (NTDs) of our time with more than 206 million individuals infected and more than 90% of those infected reside in Sub-Saharan Africa (WHO 2017). Chemotherapy based control programs play an essential role in contributing to the elimination of human schistosomiasis; however, there is an increasing consensus that chemotherapy needs to be supplemented by other means if interruption of transmission and elimination are to be achieved. Given this situation, the focus of this dissertation was to better understand transmission dynamics in a hyperendemic setting in western Kenya and to find alternative measures to supplement ongoing mass drug administration (MDA) using indigenous resources that disrupt the development of *Schistosoma mansoni* (the causative agent of intestinal schistosomiasis in Africa) within its obligatory aquatic snail intermediate host, *Biomphalaria*.

The discipline of disease ecology emphasizes understanding the biotic context in which disease transmission occurs. *S. mansoni* and *Biomphalaria* exist

within a complex ecological milieu in streams, ponds and lakes in Kenya. The research in this dissertation combined DNA barcodes, phylogenetics, host use patterns and morphology to determine the diversity of trematodes that use Kenyan *Biomphalaria* as an intermediate host. Along with *S. mansoni*, we found 21 additional digenetic trematodes that also use *Biomphalaria* species in Kenya as an intermediate host. The presence of other trematode species in *Biomphalaria* affects *S. mansoni* by causing competition for access to snail resources. Furthermore, we used experimental approaches to understand the competitive dynamics among these trematodes and to generate a dominance hierarchy among them. We found that several trematode species are dominant to *S. mansoni* and long-term agricultural practices have created a situation where an amphistome parasite of cattle relies on a facilitating effect by *S. mansoni* for its own successful development in the snail host. Coupled with these data are four years of observational survey data to predict how these trematodes influence *S. mansoni's* prevalence in *Biomphalaria* and consequently the likelihood of influencing human infections.

TABLE OF CONTENTS

TABLE OF CONTENTS……………………………………………………………....vii

INTRODUCTION…………………………………………………………………..1

References……………………………………………………………………....5

CHAPTER 1: Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants………………………………………8

Abstract……………………………………………………………………..9

Introduction………………………………………………………………..10

Materials and Methods…………………………………………………..…13

Results…………………………………………………………………..……18

Discussion……………………………………………………………………22

Acknowledgements ………………………………………………………...31

Financial Support…………………………………………………………...31

References ……………………………………………………………………33

Figures and Tables……………………………………………………………46

CHAPTER 2: The diverse echinostomes from East Africa: with a focus on species that use schistosome transmitting snails as intermediate hosts...59

Abstract……………………………………………………………………....60

Introduction…………………………………………………………………..60

Materials and Methods……………………………………………………….65

Results……………………………………………………………………68

Discussion……………………………………………………………...77

Acknowledgements………………………………………………………86

Financial Support…………………………………………………………86

References …………………………………………………………………88

Figures and Tables…………………………………………………….....99

CHAPTER 3: Cascading effects of pastoralism, biodiversity and habitat stability on the transmission of one of the world's most abundant neglected tropical diseases, schistosomiasis……………………………...107

Abstract………………………………………………………………..108

Introduction……………………………………………………….….108

Results……………………………………………………………...110

Discussion………………………………………………………………123

Methods…………………………………………………………………130

Acknowledgements……………………………………………………..135

References………………………………………………………………137

Figures and Tables……………………………………………………..146

CONCLUSIONS………………………………………………………..159

References……………………………………………………………………163

INTRODUCTION

African countries have relied extensively on mass administration of the drug, praziquantel (PZQ) in attempt to control human schistosomiasis. However, mounting evidence suggests that even after 5-10 years of annual treatments there is little reduction in disease prevalence (Lamberton et al., 2014; Andrade et al., 2017), or in schistosome genetic diversity (Lelo et al., 2014), calling for the need of alternative measures to control human schistosomiasis (Rollinson et al., 2013; Loker et al., 2013). Interventions such as education, increased hygiene, water sanitation, and particularly snail control should be used as supplemental control strategies along with PZQ administration (Rollinson et al, 2013; Loker et al., 2013; Sokolow et al., 2017).

Along these lines, we were interested in the finding alternative indigenous measures to control snails, because molluscicides (chemicals that kill snails) used for snail control are expensive and can have detrimental effects on fish and non-target gastropods (Rollinson et al., 2013). In recent years biologists have gained an increased appreciation for the reality that infectious diseases exist in complex ecological systems (Johnson and Thieltges, 2010; Keesing et al., 2010; Civitello et al., 2015). For example, according to the dilution hypothesis, a diverse community may interfere with transmission of a particular parasite by diverting the parasite into inappropriate hosts or by regulating the populations of susceptible hosts. On the other hand, transmission may be favored in diverse communities by providing more competent host species and thus more transmission options. Therefore, the biotic context in which disease transmission occurs must be considered carefully because not all host-parasite systems are

the same and because diversity can have both positive and negative effects in a given host-parasite system, particularly for parasites that use multiple hosts to complete their life cycles.

The focus of this dissertation was to determine the diversity of trematodes in Kenyan freshwater bodies where human schistosomiasis transmission occurs (chapters 1-2). We were interested in their diversity because certain species of trematodes may compete with larval stages of *S. mansoni* for access to obligatory snail hosts. Not all larval trematodes have the same pattern of development within their host snail. For example, some trematodes (like *S. mansoni*) develop into sporocysts within the snail. Sporocysts are sac-like structures that absorb their nutrients across their body wall. They can nevertheless release materials that not only interfere with their host's reproductive activities, but also in some cases interfere with development of larvae of other digenean species, what has been called indirect antagonism (Lie et al., 1965). Other trematode parasites develop rediae that have a mouth, pharynx, and gut. Rediae often move throughout the snail actively ingesting host tissue, including gonadal tissue (Lim and Lie 1969), and can actively attack and kill the larvae of other trematode species, during what is referred to as direct antagonism (Lim and Heyneman, 1972). There is growing evidence that for at least some species, particularly echinostomes that some species are dominant over other trematodes because they develop rediae in the snail host (Lim and Heyneman 1972; Hechinger et al., 2010). Certain rediae can become specialized for the purpose of attacking and killing the larvae of competing digenean species

within the body of their molluscan hosts, while other rediae are more specialized for reproduction (Garcia et al., 2016). Therefore, we were interested in how many different trematodes use *Biomphalaria* as an intermediate host and which species were more dominant against *S. mansoni*.

A collecting program was initiated in January 2014 to identify freshwater snails and their parasites (Chapter 3). We used morphological features, host use, molecular markers and phylogenetics to delineate East African representatives of two major groups of trematodes, the amphistomes (Chapters 1) and the echinostomes (Chapter 2). We wanted to know more about both of these common groups of trematodes because they often infect the same snails hosts as *S. mansoni*, and because they are typically dominant to *S. mansoni* when found in the same individual snail. Molecular markers were used because cryptic species often exist, which can create issues when delineating specific clades of amphistome or echinostome parasites (Detwiler et al., 2010). We used the information from our molecular and survey data to determine which trematode species should be used for experimental exposures. Experimental exposures were done to determine the impact these trematodes may have on the *Biomphalaria*-*S. mansoni* system because they may 1) directly increase snail mortality, 2) reduce compatibility with *S. mansoni,* and 3) compete with *S. mansoni* infections. Ironically, virtually none of the previous work focusing on interspecific antagonism between schistosomes and other trematodes as a potential control strategy has been undertaken with species transmitted in sub-Saharan Africa, where schistosomiasis assumes its greatest public health

significance (Combes 1982; Banes et al., 1974; Pointier and Jourdane 2000; Toledo and Fried, 2011).

Using molecular markers, we found over twenty different trematodes that use *Biomphalaria* as an intermediate host. We also developed a dominance hierarchy among the various trematodes and found that certain species like amphistomes and echinostomes were more dominant than *S. mansoni,* but some, like xiphidiocercariae and strigeids, were subordinate and taken over by *S. mansoni* in *B. pfeifferi.* Lastly, we coupled our survey and experimental data to develop a model (Chapter 3) in the context of a hyper-endemic setting (west Kenya) to predict how one commonly transmitted parasite, an amphistome species provisionally identified as *Calicophoron sukari* influenced the outcomes of *S. mansoni* infections in *B. pfeifferi.* Our mathematical analyses found that in the absence of *C. sukari,* the number of *B. pfeifferi* infected with *S. mansoni* significantly increases (3-fold increase). Moreover, if we were to artificially increase *C. sukari'*s input into the system this significantly dampens the number of *S. mansoni* cercariae in the water, thereby reducing human infections.

References:

Andrade, G., Bertsch, D.J., Gazzinelli, A., King, C.H., 2017. Decline in infection-related morbidities following drug-mediated reductions in the intensity of Schistosoma infection: A systematic review and meta-analysis. PLoS Negl Trop Dis. 11: e0005372.

Banes, V., Rysavy, B., Yousif, F., 1974. Observations on the development of two echinostomes, *Echinoparyphium reaurvatum* and *Echinostoma revolutum*, the antagonists of human schistosomes in Egypt. Folia Parasitol 21: 143-154

Civitello, D.J., Cohen, J., Fatima, H., Halstead, N.T., Liriano, J., McMahon, T.A., Ortega, C.N., Sauer, E.L., Sehgal, T., Young, S., Rohr, J.R., 2015. Biodiversity inhibits parasites: Broad evidence for the dilution effect. Proc Natl Acad Sci U S A 112: 8667-8671.

Combes, C., 1982. Digeneans: antagonism between species and sterilizing effects on snails in biological control. Parasitology. 84:151-175

Detwiler, J.T., Bos, D.H., Minchella, D.J., 2010. Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America. Mol Phylogenet Evol 55: 611-620.

Keesing, F., Belden, L.K., Daszak, P., Dobson, A., Harvell, C.D., Holt, R.D., Hudson, P., Jolles, A., Jones, K.E., Mitchell, C.E., Myers, S.S., Bogich, T., Ostfeld, R.S., 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468: 647-652.

Johnson, P.T.J., and Thieltges, D.W., 2010. Diversity, decoys and the dilution effect: how ecological communities affect disease risk. J. Exp. Biol. 213: 961-970

Lamberton, P.H., Kabatereine, N.B., Oguttu, D.W., Fenwick, A., Webster, J.P., 2014. Sensitivity and specificity of multiple Kato-Katz thick smears and a circulating cathodic antigen test for Schistosoma mansoni diagnosis pre- and post-repeated-praziquantel treatment. PLoS Negl Trop Dis 8: e3139.

Lelo, E.S., D.N Mburu, G.N. Magoma, B.N. Mungai, J.H. Kihara, I.N. Mwangi, G.M. Maina, J.M. Kinuthia, M.W. Mutuku, E.S. Loker, G.M. Mkoji, and M.L., Steinauer. 2014.No apparent reduction in schistosome burden or genetic diversity following four years of school-based mass drug administration in Mwea, Central Kenya, a heavy transmission area. PLoS Negl Trop Dis. 8: 1-11

Lie, J.K., P.F. Basch, and Umathevy, T., 1965. Antagonism between two species of larval digeneans in the same snail. Nature, London. 208: 422-423

Lim, HK. and Lie, J.K., 1969. The redial population of *Paryphostomum segregatum* (Trematoda: Echinostomatidae) in the snail *Biomphalaria glabrata.* Z Parasitenkd. 32: 112-119

Lim, HK, and Heyneman, D., 1972. Intramolluscan inter-trematode antagonism: a Review of factors influencing the host-parasite system and its possible role in biological control. Advances in Parasitology. 10:191-268

Loker, E.S., This De-Wormed World? J. Parasitol. 99: 933-942

Pointier, J.P., Jourdane, J., 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. Acta Trop, 77, 53–60

Rollinson, D., Knopp, S., Levitz, S., Stothard, J.R., Tchuem Tchuente, L.A.,

Garba, A., Mohammed, K.A., Schur, N., Person, B., Colley, D.G., Utzinger, J., 2013. Time to set the agenda for schistosomiasis elimination. Acta Trop 128: 423-440.

Sokolow, S.H., Wood, C.L., Jones, I.J., Lafferty, K.D., Kuris, A.M., Hsieh, M.H., De Leo, G.A., To Reduce the Global Burden of Human Schistosomiasis, Use 'Old Fashioned' Snail Control. Trends in Parasitology 34: 23-40.

Toledo, R., and B. Fried. 2011. *Biomphalaria* snails and larval trematodes. Springer.

Chapter 1

Loads of trematodes: Discovering hidden diversity of paramphistomoids in Kenyan ruminants

Laidemitt, M.R., Zawadzki, E.T., Brant, S.V., Mutuku, M.W., Mkoji, G.M., Loker, E.S., 2017. Loads of trematodes: discovering hidden diversity of paramphistomoids in Kenyan ruminants. Parasitology 144, 131-147

Abstract

Paramphistomoids are ubiquitous and widespread digeneans that infect a diverse range of definitive hosts, being particularly speciose in ruminants. We collected adult worms from cattle, goats, and sheep from slaughterhouses, and cercariae from freshwater snails from ten localities in central and west Kenya. We sequenced *cox*1 (690 bp) and ITS2 (385 bp) genes from a small piece of 79 different adult worms and stained and mounted the remaining worm bodies for comparisons with available descriptions. We also sequenced *cox*1 and ITS2 from 41 cercariae/rediae samples collected from four different genera of planorbid snails. Combining morphological observations, host use information, genetic distance values, and phylogenetic methods, we delineated sixteen distinct clades of paramphistomoids. For four of the 16 clades, sequences from adult worms and cercariae/rediae matched, providing an independent assessment for their life cycles. Much work is yet to be done to resolve fully the relationships among paramphistomoids, but some correspondence between sequence- and anatomically based classifications were noted. Paramphistomoids of domestic ruminants provide one of the most abundant sources of parasitic flatworm biomass, and because of the predilection of several species use *Bulinus* and *Biomphalaria* snail hosts, have interesting linkages with the biology of animal and human schistosomes in Africa.

Introduction

The Superfamily Paramphistomoidea is a prominent group of digeneans where adults are characterized by the absence of an oral sucker and the presence of an acetabulum at or near the posterior end of the body. The systematics of this group of digeneans is a work in progress. Sey (1991) concluded it is comprised of eight families, whereas Jones (2005a) concluded there are 12 families. Paramphistomoids are often called rumen flukes because many of the best-known representatives live in this habitat in domestic ruminants. However, many species also inhabit the intestines of fish, amphibians, reptiles, birds and non-ruminant mammals. They feature a life cycle in which cercariae produced in rediae emerge from snails and encyst on vegetation as metacercariae which are later ingested by the definitive host (Jones, 2005a). As part of a larger study to determine how digenean community diversity influences the transmission of schistosomes in Kenya, we provide new results regarding the overall diversity and host relationships of paramphistomoids in Kenya, based on cercariae collected from snails and adult worms from domestic animals from abattoirs.

Paramphistomoids are of interest to parasitologists in several contexts. They are diverse in number of species and provide an understudied model group for those focused on revealing patterns and mechanisms of diversity. Of the 12 recognized paramphistomoid families recognized by Jones (2005a), representatives of nine occur in Africa. The diversity of paramphistomoids in Africa reflects the presence of many species of terrestrial mammals, including

elephants, rhinoceroses, hippopotami, and a rich diversity of wild and domestic ruminants. Three families in particular (Paramphistomidae, Gastrodiscidae, and Gastrothylacidae) are speciose in Africa. The distribution of diversity in rumen hosts can partly be explained by characters (e.g. regressed pharyngeal appendages) that are apomorphic which have allowed them to colonize the forestomach (Sey 1991). The three families comprise over 40% of all known paramphistomoids, the majority of which use ruminants as their definitive hosts (Sey, 1991).

Paramphistomoids have thick bodies which make detailed morphological characterization of adult features and species identification challenging (Horak, 1971; Jones, 1991; Mage *et al.* 2002; Rinaldi *et al.* 2005). The bodies of paramphistomoid cercariae are also relatively thick and typically filled with cystogenous material or pigment, also rendering identification difficult. Nonetheless, a meticulous framework for paramphistomoid identification and classification has been developed (see reviews by Sey, 1991; Jones, 2005a). Given the inherent difficulties in identification, coupled with a growing list of studies from other digenean groups documenting the presence of cryptic species (Detwiler *et al.* 2012; Herrmann *et al.* 2014; McNamara *et al.* 2014), paramphistomoids are ideal for studies attempting to meld traditional morphological identification with sequence data characterization provided by molecular approaches. The number of studies that use molecular techniques to provide assessments of the diversity of paramphistomoids have in general been

limited, especially so for African species (Lotfy *et al.* 2010; Mansour *et al.* 2014; Sibula *et al.* 2014; Titi *et al.* 2014; Dube *et al.* 2015).

In addition to being speciose, paramphistomoids are often remarkably abundant (Horak, 1971; Cheruiyot and Wamae, 1988; Rolfe *et al.* 1994; Sanabria and Romero, 2008). In fact, one might be hard pressed to find a larger source of sheer digenean biomass than is presented routinely at abattoirs by ruminant paramphistomoids. Given the large worm populations that can occur in individual cattle, goats or sheep, vast numbers of paramphistomoid eggs are regularly passed into the environment. In rural west Kenya, we can routinely collect 10,000 paramphistomoid eggs from a single cow dung sample. As domestic ruminants regularly seek water from natural habitats, it is not surprising that many paramphistomoid eggs enter freshwater, creating the potential for high levels of infection in their snail hosts (Chingwena *et al.* 2002a; Mohammed *et al.* 2016).

A review of the East African paramphistomoid literature reveals that many of the described species are transmitted by *Biomphalaria* and *Bulinus*, the snail genera also of concern with respect to their role in transmission of human schistosomiasis in Africa (Dinnik, 1954; Dinnik and Dinnik, 1957; Dinnik, 1961; Eduardo, 1983; Brown, 1994; Chingwena et al. 2002b; Jones, 2005b; Jones, 2005c). In some areas, *Bulinus* and *Biomphalaria* are the most commonly implicated snail hosts for paramphistomoids (Dinnik, 1965; Wright *et al.* 1979; Chingwena *et al.* 2002b; Ahmed *et al.* 2006; Mohammad *et al.* 2016). The presence of other digenean species utilizing the same snail species as schistosomes could be a factor that influences the overall success of animal and

human schistosome transmission (Lim and Heyneman, 1972; Combes, 1982; Hechinger *et al.* 2011; Spatz *et al.* 2014). This is particularly so for species like paramphistomoids that produce rediae as larval stages within their snail hosts, because rediae may attack, damage and consume schistosome sporocysts (Lim and Heyneman, 1972).

We collected cercariae and adult worms from ten localities in Kenya. We provide stained whole mounts and provisional identification of adults that are linked to sequence data for cytochrome oxidase 1 (*cox*1) and the internal transcribed region 2 (ITS2). In some cases we provide matches with sequences obtained from cercariae and adult worms thus providing probable life cycle linkages. We also provide new hypotheses for phylogenetic relationships among the paramphistomoids that include available sequences from NCBI GenBank, which show that some species of paramphistomoids are geographically widespread throughout Africa. Data presented here will contribute to an increased understanding of the superfamily Paramphistomoidea, including providing greater clarification for how these worms are distributed among hosts, their potential roles if any in causing disease in domestic or wild animals, and their interactions with other digeneans, including schistosomes.

Materials and Methods

Sampling

We collected larval and adult paramphistomoids from ten different localities in central and especially western Kenya between 2005-2015 (Table 1).

All species of field-collected aquatic snails were brought to the lab at Kisian, near Kisumu, Kenya. The snails were cleaned and then placed individually into 12-well tissue culture plates in 3 ml of aged tap water. The tissue culture plates were placed in natural light for two hours to induce shedding of cercariae. Snails shedding cercariae were identified using keys and information in Brown and Kristensen (1989) and Brown (1994), and cercariae were preliminarily identified using keys (Frandsen and Christensen, 1984; Schell, 1985) and by reference to regional monographs (e.g. Fain, 1953). All cercariae designated as paramphistomoids were confirmed as such according to Sey (1991). Snails were either dissected at the time of collection to procure rediae, or re-shed two and four weeks later to determine if snails were harboring prepatent infections at the time of collection. Snails were kept in 20 L plastic tanks and fed red leaf lettuce following collection. Cercariae and rediae were preserved in 95% ethanol for later molecular analysis.

Adults were collected from the rumen or reticulum of *Bos indicus*, *Capra aegagrus hircus*, and *Ovis aries* from one slaughterhouse in central Kenya and three in western Kenya (Table 1). Adults were preserved in 95% ethanol for later molecular and morphological identification.

Staining adult worms

Adult worms were placed into 70% ethanol for 24 hours prior to staining. Sections of the adult worms were stained and mounted according to Eduardo (1982). Because of their thickness, each adult was sectioned frontally using a

razor blade. Part of the posteroterminally placed acetabulum was severed and used for molecular analysis.

Collection of molecular data

A partial sequence of cytochrome oxidase 1 (*cox*1) mtDNA and internal transcribed spacer two (ITS2) were amplified by polymerase chain reaction (PCR) to facilitate differentiation among paramphistomoid specimens. One to six cercariae, one to three rediae, or a portion of the acetabulum from adults were used for DNA extraction. Genomic DNA was extracted from 120 paramphistomoid samples (Table 2) by the alkaline-lysis (HOT-SHOT) method (Truett *et al.* 2000), or by the QIAamp DNA Micro Kit following the manufacturer's instructions, with a final elution volume of 30 µl (Qiagen, Valencia, CA). Although not the equal of the QIAamp Kit with respect to absolute quality of the DNA produced, the HOT-SHOT method also produced DNA of quality and proved more amenable for use under conditions where controlled conditions were less available.

*Cox*1 oligonucleotide primers were designed based on the barcode region (Folmer *et al.* 1994) and on conserved regions in the *Fasciola hepatica* (NC_002546*), Paragonimus westermani (*AF219379), and *Paramphistomum cervi* (NC_023095) mitochondrial genomes. *Cox*1 was amplified using primers 123F [5'-ATTCGTTTGAACTATATGGA-3'] and 858R [5'-CATATGATGAGCCCAAACAAC-3']. The volume of each PCR reaction was 25 µl with 1 µl of 100 ng of DNA, 0.8mM/L dNTPs, 2.5 mM/L MgCl2, 0.25 units of Ex Taq DNA (Clontech, Mountain View, CA), and 0.4 µM/L of each primer. PCR

cycles were programmed as follows: 2 minute denaturation hold at 94°C; 94°C for 1 min, 46°C for 30 s, and 72°C for 1 min for 3 cycles, 94°C for 1 min, 45°C for 30 s, and 72°C for 1 min for 3 cycles, 94°C for 1 min, 44°C for 30 s, and 72°C for 1 min for 3 cycles, 94°C for 1 min, 44°C for 30 seconds, and 72°C for 1 min for 20 cycles, and followed by an extension step for 7 min at 72°C.

ITS2 was amplified using GA1 [5'-AGA ACA TCG ACA TCT TGA AC-3'] (Anderson and Barker, 1998) and BD2 primers [5'-TAT GCT TAA ATT CAG CGG GT-3'] (Bowles *et al.*1995). The volume of each reaction was 25 µl, with 12.5 µl of Premix Taq™ (Clontech, Mountain View, CA), 0.4 µM/L of each primer, and one µl of 55 ng of DNA. PCR cycles were performed on Eppendorf Mastercycler epigradient machines which were programmed as follows: 1 C/s rate of change, one cycle at 98°C for 10 s, followed by 30 cycles of 98°C for 1 min, 52°C for 2 min, and 72°C for 1 min with an extension step for 7 min at 72°C.

PCR fragments were separated by agarose gel electrophoresis and visualized with 0.5% GelRed™ Nucleic acid gel stain (Biotium, Hayward, CA, USA). PCR products were purified using the QIAquick purification kit (Qiagen, Valencia, CA) or by ExoSap-IT® (Affymetrix, Santa Clara, CA). Both strands were sequenced using an Applied Biosystems 3130 automated sequencer and BigDye terminator cycle sequencing kit Version 3.1 (Applied Biosystems, Foster City, CA). DNA sequences were verified by aligning reads from the 5' and 3' directions using Sequencher 5.0 and manually corrected for ambiguous base calls (Gene Codes, Ann Arbor, Michigan)

Outgroup determination

To determine the most appropriate outgroup available for our data, we reconstructed trees with the most likely outgroups based on Lockyer et al. (2003) and chose the sister group to the paramphistomoids (ingroup). Species from the following nine families were used from twelve digenean mitochondrial genomes for Maximum Likelihood analysis: *Dicrocoelium dendriticum* (NC_025280), *Fasciola gigantica* (NC_024025), *Paramphistomum cervi* (NC_023095), *Opisthorchis felineus* (NC_011127), *Clonorchis sinensis* (NC_012147), *Orthocoelium streptocoelium* (NC_028071), *Echinostoma hortense* (NC_028010), *Fischoederius elgonatus* (NC_028001), *Paragonimus westermani* (NC_027673), *Eurytrema pancreaticum* (NC_026916), *Fasciola hepatica* (NC_002546), and *Ogmocotyle sikae* (NC_027112).

Sequence alignment and phylogenetic analyses

Phylogenetic analyses were done with *cox*1 and ITS2 sequences using Maximum Likelihood (ML) and Bayesian interferences (BI). The analysis included 4 specimens from NCBI-GenBank for *cox*1 and 43 for ITS2 (Table 2). Non-identical haplotypes of *cox*1 and ITS2 sequences were aligned by eye and edited in MEGA6 (Tamura *et al.* 2013). A total of 690 bases were used for cox1 alignment and 385 bases for ITS2 alignments. Sequences generated in this study were submitted to GenBank (Table 2). ML analyses used PAUP* 4.0 b10 (Wilgenbusch and Swofford, 2003) and BI analyses were carried out using MrBayes (v 3.12) (Ronquist and Huelsenbeck, 2003). MrModeltest 2.0 (Nylander, 2004) was used to find the best fit model of substitution for BI and ML for both

genes. Heuristic searchers were utilized for ML analyses (excluding the third codon for *cox*1) and 100 bootstrap replicates were run for each dataset. For BI analyses of the *cox*1 dataset (excluding the third codon for *cox*1), the parameters were: nst=6, rates =invgamma, and ngammacat= 4. Four heated chains were run simultaneously for 1,000,000 generations. For BI analyses of the ITS2 dataset, the parameters were: nst=6, rates =gamma, and ngammacat= 4. Four heated chains were run simultaneously for 1,400,000 generations. In both datasets the trees were sampled every 100 cycles, and the first 25% of trees with pre-asymptotic likelihood scores were discarded as burn-in. The number of generations were determined sufficient because the SD dropped below 0.01 at the end of the runs.

Nucleotide substitution saturation at the third codon was tested in DAMBE5 (Xia, 2013) for *cox*1. Uncorrected pairwise distances values were calculated in MEGA6 (Tamura *et al.* 2013). Data were summarized within and between groups (Tables 3 and 4). We used similar criteria of other studies that used a *p*-distance value > 5% difference with *cox*1 and *nd*1 mtDNA markers and > 1.0% for ITS to indicate separate species (Vilas *et al.* 2005; Brant and Loker, 2009; Detwiler *et al.* 2010).

Results

Samples

Paramphistomoid adults were collected from three species of ruminants and cercariae and/or rediae were collected from four different genera of planorbid

snails (*Biomphalaria*, *Bulinus, Ceratophallus, Segmentorbis*) from ten localities in central and west Kenya (Tables 1 and 2). Paramphistomoid cercariae were not found in other snail species examined (*Melanoides tuberculata*, *Radix natalensis*, *Physa acuta* and *Bellamya unicolor*). Ruminants were typically heavily infected, and often hundreds of adult worms could be quickly collected per host. From our samples collected, we examined and sequenced 79 adults and 41 cercariae specimens (120 total specimens) that represented obvious variants. To facilitate sampling, if a large numbers of adult worms were acquired from a single host, we separated them by differences in adult host morphology (size and presence of a pouch or a genital sucker). To further assure collection of a diversity of specimens, we sampled both adult worms and rediae/cercariae from different localities

Outgroup determination

With the diversity of sequence data available in GenBank, our analysis revealed that *Ogmocotyle sikae* (Notocotylidae) is more closely related to paramphistomoids than members of Echinostomatidae or Fasciolidae used as outgroups for other paramphistomoid molecular phylogenies (Lotfy *et al.* 2010; Shylla *et al.* 2011; Ghatani *et al.* 2012). For phylogenetic analyses of both genes, we used three species of notocotylids as outgroup taxa.

Cox1 phylogenetic analyses and pairwise distance divergences

In general, trees were first constructed incorporating all 120 specimens (Supplementary Figs. S1 and S2). Because some clades were represented by

multiple specimens (haplotypes with a 1-4 bp difference for *cox*1) we reduced the number of specimens per clade to simplify the trees for display purposes (Figs. 1 and 2). Many of the deeper nodes were not supported, however, the trees nonetheless provided a useful way to visualize the overall diversity of specimens found, and to provide comparisons with available systematic treatments. The specific clades identified (names next to the bolded black vertical lines) on the *cox*1 tree represent conspecifics (Fig. 1).

Partial sequences of *cox*1 (690 bp) were obtained for all 120 samples (Supplementary Fig. S1). ML and BI (Supplementary Fig. S3) trees were created for the *cox*1 alignment, and the ML tree is shown (Fig. 1). MrModeltest 2.3 selected the GTR+I+G model of nucleotide substitution. Based on bootstrap and posterior probabilities in Table 3, 16 distinct *cox*1 clades were identified among Kenyan specimens and are portrayed alongside the tree in Fig. 1 (vertical black lines or arrows). We used genetic distance data to determine if a clade was comprised of more than one species. A single species was determined for specimens with genetic distance values <1.3%, and species were designated as distinct when genetic distance values were >6.2% (Table 3). Most interclade pairwise distance values were >10.0% and they ranged up to 19.9%. These same clade numbers or scientific names were also used adjacent to the ITS2 tree in Fig. 2.

ITS2 phylogenetic analyses and pairwise distance divergences

For ITS2, sequences were obtained from all 120 samples and our phylogenetic analyses also included 46 samples from GenBank (Supplementary

Fig. S2). The ITS2 alignment included 61 bp of 5.8S, 283 bp of ITS2, and 46 bp of 28S. The average intraclade pairwise distance was 0.30% and the average interclade pairwise distance was 3.9% (Table 4). MrModeltest 2.3 selected the GTR+G model of nucleotide substitution for ITS2. Both BI and ML analyses were run using 33 or 46, respectively, additional relevant species sequences from GenBank, with the ML tree shown (Figs 2 and Fig S4). Not surprisingly, the degree of resolution provided by phylogenetic analysis of ITS2 sequences was not high given the more conservative rate of change of this widely-used nuclear gene marker (Locke *et al.* 2010). Based on ML and BI analyses, 12 ITS2 clades were identified among our Kenyan specimens (Fig. 2 and Supplementary Fig. S4.) Intraclade genetic distance values were <0.6%, and interclade genetic distance values were >1.0%.

Further comparisons of the cox1 *and ITS2 datasets*

*Cox*1 and ITS2 trees did not conflict, but the ITS2 trees did not have as much support for the deeper nodes as *cox*1 (Figs 1 and 2). All 12 clades from ITS2 were represented in the *cox*1 data set. The *cox1* genetic distance data enabled differentiation among some of the worms clustered with *Cotylophoron cotylophorum* in the ITS2 data set, and also clearly differentiated clades 14 and 15 (Fig. 2).

In three cases (clades 4, 10, 16), *cox*1 sequence matches (<1.3%) were obtained between worms from ruminants and cercariae from snails (Fig. 1, orange stars). Clade 2 matched an ITS2 sequence from GenBank of cercariae from *Ceratophallus natalensis*, thus also confirming the intermediate host for this

clade (Fig. 1, yellow star). In four cases (clades 1, 5, 10, 12), sequences were found from cercariae with no matches from adult worms for either sequence (Fig. 1). In at least five cases (PA7, PA26, PA27, PA35, and PA42), the ITS2 nuclear sequences obtained clustered in different clades than what is seen in the *cox*1 trees (clades highlighted with red star in Fig. 2). These samples appear to have nuclear mitochondrial discordance (NMD) and are identified as worms with likely hybrid ancestry (see discussion).

Provisional identification of the paramphistomoids

Provisional identifications were based on the paramphistomoid systematics literature (Eduardo, 1983; Sey, 1991; Jones, 2005b,c,d) pertaining to intermediate or definitive host use, and descriptions of adult worms in comparison to our mounted adult specimens (Table 5, Fig. 3). Some of the sequences we obtained matched sequences from named species in GenBank, and in those cases the names we provide here are the ones from GenBank (clades 4, 8, and 16). Four clades were represented only by cercariae and did not match any sequences derived from adult worms in this study or from GenBank. These included two clades from *B. pfeifferi* (clades 1 and 12), one from *Segmentorbis kanisaensis* (clade 5), and one from *C. natalensis* (clade 10). Our 16 clades represented three different families of Paramphistomoidea: Gastrothylacidae, Paramphistomidae and Stephanopharyngidae. Species names in quotation marks in Fig. 1 were assigned based on our morphological identification from species descriptions.

Discussion

Paramphistomoid flukes are speciose in sub-Saharan Africa, reflective of the presence there of many mammal species, particularly wild and domestic ruminants. These flukes are also ubiquitous and can have a high prevalence among domestic ruminants reaching 100% in some villages (Chingwena *et al.* 2002a; Nzalawahe *et al.* 2015). During our sampling of Kenyan slaughterhouses, we found up to 90% percent of the domestic ruminants infected, and many individual animals harbored hundreds of adult worms. Of the many adult worm and cercariae samples collected, we further investigated 120 samples (79 adult worms, 41 cercariae) determined most likely to be genetically distinctive. We found 16 distinct clades in three families of the Paramphistomoidea. For future comparisons, all of our specimens are available as vouchers at the Parasite Division, Museum of Southwestern Biology (MSB) or at the Kenyan Medical Research Institute (KEMRI).

Previous studies have used the easily-obtained ITS2 sequence as a molecular marker to distinguish among paramphistomoid species (Itagaki *et al.* 2003; Rinaldi *et al.* 2005; Goswami *et al.* 2009; Lotfy *et al.* 2010; Sanabria *et al.* 2011; Shylla *et al.* 2013; Ichikawa *et al.* 2013; Ghatani *et al.* 2014; Dube *et al.* 2015). ITS2 is helpful for distinguishing paramphistomoid genera and differentiating more divergent species within a genus (Rinaldi *et al.* 2005; Ghatani *et al.* 2012). Because mitochondrial DNA accumulates substitutions more frequently than the internal transcribed spacers, it is more useful to differentiate among closely related species, particularly cryptic species (Blouin, 2002; Vilas *et al.* 2005; Locke *et al.* 2015), or to reveal intraspecific variation (Ghatani *et al.*

2014). Consequently, we used genetic distance values for *cox*1 sequence data as the primary means to delineate species. For *cox*1, interclade *p*-distance values were > 6.2%, although the majority of pairwise comparisons were >10.0%. In contrast, intraclade pairwise divergence values were less <1.3%. Other studies have used a *p*-distance value > 5% difference with *cox*1 and *nd*1 mtDNA markers to indicate separate species (Vilas *et al.* 2005; Brant and Loker, 2009; Detwiler *et al.* 2010). Our data suggests that ITS2 should not be used alone to differentiate species for paramphistomoids.

We also examined the delineated clades with respect to where they grouped in either ML or BI phylogenetic analyses based on either *cox1* or ITS2 sequences. In general, there was low bootstrap/posterior probability support for many of the deeper nodes in either ML or BI trees, suggesting that broader taxon sampling, along with sequencing of additional markers, is needed to more definitively support or refute the morphologically based systematic framework developed for paramphistomoids (Sey, 1991; Jones, 2005a). The phylogenetic trees were useful, however, in providing preliminary hypotheses for how the various clades were related to one another (see paragraph below). Relative to other paramphistomoid molecular phylogenetic studies involving specimens from African ruminants and snails, we recovered 5 out of the 6 previously reported taxa from Kenya, Egypt, and Tanzania noted by Lotfy et al. (2010), 3 out of the 3 identified taxa from Zimbabwe, Zambia, and Botswana (Dube *et al.* 2015) and 1 of the 2 identified taxa from Algeria (Titi *et al.* 2014). The extent of overlap among specimens recovered from all four studies suggests that at least some of the

species have broad distributions in Africa. Additional sampling is needed to provide a more comprehensive picture of African paramphistomoid diversity, particularly from Central and West Africa.

The phylogenetic trees provided support for anatomically-based taxon delineations as four clades identified as *Calicophoron* grouped together, as did three clades of *Carmyerius* and four clades of *Cotylophoron*. Furthermore, worms in the Stephanopharyngidae (*Stephanopharynx)* formed a clade, as did presumptive members of the Gastrothylacidae. However, all presumptive members of the Paramphistomidae did not group together. It is possible that this is a paraphyletic group or that certain genera, such as *Cotylophoron* belong in a different family. Clade 1 is quite divergent from the other specimens discussed and it is possible it represents a different family or superfamily. The trees also show some incongruences between nuclear and mitochondrial sequences (discussed further below).

With respect to host use, specimens from a particular clade were reported from the same snail host species or genus, and different clades that group together tend to share the same genus of snail host (*Calicophoron*, in clades 13-16, in *Bulinus*) or snail genera in related tribes (*Carymerius* in clades 2, 3 and 5 in *Segmentorbis* and *Ceratophallus*). For 10 of 11 clades for which snail host usage could be identified, those snails belong in the family Planorbidae. Snail host use may thus have had an important impact on paramphistomoid diversification, which has also been suggested for other digenean groups (Brant and Loker, 2013). In only one instance have we found cercariae that we have

assigned to the same clade (clade 10) that derive from two different snail genera: cercariae from *Ceratophallus natalensis* collected from this study and cercariae from *Biomphalaria sudanica* collected by Lotfy *et al.* (2010). Many other digenean groups also indicate high first intermediate host specificity (Shoop, 1988; Donald *et al.* 2004; Detwiler *et al.* 2010; Brant and Loker, 2013). By contrast, adult worms of a particular clade were often recovered from more than one definitive host species, and we recovered up to three different taxa of paramphistomoids from an individual bovine.

Sequence data derived from life cycle stages from different hosts provide an important alternative way to piece together the complex life cycles of digeneans, especially when experimental exposures are not possible (Chibwana *et al.* 2015). We provide supportive evidence for the life cycles of four of our identified clades (Fig. 1) by matching genetic sequences (< 0.6% for ITS2 and < 1.3% *cox*1) collected from cercariae and adults: 1) ITS2 sequences from cercariae from *Ceratophallus natalensis* (GU735645) collected in Kenya grouped with sequences from adult worms we recovered from cattle (clade 2), provisionally identified as *Carmyerius exporous* (Dinnik and Dinnik, 1960). 2) Cercariae (clade 4) we collected from *C. natalensis* matched adults collected in this study as well as two adults from Botswana (KP639636) and Kenya (GU735658) identified as *Carmyerius dollfusi* by Dube et al. (2015). The latter species was synonymized with *C. mancupatus* (Sey, 1991), a species known to be transmitted by *C. natalensis* (Dinnik, 1965). 3) Sequences from seven adults we obtained (clade 15) matched sequences collected from a cercariae sample

from *B. forskalii*. We provisionally identified the adults as *C. phillerouxi*, which is known to be transmitted by *B. forskalii* (Dinnik, 1961). 4) Lastly, two cercariae samples we collected from *B. forskalii* matched with 23 adults collected in this study, and with one cercariae sample from *B. forskalii* and 18 adults in GenBank, all of which were identified as *C. microbothrium* (clade 16). As the host record and sequence databases grow, the probabilities that more matches will be found also increases, providing a way forward in working out life cycles that will help offset increasing difficulties in doing so with more conventional experimental infections.

The most common paramphistomoid genus we collected was *Calicophoron* (40 out of 120 specimens examined), and the most abundant species was *Calicophoron microbothrium* which is transmitted by bulinid snails. This species is the most geographically widespread paramphistome in Africa, its presence confirmed with molecular markers from Egypt, Kenya, Tanzania, Zambia, Zimbabwe, South Africa, Algeria, and Botswana (Lotfy *et al.* 2010; Titi *et al.* 2014; Dube *et al.* 2015). Given the difficulties in discriminating this species from others based on morphology alone, the broad geographic distribution, and the diversity of different bulinid snails reported as hosts, this species is a good candidate for further inspection as a possible complex of cryptic species. Presently the best sequence available to evaluate this possibility is *cox*1, but most of the data in the literature thus far for this species are for ITS2. Our ML analysis based on 354 bp of ITS2 (figure not shown) suggests there are distinct clades among the samples identified as *C. microbothrium* in GenBank, with an

average distance among them of 0.75%. Other sequence markers are needed to determine if *C. microbothrium* is a complex of cryptic species, and how well differentiated they prove to be from the other *Calicophoron* clades (13-15) identified in this study.

We found some specimens with discordant nuclear and mitochondrial sequences, consistent with the possibility of hybrid origins (red stars, Fig. 2). For example, two samples (PA12 and PA24) grouped with *C. microbothrium* in the ITS2 trees, but fell in their own clade (3) in the *cox*1 trees. PA12 and PA24 were also morphologically distinct from *C. microbothrium*, being provisionally identified as members of the gastrothylacid genus *Carmyerius*. As we have noted, multiple species of paramphistomoids are frequently recovered from a single ruminant host, creating circumstances conducive for potential hybridization. The putative parental species and hybrids (PA7, PA12, PA24 PA27, PA35) all use *Bulinus* as intermediate hosts. It seems possible that the likelihood of successful hybridization would be increased if both parental species use the same genus or species of intermediate host, if as appears intermediate host use is more specific than definitive host use among the paramphistomoids. Other examples of sequence discordance in digeneans also involve groups with closely related species that can hybridize, and that share snail hosts, such as with some species of fasciolids and schistosomes (Steinauer *et al.* 2008; Peng *et al.* 2009). Further studies using microsatellite markers or RADSeq technology will be needed to verify a hybrid origin for paramphistomoids with discordant sequences.

Members of the basommatophoran family Planorbidae are the most common intermediate hosts transmitting paramphistomoids in Kenya, although snails of the Family Lymnaeidae have also been identified as hosts for paramphistomoids in East Africa (Sey, 1991). The snail hosts for some of the clades we have identified such as clades 3, 6, 7, 8 (*Cotylophoron cotylophorum),* 9 and 11 (*Stephanopharynx sp.*) are unknown or require additional sequence-based verification. *Bulinus* snails, with an ancient history and diversification in Africa (Van Damme 1984; Brown, 1994; De Groeve 2005), are particularly prominent as African paramphistomoid hosts (Sey, 1991). By contrast, *Biomphalaria* supports fewer paramphistomoid species and has a much shorter evolutionary history in Africa, with estimates ranging from <1-5 mya (Woodruff and Mulvey, 1997; Campbell *et al.* 2000; DeJong *et al.* 2001). It is noteworthy that clade 1, which is known only from cercariae from *B. pfeifferi,* is one of the most divergent clades we recovered. Clade 1 cercariae are also much larger than the other paramphistomoid cercariae we recovered (about 2.0X longer in combined body and tail length). This raises a possibility that the diversification of paramphistomoids is more recent than the longer evolutionary history of *Bulinus* in Africa might suggest. More data are needed to resolve the phylogenetic position of this and other paramphistomoid clades, including those found in non-ruminant species.

In Kenya, *Bulinus globosus*, *B. nasutus, B. africanus*, *B. tropicus*, *B. forskalii,* and *Biomphalaria pfeifferi,* are known to transmit paramphistomoids as well as ruminant and/or human schistosomes (Brown, 1994; Southgate *et al.*

1989). The overlap in use of snail hosts creates opportunities for distinctive interactions between the two common digenean groups. For example, in Kenya, Southgate et al. (1989) found that *Bulinus tropicus* was capable only of supporting the development of *Schistosoma bovis* to production of cercariae if it was first exposed to *C. microbothrium.* Similarly, in South America, *Biomphalaria oligoza* and *Biomphalaria orbignyi* are naturally resistant to *S. mansoni*, but become susceptible to *S. mansoni* if first exposed to *Zygocotyle lunata* (Spatz *et al.* 2012). Paramphistomoids can also have the opposite influence on the success of other digeneans during co-infections. For example, as compared to snails exposed only to *Fasciola hepatica*, significantly fewer *Pseudosuccinea columella* produced *F. hepatica* cercariae if first exposed to *Calicophoron daubneyi* and then later exposed to *F. hepatica* (Dreyfuss *et al.* 2016).

This study has shown that even in a fairly circumscribed area within one East African country that a considerable diversity of paramphistomoid flukes is present and that several of these fluke species are abundantly represented. Paramphistomoids are of veterinary interest because of their ubiquitous presence in herds of cattle, sheep and goats that are routinely watered in natural habitats where the presence of susceptible species of snails ensures their transmission. Whether the species we have encountered have long parasitized domestic livestock or represent recent acquisitions from the region's many wild ruminants is an interesting question for future study. Studies currently underway in Kenya indicate that paramphistomoid infections are very common in some snail populations, so much so that they may represent significant impediments to the

ongoing transmission of schistosomes using the very same snail hosts in the same aquatic habitats (Laidemitt, personal communication). Furthermore, the spectra of freshwater snails used by these two common digenean groups are broadly overlapping, further increasing the likelihood that interesting interactions and accommodations have been made over evolutionary time. It will be interesting to more fully ascertain how these two major groups of digeneans influence one another's abundance. It is clear though that the domestication of livestock ensures that both paramphistomoid and schistosome (both human and ruminant schistosome species) life cycles are perpetuated side-by-side in the same habitats year after year. Livestock domestication may well prove to have had multiple downstream effects - mediated by the digeneans of livestock - on the present-day transmission of the all-too-common human blood flukes of sub-Saharan Africa.

Acknowledgements

We thank Sarah K. Buddenborg, Dr. Si-Ming Zhang, and Dr. Ben Hanelt for assistance with collection of field samples; Kylie Greider for help with sequencing preparation; and to the International Livestock Research Institute (ILRI), Nairobi, Kenya for sequencing a number of our samples. This research was undertaken with the approval of the National Commission for Science, Technology and Innovation, Permit Number NACOSTI/P/15/9609/4270.

Financial Support

Technical assistance at the University of New Mexico Molecular Biology Facility was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P30GM110907. We thank the following agencies for their financial support: The National Institute of Health (NIH) grant RO1 AI101438, and the Bill and Melinda Gates Foundation for the Grand Challenges Explorations Initiative grant. The content for this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This paper was published with the approval of the Director of KEMRI.

References

Ahmed, A.A.M., Ibrahim, N.A. and Idris, M.A. (2006). Laboratory studies on the prevalence and cercarial rhythms of trematodes from *Bulinus truncatus* and *Biomphalaria pfeifferi* snails from Khartoum State, Sudan. *Sultan Qaboos University Medical Journal,* 6, 65-69.

Anderson, G.R. and Barker, S.C. (1998). Inference of phylogeny and taxonomy within the Didymozoidae (Digenea) from the second internal transcribed spacer (ITS2) of ribosomal DNA. *Systematic Parasitology*, 41, 87–94.

Blouin, M. S. (2002). Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal of Parasitology,* 32, 527-531.

Bowles, J., Blair, D. and Mcmanus, D.P. (1995). A molecular phylogeny of the human schistosomes. *Molecular Phylogenetics and Evolution*. 4, 103-109.

Brant, S. V. and Loker, E. S. (2009). Molecular systematics of the avian schistosome genus *Trichobilharzia* (Trematoda: Schistosomatidae) in North America. *Journal of Parasitology,* 95, 941-963.

Brant, S. V. and Loker, E. S. (2013). Discovery-based studies of schistosome diversity stimulate new hypotheses about parasite biology. *Trends in Parasitology,* 29, 449-459.

Brown, D. S. (1994). *Freshwater snails of Africa and their medical importance,* 2nd edn. Taylor & Francis, London; Bristol, PA.

Brown, D.S. and Kristensen, T.K. (1989). A field guide to African freshwater snails, southern African species. Danish Bilharziasis Laboratory Publication number 383.

Campbell, G., Jones, C. S., Lockyer, A. E., Hughes, S., Brown, D., Noble, L. R. and Rollinson, D. (2000). Molecular evidence supports an African affinity of the Neotropical freshwater gastropod, *Biomphalaria glabrata*, Say 1818, an intermediate host for *Schistosoma mansoni*. *Proceedings of the Royal Society B-Biological Sciences,* 267, 2351-2358.

Cheruiyot, H.K. and Wamae, L.W. (1988). Incidence of bovine paramphistomiasis in Kenya. *Bulletin of Animal Health and Production in Africa*, 36, 55-57.

Chibwana, F. D., Nkwengulila, G., Locke, S. A., McLaughlin, J. D. and Marcogliese, D. J. (2015). Completion of the life cycle of *Tylodelphys mashonense* (Sudarikov, 1971) (Digenea: Diplostomidae) with DNA barcodes and rDNA sequences. *Parasitology Research,* 114, 3675-3682.

Chingwena, G., Mukaratirwa, S., Kristensen, T. K. and Chimbari, M. (2002a). Larval trematode infections in freshwater snails from the highveld and lowveld areas of Zimbabwe. *Journal of Helminthology,* 76, 283-293.

Chingwena, G., Mukaratirwa, S., Kristensen, T. K. and Chimberi, M. (2002b). Susceptibility of freshwater snails to the amphistome *Calicophoron microbothrium* and the influence of the species on susceptibility of *Bulinus tropicus* to *Schistosoma haematobium* and *Schistosoma mattheei* infections. *Journal of Parasitology,* 88, 880-883.

Combes, C. (1982). Trematodes - Antagonism between species and sterilizing effects on snails in biological-control. *Parasitology,* 84, 151-175.

De Groeve, E. (2005). The late Cenozoic freshwater mollusca of the Tugen Hills (Kenya): Taxonomy, palaeoecology and palaeozoogeography. Msc. Thesis, Gent: University of Gent, 117 pp.

DeJong, R. J., Morgan, J. A., Paraense, W. L., Pointier, J. P., Amarista, M., Ayeh-Kumi, P. F., Babiker, A., Barbosa, C. S., Bremond, P., Pedro Canese, A., de Souza, C. P., Dominguez, C., File, S., Gutierrez, A., Incani, R. N., Kawano, T., Kazibwe, F., Kpikpi, J., Lwambo, N. J., Mimpfoundi, R., Njiokou, F., Noel Poda, J., Sene, M., Velasquez, L. E., Yong, M., Adema, C. M., Hofkin, B. V., Mkoji, G. M. and Loker, E. S. (2001). Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni*. *Molecular Biology and Evolution,* 18, 2225-2239.

Detwiler, J. T., Bos, D. H. and Minchella, D. J. (2010). Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America. *Molecular Phylogenetics and Evolution,* 55, 611-620.

Detwiler, J. T., Zajac, A. M., Minchella, D. J. and Belden, L. K. (2012). Revealing cryptic parasite diversity in a definitive host: echinostomes in muskrats. *Journal of Parasitology,* 98, 1148-1155.

Dinnik, J. A. (1954). *Paramphistomum sukari n. sp.* from Kenya cattle and its intermediate host. *Parasitology,* 44, 414-421.

Dinnik, J. A. (1961). *Paramphistomum phillerouxi sp.* nov. Paramphistomatidae) and its development in *Bulinus forskalii*. *Journal of Helminthology,* Trematoda 35, 69-90.

Dinnik, J. A. (1965). The snail hosts of certain Paramphistomatidae and Gastrothylacidae (Trematoda) discovered by the late Dr. P. L. LeRoux in Africa. *Journal of Helminthology,* 39, 141-150.

Dinnik, J. A. and Dinnik, N. N. (1954). The life cycle of *Paramphistomum microbothrium* Fischoeder, 1901 (Trematoda, Paramphistomidae). *Parasitology,* 44, 285-299.

Dinnik, J. A. and Dinnik, N. N. (1957). Development of *Paramphistomum sukari* Dinnik, 1954 (Trematoda: Paramphistomidae) in a snail host. *Parasitology,* 47, 209-216.

Dinnik, J. A. and Dinnik, N. N. (1960). Development of *Carmyerius-Exoporus Maplestone* (Trematoda, Gastrothylacidae) in a snail host. *Parasitology,* 50, 469-480.

Donald, K. M., Kennedy, M., Poulin, R. and Spencer, H. G. (2004). Host specificity and molecular phylogeny of larval Digenea isolated from New Zealand and Australian topshells (Gastropoda: Trochidae). *International Journal for Parasitology,* 34, 557-568.

Dreyfuss, G., Vignoles, P. and Rondelaud, D. (2016). *Pseudosuccinea columella*: experimental co-infections of juvenile and pre-adult snails with the digeneans *Calicophoron daubneyi* and *Fasciola hepatica*. *Journal of Helminthology*, 23, 1-7.

Dube, S., Sibula, M. S. and Dhlamini, Z. (2015). Molecular analysis of selected paramphistome isolates from cattle in southern Africa. *Journal of Helminthology*, 1-5.

Eduardo, S. L. (1982). The Taxonomy of the family Paramphistomidae Fischoeder, 1901 with special reference to the morphology of species occurring in ruminants .2. Revision of the genus *Paramphistomum* Fischoeder, 1901. *Systematic Parasitology,* 4, 189-238. doi 10.1007/Bf00009622.

Eduardo, S.L. (1983). The taxonomy of the family Paramphistomidae Fischoeder, 1901 with special reference to the morphology of species occurring in ruminants .3. Revision of the genus *Calicophoron* Nasmark, 1937. *Systematic Parasitology.* 5, 25-79.

Fain, A. (1953). Contribution à l'étude des formes larvaires des trématodes au Congo belge et spécialement de la larve de *Schistosoma Mansoni*, Bruxelles.

Folmer, O. (1994). DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metasoan invertebrates. *Molecular Marine Biology and Biotechnology,* 3, 294-299

Frandsen, F. and Christensen, N. O. (1984). An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. *Acta Trop,* 41, 181-202.

Ghatani, S., Shylla, J. A., Roy, B. and Tandon, V. (2014). Multilocus sequence evaluation for differentiating species of the trematode Family Gastrothylacidae,

with a note on the utility of mitochondrial COI motifs in species identification. *Gene,* 548, 277-284.

Ghatani, S., Shylla, J. A., Tandon, V., Chatterjee, A. and Roy, B. (2012). Molecular characterization of pouched amphistome parasites (Trematoda: Gastrothylacidae) using ribosomal ITS2 sequence and secondary structures. *Journal of Helminthology,* 86, 117-124.

Goswami, L. M., Prasad, P. K., Tandon, V. and Chatterjee, A. (2009). Molecular characterization of *Gastrodiscoides hominis* (Platyhelminthes: Trematoda: Digenea) inferred from ITS rDNA sequence analysis. *Parasitology Research,* 104, 1485-1490. doi: 10.1007/s00436-009-1354-8.

Gretillat, S. (1964). Valeur taxonomique des caracte`res morphologiques et anatomiques du pore ge´nital chezles Tre´matodes du genre *Carmyerıˆus* (Gastrothylacidae). *Revue d'e´levage et de me´decine ve´te´rinaire des pays tropicaux,* 17, 421–428.

Hechinger, R. F., Wood, A. C. and Kuris, A. M. (2011). Social organization in a flatworm: trematode parasites form soldier and reproductive castes. *Proceedings of the Royal Society B: Biological Sciences,* 278, 656-665.

Herrmann, K. K., Poulin, R., Keeney, D. B. and Blasco-Costa, I. (2014). Genetic structure in a progenetic trematode: signs of cryptic species with contrasting reproductive strategies. *International Journal of Parasitology,* 44, 811-818. S0020-7519(14)00166-0

Horak, I.G. (1971). Paramphistomiasis of domestic ruminants. *Advances in Parasitology*, 9, 33-72.

Ichikawa, M., Kondoh, D., Bawn, S., Maw, N. N., Htun, L. L., Thein, M., Gyi, A., Sunn, K., Katakura, K. and Itagaki, T. (2013). Morphological and molecular characterization of *Explanatum explanatum* from cattle and buffaloes in Myanmar. *Journal of Veterinary Medicine Science,* 75, 309-314. doi: DN/JST.JSTAGE/jvms/12-0389

Itagaki, T., Tsumagari, N., Tsutsumi, K. and Chinone, S. (2003). Discrimination of three amphistome species by PCR-RFLP based on rDNA ITS2 markers. *Journal of Veterinary Medicine Science,* 65, 931-933.

Jones, A. (1991). Characterisation of the muscular organs of paramphistomes from Asian fishes. *Systematic Parasitology*, 18: 9-16

Jones, A. (2005a). Superfamily Paramphistomoidea Fischoeder, 1901. In *Keys to the Trematoda* (ed. Jones, A., Bray, R.A. and Gibson, D.I.), pp. 221–327.CABI Publishing and the Natural History Museum, New York.

Jones, A. (2005b). Family Paramphistomidae Fischoeder, 1901. In *Keys to the Trematoda* (ed. Jones, A., Bray, R.A. and Gibson, D.I.), pp. 229–246. CABI Publishing and the Natural History Museum, New York.

Jones, A. (2005c). Family Gastrothylacidae Stiles & Goldberger 1910. In *Keys to the Trematoda* (ed. Jones, A., Bray, R.A. and Gibson, D.I.), pp. 337-341. CABI Publishing and the Natural History Museum, New York.

Jones, A. (2005d). Family Stephanopharyngidae Stiles & Goldberger 1910. In *Keys to the Trematoda* (ed. Jones, A., Bray, R.A. and Gibson, D.I.), pp. 347–348. CABI Publishing and the Natural History Museum, New York.

Le, T. H., De, N. V., Agatsuma, T., Thi Nguyen, T. G., Nguyen, Q. D., McManus, D. P. and Blair, D. (2008). Human fascioliasis and the presence of hybrid/introgressed forms of F*asciola hepatica* and *Fasciola gigantica* in Vietnam. *International Journal for Parasitology,* 38, 725-730.

Lim, H. K. and Heyneman, D. (1972). Intramolluscan inter-trematode antagonism: a review of factors influencing the host-parasite system and its possible role in biological control. *Advances in Parasitology,* 10, 191-268.

Locke, S. A., Al-Nasiri, F. S., Caffara, M., Drago, F., Kalbe, M., Lapierre, A. R., McLaughlin, J. D., Nie, P., Overstreet, R. M., Souza, G. T., Takemoto, R. M. and Marcogliese, D. J. (2015). Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *International Journal of Parasitology,* 45, 841-855.

Locke, S. A., McLaughlin, J. D., Dayanandan, S. and Marcogliese, D. J. (2010). Diversity and specificity in *Diplostomum spp.* metacercariae in freshwater fishes revealed by cytochrome c oxidase I and internal transcribed spacer sequences. *International Journal of Parasitology,* 40, 333-343.

Lockyer, A.E., Olson, P.D. and Littlewood, D.T.J. (2003). Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory*. Biological Journal of the Linnean Society*, 78, 155–171

Loker, E. S., Moyo, H. G. and Gardner, S. L. (1981). Trematode-Gastropod Associations in 9 Non-Lacustrine Habitats in the Mwanza Region of Tanzania. *Parasitology,* 83, 381-399.

Looss, A. (1896). Recherches sur la faune parasitaire d'Egypte. In *Mémoires présentés à l'Institut égyptien, t. 3* Le Caire.

Lotfy, W. M., Brant, S. V., Ashmawy, K. I., Devkota, R., Mkoji, G. M. and Loker, E. S. (2010). A molecular approach for identification of paramphistomes from Africa and Asia. *Veterinary Parasitology,* 174, 234-240.

Mage, C., Bourgne, H., Toullieu, J. M., Rondelaud, D. and Dreyfuss, G. (2002). *Fasciola hepatica* and *Paramphistomum daubneyi*: changes in prevalences of natural infections in cattle and in *Lymnaea truncatula* from central France over the past 12 years. *Veterinary Research,* 33, 439-447.

Mansour, M.F.A., Ellazek, Y.O. and Madkour, M.M. (2014). Molecular characterization of five digenetic trematode species from different hosts in Egypt. *The Egyptian Society of Experimental Biology Journal,* 10, 1-8.

McNamara, M. K., Miller, T. L. and Cribb, T. H. (2014). Evidence for extensive cryptic speciation in trematodes of butterflyfishes (Chaetodontidae) of the tropical Indo-West Pacific. *International Journal of Parasitology,* 44, 37-48.

Mohammed, N.A.I., Madsen, H. and Ahmed, A.A.M. (2016). Types of trematodes infecting freshwater snails found in irrigation canals in the East Nile locality, Khartoum, Sudan. *Infectious Diseases of Poverty*, 5, 16-26.

Nzalawahe, J., Kassuku, A. A., Stothard, J. R., Coles, G. C. and Eisler, M. C. (2015). Associations between trematode infections in cattle and freshwater snails in highland and lowland areas of Iringa Rural District, Tanzania. *Parasitology,* 142, 1430-1439.

Rinaldi, L., Perugini, A. G., Capuano, F., Fenizia, D., Musella, V., Veneziano, V. and Cringoli, G. (2005). Characterization of the second internal transcribed spacer of ribosomal DNA of *Calicophoron daubneyi* from various hosts and locations in southern Italy. *Veterinary Parasitology,* 131, 247-253.

Rolfe, P. F., Boray, J. C. and Collins, G. H. (1994). Pathology of infection with *Paramphistomum ichikawai* in sheep. *International Journal of Parasitology,* 24, 995-1004.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* 19, 1572-1574.

Sanabria, R. E. F. and Romero, J. R. (2008). Review and update of paramphistomosis. *Helminthologia,* 45, 64-68.

Sanabria, R., Moré G. and Romero J. R. (2011). Molecular characterization of the ITS-2 fragment of *Paramphistomum leydeni* (Trematoda: Paramphistomidae). *Veterinary Parasitology*, 177, 182–185.

Schell, S. C. (1985). *Handbook of trematodes of North America north of Mexico,* University Press of Idaho, Moscow, Idaho.

Sey, O. (1991). *CRC handbook of the zoology of amphistomes,* CRC Press, Boca Raton, Fla.

Shoop, W. L. (1988). Trematode transmission patterns. *Journal of Parasitology,* 74, 46-59.

Shylla, J. A., Ghatani, S., Chatterjee, A. and Tandon, V. (2011). Secondary structure analysis of ITS2 in the rDNA of three Indian paramphistomid species found in local livestock. *Parasitol Res,* 108, 1027-1032.

Shylla, J. A., Ghatani, S. and Tandon, V. (2013). Utility of divergent domains of 28S ribosomal RNA in species discrimination of paramphistomes (Trematoda: Digenea: Paramphistomoidea). *Parasitology Research,* 112, 4239-4253. doi: 10.1007/s00436-013-3616-8.

Sobrero, R. (1962). Ricostruzione del ciclo di vita di *Paramphistomum clavula* (Näsmark, 1937), parassita dei ruminanti in Somalia. *Parassitologia*, 4, 165-167.

Southgate, V. R., Brown, D. S., Rollinson, D., Ross, G. C. and Knowles, R. J. (1985). *Bulinus tropicus* from Central Kenya acting as a host for *Schistosoma bovis*. *Z Parasitenkd,* 71, 61-69.

Southgate, V. R., Brown, D. S., Warlow, A., Knowles, R. J. and Jones, A. (1989). The influence of *Calicophoron microbothrium* on the susceptibility of *Bulinus tropicus* to *Schistosoma bovis*. *Parasitology Research,* 75, 381-391.

Spatz, L., Cappa, S. M. and de Nunez, M. O. (2012). Susceptibility of wild populations of *Biomphalaria spp.* from neotropical South America to *Schistosoma mansoni* and interference of *Zygocotyle lunata*. *Journal of Parasitology,* 98, 1291-1295.

Steinauer, M. L., Hanelt, B., Mwangi, I. N., Maina, G. M., Agola, L. E., Kinuthia, J. M., Mutuku, M. W., Mungai, B. N., Wilson, W. D., Mkoji, G. M. and Loker, E. S. (2008). Introgressive hybridization of human and rodent schistosome parasites in western Kenya. *Molecular Ecology,* 17, 5062-5074.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution,* 30, 2725-2729. Titi, A., Mekroud, A., Chibat Mel, H.,

Boucheikhchoukh, M., Zein-Eddine, R., Djuikwo-Teukeng, F. F., Vignoles, P., Rondelaud, D. and Dreyfuss, G. (2014). Ruminal paramphistomosis in cattle from northeastern Algeria: prevalence, parasite burdens and species identification. *Parasite,* 21, 50.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A. and Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques,* 29, 52, 54.

Van Damme, D. (1984). *The Freshwater Mollusca of Northern Africa: Distribution, Biogeography and Paleoecology*. Dr W. Junk Publishers, Kluwer Academic Publishers, Dordrecht.

Vilas, R., Criscione, C. D. and Blouin, M. S. (2005). A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. *Parasitology,* 131, 839-846.

Wilgenbusch, J. C. and Swofford, D. (2003). Inferring evolutionary trees with PAUP*. *Current Protocols in Bioinformatics,* Chapter 6, Unit 6 4.

Woodruff, D. S. and Mulvey, M. (1997). Neotropical schistosomiasis: African affinities of the host snail *Biomphalaria glabrata* (Gastropoda: Planorbidae). *Biological Journal of the Linnean Society,* 60, 505-516.

Wright, C. A., Rollinson, D. and Goll, P. H. (1979). Parasites in *Bulinus senegalensis* (Mollusca: Planorbidae) and their detection. *Parasitology,* 79, 95-105.

Xia, X. (2013). DAMBE5: a comprehensive software package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution,* 30, 1720-1728.

Figures and Tables

Table 1. Collection Localities in central and west Kenya

Site name	Lat.	Long.
Asao Stream	−0·3181	35·0069
Katito Slaughterhouse	−0·2700	34·9719
Sondu Slaughterhouse	−0·3927	35·018
Kasabong Stream	−0·1519	34·3355
Mgosi Slaughterhouse	−0·0768	34·7754
Mwea	−0·8180	37·6220
Ng'alalia	−1·5357	37·2361
Kibwezi Slaughterhouse	−2·4167	37·9667
Nyabera Swamp	−0·1091	34·7750
Powerhouse Lake Victoria	−0·0941	34·7076

Table 2. Specimen name, host collected from, collection locality, provisional identification, Museum of Southwestern Biology/KEMRI voucher numbers, and GenBank accession numbers of paramphistomoid specimens used in this study. PA1-PA44 contain representatives of the 16 different clades used to construct the Maximum Likelihood and Bayesian trees. PA45-PA120 were included in the preliminary trees. An (*) denotes samples that are in Kenya.

Specimen name host	Provisional ID	Stage	Locality	Year	MSB/KEMRI Voucher	GenBank ITS2	GenBank *cox*1
PA1 Goat	*Calicophoron microbothrium*	Adult	Asao Stream	Aug-12	MSB:Para:25079	KX668901	KX670098
PA2 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25101	KX668933	KX670128
PA3 Cattle	*Calicophoron clavula*	Adult	Mgosi	Jan-10	MSB:Para:25088	KX668944	KX670139
PA4 Sheep	*Calicophoron raja*	Adult	Mgosi	Feb-13	MSB:Para:25078	KX668955	KX670150
PA5 Cattle	*Calicophoron raja*	Adult	Mgosi	Oct-13	MSB:Para:25051	KX668966	KX670161
PA6 Goat	*Calicophoron phillerouxi*	Adult	Asao Stream	Aug-12	MSB:Para:25080	KX668977	KX670172
PA7 Goat	*Calicophoron microbothrium*	Adult	Mgosi	Oct-13	MSB:Para:25050	KX668988	KX670183
PA8 Sheep	Paramphistomoidea	Adult	Mgosi	Nov-13	MSB:Para:25047	KX668999	KX670194
PA9 Sheep	Paramphistomoidea	Adult	Mgosi	Dec-13	MSB:Para:25053	KX669010	KX670205
PA10 Cattle	*Carmyerius mancupatus*	Adult	Mgosi	Jan-14	*MSB:Para:25300/KEMRI:Para:1	KX668902	KX670099
PA11 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Jan-14	*MSB:Para:25045/KEMRI:Para:2	KX668913	KX670108
PA12 Cattle	*Carmyerius gregarius*	Adult	Mgosi	Jan-14	*MSB:Para:25055/KEMRI:Para:3	KX668924	KX670119
PA13 Goat	*Cotylophoron* sp.	Adult	Mgosi	Jan-14	*MSB:Para:25157//KEMRI:Para:4	KX668926	KX670121
PA14 Sheep	*Calicophoron raja*	Adult	Mgosi	Jan-14	*MSB:Para:25153/KEMRI:Para:5	KX668927	KX670122
PA15 *Ceratophallus natalensis*	Paramphistomoidea	Cercariae	Nyabera	Jan-05	MSB:Para:25059	KX668928	KX670123
PA16 *Ceratophallus natalensis*	Paramphistomoidea	Cercariae	Nyabera	Jan-05	MSB:Para:25060	KX668929	KX670124
PA17 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jan-14	*MSB:Para:25138/KEMRI:Para:6	KX668930	KX670125
PA18 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao Stream	Feb-13	MSB:Para:25065	KX668931	KX670126
PA19 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao Stream	Jan-15	*MSB:Para:25287/KEMRI:Para:7	KX668932	KX670127
PA20 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao Stream	Jan-15	*MSB:Para:25288/KEMRI:Para:8	KX668934	KX670129
PA21 *Bulinus forskalii*	*Calicophoron phillerouxi*	Cercariae	Mwea	Feb-13	MSB:Para:25064	KX668935	KX670130
PA22 *Bulinus forskalii*	*Calicophoron microbothrium*	Cercariae	Ng'alalia	May-10	MSB:Para:25150	KX668936	KX670131
PA23 *Biomphalaria pfeifferi*	Unknown	Cercariae	Asao Stream	Jul-15	*MSB:Para:25289/KEMRI:Para:9	KX668937	KX670132
PA24 Cattle	*Carmyerius gregarius*	Adult	Mgosi	May-10	MSB:Para:25113	KX668938	KX670133
PA25 Cattle	*Carmyerius mancupatus*	Adult	Mgosi	Jun-14	*MSB:Para:25070/KEMRI:Para:10	KX668939	KX670134
PA26 Cattle	*Carmyerius exporous*	Adult	Mgosi	Jun-14	*MSB:Para:25071/KEMRI:Para:11	KX668940	KX670135
PA27 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jun-14	*MSB:Para:25073/KEMRI:Para:12	KX668941	KX670136
PA28 Cattle	*Calicophoron raja*	Adult	Mgosi	Jan-10	MSB:Para:25085	KX668942	KX670137
PA29 Cattle	*Calicophoron microbothrium*	Adult	Kibwezi	Oct-13	MSB:Para:25092	KX668943	KX670138
PA30 Cattle	*Calicophoron microbothrium*	Adult	Kibwezi	Oct-13	MSB:Para:25093	KX668945	KX670140
PA31 *Segmentorbis*	Gastrothylacidae	Cercariae	Lake Victoria	Oct-13	MSB:Para:25094	KX668946	KX670141
PA32 *Segmentorbis*	Gastrothylacidae	Cercariae	Lake Victoria	Oct-13	MSB:Para:25095	KX668947	KX670142
PA33 Cattle	*Carmyerius exporous*	Adult	Mgosi	Jan-10	MSB:Para:25114	KX668948	KX670143
PA34 *Segmentorbis*	Gastrothylacidae	Cercariae	Lake Victoria	Oct-13	MSB:Para:25096	KX668949	KX670144
PA35 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jan-10	MSB:Para:25115	KX668950	KX670145
PA36 *Ceratophallus natalensis*	*Carmyerius mancupatus*	Cercariae	Nyabera	Jan-15	*MSB:Para:25290/KEMRI:Para:13	KX668951	KX670146
PA37 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25109	KX668952	KX670147
PA38 Cattle	*Carmyerius exporous*	Adult	Mgosi	Feb-13	MSB:Para:25145	KX668953	KX670148
PA39 Cattle	*Calicophoron phillerouxi*	Adult	Mgosi	Feb-13	MSB:Para:25108	KX668954	KX670149
PA40 Cattle	*Calicophoron clavula*	Adult	Mgosi	Jan-10	MSB:Para:25081	KX668956	KX670151
PA41 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jan-14	*MSB:Para:25048/KEMRI:Para:14	KX668957	KX670152
PA42 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Jan-14	*MSB:Para:25054/KEMRI:Para:15	KX668958	KX670153

PA43 Cattle	*Cotylophoron cotylophorum*	Adult	Mgosi	Jan-10	MSB:Para:25083	KX668959	KX670154
PN1 Notocotylidae	Notocotylidae	Cercariae	Lake Victoria	Jan-15	*MSB:Para:25299/KEMRI:Para:16	KX669021	KX670216
PA44 Goat	*Cotylophoron* sp.	Adult	Mgosi	Jan-14	*MSB:Para:25302/KEMRI:Para:17	KX668960	KX670155
PA45 Cattle	*Calicophoron phillerouxi*	Adult	Mgosi	Oct-13	MSB:Para:25057	KX668961	KX670156
PA46 Cattle	*Calicophoron phillerouxi*	Adult	Katito	Mar-13	MSB:Para:25142	KX668962	KX670157
PA47 Sheep	*Calicophoron phillerouxi*	Adult	Mgosi	Jan-13	MSB:Para:25105	KX668963	KX670158
PA48 Goat	*Calicophoron phillerouxi*	Adult	Asao	Aug-12	MSB:Para:25075	KX668964	KX670159
PA49 Goat	*Calicophoron phillerouxi*	Adult	Asao	Aug-12	MSB:Para:25076	KX668965	KX670160
PA50 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jan-15	*MSB:Para:25291/KEMRI:Para:18	KX668967	KX670162
PA51 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25107	KX668968	KX670163
PA52 Cattle	*Cotylophoron* sp.	Adult	Katito	Feb-13	MSB:Para:25144	KX668969	KX670164
PA53 Sheep	*Cotylophoron* sp.	Adult	Sondu	Feb-13	MSB:Para:25058	KX668970	KX670165
PA54 Goat	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25102	KX668971	KX670166
PA55 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25041	KX668972	KX670167
PA56 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25074	KX668973	KX670168
PA57 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25099	KX668974	KX670169
PA58 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Jan-14	*MSB:Para:25292/KEMRI:Para:28	KX668975	KX670170
PA59 Cattle	*Cotylophoron* sp.	Adult	Mgosi	Feb-13	MSB:Para:25106	KX668976	KX670171
PA60 *Segmentorbis*	Gastrothylacidae	Cercariae	Lake Victoria	Oct-13	MSB:Para:25097	KX668978	KX670173
PA61 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25143	KX668979	KX670174
PA62 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25110	KX668980	KX670175
PA63 Cattle	*Carmyerius exporous*	Adult	Mgosi	Jun-14	*MSB:Para:25069/KEMRI:Para:33	KX668981	KX670176
PA64 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25038	KX668982	KX670177
PA65 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25036	KX668983	KX670178
PA66 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25112	KX668984	KX670179
PA67 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25140	KX668985	KX670180
PA68 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25135	KX668986	KX670181
PA69 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Mar-13	MSB:Para:25293	KX668987	KX670182
PA70 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jun-14	*MSB:Para:25297/KEMRI:Para:34	KX668989	KX670184
PA71 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25120	KX668990	KX670185
PA72 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25124	KX668991	KX670186
PA73 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Aug-12	MSB:Para:25068	KX668992	KX670187
PA74 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Aug-12	MSB:Para:25116	KX668993	KX670188
PA75 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25121	KX668994	KX670189
PA76 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jul-14	*MSB:Para:25294/KEMRI:Para:35	KX668995	KX670190
PA77 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25118	KX668996	KX670191
PA78 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25134	KX668997	KX670192
PA79 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Feb-13	MSB:Para:25295	KX668998	KX670193
PA80 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jan-15	*MSB:Para:25296/KEMRI:Para:19	KX669000	KX670195
PA81 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25136	KX669001	KX670196
PA82 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25137	KX669002	KX670197
PA83 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25119	KX669003	KX670198
PA84 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Feb-13	MSB:Para:25063	KX669004	KX670199
PA85 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25122	KX669005	KX670200
PA86 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25123	KX669006	KX670201
PA87 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25125	KX669007	KX670202
PA88 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25126	KX669008	KX670203
PA89 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25127	KX669009	KX670204
PA90 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25128	KX669011	KX670206
PA91 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Asao	Mar-13	MSB:Para:25130	KX669012	KX670207
PA92 Cattle	*Carmyerius exporous*	Adult	Sondu	Feb-13	MSB:Para:25301	KX669013	KX670208
PA93 Cattle	*Carmyerius exporous*	Adult	Sondu	Feb-13	MSB:Para:25141	KX669014	KX670209
PA94 Cattle	*Carmyerius exporous*	Adult	Sondu	Feb-13	MSB:Para:25151	KX669015	KX670210
PA95 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25043	KX669016	KX670211
PA96 Cattle	*Carmyerius exporous*	Adult	Sondu	Feb-13	MSB:Para:25111	KX669017	KX670212
PA97 Cattle	*Carmyerius exporous*	Adult	Katito	Feb-13	MSB:Para:25037	KX669018	KX670213
PA98 Cattle	*Carmyerius exporous*	Adult	Sondu	Feb-13	MSB:Para:25044	KX669019	KX670214
PA99 *Biomphalaria pfeifferi*	Paramphistomoidea	Cercariae	Kasabong	Jun-14	*MSB:Para:25298/KEMRI:Para:20	KX669020	KX670215
PA100 Sheep	*Carmyerius mancupatus*	Adult	Sondu	Oct-13	MSB:Para:25154	KX668903	KX670100
PA101 Goat	*Carmyerius mancupatus*	Adult	Mgosi	Jan-14	*MSB:Para:25067/KEMRI:Para:21	KX668904	KX670101
PA102 Goat	*Calicophoron raja*	Adult	Mgosi	Jan-14	*MSB:Para:25052/KEMRI:Para:22	KX668905	KX670102
PA103 Goat	*Calicophoron raja*	Adult	Mgosi	Jan-14	*MSB:Para:25046/KEMRI:Para:23	KX668906	KX670103
PA104 Cattle	*Calicophoron raja*	Adult	Mgosi	Jan-14	*MSB:Para:25100/KEMRI:Para:24	KX668907	KX670104
PA105 Cattle	*Calicophoron raja*	Adult	Mgosi	Jan-14	*MSB:Para:25281/KEMRI:Para:25	KX668908	KX670105
PA106 Goat	*Calicophoron raja*	Adult	Asao	Jan-14	*MSB:Para:25077/KEMRI:Para:26	KX668909	KX670106
PA107 Sheep	*Calicophoron microbothrium*	Adult	Katito	Jan-14	*MSB:Para:25049/KEMRI:Para:27	KX668910	KX670107
PA108 Cattle	*Calicophoron microbothrium*	Adult	Sondu	Feb-13	MSB:Para:25039	KX668911	KX670096
PA109 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Feb-13	MSB:Para:25282	KX668912	KX670097
PA110 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jan-10	MSB:Para:25089	KX668914	KX670109
PA111 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jan-14	*MSB:Para:25139/KEMRI:Para:29	KX668915	KX670110
PA112 *Bulinus forskalii*	*Calicophoron microbothrium*	Cercariae	Kasabong	Jan-15	*MSB:Para:25283/KEMRI:Para:36	KX668916	KX670111
PA113 Cattle	*Calicophoron microbothrium*	Adult	Kibewze	Oct-13	MSB:Para:25091	KX668917	KX670112
PA114 Goat	*Calicophoron microbothrium*	Adult	Mgosi	Jan-14	*MSB:Para:25284/KEMRI:Para:30	KX668918	KX670113
PA115 Goat	*Calicophoron microbothrium*	Adult	Mgosi	Jan-14	*MSB:Para:25056/KEMRI:Para:31	KX668919	KX670114
PA116 Cattle	*Calicophoron microbothrium*	Adult	Mgosi	Jan-14	*MSB:Para:25285/KEMRI:Para:32	KX668920	KX670115
PA117 Sheep	*Calicophoron microbothrium*	Adult	Katito	Feb-13	MSB:Para:25152	KX668921	KX670116
PA118 Goat	*Calicophoron microbothrium*	Adult	Mgosi	Jan-10	MSB:Para:25090	KX668922	KX670117
PA119 Cattle	*Calicophoron microbothrium*	Adult	Sondu	Feb-13	MSB:Para:25286	KX668923	KX670118
PA120 Cattle	*Calicophoron microbothrium*	Adult	Sondu	Feb-13	MSB:Para:25040	KX668925	KX670120

PA1-PA44 contain representatives of the 16 different clades used to construct the ML and Bayesian trees. PA45-PA120 were included in the preliminary trees. An (*) denotes samples that are in Kenya.

Table 3. Intra- and interclade *p*- distance values of *cox*1 amplified from paramphistomoids from Kenya. Values in bold are intraclade divergences. Note that "-" indicates only a single specimen was collected and within distances could not be calculated.

Clade	*N*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Clade 1	1	_															
2. Clade 2	17	0.185	**0.011**														
3. Clade 3	2	0.199	0.108	**0.003**													
4. Clade 4	5	0.175	0.127	0.132	**0.003**												
5. Clade 5	4	0.166	0.131	0.143	0.126	**0.010**											
6. Clade 6	1	0.157	0.155	0.162	0.133	0.120	_										
7. Clade 7	1	0.164	0.165	0.158	0.134	0.129	0.098	_									
8. Clade 8	1	0.167	0.146	0.163	0.132	0.128	0.062	0.105	_								
9. Clade 9	13	0.158	0.138	0.148	0.124	0.109	0.061	0.099	0.063	**0.010**							
10. Clade 10	2	0.167	0.155	0.160	0.138	0.133	0.126	0.140	0.126	0.128	**0.000**						
11. Clade 11	2	0.177	0.155	0.175	0.164	0.151	0.152	0.155	0.155	0.155	0.140	**0.001**					
12. Clade 12	30	0.171	0.156	0.157	0.138	0.122	0.135	0.144	0.145	0.136	0.130	0.160	**0.009**				
13. Clade 13	9	0.151	0.164	0.169	0.149	0.138	0.132	0.131	0.140	0.123	0.141	0.167	0.123	**0.012**			
14. Clade 14	2	0.158	0.162	0.176	0.144	0.131	0.130	0.126	0.132	0.121	0.138	0.145	0.129	0.088	**0.004**		
15. Clade 15	8	0.161	0.170	0.170	0.150	0.124	0.121	0.135	0.127	0.122	0.134	0.165	0.130	0.098	0.109	**0.010**	
16. Clade 16	22	0.165	0.151	0.179	0.145	0.130	0.142	0.132	0.130	0.140	0.142	0.157	0.131	0.100	0.119	0.111	**0.013**

Table 4. Intra- and interclade *p*- distance values of ITS2 amplified from paramphistomoids from Kenya. Values in bold are intraclade divergences. Note that “-“ indicates only a single specimen was collected and within distances could not be calculated.

Clade	*N*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Clade 1	1	-															
2. Clade 2	17	0.063	**0.003**														
3. Clade 3	2	0.065	0.009	**0.000**													
4. Clade 4	5	0.064	0.009	0.016	**0.005**												
5. Clade 5	4	0.061	0.010	0.015	0.011	**0.002**											
6. Clade 6	1	0.077	0.042	0.045	0.043	0.038	-										
7. Clade 7	1	0.073	0.038	0.042	0.039	0.034	0.004	-									
8. Clade 8	1	0.073	0.038	0.042	0.039	0.034	0.004	0.001	-								
9. Clade 9	13	0.075	0.040	0.044	0.042	0.036	0.007	0.003	0.003	**0.003**							
10. Clade 10	2	0.068	0.017	0.023	0.018	0.014	0.040	0.036	0.036	0.039	**0.003**						
11. Clade 11	2	0.058	0.018	0.025	0.019	0.015	0.041	0.038	0.038	0.040	0.019	**0.006**					
12. Clade 12	30	0.067	0.036	0.042	0.038	0.034	0.050	0.046	0.046	0.048	0.038	0.021	**0.003**				
13. Clade 13	9	0.061	0.021	0.027	0.022	0.018	0.042	0.038	0.038	0.040	0.025	0.018	0.026	**0.003**			
14. Clade 14	2	0.057	0.022	0.029	0.023	0.019	0.035	0.031	0.031	0.034	0.026	0.017	0.025	0.006	**0.001**		
15. Clade 15	8	0.060	0.020	0.026	0.021	0.017	0.038	0.034	0.034	0.037	0.024	0.015	0.023	0.004	0.003	**0.001**	
16. Clade 16	22	0.061	0.027	0.033	0.028	0.023	0.040	0.035	0.035	0.038	0.030	0.021	0.029	0.011	0.004	0.007	**0.003**

Table 5. Provisional identification of the paramphistomoids was based on species descriptions and intermediate host use from the literature and on position in phylogenetic trees. Cercariae (C), adults (A) and their associated hosts are listed. Ventral pouch, acetabulum type and genital sucker were useful morphological features for genus and species placement.

Clade	Provisional Identification	Stage	Ventral Pouch	Acetabulum Type	Genital Sucker	Known Intermediate Hosts	Hosts from this Study	References
1	Unknown	C	n/a	n/a	n/a	n/a	*B. pfeifferi*	Sey, 1991, Jones, 2005a
2	*Carmyerius exporus*	C, A	Yes	*Carmyerius*	No	*Ceratophallus natalensis*	*C. natalensis* and cattle	Dinnik 1965; Sey 1991; Jones, 2005c
3	*Carmyerius gregarius*	A	Yes	*Carmyerius*	No	*Bulinus* species	Cattle	Looss, 1896; Sey 1991
4	*Carmyerius mancupatus*	C, A	Yes	*Gastrothylax*	No	*Ceratophallus natalensis*	*C. natalensis*, cattle, sheep and goats	Gretillat, 1964; Dinnik 1965; Sey 1991, Jones 2005c
5	Unknown	C	n/a	n/a	n/a	n/a	*S. kanisaensis*	Sey, 1991, Jones, 2005c
6	*Cotylophoron sp.*	A	No	*Cotylophoron*	Yes	Unknown	Cattle	Sey, 1991, Jones, 2005b
7	*Cotylophoron sp.*	A	No	*Cotylophoron*	Yes	Unknown	Cattle	Sey, 1991, Jones, 2005b
8	*Cotylophoron cotylophorum*	A	No	*Cotylophoron*	Yes	Unknown	Cattle	Sey, 1991; Eduardo, 1983; Jones, 2005b
9	*Cotylophoron sp.*	A	No	*Cotylophoron*	Yes	Unknown	Cattle, sheep and goats	Sey, 1991, Jones, 2005b
10	Unknown	C	n/a	n/a	n/a	*Ceratophallus natalensis*	*C. natalensis*	Sey, 1991, Jones, 2005a
11	*Stephanopharynx sp.*	A	No	*Stephanopharynx*	No	Unknown	Sheep	Sey 1991; Jones, 2005d
12	Unknown	C	n/a	n/a	n/a	n/a	*B. pfeifferi*	Sey, 1991, Jones, 2005a
13	*Calicophoron raja*	A	No	*Calicophoron*	No	*Bulinus globosus*	Cattle, sheep and goats	Dinnik and Dinnik, 1955; Sey, 1991; Eduardo, 1983
14	*Calicophoron clavula*	A	No	*Calicophoron*	No	*Bulinus abyssinicus*	Cattle	Sobrero, 1962; Eduardo, 1983; Sey, 1991
15	*Calicophoron phillerouxi*	C, A	No	*Calicophoron*	No	*Bulinus forskalii*	*B. forskalii*, cattle, sheep and goats	Dinnik, 1961; Sey 1991, Eduardo 1983
16	*Calicophoron microbothrium*	C, A	No	*Calicophoron*	No	*Bulinus* species	*B. forskali*, cattle, sheep and goats	Dinnik and Dinnik, 1954; Sey, 1991, Eduardo, 1983

Fig. 1. Phylogenetic relationships of 44 samples of paramphistomoids from this study and from GenBank based on *cox*1 (690 bp) sequences inferred from Maximum Likelihood (bootstrap values) analysis. Specimens are named based on sample name, the host it was collected from and are color coded based on intraclade *p*- distance values <1.3% and interclade values > 6.5%. An orange star represents clades where we matched cercariae and adult sequences. A yellow star represents clades where cercariae and adult ITS2 sequences matched. Identifications were made based on GenBank sequences and on the species descriptions in the literature (parentheses). An (*) denotes intermediate host use from studies in the literature that have not been sequenced confirmed.

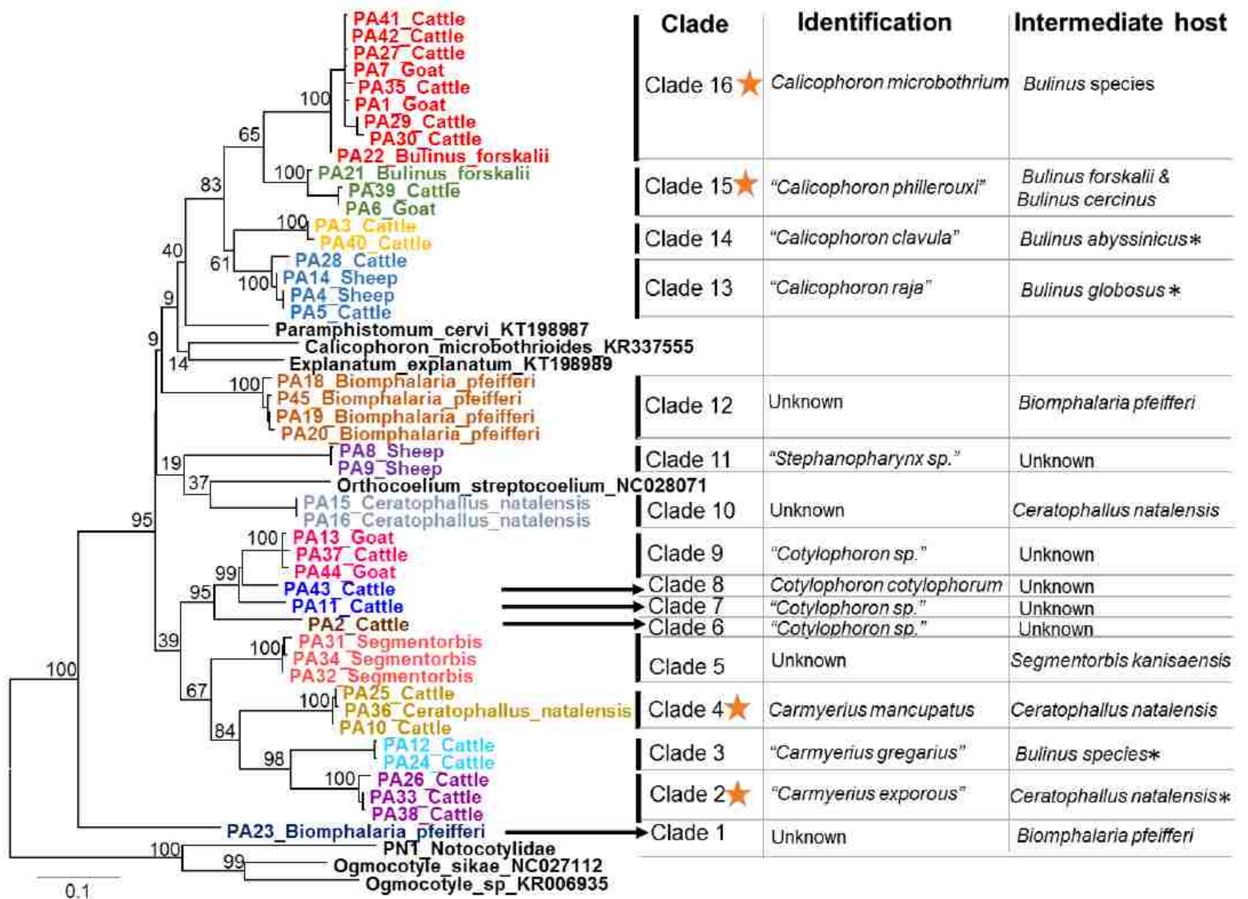
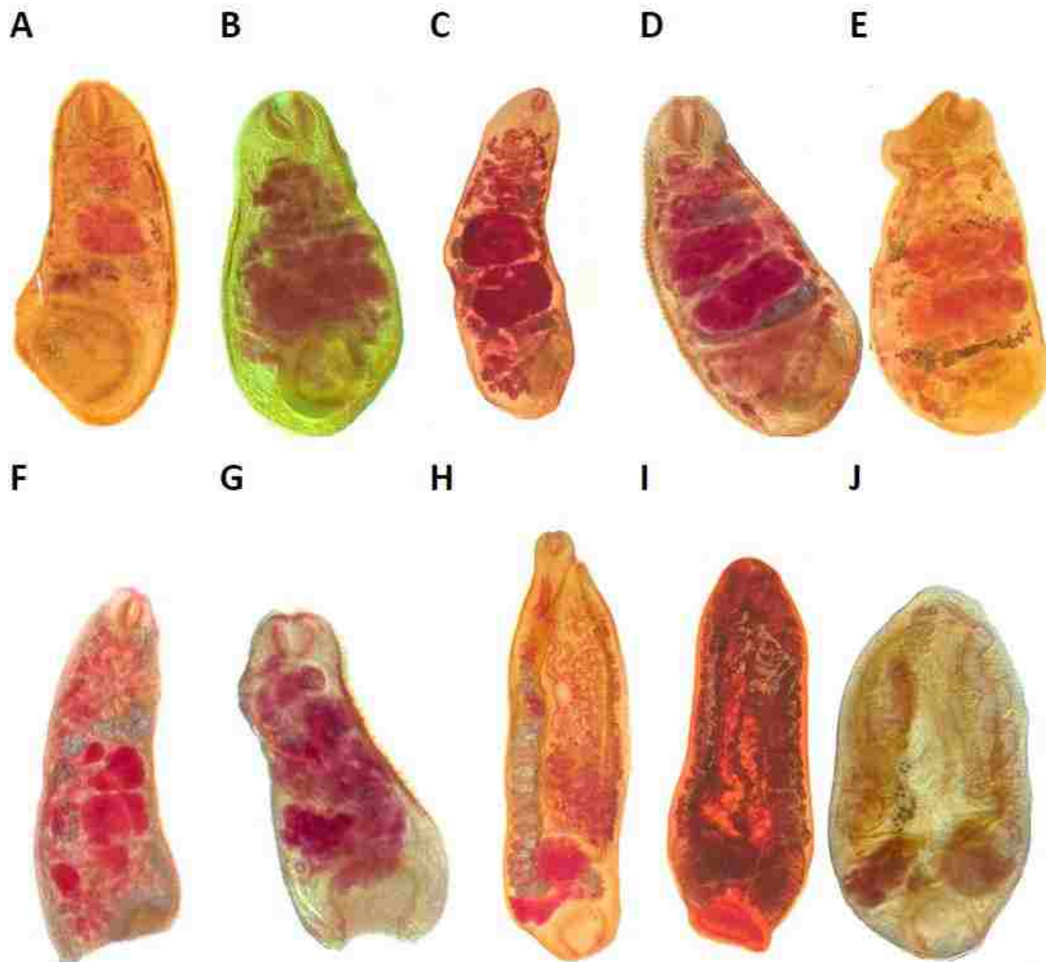
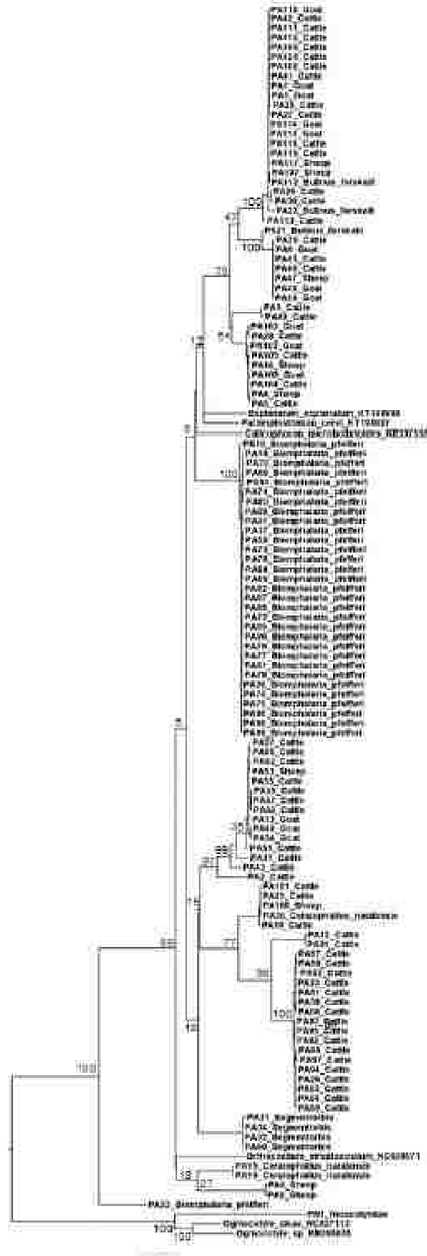


Fig. 2. Phylogenetic relationships of 44 samples of paramphistomoids from this study and from GenBank based on ITS2 (385 bp) sequences inferred from Maximum Likelihood (bootstrap values) analysis. Specimens are named based on sample name, the host it was collected from, and color coded based on clade designation from *cox*1 distance values. A red star represents clades where we have found evidence of putative hybrids. Adjacent to these indicated clades, are clade numbers that correspond to the same specimens and clade numbers as appearing on the *cox*1 tree (Fig. 1).

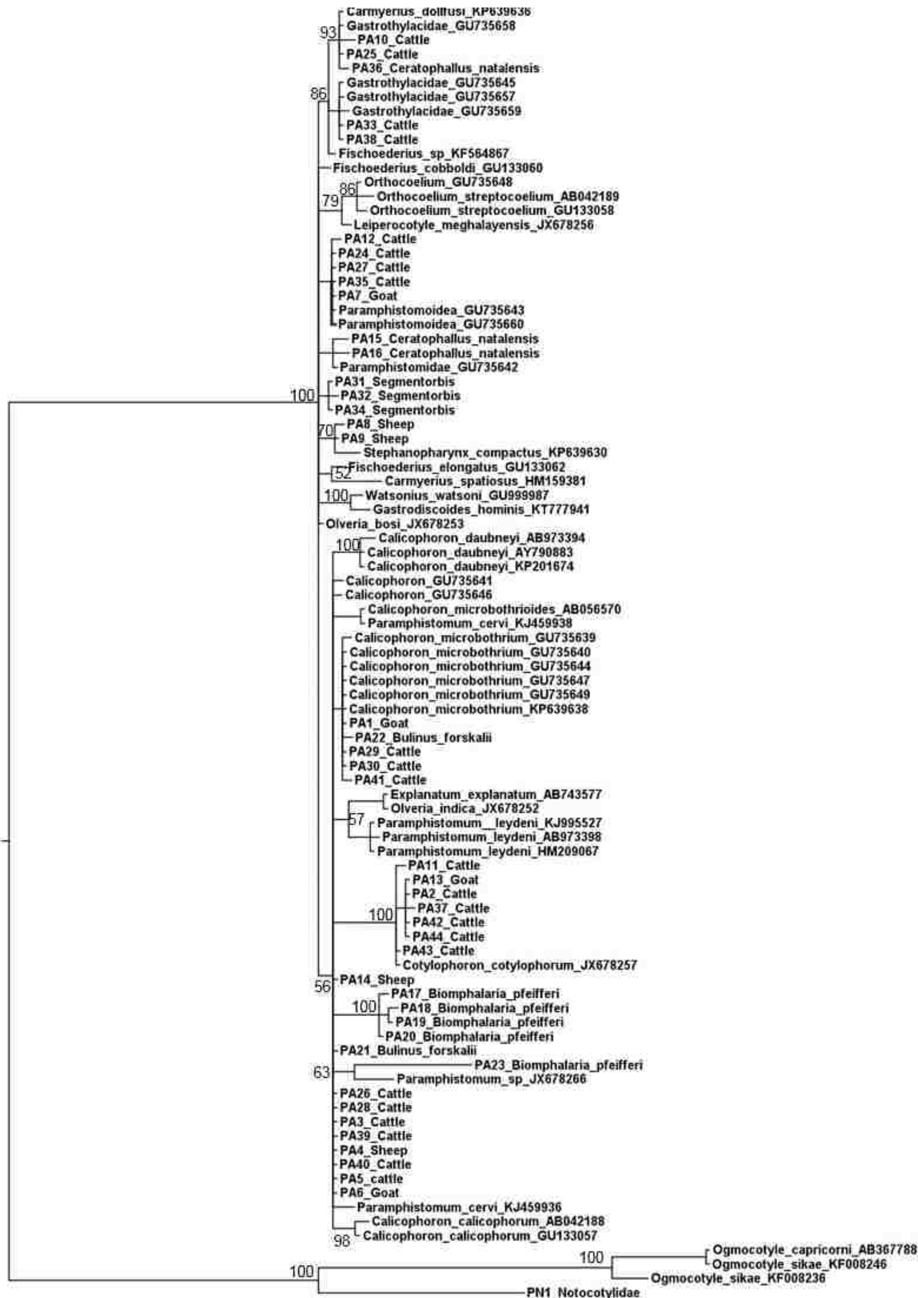
Fig. 3. Sections of adult paramphistomoids collected from domestic ruminants in Kenya and their provisional identifications. A. *Calicophoron phillerouxi* B. *Calicophoron raja* C. *Calicophoron clavula* D. *Calicophoron microbothrium* E. *Cotylophoron sp.* F. *Cotylophoron cotylophorum* G. *Cotylophoron sp.* H. *Carmyerius exporous* I. *Carmyerius gregarius* J. *Carmyerius mancupatus*. Note that the photographed specimens represent sections of adults, and presence of some organs like the testes (T), or genital sucker (GS) are indicated. For the genus *Carmyerius*, a ventral pouch was present, but is not visible in the sections chosen for presentation.



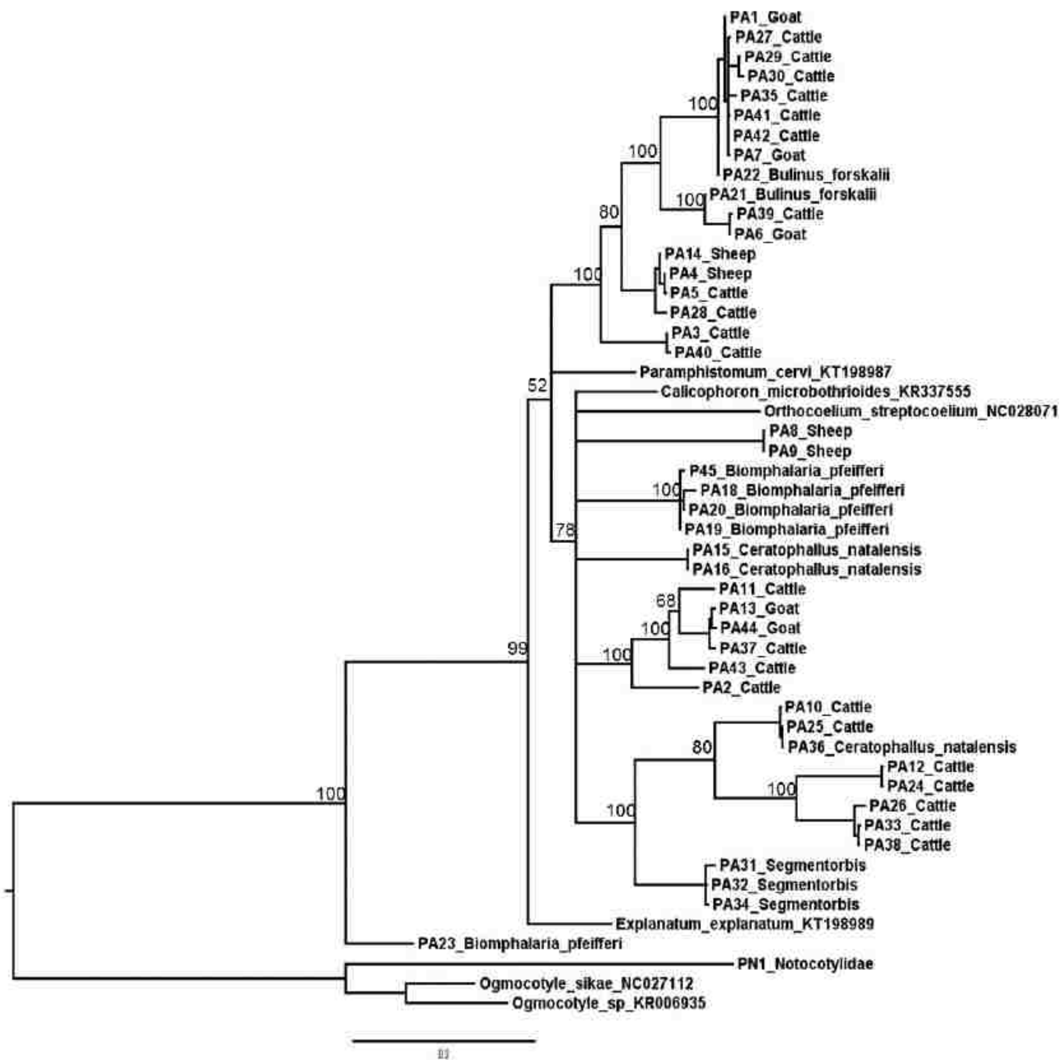
Supplementary Fig. S1. Phylogenetic relationships of 120 samples of paramphistimoids from this study and from GenBank based on *cox*1 (690 bp) sequences inferred from Maximum Likelihood analysis. Specimens are named based on sample name and the host it was collected.



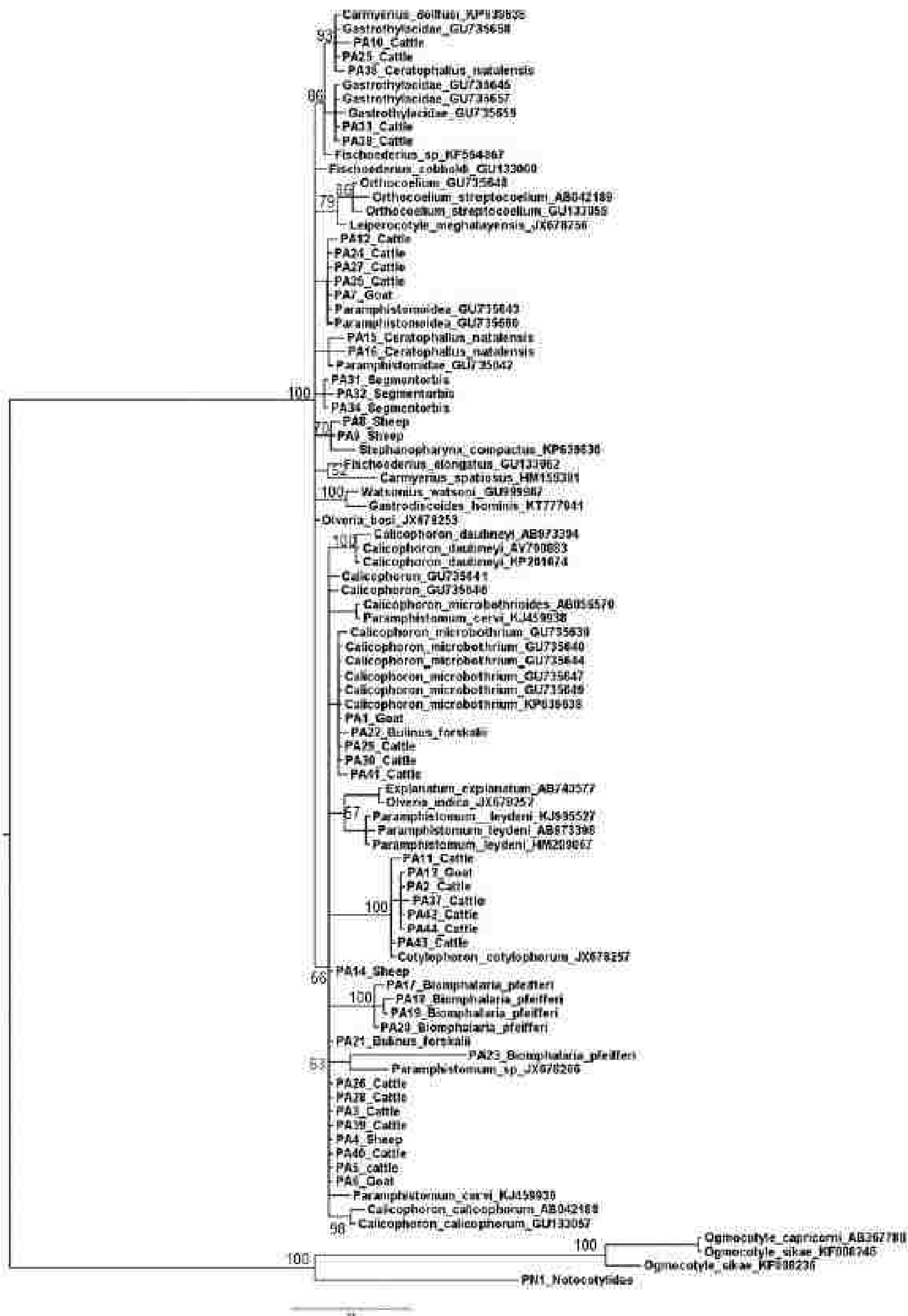
Supplementary Fig. S2. Phylogenetic relationships of 120 samples of paramphistimoids from this study and from GenBank based on ITS2 (385 bp) sequences inferred from Maximum Likelihood analysis. Specimens are named based on sample name and the host it was collected. Bootstrap values are listed if greater than 70%.



Supplementary Fig. S3. Phylogenetic relationships of 44 samples of paramphistomoids from this study and from GenBank based on *cox*1 (690 bp) sequences inferred from Bayesian Interference analysis. Specimens are named based on sample name and the host it was collected.



Supplementary Fig. S4. Phylogenetic relationships of 44 samples of paramphistomoids from this study and from GenBank based on ITS2 (385 bp) sequences inferred from Bayesian Interference analysis. Specimens are named based on sample name and the host it was collected.



Chapter 2. The diverse echinostomes from East Africa: with a focus on species that use schistosome transmitting snails as intermediate hosts

Martina R. Laidemitt[1], Sara V. Brant[1], Martin W. Mutuku[2], Gerald M. Mkoji[2], and Eric S. Loker[1]

1 Department of Biology, Center for Evolutionary and Theoretical Immunology, Parasite Division Museum of Southwestern Biology, University of New Mexico, 167 Castetter MSCO3 2020 Albuquerque, New Mexico 87131, USA

2 Center for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI), P.O. Box 54840-00200, Nairobi, Kenya

Key words: Biodiversity, Echinostomatidae, DNA Barcode, Echinostomiasis, *Biomphalaria*, *Bulinus*, Schistosomes

Abstract

Echinostomes are a diverse group of digenetic trematodes that are globally distributed. The diversity of echinostomes in Africa remains largely unknown, particularly in analyses using molecular markers. Therefore, we were interested in the composition and host usage patterns of African echinostomes, especially those that also use schistosome transmitting snails as intermediate hosts. We collected adults and larval stages of echinostomes from 19 different localities in East Africa. In this study we provide locality information, host use, museum vouchers, and genetic data for two loci (28S and *nad*1) from 98 samples of echinostomes from East Africa. Combining morphological features, host use information, and phylogenetic analyses we found 17 clades of echinostomes in East Africa. Four clades were found to use more than one genus of freshwater snails as their first intermediate hosts, and we determined at least partial life cycles of four clades using molecular markers. The cercariae of many of these clades had peculiar structures (granules or spines) near their anterior end that are likely used for light gathering. Of the 17 clades, 13 use *Biomphalaria* or *Bulinus* as a first intermediate host. The overlap in host usage creates opportunities for competition, including against human schistosomes, and although echinostomes are diverse, future studies need to be done to ascertain the interactions between schistosomes in their respective intermediate hosts.

1. Introduction

The Echinostomatoidea is a diverse superfamily of trematodes that includes nine different families and 105 genera (Tkach et al., 2016). Here we

discuss representatives of one family of that group, the Echinostomatidae, referred to hereafter as echinostomes.

Echinostomes are characterized by having a distinctive crown of collar spines, a ventral sucker larger than the oral sucker, two testes tandemly or symmetrically arranged, a pretesticular ovary, and a cirrus sac (Kostadinova and Jones 2005). Echinostomes have a multi-host life cycle that involves a vertebrate definitive host, a molluscan first intermediate host, and a second intermediate host that is typically a mollusc, amphibian, or fish. The family Echinostomatidae (with a recent reclassification to now include the former Rhopaliidae, Looss, 1899; Cathaemasiidae Fuhrmann, 1928; and Ribeiroiinae Travassos, 1951) is the most speciose family in the superfamily (Tkach et al., 2016). Delineation of genera has traditionally been based extensively on characteristics of adult worms and has included consideration of definitive host use, the morphology of the cephalic collar, number and arrangement of the collar spines, position of the testes and ovary, and location and structure of the vitellaria (Kostadinova 2005). Characteristics of the larval stages, especially of cercariae, have received less consideration. A recent molecular phylogenetic study focused on 28S rDNA sequences and incorporated a broad array of echinostome species has provided a new framework to organize our thinking about echinostomes (Tkach et al., 2016).

Echinostomes are of interest to parasitologists not only for their diversity, but also for their distinctive morphology and life cycles, their systematic inter-relationships and their interactions with their intermediate hosts. Echinostomes

are often easily maintained in the laboratory and thus provide excellent subjects to pursue studies of parasite-host relationships (Fried and Graczyk 2000; Fried 2001; Toledo et al., 2009). Many questions remain as to how these species diversify and remain distinct in nature. Moreover, their morphological innovations and peculiar patterns of host use have yet to be fully elucidated. Thus, parasitologists with interests in evolutionary and ecological processes have found echinostomes to be intriguing subjects for study, especially given that recent studies have documented that some echinostome "species" are actually complexes of cryptic species (Detwiler et al., 2010; Detwiler et al., 2012; Georgieva et al., 2013). Epidemiologists are also interested in studying echinostomes because they can cause disease in humans and animals (Fried and Toledo; Noikong et al., 2014). Immunologists too have adopted echinostomes for study because adult worms can modulate the immune responses of their definitive hosts (Cortes et al., 2017) and echinostome sporocysts and rediae can actively interfere with the defense responses of their snail intermediate hosts (Loker et al., 1992) in ways that differ from the evasive responses of schistosome sporocysts in snails (Bayne 2009).

Echinostomes are of interest to parasitologists not only for their diversity, but also for their distinctive morphology and life cycles, their systematic inter-relationships and their interactions with their intermediate hosts They are often easily maintained in the laboratory and thus provide excellent subjects to pursue studies of parasite-host relationships (Fried and Graczyk 2000; Fried 2001; Toledo et al., 2009). Many questions remain as to how these species diversify

and remain distinct in nature. Fundamental questions remain for echinostomes, including unraveling their morphological innovations and several peculiar aspects in their patterns of host use.Parasitologists with interests in evolutionary and ecological processes have also found echinostomes to be intriguing subjects for study. It has been suspected that some echinostome “species” are actually complexes of cryptic species, and this has been documented in recent studies (Detwiler et al., 2010; Detwiler et al., 2012; Georgieva et al., 2013). Epidemiologists are also interested in studying echinostomes because they can cause disease in humans and animals (Fried and Toledo; Noikong et al., 2014). Immunologists too have adopted echinostomes for study because adult worms can modulate the immune responses of their definitive hosts (Cortes et al., 2017) and echinostome sporocysts and rediae can actively interfere with the defense responses of their snail intermediate hosts (Loker et al., 1992) in ways that differ from the evasive responses of schistosome sporocysts in snails (Bayne 2009).

Of interest to this study are echinostomes transmitted in Africa, for which little is known with respect to biogeography, phylogenetic placement (especially using molecular markers), and host use. The majority of echinostome descriptions from Africa are of adults that use birds as a definitive host (Dietz 1909; Ohdner 1910; Faust 1921; Himly 1949; Dollfus 1950; Bisseru 1957; Appleton et al., 1983; King and As 2000). One of our motivations is to learn how other digenean species, particularly echinostomes, can influence schistosomiasis transmission in Sub-Saharan Africa by competing with schistosome sporocysts for access to their required snail hosts. Studies have shown that when multiple

species of digenetic trematodes colonize the same snail host, echinostomes have usually proven to be dominant to other species (Lim and Heyneman 1972; Hechinger et al., 2011). There is growing evidence that for at least some species, echinostome rediae become specialized for the purpose of attacking and killing the larvae of competing digenean species within the body of their molluscan hosts, while other rediae are more specialized for reproduction (Garcia et al., 2016). An important first step in understanding echinostome-schistosome interactions is the need to gain a full appreciation for the biodiversity of echinostomes extant in East Africa to relate our work to the growing body of work that highlights the relevance between biodiversity and human disease transmission (Johnson and Thieltges, 2010; Civitello et al., 2015).

In addition, our survey data from under-sampled locations coupled with published works will provide additional groundwork for a broader understanding of echinostome biology. This will include several attributes such as the distinctive morphological features among larval stages and patterns of intermediate host usage including the extent to which echinostomes prove to be host specific relative to other digenean groups. Better sequence-based analyses of echinostome diversity will also contribute to understanding broad biogeographical patterns and will enable new perspectives to emerge on phenomena like cryptic speciation.

Towards this end, we collected and characterized different species of echinostomes that are transmitted in East Africa, primarily from western Kenya, with an emphasis on species that use *Biomphalaria* or *Bulinus* as their first

intermediate hosts. These two snail genera host *Schistosoma mansoni,* and *Schistosoma haematobium* and its close relatives, respectively. Our goal is to learn how many echinostome species use these snail hosts in order to provide context for future experiments to determine their ability to compete with and prey upon the sporocysts of schistosomes in their snail hosts. Here we provide locality information, sequence data, provisional species identification, museum vouchers and host use information for the African echinostome adults and larvae collected.

2. Materials and methods

2.1. Sampling

All field-collected aquatic snails were brought to the lab and were individually placed into 12-well tissue culture plates in 3 ml of aged tap water. The tissue culture plates were placed in natural light for two hours to induce shedding of cercariae. Available keys were used for preliminary identification of African snails and their trematodes (Fain, 1953; Brown and Kristensen; 1989; Brown 1994; Frandsen and Christensen, 1984; Schell, 1985). Cercariae and rediae were fixed in 95% ethanol for later molecular analysis.

2.2. Staining adult worms

Adult worms were preserved in 95% ethanol and then were placed into 70% ethanol for 24 hours prior to staining. Part of the posterior portion of the adult was severed and used for molecular work. For the voucher, the remaining part of the adult was stained according to Fried and Manger (1992).

2.3. Molecular Characterization

Partial sequences of the 28S ribosomal gene and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) from 98 echinostome specimens were amplified by polymerase chain reaction (PCR). Samples were chosen based on host usage, locality, and sampling time. One or two cercariae, one rediae, or a partial portion of the posterior end of an adult were used for DNA extraction. Genomic DNA was extracted using the QIAamp DNA Micro Kit following the manufacturer's instructions, with a final elution volume of 35 μl (Qiagen, Valencia, CA).

The 28S gene was amplified using forward primer, dig12 (5'-AAG CAT ATC ACT AAGCGG-3') and reverse primer 1500R (5'-GCT ATC CTG AGGGAA ACT TCG-3') (Tkach et al., 2003). The volume of each PCR reaction was 25 μl with 1 μl of 100 ng of DNA, 0.8mM/L dNTPs, 2.5 mM/L MgCl2, 0.25 units of Ex Taq DNA (Clontech, Mountain View, CA), and 0.4 μM/L of each primer. PCR cycles were followed according to Tkach et al. (2016).

The *nad*1 gene was amplified using forward primer NDJ11 (Morgan & Blair, 1998) (5' -AGA TTCGTA AGG GGC CTA ATA-3') and the reverse primer NDJ2a (5'-CTT CAG CCT CAG CAT AAT-3') (Kostadinova et al., 2003). The volume of each PCR reaction was 25 μl with 1 μl of 100 ng of DNA, 0.8mM/L dNTPs, 2.5 mM/L MgCl2, 0.25 units of Ex Taq DNA (Clontech, Mountain View, CA), and 0.4 μM/L of each primer PCR cycles were performed on Eppendorf Mastercycler epigradient machines which were programmed as follows: 2 min denaturation at 94 °C; 94 °C for 1 min, 54 °C for 30 s and 72 °C for 1 min for three cycles; 94 °C for 1 min, 53 °C for 30 s, and 72 °C for 1 min for three cycles;

94 °C for 1 min, 52 °C for 30 s and 72 °C for 1 min for three cycles; 94 °C for 1 min, 51 °C for 30 s and 72 °C for 1 min for 20 cycles, and followed by an extension step for 7 min at 72 °C.

For some of the samples, only cercariae were saved. As cercariae may have adherent snail macromolecules on them including amplifiable DNA (Devkota et al., 2015), we used snail *cox1* primers to generate amplicons from those cercariae derived from *Bulinus*. This was done in attempt to verify the original identification of the *Bulinus* species from which the cercariae were derived, because identification based only on snail keys is difficult for this genus. Many of samples yielded amplicons; however, in some cases, we were unable to amplify snail DNA from the cercariae samples, therefore we did not designate a species.

PCR fragments were separated by agarose gel electrophoresis and visualized with 0.5% GelRed™ Nucleic acid gel stain (Biotium, Hayward, CA, USA). PCR products were purified using the Illustra ExoProStar (GE Healthcare Life Sciences, Pittsburgh, PA). Both strands were sequenced using an Applied Biosystems 3130 automated sequencer and BigDye terminator cycle sequencing kit Version 3.1 (Applied Biosystems, Foster City, CA). DNA sequences were verified by aligning reads from the 5’ and 3’ directions using Sequencher 5.0 and manually corrected for ambiguous base calls (Gene Codes, Ann Arbor, Michigan).

2.4. Sequence alignment and phylogenetic analyses

28S and *nad*1 sequences were used in phylogenetic analyses using Maximum Likelihood (ML) and Bayesian interferences (BI). The analysis included 47 specimens from NCBI-GenBank for 28S and 41 for *nad*1. Non-redundant sequences were aligned by eye and edited in MEGA7 (Kumar et al., 2016). A total of 1,143 bases were used for 28S alignment and 493 bases for *nad*1 alignments. Sequences generated in this study were submitted to GenBank (Table 2). ML and BI analyses were carried out using PAUP* 4.0 b10 (Swofford, 2003) and MrBayes v 3.12 (Ronquist and Huelsenbeck, 2003) respectively. jModelTest 2.0 (Darriba et al., 2012) was used to find the best fit model of substitution for BI and ML for both genes. Heuristic searchers were utilized for ML analyses and 1,000 bootstrap replicates were run for each dataset. For BI analyses the parameters were unlinked: In both datasets the trees were sampled every 100 cycles, and the first 25% of trees with pre-asymptotic likelihood scores were discarded as burn-in.

Uncorrected pairwise distance values were calculated in MEGA7 (Kumar et al., 2016). Data were summarized within and between groups (Tables 3, 4). We followed other studies in using a *p*-distance value >5% in mtDNA markers to provisionally designate our specimens as distinct species (Vilas et al. 2005; Brant and Loker, 2009; Detwiler et al. 2010; Laidemitt et al., 2017).

3. Results

3.1. Samples

We collected echinostome adults and larva between 2002-2017 from 19 localities (Table 1). Cercariae or rediae were collected from 9 species of snail hosts and adults were collected from two species of birds. We sequenced 28S and *nad*1 from 92 different cercariae, 4 metacercariae, and 2 adult samples. Although we attempted to sequence *nad*1 from all 98 samples, 4 samples would not amplify using the *nad*1 primers. Our specimens were deposited as vouchers in the Museum of Southwestern Biology (MSB).

3.2. 28S Phylogenetic analyses

Forty-seven samples from GenBank and 98 specimens from this study were used in analyses to determine into which clades our specimens fell. Because some clades had multiple representatives, we chose two or three specimens per clade to simplify the display of echinostome diversity. Sequences (1,243 bp) were obtained for all 98 samples of which 1,143 bp were used for Maximum Likelihood (Fig 1.) and BI (not shown) analyses. Analyses were run using the G+I+F model of nucleotide substitution by the Akaike Information Criterion (AIC) jModelTest 2.1 (Darriba et al., 2012). *Caballerotrema sp*. was used as the outgroup because it is the most related family to Echinostomatidae that has GenBank records. (Tkach et al., 2016). ML and BI topologies were identical and overall the BI analysis had higher nodal support than the ML analysis. These analyses revealed 17 clades, the names for which are shown in Fig 1. Clades were color coded (Figs 2 and 3) based on intraclade *nad*1 *p-distance* value of less than 1.5% (see below).

3.3. nad*1 Phylogenetic Analyses*

Forty-one samples from GenBank and the same specimens from this study were used to generate the 28S tree in this study were used in the analysis. Four of the *Ribeiroia* samples did not amplify or the quality of the sequences was poor. Therefore, 94 samples were used in the original analyses and to determine *p-distance* values. *Fasciolopsis buski* (EF612501) was used as the outgroup instead of *Caballerotrema sp.* because *nad*1 sequences for *Caballerotrema sp.* are not represented in GenBank (Tkach et al., 2016). ML and BI analyses were run using the GTR+I+G model of nucleotide substitution by the Alkaike Infromation Criterion (AIC) jModelTest 2.1 (Darriba et al., 2012). The ML and BI topologies were identical and overall the BI tree had higher nodal support than the ML tree. *Nad*1 sequences revealed two additional clades that were not found from the 28S analysis (see below under *Patagifer*).

3.4. Clade 1 (Echinostoma caproni*)*

Two of our specimens (PE79 and PE89) were representatives of *Echinostoma caproni* (*p*-distance value 0.005) based on GenBank accession number, AF025829 from Madagascar (Morgan et al., 1998).

*3.5. Clades 2-3 (*Patagifer*)*

*3.5. Clades 2-3 (*Patagifer*)*

Representatives of *Patagifer* were known to use ibises as definitive hosts and snails as first and second intermediate hosts (Faltynkova et al., 2008). Many of our samples (43) grouped into clades 2 and 3. Thirty-one specimens grouped into clade 2 *(Patagifer* sp. 1) and 12 specimens grouped into clade 3 (*Patagifer*

sp. 2). There was a 0.077 (7.7%) *p*-distance value between these two clades. We completed the life cycle of worms from clade 2. We acquired eggs from fecal samples from a sacred ibis (*Threskiornis aethiopicus*), hatched the eggs and experimentally exposed *Biomphalaria sudanica* to the miracidia. We then used cercariae from successful experimental infections to expose *B. sudanica* to obtain metacercariae. We sequenced representatives of each life cycle stage for clade 2 and found them to be identical or to differ by less than 1.0% from one another. Clade 2 cercariae had tail fins and 58-62 collar spines. The larvae also possessed a structure we termed the spine pocket containing approximately 20 spines that was located mid-ventrally just posterior to the oral sucker. Other descriptions called this unit a “brush of needles” (Appleton et al., 1983) or a “rosette of spines” (Ostrowski de Nunez 1997). These cercariae were also noteworthy for possessing diverticuli (greater than 16/side) along the length of their major excretory canals and for possessing numerous calcareous corpuscles (90-100 granules/side) in each major excretory canal (Fig. 3B). Clade 2 closely grouped with a 28S GenBank sample of an adult *Patagifer vioscai* worm which had 53 collar spines (Falynkova et al., 2008). Acquisition of *nad*1 sequences for *P. vioscai* would help clarify the relationship to our clade 2 specimens. *P. vioscai* is from the American white ibis (*Eudocimus albus*) which is endemic to the Americas. We also noted that cercariae of our clade 2 resembled cercariae from two South American species of *Biomphalaria*: 1) cercariae of *B. tenagophila* from the Uruguay River that transmitted an echinostome cercaria with 58 spines and 16 excretory diverticuli/side (Martorelli et al., 2013), and 2) cercariae from

Biomphalaria straminea from Argentina have been reported with 53-54 collar spines, a spine pocket, diverticuli and tail fins (Fernandez et al., 2014).

Clade 3 cercariae have tail fins, 54 collar spines, a spine pocket posterior to the oral sucker containing a cluster of 25 spines (Fig 3. A2), fewer diverticuli (less than 16/side) along each major excretory canal, and less than 60 calcareous corpuscles within each excretory canal. Appleton et al. (1983) established the life cycle of *Echinoparyphium montgomeriana* from South Africa. He found this species to be transmitted by *Bulinus africanus* and reported it to have 48-54 collar spines and a brush of spines posterior to the oral sucker and the species was named *E. montgomeriana* which we believe does not correspond to that genus as defined by (Tkach et al., 2016). Ostrowski de Nunez et al. (1996) described a similar cercaria (including with a spine pocket) transmitted by *Biomphalaria orbignyi* from Argentina with 50 collar spines and less than 16 diverticuli/side associated with each main excretory canal. Lie and Umathevy (1966) described cercariae of *Echinostoma hystricosum* from the lymnaeid snail, *Radix* (*Lymnaea*) *rubiginosa* as having 60 collar spines and a spine pocket as well, but excretory diverticuli were not present.

3.6. Clades 4-6 (Echinostomatidae sp. 1-3)

These three clades did not group closely with any other specimens in GenBank, in either 28S or *nad*1 trees. Clades 4 and 5 (Echinostomatidae sp. 1-2) did not have prominent tail fins and have 33 collar spines. Four specimens grouped in clade 4. We found cercariae from this clade to be transmitted by both *Ceratophallus natalensis* and *Segmentorbis kanisaensis*. Clade 4 cercariae have

a cluster of approximately 20 granules just posterior to the oral sucker and approximately forty calcareous corpuscles within each main excretory canal (Fig. 3D).

Ten specimens grouped into clade 5. We found only *B. pfeifferi* from a single locality to be shedding this cercaria (Fig. 3C). We also collected an adult worm from a hadada ibis (*Bostrychia hagedash*) that matched the cercariae samples in sequence.

Clade 6 was represented by a single sample of cercariae (PE73) from *Ceratophallus natalensis*, designated Echinostomatidae sp. 3. These cercariae had approximately 18 collar spines on each side and a cluster of about 30 small granules posterior to the oral sucker. Tail fins were not prominent, and many small lipid drops were evident in the body. These cercariae also had approximately 60 small excretory granules in each main canal of the excretory system.

*3.7. Clade 7 (*Echinoparyphium*)*

A single specimen of a cercaria from *Bulinus tropicus* (PE68) comprised clade 7. The specimen was preserved in ethanol and not maintained in adequate shape to determine the number of collar spines or other morphological features; however, it grouped within *Echinoparyphium* from other GenBank samples. There were multiple species descriptions of *Echinoparyphium* from *Bulinus* from Africa; however, some of the descriptions matched more closely species in *Patagifer* than in *Echinoparyphium* (Appleton et al., 1983). Two species, *E.*

elegans and *E. ralphaudyi* were known to be transmitted by *Bulinus* from Africa. Molecular signatures for these two species were not represented in GenBank and no adult specimens are available for study. It is possible that our specimen was one of these two previously described bulinid-transmitted species based on geography and host-use, but molecular sequences of the two species would be required to validate this hypothesis.

3.8. Clades 8-10 (Ribeiroia)

3.8. Clades 8-10 (Ribeiroia)

Five samples from our dataset grouped into three clades of *Ribeiroia* flukes that typically use birds as definitive hosts, planorbids as first intermediate hosts, and amphibians as second intermediate hosts, where they often cause limb deformities in such amphibians (Johnson et al., 2004). Cercariae from *B. sudani*ca representing clade 9 (*Ribeiroia sp.* 2) resembled Fain's (1953) description of *Cercaria lileta* from *Biomphalaria stanleyi*, notable for its possession of a distinctive rose-colored organ placed just posterior to the oral sucker. Based on ITS2 sequences (tree not shown), our cercariae representing clade 9 also grouped with sequences derived from cercariae from *B. sudanica* (GenBank AY761143) that also resembled *Cercaria lileta* and possessed the rose-colored organ (Wilson et al., 2005). Our clade 9 samples were from *B. pfeifferi* and *B. sudanica* from central and west Kenya, which resemble earlier descriptions of cercariae from *B. sudanica* (Fain 1953) and *R. congolensis* which was transmitted by the goliath heron (*Ardea goliath*) from the Democratic Republic of the Congo (Dollfus 1950; Wilson et al., 2005). In addition, we found

matching sequences from metacercariae of clade 9 obtained from *B. sudanica*, which was not infected with other larval stages. This was not expected because species of *Ribeiroia* was not known for using snails as second intermediate hosts (Johnson et al., 2004).

Cercariae representing clades 8 and 10 developed in *B. sudanica*. Clade 8 (*Ribeiroia* sp. 1, Fig. 3H) was from a single sample (PE19) collected 15 years ago in west Kenya. It had fewer granules in the excretory system than did cercariae of clade 10 (*Ribeiroia sp.* 3). Clade 10 was also represented by a single sample (PE52) of cercariae. These cercariae had a small pharynx and over 120 large, densely packed calcareous corpuscles in each main excretory canal, with some of the corpuscles appearing to be composed of two partially fused corpuscles. These cercariae also had a peculiar organ just posterior to the pharynx. However, this organ lacked the distinctive rose color observed in cercariae of clade 9 (Fig. 3G).

3.9. Clade 11 (Isthmiophora)

One sample, (PE30) of cercariae from *Radix natalensis* grouped closely with GenBank records for the genus *Isthmiophora*, which infected small mammals while using molluscs, including lymnaeids, as 1st intermediate hosts and fish or amphibians as 2nd intermediate hosts (Kostadinova and Gibson 2002). To our knowledge, this was the first genetic evidence of the genus in Africa

3.10. Clades 12-17 (Petasiger)

We found six different clades that likely belonged to the genus *Petasiger.* Members of this genus were known for using snails as first intermediate hosts, fish or tadpoles as second intermediate hosts and birds (mainly cormorants) as definitive hosts (Faltynkova et al., 2008). Cercariae representing all six of the clades we identified had 27 collar spines, which was considered a trait of the genus (Faltynkova et al., 2008). Cercariae representing these clades had two conspicuous refractile granules situated immediately posterior to the oral sucker, an inflated gut and no tail fins. None of these clades matched any GenBank records.

Clade 12 (*Petasiger* sp. 1) was represented by one cercaria (PE36), from *R. natalensis* occurring in central Kenya. The specimen had been preserved for 4 years and was not in good condition. Although it was difficult to make out many of its morphological features, we were able to obtain sequences from it.

Eight samples of cercariae from *Bulinus* grouped into clade 13 (*Petasiger* sp. 2). These cercariae had 7-10 calcareous corpuscles per main excretory canal, a small oral sucker and two refractile granules posterior to the oral sucker (Fig. 3F).

Two specimens, PE39 and PE5 from *R. natalensis* and *Bulinus* sp., respectively made up clade 14 (*Petasiger* sp. 3). Both specimens were collected from central Kenya. The *nad*1 *p*-distance value between these two specimens was 0.014, suggesting that these two specimens were the same species. The cercaria from *R. natalensis* resembled that of an echinostome cercariae from South Africa also transmitted by *R. (Lymnaea) natalensis* (Moema et al., 2008).

Cercariae from both snail hosts had two large granules just posterior to the oral sucker.

The cercariae comprising clade 15 *(Petasiger* sp. 4) that were recovered from *B. pfeifferi* and *B. sudanica* also had two granules just posterior to the oral sucker and 17 calcareous corpuscles in each main excretory canal. Sequences from these cercariae also matched those from an adult worm (PE38) recovered from a reed cormorant (*Microcarbo africanus*).

Clade 16 (*Petasiger* sp. 5) likely corresponded to what was described as *Petasiger variospinosus* (King and Van As 2000) and *Cercaria decora* (Fain 1953). Cercariae from the two samples representing this clade were both recovered from *Bulinus* sp. Such cercariae had 27 collar spines, two large granules posterior to the oral sucker, and 19-20 calcareous corpuscles in each main excretory canal. The life cycle was completed by experimentally exposing laboratory raised reed cormorants (*Microcarbo africanus*) to metacercariae from *Xenopus* that had been experimentally exposed to cercariae from *B. tropicus* (King and Van As 2000).

Only one cercaria (PE4) obtained from *Bulinus* sp. comprised clade 17 (*Petasiger sp.* 6). This specimen was from a preserved specimen and it was difficult to make out distinct morphological features.

4. Discussion

Echinostomes are a diverse group of digenetic trematodes that are globally distributed, commonly represented in ecosystems and easily recognized

because of their collar spines. Species descriptions of echinostomes have been primarily based on their collar spine counts and arrangements, reproductive features of adult worms and their host use. As is the case with many other parasites that use multiple hosts, species descriptions of echinostomes rarely encompass all stages of the parasite's life cycle, and this is particularly true for echinostomes from Africa. Even meticulous species descriptions, if based on adult morphology alone, can lead to confusion in their systematics. Likewise, use of other life cycle stages like cercariae or metacercariae by themselves may also prove difficult or unreliable as a basis for species descriptions and identifications. Also, in light of evidence that cryptic species exist among echinostomes (Detwiler et al., 2010) we used molecular markers for life cycle stages we collected, which allowed us to link certain life cycle stages in order to better understand host usage patterns and to differentiate clades of echinostomes from East Africa.

Sequence data of all life cycle stages accompanied by morphological features, host and location data and that have been vouchered in museums provide a way forward for teasing apart differences among morphologically similar specimens. Sequence data from collected specimens from underrepresented areas is also important in building more comprehensive phylogenies and providing invaluable reference points that may eventually allow complete life cycles to be inferred on the basis of shared sequences. This is important in an age when collecting permits to work with many host species are becoming more difficult to obtain, and the hosts themselves are becoming rarer.

Analysis of 98 East African echinostome specimens, mostly of cercariae, using 28S and *nad*1 molecular markers revealed 17 clades from 5 genera of freshwater gastropods collected from 19 localities. We sequenced the 28S gene because such data are available for many of the echinostomes listed in GenBank, and can thus facilitate proper placement of our specimens into genera according to the scheme of Tkach et al. (2016). We sequenced the *nad*1 gene to provide additional resolution for some of the more-closely related representatives we obtained. The boundaries we used to delineate the 17 clades were intraspecific *p*-distance values less 1.5% and interspecific differences greater than 5% (Vilas et al., 2005). For instance, using *p*-distance values from the *nad*1 gene we could distinguish two distinct species of *Patagifer* (7.7% difference), whereas this distinction was not apparent in our 28S tree or distance matrix.

One of the distinct challenges posed by this collecting effort is that many of the classic species descriptions were done prior to, or without reference to molecular markers. Without access to adult specimens from type localities from which confirmatory morphological and sequence data could be obtained, this poses problems when relating newly-acquired sequence-based data to the classic descriptions. Also, some of the original descriptions are clearly at variance with evolving sequence- or complete evidence-based descriptions or phylogenies. Additionally, some sequence data provided in GenBank is associated with formal names for which the growing molecular and complete evidence databases strongly suggests the formal name used is incorrect. As we move forward and provide more sequence data for vouchered specimens, there

will continue to be some discrepancies created among different sources of information.

To reduce these complications among our samples we used ML and BI analyses to determine how our specimens grouped relative to each other and to echinostomes represented in GenBank. From our analyses, three clades (4-6) did not group with any GenBank records. Specimens from clades 4 and 5 possessed 33 collar spines and those from clade 6 had 36 collar spines. There are few previous descriptions of echinostomes with 33 collar spines (Dietz 1909; Lumsden and Hugg 1965; Premvati 1968; Kanev et al., 2009), some of which placed 33-spined echinostomes in either *Echinostoma* or *Petasiger*. However, species of *Echinostoma* have 37 spines (Huffman et al., 1990) and *Petasiger* has 27 collar spines (Faltynkova et al., 2008), but our 33-spined samples did not group with either genus (Tkach et al., 2016).

From the addition of our specimens from our survey work in East Africa, we confirmed that *E. caproni* (37-collar-spined group) has a broad distribution throughout Africa (Morgan et al., 1998). It is of interest that this species was found because many studies have been done on the immunobiology of *Biomphalaria* and *E. caproni* and others that have shown *E. caproni* rediae move toward intramolluscan stages of other trematodes (Reddy and Fried 1996). Also, *E. caproni* was dominant against *S. mansoni* in co-infections in *B. glabrata*, and *E. caproni* had enhanced virulence when *B. glabrata* were exposed to both parasites (Sandland et al., 2007). Even though these studies used *B. glabrata*

(Neotropical snail), this species is from Africa and uses African *Biomphalaria* as intermediate hosts in nature.

One surprising and previously unappreciated aspect of echinostome biology that emerged from examining a broad spectrum of cercariae was the presence of a variety of peculiar structures lying posterior to the oral sucker. Clades 2 and 3 have a distinctive concentration of spines that appear mid-ventrally, a short distance posterior to the posterior margin of the oral sucker in what we have termed a spine pocket. The 20-30 spines contained in the pocket are similar in size and appearance to the collar spines and are arranged with their bases overlapping centrally and with their bases overlapping centrally and with their sharp distal tips fanning outward and anteriorly. They appear refractile as do the associated collar spines, but the number of collar spines for both clades is much greater (54-62). A role for the spines in the spine pocket as holdfast structures does not seem likely. Appleton et al. (1983) found the spine pocket of cercariae from *Bulinus africanus* to be lost once the cercariae encyst as metacercariae. Perhaps these spines are somehow moved to a position on the collar to replace spines lost during subsequent encystment as metacercariae or when excysted worms develop into adults in their definitive hosts. One possibility is that the spines in the spine pocket function as a light-harvesting organ to facilitate orientation to light by cercariae once they leave their snail host. As discussed further below, cercariae with spine pockets have also been recovered from South American echinostomes.

Four more peculiar structures were found just posterior to the oral sucker. The second type of peculiar refractile structure was found in clades 4 and 5. The enclosed structure lying just posterior to the oral sucker contains a cluster of granules (20-24), some of which are fused and similar to what were described by Fain 1953, Lie 1963 and Fernandez et al., 2014. A third type of refractile structure is exhibited by clades 13-16, also which have an enclosed structure located just posterior to the oral sucker. But in the case of clades 13-16, the structure contains only two larger granules, similar to what was described by Fain 1953, King and Van as 1996, King and Van As 2000, and Moema et al., 2008. A fourth type is found in clade 9, a species of *Ribeiroia* with its cercaria corresponding to *C. lileta* of Fain (1953). Fain (1953) observed a distinctive oval-shaped rose-colored organ just posterior to the oral sucker, the presence of which was confirmed by Wilson et al. (2005) and in the present study. A fifth type, represented by Clade 10, likely a previously unappreciated *Ribeiroia* species, also possessed an identifiable oval structure lying in a comparable position to that seen for *C. lileta,* but it lacked any distinctive coloration. Similar structures have not been noted from the many echinostome cercariae described from North America or Eurasia; however, there are striking similarities between cercariae transmitted by *Biomphalaria* from Africa and South America (Ostrowski de Núñez et al., 1997; Martorelli et al., 2013; Fernandez et al., 2014).

Some of these similarities were from cercariae recovered from Neotropical and African representatives of *Biomphalaria* which are of interest because of its role in vectoring *Schistosoma mansoni*. Several phylogenetic studies of the

genus have indicated that *Biomphalaria* originated in the Neotropics and later colonized Africa (DeJong et al., 2001). The presence of *Biomphalaria* in South America probably dates to 55-65 million years ago (MYA), whereas its appearance in Africa is relatively recent, <1-5 MYA (Woodruff and Mulvey, 1997; Campbell et al., 2000; DeJong et al., 2001). Given that many echinostome species are hosted by aquatic birds, they may have provided a conduit for dispersal of Neotropical echinostomes to Africa and vice versa (Woodruff and Mulvey 1997). This idea is supported by the fact that similar cercariae from opposite sides of the Atlantic use related, but distinct species of avian definitive hosts. For example, members of clade 2 from *Biomphalaria* in Africa are known to use sacred ibises as definitive hosts. Their cercariae are remarkably similar to, though distinct from echinostome cercariae from *Biomphalaria straminea* in South America (Ostrowski et al., 1997). There are very few GenBank records of South American echinostomes and further comparisons of sequence data among morphologically similar cercariae between the two continents will help to unravel patterns of intercontinental dispersal or to provide insight if they were part of Gondwanaland.

Another interesting aspect is the involvement of other planorbid genera and species from both continents as additional first intermediate hosts of some of the echinostome recovered. Species recovered from African *Biomphalaria* were sometimes also recovered in another important schistosome-transmitting planorbid genus, *Bulinus*. *Bulinus* has been extant in Africa significantly longer than *Biomphalaria* (Morgan et al., 2002). Do these cases then represent

echinostomes that originated in *Bulinus*, then switched to *Biomphalaria* upon the arrival of *Biomphalaria* in Africa, followed by later colonization of South America where they infected local *Biomphalaria* species? Or did these echinostomes originate in South America *Biomphalaria*, then colonize Africa and exploit *Biomphalaria* there, and only later switch into *Bulinus* which commonly shares habitats with *Biomphalaria*?

Using molecular markers, we confirmed that four clades (2, 4, 13, and 14) use more than one genus of snails (and sometimes multiple families of snails) as first intermediate hosts. For example, clade 2 was composed of cercariae samples from *Ceratophallus*, *Bulinus*, and *Biomphalaria* that all grouped into the same clade. We relied on the genetic markers to show what clade these specimens grouped into because relying on host usage alone cannot always determine species. This is in line with other studies that used molecular markers that have shown some echinostomes to have broad host specificity (using multiple genera and families of snails) even with respect to their first intermediate hosts (Detwiler et al., 2010).

In many cases, it is difficult to complete species life cycles because collecting all necessary hosts in a life cycle and experimentally exposing those hosts is unfeasible in many settings. However, using molecular markers we were able to connect at least two hosts (2/3) in the life cycles for four clades of echinostomes. We sequenced certain life cycle stages (cercariae, metacercariae, or adults) and compared them to one another and if two life cycle stages fell into the same clade in the *nad*1 tree (less than 1.5% pairwise difference) we

considered them to be conspecifics. For example, in clade 5, we collected an adult worm from a hadada ibis which fell into the same clade as cercariae from *B. pfeifferi*. Clade 9 was composed of cercariae from *B. sudanica* and *B. pfeifferi* which grouped with metacercariae from *B. sudanica*. We collected an adult from a reed cormorant which grouped with cercariae from *B. sudanica* and *B. pfeifferi* from clade 15.

With respect to transmission of human schistosomiasis, 15 of the 17 clades we found were transmitted by planorbids, suggesting that planorbids are being heavily exploited by these echinostomes even though we collected other snail families including Physidae, Viviparidae, Thiaridae and Bithyniidae for which we did not find any infected with echinostomes. Of the 17 clades, 13 use the same (first) intermediate hosts as human schistosomes (*Biomphalaria* and *Bulinus*). Seven clades are transmitted by *Biomphalaria* and 6 of the clades are transmitted by *Bulinus*. Approximately 44% of the specimens we collected fell into clades 2 and 3 and these clades were transmitted by *B. pfeifferi* and *B. sudanica.* Even though many clades were found to be transmitted by planorbids, we also found 3 of the clades to be transmitted by *Radix natalensis* which is an intermediate host for *Fasciola gigantica* and *F. hepatica*, which causes fascioliasis (Correa et al., 2010). Further investigations should be done on their interactions within *R. natalensis*.

The presence of echinostomes in these snails creates opportunities for competition between other trematode species. Although it is well known that a single snail species can be utilized by multiple different species of digeneans,

double infections are rare in nature, and some digenean species interfere with one another's development within the same intermediate host (Lim and Heyneman 1972). Dominance hierarchies among digenean species have been documented and certain species of echinostomes have been shown to be dominant among other trematode species (Kuris 1990; Hechinger et al., 2011). Since 13 of the 17 clades of echinostomes use the same intermediate hosts (first) as human schistosomes, this creates issues for schistosomes because echinostomes have been shown to be strong competitors against human schistosomes (Lim and Hyneman 1972; Banes et al., 1974; Rashed 2002). Because certain echinostome species can be dominant, particularly against human schistosomes it has been suggested that other larval digeneans can be integrated into schistosome control programs (Bayer 1954; Lim and Heyneman 1972; Banes et al., 1974; Pointier and Jourdane 2000). The use of indigenous echinostome species for control of human schistosomes deserves further consideration, and supplemental studies are needed to ascertain how these African species may affect schistosome abundance.

Acknowledgments

We thank Sarah K. Buddenborg, Joesph Kinuthia, Geoffery Maina and Ibrahim Mwangi for assistance with collection of field samples. This research was undertaken with the approval of the National Commission for Science, Technology and Innovation, Permit Number NACOSTI/P/15/9609/4270 and approval from KWS permit number 0004754.

Financial Support

Technical assistance at the University of New Mexico Molecular Biology Facility was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P30GM110907. We gratefully acknowledge the following agencies for their financial support: The National Institute of Health (NIH) grant R37AI101438, and the Bill and Melinda Gates Foundation for the Grand Challenges Explorations Initiative grant. The content for this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This paper was published with the approval of the Director of KEMRI.

References

Appleton, C.C., Donnely, F.A., and Eriksson, I.M., 1983. The life-cycle and seasonal abundance of *Echinoparyphium montgomeriana n*. sp. (Trematoda: Echinostomatidae) in Natal, South Africa. Afr. Zool, 18, 320-325.

Banes, V., Rysavy, B., Yousif, F. 1974. Observations on the development of two echinostomes, *Echinoparyphium reaurvatum* and *Echinostoma revolutum*, the antagonists of human schistosomes in Egypt. Folia Parasitol 21, 143-154

Bayer, F.A.H., 1954. Larval trematodes found in some freshwater snails: a suggested biological method of bilharzia control. Trans R Soc Trop Med Hyg. 48, 414-418.

Bayne, C.J., 2009. Successful parasitism of vector snail *Biomphalaria glabrata* by the human blood fluke (trematode) *Schistosoma mansoni*: a 2009 assessment. Mol Biochem Parasitol 165, 8-18.

Bisseru, B., 1957. On three known trematodes from African birds, with notes on the genera *Typhlocoelum*, *Paryphostomum* and *Petasiger*. J Helminthol. 3, 173-186

Brant, S.V., Loker, E.S., 2009. Molecular systematics of the avian schistosome genus Trichobilharzia (Trematoda: Schistosomatidae) in North America. J Parasitol 95, 941-963.

Brown D.S., 1984. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Schwabe & Co LTD.

Brown, D.S., 1994. Freshwater snails of Africa and their medical importance, 2nd ed. Taylor & Francis, London; Bristol, PA.

Brown, D.S. & Kristensen, T.K.,1989. A field guide to African freshwater snails, southern African species. Danish Bilharziasis Laboratory Publication number 383.

Campbell, G., Jones, C.S., Lockyer, A.E., Hughes, S., Brown, D., Noble, L.R., Rollinson, D., 2000. Molecular evidence supports an African affinity of the neotropical freshwater gastropod, *Biomphalaria glabrata,* say 1818, an intermediate host for *Schistosoma mansoni.* Proc Biol Sci 267, 2351-2358.

Chibwana, F., Nwengulila, G., 2017. A faunistic survey of digenean larvae infecting freshwater snails *Biomphalaria*, *Radix* and *Bulinus* species in the Lake Victoria and Mindu dam, Morogoro in Tanzania. Tanz J Sci Vol 43, 1-13.

Civitello, D.J., Cohen, J., Fatima, H., Halstead, N.T., Liriano, J., McMahon, T.A., Ortega, C.N., Sauer, E.L., Sehgal, T., Young, S., Rohr, J.R., 2015. Biodiversity inhibits parasites: Broad evidence for the dilution effect. Proc Natl Acad Sci U S A 112, 8667-8671.

Correa, A.C., Escobar, J.S., Durand, P., Renaud, F., David, P., Jarne, P., Pointier, J.P., Hurtrez-Bousses, S., 2010. Bridging gaps in the molecular

phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fascioliasis. BMC Evol Biol 10, 381-393.

Cortes, A., Sotillo, J., Munoz-Antoli, C., Molina-Duran, J., Esteban, J.G., Toledo, R., 2017. Antibody trapping: A novel mechanism of parasite immune evasion by the trematode *Echinostoma caproni*. PLoS Negl Trop Dis 11, e0005773.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9, 772.

Dietz, E. 1909. Die Echinostomiden der Vögel. Zool. Anz, 34, 180–192.

DeJong, R.J., Morgan, J.A., Paraense, W.L., Pointier, J.P., Amarista, M., Ayeh-Kumi, P.F., Babiker, A., Barbosa, C.S., Bremond, P., Pedro Canese, A., de Souza, C.P., Dominguez, C., File, S., Gutierrez, A., Incani, R.N., Kawano, T., Kazibwe, F., Kpikpi, J., Lwambo, N.J., Mimpfoundi, R., Njiokou, F., Noel Poda, J., Sene, M., Velasquez, L.E., Yong, M., Adema, C.M., Hofkin, B.V., Mkoji, G.M., Loker, E.S., 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni.* Mol Biol Evol 18, 2225-2239.

Detwiler, J.T., Bos, D.H., Minchella, D.J., 2010. Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostomoid parasites in North America. Mol Phylogenet Evol 55, 611-620.

Detwiler, J.T., Zajac, A.M., Minchella, D.J., Belden, L.K., 2012. Revealing cryptic parasite diversity in a definitive host: echinostomoids in muskrats. J Parasitol 98, 1148-1155.

Devkota, R., Brant, S.V., Loker, E.S., 2015. The *Schistosoma indicum* species group in Nepal: presence of a new lineage of schistosome and use of the *Indoplanorbis exustus* species complex of snail hosts. Int J Parasitol 45, 857-870.

Dollfus, R. P., 1950. Trematodes re´colte´s au Congo Belge par le Professeur Paul Brien (mai-aouˆt, 1937). Annales de la Musee Royale de Congo Belge (Zoologie), ser 5, 1: 136 p.

Faltynkova, A., Gibson, D.I., Kostadinova, A., 2008. A revision of *Patagifer* Dietz, 1909 (Digenea: Echinostomatidae) and a key to its species. Syst Parasitol 70, 159-183.

Fernandez, M.V., Hamann, M.I., Ostrowski-de Nunez, M.O., 2016. New larval trematodes in *Biomphalaria* species (Planorbidae) from Northeastern Argentina. Acta Parasitol 61, 471-492.

Fernandez, M.V., Hamann, M.I., Ostrowski-de Nunez, M., 2014. Echinostomoid cercariae from *Biomphalaria straminea* (Mollusca: Planorbidae) in a ricefield from northeastern Argentina. Rev Mex Biodivers 85, 1024-1031.

Fain, A., 1953. Contribution à l'étude des formes larvaires des trématodes au Congo belge et spécialement de la larve de *Schistosoma mansoni*, Bruxelles.

Faust, C.F., 1921. Notes on South African Larval Trematodes. J. Parasitol. 8, 11-21

Frandsen, F., Christensen, N.O., 1984. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Trop. 41, 181-202.

Fried, B., 2001. Biology of echinostomoids except *Echinostoma*. Adv Parasitol 49, 163-210.

Fried, B., Manger, P.M., Jr., 1992. Use of an aceto-carmine procedure to examine the excysted metacercariae of *Echinostoma caproni* and *E. trivolvis*. J Helminthol 66, 238-240.

Fried, B., Toledo, R., 2009. The Biology of Echinostomes Springer-Verlag New York

Garcia-Vedrenne, A.E., Quintana, A.C., DeRogatis, A.M., Martyn, K., Kuris, A.M., Hechinger, R.F., 2016. Social Organization in Parasitic Flatworms--Four Additional Echinostomoid Trematodes Have a Soldier Caste and One Does Not. J Parasitol 102, 11-20.

Georgieva, S., Selbach, C., Faltynkova, A., Soldanova, M., Sures, B., Skirnisson, K., Kostadinova, A., 2013. New cryptic species of the *'revolutum'* group of *Echinostoma* (Digenea: Echinostomatidae) revealed by molecular and morphological data. Parasit Vectors 6, 64.

Hechinger, R.F., Wood, A.C., Kuris, A.M., 2011. Social organization in a flatworm: trematode parasites form soldier and reproductive castes. Proc Biol Sci 278, 656-665.

Hilmy, I. S., 1949. *Patagifer skrjabini* n. sp. (Echinostomatidae) from the glossy ibis, *Plegadis facinellus facinellus*, with a note on the genus. Proc Egypt Acad Sci 4, 20-23

Huffman, J.E., Fried, B., 1990. *Echinostoma* and Echinostomiasis, in: Baker, J.R., Muller, R. (Eds.), Advances in Parasitology. Academic Press, 29, 215-269.

Johnson, P.T.J., Sutherland, D.R., Kinsella, J.M., Lunde, K.B., 2004. Review of the trematode genus *Ribeiroia* (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Adv Parasit 57, 191-253.

Johnson, P.T.J., and D.W. Thieltges. 2010. Diversity, decoys and the dilution effect: how ecological communities affect disease risk. J. Exp. Biol 213, 961-970

Kanev, I., Fried, B., Radev, V., 2009. Collar spine models in the genus *Echinostoma* (Trematoda: Echinostomatidae). Parasitol Res 105, 921-927.

King, P.H., Van As, J.G., 1996. A description of the life stages of *Echinoparyphium elegans* (Trematoda: Echinostomatidae). Afr. Zool 31, 145-153.

King, P.H., Van As J.G., 2000. Morphology and Life History of *Petasiger variospinosus* (Trematoda: Echinostomatidae) in the Free State, South Africa. J. Parastiol 86, 312-318.

Kostadinova, A., Gibson, D.I., 2002. *Isthmiophora* Luhe, 1909 and *Euparyphium* Dietz, 1909 (Digenea: Echinostomatidae) re-defined, with comments on their nominal species. Syst. Parasitol 52, 205-217.

Kostadinova, A., and Jones, A., 2005a. Superfamily Echinostomatoidea Looss, 1899. In: Jones, A., Bray, R.A., Gibson, D.I. (Eds.), Keys to the Trematoda. CABI Publishing and the Natural History Museum, New York, pp. 5–8.

Kostadinova, A., 2005b. Family Echinostomatidae Looss, 1899. In: Jones, A., Bray, R.A., Gibson, D.I. (Eds.), Keys to the Trematoda. CABI Publishing and the Natural History Museum, New York, pp. 9-64.

Kuris, A., 1990. Parasite Communities. Patterns and Processes. Gerald W. Esch, Albert O. Bush, and John M. Aho, Eds. Chapman and Hall (Routledge, Chapman and Hall), New York, 1990.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.

Laidemitt, M.R., Zawadzki, E.T., Brant, S.V., Mutuku, M.W., Mkoji, G.M., Loker, E.S., 2017. Loads of trematodes: discovering hidden diversity of paramphistomoids in Kenyan ruminants. Parasitology 144, 131-147.

Lim, H.K., Heyneman, D., 1972. Intramolluscan inter-trematode antagonism: a review of factors influencing the host-parasite system and its possible role in biological control. Adv Parasitol 10, 191-268.

Loker, E.S., Cimino, D.F., Hertel, L.A., 1992. Excretory-secretory products of *Echinostoma paraensei* sporocysts mediate interference with *Biomphalaria glabrata* hemocyte functions. J Parasitol 78, 104-115.

Lumsden, R.D., Hugg, D., 1965. A redescription of avian echinostome trematode *Echinostoma operosum* Dietz, 1909. Zool. Anz 174, 222-227.

Martorelli, S.R., Alda, P., Marcotegui, P., La Sala, L.F., and Montes M.M., 2013. Larval digeneans in *Biomphalaria* snails from the Salto Grande Dam area in the Uruguay River. Laboratorio de Helmintos y Parásitos de Crustáceos. 2013, 1-3.

Moema, E.B.E., King, P.H., Baker, C., 2008. Cercariae developing in *Lymnaea natalensis* Krauss, 1848 collected in the vicinity of Pretoria, Gauteng Province, South Africa. Onderstepoort J Vet Res. 75, 215-223.

Mohammed, N.A., Madsen, H., Ahmed, A.A., 2016. Types of trematodes infecting freshwater snails found in irrigation canals in the East Nile locality, Khartoum, Sudan. Infect Dis Poverty 5, 16.

Morgan, J.A., Blair, D., 1998. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). Parasitology 116 (Pt 3), 289-297.

Morgan, J.A., DeJong, R.J., Jung, Y., Khallaayoune, K., Kock, S., Mkoji, G.M., Loker, E.S., 2002. A phylogeny of planorbid snails, with implications for the evolution of *Schistosoma* parasites. Mol Phylogenet Evol 25, 477-488.

Nkwengulila G., Kigadye, E.S.P., 2005. Occurrence of digenean larvae in freshwater snails in Ruvu Basin, Tanzania. Tanz. J. Sci 31, 23-30.

Odhner, T. 1910. Nordafrikanische trematoden größtenteils vom weißen Nil. Results of the Swedish zoological expedition to Egypt and the White Nile, 1901, 23, 1-170.

Ostrowski-de Núñez, M., H. I. Hamann and A. Rumi. 1997. Estudios de trematodes larvales en *Biomphalaria spp*. (Mollusca, Planorbidae) de la localidad de San Roque, provincia de Corrientes, Argentina. Physis 54, 7-15.

Premvati G., 1968. Echinostomoid trematodes from Florida birds. Proc. Helminthol. Soc. Wash 35, 197-200.

Pointier, J.P., Jourdane, J. 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. Acta Trop, 77, 53–60

Rashed, A.A., 2002. Biological studies on the snail intermediate hosts of schistosomiasis with a special emphasis on using larval echinostomes as biocontrol agent against larval schistosomes and snail. J Egypt Soc Parasitol 32, 775-784

Reddy, A., Fried, B., 1996. In vitro studies on intraspecific and interspecific chemical attraction in daughter rediae of *Echinostoma trivolvis* and *E. caproni*. Int J Parasitol 26, 1081-1085.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

Sandland, G.J., Rodgers, J.K., Minchella, D.J., 2007. Interspecific antagonism and virulence in hosts exposed to two parasite species. J Invertebr Pathol 96, 43-47.

Schell, S. C., 1985. Handbook of trematodes of North America north of Mexico. University Press of Idaho, Moscow, Idaho

Toledo, R., Esteban, J.G., Fried, B., 2009. Chapter 3. Recent advances in the biology of echinostomoids. Adv Parasitol 69, 147-204.

Tkach, V.V., Kudlai, O., Kostadinova, A., 2016. Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). Int J Parasitol 46, 171-185.

Vilas, R., Criscione, C.D., Blouin, M.S., 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology 131, 839-846.

Wilgenbusch, J.C., Swofford, D., 2003. Inferring evolutionary trees with PAUP*. Curr Protoc Bioinformatics Chapter 6, Unit 6 4.

Wilson, W.D., Johnson, P.T.J., Sutherland, D.R., Mone, H., Loker, E.S., 2005. A molecular phylogenetic study of the genus *Ribeiroia* (Digenea): Trematodes known to cause limb malformations in amphibians. J. Parasitol 91, 1040-1045.

Woodruff, D. S. and Mulvey, M. (1997). Neotropical schistosomiasis: African affinities of the host snail *Biomphalaria glabrata* (Gastropoda: Planorbidae). Biol J Linnean Soc 60, 505–516.

Tables and Figures

Table 1. Collections localities

Locality Name	Lat	Long
Sirikwa dam	0.46713	35.35170
Anyanga Beach	-0.05364	34.05149
Asao Stream	-0.31810	35.00690
Dunga Beach	-0.14532	34.736330
Kasabong Stream	-0.15190	34.33550
Powerhouse Beach	-0.09410	34.70760
Carwash Beach	-0.09587	34.74850
Kazinga Channel	-0.191928	29.89807
Kameta Dam	-0.109979	34.77456
Nawa Beach	-0.10194	34.71333
Forest Beach	-0.356594	34.68358
Kabuong beach	-0.336198	34.356155
Kotieno Beach	-0.35250	34.66733
Mwea Rice Field	-0.81800	37.62200
Kagwa Beach	-0.356594	34.68358
Kobala Beach	-0.34864	34.689057
Alara Beach	-0.350466	34.753866

Table 2. Provisional identification, sample name, host it was collected from, life cycle stage, collection locality, date, Museum of Southwestern Biology voucher number, and GenBank accession numbers of echinostome specimens used in this study

Clade	Sample Name	Host	Stage	Locality	Date Collected	MSB Voucher Number	GenBank 28S	GenBank *nad*1
Petasiger sp. 5	PE1	*Bulinus sp.*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26602	xxxxxx	xxxxxx
Petasiger sp. 5	PE2	*Bulinus sp.*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26620		
Petasiger sp. 5	PE3	*Bulinus sp.*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26644		
Petasiger sp. 6	PE4	*Bulinus sp.*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26655		
Petasiger sp. 3	PE5	*Bulinus sp.*	Cercariae	Sirikwa Dam	Jan-14	MSB:Para:26666		
Petasiger sp. 4	PE6	*Biomphalaria pfeifferi*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26677		
Petasiger sp. 5	PE7	*Bulinus truncatus trigonus*	Cercariae	Anyanga Beach	Jan-17	MSB:Para:26688		
Patagifer sp. 1	PE8	*Biomphalaria sudanica*	Cercariae	Dunga Beach	Apr-17	MSB:Para:26601		
Patagifer sp. 1	PE9	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jul-15	MSB:Para:26626		
Echinostomatidae sp. 1	PE10	*Ceratophallus natalensis*	Cercariae	Asao Stream, Kenya	Jun-15	MSB:Para:26603		
Echinostomatidae sp. 2	PE11	*Biomphalaria pfeifferi*	Cercariae	Asao Stream, Kenya	Jun-15	MSB:Para:26604		
Patagifer sp. 1	PE12	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-13	MSB:Para:26605		
Ribeiroia sp.2	PE13	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-14	MSB:Para:26606		
Echinostomatidae sp. 1	PE14	*Ceratophallus natalensis*	Cercariae	Carwash Beach	Aug-12	MSB:Para:26607		
Patagifer sp. 1	PE15	*Ceratophallus natalensis*	Cercariae	Carwash Beach	Aug-12	MSB:Para:26608		
Patagifer sp. 2	PE16	*Biomphalaria sudanica*	Cercariae	Dunga Beach	Apr-17	MSB:Para:26616		
Patagifer sp. 1	PE17	*Biomphalaria sudanica*	Cercariae	Kazing Channel	May-02	MSB:Para:26617		
Patagifer sp. 2	PE18	*Biomphalaria sudanica*	Cercariae	Kazing Channel	May-02	MSB:Para:26618		
Ribeiroia sp. 1	PE19	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	May-02	MSB:Para:26619		
Patagifer sp. 1	PE20	*Biomphalaria sudanica*	Cercariae	Dunga Beach	May-17	MSB:Para:26621		
Patagifer sp. 1	PE21	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Dec-10	MSB:Para:26622		
Patagifer sp. 1	PE22	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jan-17	MSB:Para:26630		
Patagifer sp. 1	PE23	*Biomphalaria sudanica*	Cercariae	Dunga Beach	Apr-17	MSB:Para:26631		
Petasiger sp. 4	PE24	*Biomphalaria pfeifferi*	Cercariae	Mwea Rice Field	Jan-13	MSB:Para:26632		
Echinostomatidae sp. 2	PE25	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Feb-13	MSB:Para:26633		
Patagifer sp. 1	PE26	*Biomphalaria sudanica*	Cercariae	Carwash Beach	Jan-12	MSB:Para:26634		
Echinostomatidae sp. 1	PE27	*Ceratophallus natalensis*	Cercariae	Powerhouse Beach	Aug-12	MSB:Para:26635		
Patagifer sp. 1	PE28	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Aug-12	MSB:Para:26636		
Ribeiroia sp.2	PE29	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Oct-13	MSB:Para:26643		
Isthmiophora sp.	PE30	*Radix natalensis*	Cercariae	Nyamo Saro	Jun-05	MSB:Para:26645		
Patagifer sp. 1	PE31	*Biomphalaria pfeifferi*	Cercariae	Kasabong Stream	Oct-13	MSB:Para:26646		
Ribeiroia sp.2	PE32	*Biomphalaria pfeifferi*	Cercariae	Mwea Rice Field	Oct-13	MSB:Para:26647		
Patagifer sp. 1	PE33	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-13	MSB:Para:26648		
Echinostomatidae sp. 2	PE34	*Microcarbo africanus*	Adult	Kameta Dam	Jan-05	MSB:Para:26649		
Echinostomatidae sp. 2	PE35	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jan-14	MSB:Para:26650		
Petasiger sp. 1	PE36	*Radix natalensis*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26651		
Petasiger sp. 4	PE37	*Biomphalaria pfeifferi*	Cercariae	Mwea Rice Field	Jan-13	MSB:Para:26652		
Echinostomatidae sp. 2	PE38	*Phalacrocorax africanus*	Adult	Kameta Dam	Jan-05	MSB:Para:26653		
Petasiger sp. 3	PE39	*Radix natalensis*	Cercariae	Monitor Lizard Pond	Jan-14	MSB:Para:26654		
Patagifer sp. 1	PE40	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jan-17	MSB:Para:26656		
Petasiger sp. 2	PE41	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26657		
Petasiger sp. 2	PE42	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26658		
Petasiger sp. 2	PE43	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26659		
Petasiger sp. 2	PE44	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26660		
Petasiger sp. 2	PE45	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26661		
Petasiger sp. 2	PE46	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26662		
Petasiger sp. 5	PE47	*Bulinus globosus*	Cercariae	Asao Stream	Jan-17	MSB:Para:26663		
Patagifer sp. 2	PE48	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jan-17	MSB:Para:26664		
Patagifer sp. 2	PE49	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jan-17	MSB:Para:26665		
Petasiger sp. 2	PE50	*Bulinus globosus*	Cercariae	Asao Stream	Apr-16	MSB:Para:26667		

Patagifer sp. 1	PE51	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jul-16	MSB:Para:26668
Ribeiroia sp. 3	PE52	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Aug-16	MSB:Para:26669
Patagifer sp. 2	PE53	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Aug-16	MSB:Para:26670
Petasiger sp. 4	PE54	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26671
Patagifer sp. 2	PE55	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26672
Patagifer sp. 2	PE56	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jun-16	MSB:Para:26673
Patagifer sp. 1	PE57	*Biomphalaria sudanica*	Cercariae	Dunga Beach	Jun-16	MSB:Para:26674
Petasiger sp. 4	PE58	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26675
Patagifer sp. 1	PE59	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jan-15	MSB:Para:26676
Petasiger sp. 2	PE60	*Pila ovata*	Cercariae	Dunga Beach	Sep-15	MSB:Para:26678
Patagifer sp. 2	PE61	*Biomphalaria pfeifferi*	Metacercariae	Asao Stream	Jun-16	MSB:Para:26679
Patagifer sp. 2	PE62	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jun-16	MSB:Para:26680
Echinostomatidae sp. 2	PE63	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jun-16	MSB:Para:26681
Patagifer sp. 1	PE64	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26682
Patagifer sp. 1	PE65	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26683
Patagifer sp. 1	PE66	*Biomphalaria pfeifferi*	Cercariae	Kasabong Stream	Jan-15	MSB:Para:26684
Echinostomatidae sp. 2	PE67	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Aug-16	MSB:Para:26685
Echinoparphium sp.	PE68	*Bulinus tropicus*	Cercariae	Mwea Rice Field	Jan-15	MSB:Para:26686
Patagifer sp. 1	PE69	*Biomphalaria sudanica*	Cercariae	Ovara Beach	Apr-16	MSB:Para:26687
Patagifer sp. 1	PE70	*Biomphalaria sudanica*	Cercariae	Kagaw Beach	Apr-16	MSB:Para:26689
Echinostomatidae sp. 2	PE71	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Aug-16	MSB:Para:26690
Echinostomatidae sp. 2	PE72	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Aug-16	MSB:Para:26691
Echinostomatidae sp. 3	PE73	*Ceratophallus natalensis*	Cercariae	Asao Stream	Aug-16	MSB:Para:26594
Patagifer sp. 1	PE74	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26595
Patagifer sp. 2	PE75	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jun-16	MSB:Para:26596
Patagifer sp. 2	PE76	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jun-16	MSB:Para:26597
Echinostomatidae sp. 2	PE77	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jul-15	MSB:Para:26598
Petasiger sp. 4	PE78	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-16	MSB:Para:26599
Echinostoma caproni	PE79	*Biomphalaria sudanica*	Cercariae	Kabuong Beach	Jan-17	MSB:Para:26600
Echinostomatidae sp. 1	PE80	*Segmentorbis kanisaensis*	Cercariae	Nawa Beach	Jun-16	MSB:Para:26609
Patagifer sp. 1	PE81	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jan-17	MSB:Para:26610
Petasiger sp. 4	PE82	*Biomphalaria sudanica*	Cercariae	Kobala Beach	Sep-16	MSB:Para:26611
Petasiger sp. 5	PE83	*Bulinus ugandae*	Cercariae	Powerhouse Beach	Jan-16	MSB:Para:26612
Patagifer sp. 1	PE84	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-16	MSB:Para:26613
Patagifer sp. 2	PE85	*Biomphalaria pfeifferi*	Cercariae	Kasabong	Jan-16	MSB:Para:26614
Patagifer sp. 1	PE86	*Biomphalaria sudanica*	Cercariae	Powerhouse Beach	Jan-16	MSB:Para:26615
Patagifer sp. 1	PE87	*Biomphalaria sudanica*	Cercariae	Nawa Beach	Feb-17	MSB:Para:26623
Petasiger sp. 4	PE88	*Biomphalaria sudanica*	Cercariae	Dunga Beach	Feb-17	MSB:Para:26624
Echinostoma caproni	PE89	*Biomphalaria sudanica*	Cercariae	Kabuong Beach	Jan-17	MSB:Para:26625
Patagifer sp. 1	PE90	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jul-15	MSB:Para:26627
Patagifer sp. 1	PE91	*Biomphalaria sudanica*	Cercariae	Forest Beach	Jan-17	MSB:Para:26628
Patagifer sp. 1	PE92	*Biomphalaria sudanica*	Metacercariae	Dunga Beach	Feb-17	MSB:Para:26629
Patagifer sp. 1	PE93	*Biomphalaria sudanica*	Metacercariae	Dunga Beach	Feb-17	MSB:Para:26642
Patagifer sp. 1	PE94	*Biomphalaria sudanica*	Metacercariae	Dunga Beach	Feb-17	MSB:Para:26637
Ribeiroia sp.2	PE95	*Biomphalaria sudanica*	Metacercariae	Dunga Beach	Feb-17	MSB:Para:26638
Patagifer sp. 1	PE96	*Biomphalaria sudanica*	Cercariae	Kotieno Beach	Jan-17	MSB:Para:26639
Echinostomatidae sp. 2	PE97	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jul-15	MSB:Para:26640
Patagifer sp. 2	PE98	*Biomphalaria pfeifferi*	Cercariae	Asao Stream	Jul-15	MSB:Para:26641

Table 3. Intra- and interclade P- distance values of 28S amplified from the 98 echinostomes in this study

Clade Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**0.001**																
2	0.020	**0.007**															
3	0.018	0.004	**0.001**														
4	0.020	0.024	0.022	**0.000**													
5	0.024	0.029	0.027	0.007	**0.004**												
6	0.024	0.028	0.026	0.009	0.013	**n/c**											
7	0.033	0.032	0.031	0.034	0.037	0.038	**n/c**										
8	0.061	0.057	0.059	0.062	0.067	0.064	0.066	**n/c**									
9	0.060	0.056	0.058	0.063	0.068	0.064	0.066	0.003	**0.000**								
10	0.063	0.060	0.061	0.066	0.071	0.068	0.068	0.012	0.008	**n/c**							
11	0.048	0.050	0.051	0.053	0.059	0.057	0.055	0.048	0.050	0.055	**n/c**						
12	0.052	0.055	0.056	0.056	0.062	0.060	0.058	0.054	0.055	0.059	0.020	**n/c**					
13	0.051	0.055	0.056	0.057	0.064	0.063	0.058	0.055	0.056	0.061	0.015	0.024	**0.001**				
14	0.049	0.052	0.052	0.053	0.060	0.059	0.055	0.054	0.056	0.056	0.018	0.024	0.020	**0.000**			
15	0.045	0.048	0.049	0.048	0.055	0.055	0.053	0.051	0.053	0.056	0.013	0.018	0.014	0.007	**0.002**		
16	0.049	0.050	0.051	0.054	0.061	0.059	0.056	0.052	0.053	0.055	0.022	0.027	0.019	0.020	0.015	**0.001**	
17	0.055	0.056	0.056	0.060	0.066	0.065	0.063	0.060	0.061	0.063	0.027	0.036	0.027	0.026	0.023	0.028	**n/c**

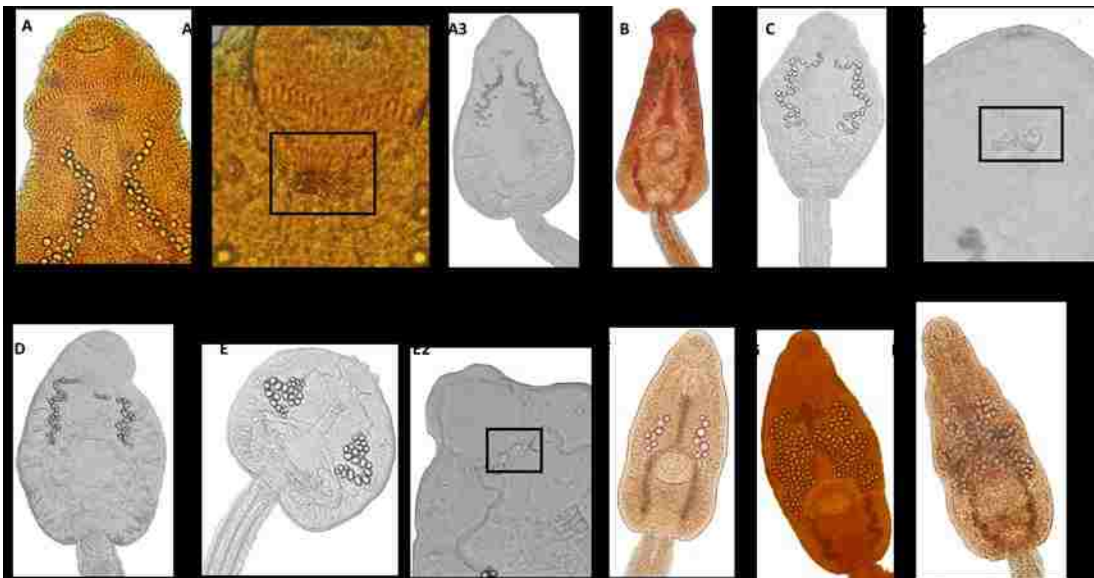
Table 4. Intra- and interclade P- distance values of *nad*1 amplified from the 94 (minus the 4 *Ribeiroia* samples) echinostomes in this study

Clade Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	**0.002**													
2	0.184	**0.007**												
3	0.200	0.077	**0.015**											
4	0.171	0.166	0.184	**0.001**										
5	0.204	0.177	0.191	0.146	**0.010**									
6	0.177	0.150	0.160	0.149	0.165	**n/c**								
7	0.214	0.224	0.230	0.223	0.229	0.204	**n/c**							
8	0.302	0.307	0.305	0.320	0.309	0.291	0.332	**n/c**						
9	0.295	0.267	0.276	0.282	0.285	0.279	0.291	0.334	**n/c**					
10	0.284	0.274	0.265	0.279	0.271	0.275	0.287	0.324	0.281	**n/c**				
11	0.286	0.259	0.258	0.258	0.246	0.235	0.275	0.315	0.259	0.242	**0.005**			
12	0.241	0.245	0.250	0.257	0.256	0.242	0.269	0.334	0.254	0.211	0.208	**0.014**		
13	0.271	0.238	0.238	0.240	0.242	0.225	0.284	0.320	0.265	0.240	0.264	0.226	**0.006**	
14	0.272	0.235	0.236	0.251	0.231	0.230	0.254	0.293	0.264	0.206	0.245	0.173	0.214	**0.004**

Figure 1. Phylogenetic relationships of echinostomes from this and study (bolded) and from GenBank (with accession numbers) based on 1,143 bp of the 28S gene inferred from ML and BI analyses. Nodes with a (*) indicate nodes that were supported (> 90%) by bootstrap values and posterior probabilities. Specimens are named based on sample name, the host and locality it was collected from, and color-coded based on clade designation from *nad*1 p-distance values of less than 2%. A black circle indicates clades where more than one genus of snails was found to be infected and a red star indicates clades where sequences from two different life-cycle stages matched.

Figure 2. Phylogenetic relationships of echinostomes from this and study (bolded) and from GenBank (with accession numbers) based on 463 bp of the *nad*1 gene inferred from ML and BI analyses. Nodes with a (*) indicate nodes that were supported by (> 90%) bootstrap values and posterior probabilities. Specimens from this study are named based on sample name, the host and locality it was collected from, and color-coded based on clade designation from *nad*1 *p*-distance values of less than 2%. A black circle indicates clades where more than one genus of snails was found to be infected and a red star indicates clades where sequences from two different life-cycle stages matched

Figure 3. Pictures of echinostomoid cercariae collected from Kenya: Clade 3, *Patagifer sp.* 2 is A1-3. A2 represents the cluster of spines posterior to the oral sucker, (B) clade 1, *Patagifer sp.* 1, (C1-2) clade 5 echinostomatidae sp. 2 and C2 displays the cluster of granules just posterior to the oral sucker, (D) clade 4 echinostomatidae sp.1, (E1-2) clade 14, *Petasiger sp.* 4 and E2 displays the two large granules posterior to the oral sucker, (F) clade 13 *Petasiger sp.* 2, (G) clade 10 *Ribeiroia sp*. 3, and clade 8 *Ribeiroia* sp. 1.



Chapter 3. Cascading effects of pastoralism, biodiversity and habitat stability on the transmission of human schistosomiasis

Martina R. Laidemitt[1], Larissa C. Anderson[1], Helen J. Wearing[1], Martin W. Mutuku[2], Gerald M. Mkoji[2], Eric S. Loker[1]

1 Center of Evolutionary and Theoretical Immunology (CETI), Museum of Southwestern Biology, Department of Biology, University of New Mexico, New Mexico, USA

2 Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya

Abstract: Human infectious agents exist within complex environmental milieu that influence the likelihood of transmission, and nowhere is this more evident than with parasites with multi-host life cycles. Among them is *Schistosoma mansoni*, a widespread causative agent of one of the world's most common neglected tropical diseases (NTDs), human schistosomiasis in tropical Africa and South America. *Biomphalaria* snails are essential to transmission serving as the vectors in which proliferative sporocysts develop that produce human-infective cercariae. Cercariae penetrate the skin of people to initiate new infections. Here we show that the force of infection of *S. mansoni* to people as estimated by the number cercariae-producing snail infections is influenced by domestic cattle and wild vertebrate hosts because of their role in transmitting digenetic trematodes with larval stages that compete with and/or displace *S. mansoni* sporocysts in snails. Furthermore, permanence of aquatic habitats influences the species composition of *Biomphalaria* and of other snails, again with indirect effects of trematode abundance and the likelihood that cercariae-producing infections of *S. mansoni* develop. Our results suggest that the predictable co-dominant exploitation of aquatic habitats by domestic animals and humans have enabled some trematodes to depend on and exploit *S. mansoni* for their transmission. Our results help expand a conceptual framework to better understand the many factors dictating the abundance of this and other endemic neglected tropical diseases.

Introduction: Infectious diseases are often conceptualized from a perspective emphasizing the routes of transmission directly to and from humans. However,

this can obscure the fact that each responsible causative agent exists in complex ecological settings that may involve sequential colonization of multiple obligatory hosts. Such obligatory hosts often must overlap closely in space and time and can be affected by multiple alternative host species. The diversity and abundance of potential host species available to an infectious agent may also vary on spatial or temporal scales. Furthermore, within host interactions among infectious agents and competitive interactions can occur. The extent to which the inherent biodiversity with which the infectious agent must interact influences the incidence or severity of human disease remains a topic of active discussion (Suzan et al., 2009, Keesing et al., 2010; Johnson et al., 2013; Wood et al., 2013; Frainer et al., 2018).

One of our interests is to understand how and why some infectious agents persistently attain high prevalence levels in their hosts such that they are designated as causing endemic and opposed to epidemic disease. Endemism in this context is indicative of ongoing and consistent success in transmission. What critical factors underlie the endemic state, and how does the ecological milieu in which the infectious agent resides influence its likelihood of success? Additionally, how might conditions exerted over longer time scales influence the evolution of infectious agents or hosts in ways that influence the level of endemicity of the infectious agent?

Digenetic trematodes of the genus *Schistosoma* are responsible for causing one of the great neglected and unconquered tropical diseases of our time, schistosomiasis. Collectively, five species of schistosomes infect over 206

million people, with most cases occurring in children within tropical Africa (WHO 2017). Our investigation centers on *S. mansoni*, one of the causative agents of intestinal schistosomiasis, near Lake Victoria in western Kenya. The Lake Victoria Basin is one of the largest hyperendemic areas of schistosomiasis in the world (Gouvras et al., 2017; Wiegand et al., 2017). Despite repeated treatments with the anthelmintic praziquantel, children living in villages near the lakeshore often exhibit prevalence of infection of >50%, with up to 90% in some areas (Woodhall et al., 2013). Here, we analyze with the aid of a mathematical model this hyper-endemic macroparasite, whose complex life cycle involves humans or other mammals as definitive hosts and freshwater snails of the genus *Biomphalaria* as intermediate hosts. Such analyses dissect and reveal how the availability of *S. mansoni* infection to people in west Kenya can be affected by various factors, including 1) availability of alternative host species; 2) animal husbandry and surrounding vertebrate biodiversity with causal influences on the availability of competitor/predator parasites; and 3) habitat stability, with attendant effects on snail host breeding systems and parasite colonization rates.

S. mansoni **transmission involves multiple hosts, multiple pathways:**

A textbook portrayal of the *S. mansoni* life cycle might convey an impression that humans are definitive hosts and an unnamed species of *Biomphalaria* serves as intermediate host. However, one of the factors contributing to the success of *S. mansoni* in the Lake Victoria Basin is that multiple options exist for transmission (Figure 1). With respect to mammalian hosts, humans are certainly the most important in maintaining transmission at a high level. Both baboons and several

small mammal species including both widespread rodents like *Mastomys natalensis* and insectivores like *Crocidura olivieri* acquire infection and can pass eggs into the environment (Fenwick 1969; Hanelt et al., 2010). For example, Hanelt et al., 2010 found that 5.4% of rodents surveyed were infected with schistosomes and 25.5% of the schistosome worms recovered were *S. mansoni*. Likewise, a recent study found the overall prevalence of schistosomes in field-captured small mammals ranged between 1.9% to 28.6% (Catalano et al., 2018). The alternative reservoir hosts for *S. mansoni* provide important options for transmission, particularly in the light of ongoing chemotherapy-based control programs targeting human transmission. Likewise, *S. mansoni* in and around the lake can infect three *Biomphalaria* taxa, including *B. pfeifferi* in streams and small impoundments, *B. sudanica* along the lakeshore, and *B. choanomphala*, now generally considered to be a distinct ecophenotype of *B. sudanica*, found anywhere from the shoreline, but especially into deeper water, up to 0.5- 20 meters in depth (Magendantz 1972; Ngupula and Kayanda 2010; Standley et al., 2011; Lang et al., 2013; Zhang et al., 2018). All three species are experimentally susceptible to *S. mansoni* infection (Figure 2), are found naturally infected with *S. mansoni* (Magendantz 1972; Standley et al., 2011; Mutuku et al., 2017), and produce cercariae that can contribute to human infections. Also noteworthy, is the fact that in some cases infected snails can live over a year and produce thousands of cercariae per day (Mutuku et al., 2014). This further adds to the formidable nature of *S. mansoni* coupled with the egg-production in adult worms

in humans that can routinely live for five years and can produce hundreds of eggs per female per day (Warren et al., 1974).

An unexpected effect of pastoralism on transmission of *S. mansoni*:

Often at *S. mansoni* transmission sites in Kenya, people are side-by-side with domestic ruminants that come to water themselves (Figure 3). Among the consequences of such visits are the deposition of large quantities of cow manure into the water, including eggs of helminth parasites transmitted by cattle. Although cattle are not themselves common hosts for *S. mansoni*, they do transmit *Schistosoma* species such as *S. bovis* that are related to and hybridize with the causative agent of human urinary schistosomiasis, *S. haematobium* (see discussion) (Webster et al., 2013). Among the helminth eggs passed in profusion (approximately 10,000 eggs/cowpat) are those of amphistome flukes, which employ a life cycle involving a freshwater snail as intermediate hosts, cercariae that encyst as metacercariae on vegetation which are then consumed by cows, goats or sheep, which then mature to egg-producing adulthood in the rumen (Figure 4). We have found at least 16 species of amphistome flukes in west Kenya, one of which is both common and employs *Biomphalaria* snails as its intermediate host (designated here provisionally as *Calicophoron sukari*) (Laidemitt et al., 2017). This creates a scenario in which the two dominant definitive host species using available stream transmission sites, humans and cattle, both predictably seed the habitats with different trematodes that converge in their use of *Biomphalaria* snails (Figure 5 - prevalence of snail infections in Asao and Kasabong).

Some amphistomes like *Calicophoron microbothrium* have attracted attention because their presence in snails like *Bulinus tropicus* can facilitate subsequent infections with *S. bovis* that would not otherwise occur in this snail species (Southgate et al., 1989). However, the pattern we have noted with *C. sukari* transmitted by *B. pfeifferi* was the opposite. We first observed *C. sukari* was common only where *S. mansoni* is present. We then noted that if snails taken from such habitats and that were determined not to be releasing cercariae of any trematode ("shedding") at the time of collection were exposed to *S. mansoni* miracidia, they surprisingly were subsequently more likely (p = < 0.001) to shed *C. sukari* than *S. mansoni* cercariae (Figure 6). This suggested the snails already had been colonized by *C. sukari* larvae, but were for some reason C. sukari were unable to complete development without a follow-up exposure to *S. mansoni*.

Additional experiments were undertaken with laboratory-bred *B. pfeifferi* exposed to *S. mansoni* and *C. sukari* miracidia, either each species or alone or both species in various combinations (Figure 7A). The results were clear in indicating that although *S. mansoni* miracidia were entirely capable of infecting *B. pfeifferi* on their own as expected (see also Mutuku et al., 2014), *C. sukari* miracidia were poorly infective to *B. pfeifferi* on their own. Examination of histological sections of *B. pfeifferi* taken 8 days after exposure only to *C. sukari* miracidia revealed the presence of sporocysts that had undergone little or no growth or development and that had host hemocytes about them (Figure 7B). However, the success of *C. sukari* miracidia increased significantly (p= <0.0091),

if *S. mansoni* miracidia were also allowed to infect the snails, particularly so if the *S. mansoni* exposures followed the exposure of snails to *C. sukari*.

Other lines of evidence are also indicative of the ability of *C. sukari* to interfere with the intramolluscan development of *S. mansoni*. An Illumina-based study of the transcriptional profiles of larval *S. mansoni* in field-derived *B. pfeifferi* showed that transcriptional activity was sharply reduced in one biological replicate that represented a snail shown to also possess a non-shedding *C. sukari* infection (Buddenborg et al., 2017). Furthermore, infections of both *S. mansoni* and *C. sukari* are common in *B. pfeifferi* in west Kenyan stream habitats, but double infections, that is snails found shedding cercariae of both species, are rare, and occur less than expected by chance ($p = < 0.001$) (Fig 5c). We have observed that some field snails found to be naturally shedding *S. mansoni* cercariae would, upon further observation in the lab, permanently switch over to producing *C. sukari* cercariae instead (Figure 11). Lastly, whereas exposure to *S. mansoni* miracidia of field-collected snails shedding *C. sukari* cercariae rarely resulted in conversion of the infections to production of *S. mansoni* cercariae, the converse situation was not the case: exposure of snails that were shedding *S. mansoni* to miracidia of *C. sukari* more commonly resulted in production of *C. sukari* cercariae (Figure 11). Consistent with these observations, *S. mansoni* larval development involves two generations of sac-like sporocysts whereas intramolluscan development of *C. sukari* progresses through an initial sporocyst generation followed by two redial generations. Rediae possess a mouth surrounded by an oral sucker and a gut, and are well-known for

their ability to attack the sporocysts of other trematode species (Lim and Hyneman 1972; Hechinger et al., 2011). The mathematical model we have developed and parameterized using values obtained from our experimental and field studies indicates that the force of infection of *S. mansoni* is reduced in the presence of the competitor/predator *C. sukari* (Figure 9). We also noted that our survey data showed that *S. mansoni* and *C. sukari* are positively correlated (r= 0.622) (Figure 5D). This is not surprising, since *C. sukari* relies on *S. mansoni* for its own transmission. Collectively, such findings indicate persistent human contamination has led to the predictable presence of *S. mansoni* in *Biomphalaria*, thereby providing opportunities for the cattle fluke *C. sukari* to initiate infections and routinely displace *S. mansoni* infections.

Wild vertebrate hosts indirectly influence the interference of the production of *S. mansoni* cercariae: Some wild rodents, including insectivores and primates can serve as viable definitive hosts for *S. mansoni*, thereby providing alternative routes for transmission, comprising a way that biodiversity can favor *S. mansoni* transmission. In the process of screening *Biomphalaria* from both stream and lake habitats for *S. mansoni* infections, we found other species of digenetic trematodes were also supported by *Biomphalaria* as first intermediate hosts (Figure 5A, B; Figure 8). For example, in Asao stream 19 species of trematode cercariae emerged from 19,914 *B. pfeifferi* and from Lake Victoria, we 21 species of cercariae emerged from 3,369 *B. sudanica*. Although some of these species like *S. mansoni* were recovered from both habitats, many were habitat specific (Figure 8). It is not uncommon for certain gastropod species to

support diverse trematode guilds though there is considerable variation among snail hosts with respect to the extent to which this happens (Kuris and Lafferty 1994).

Extrapolating what is known about trematode life cycles, all the *Biomphalaria*- dependent species of trematodes (determined from cercariae morphology and sequence data) we collected require a vertebrate definitive host, and most require a second intermediate host in which cercariae encyst to form metacercariae. Thus, the presence of these complex guilds of cercariae in *Biomphalaria* provides a distinctive means to assess the variety of definitive and intermediate host species that must be present to support the various trematode life cycles. Although many of the specific definitive host species that go with the cercariae species recovered from *Biomphalaria* are as yet unknown, it is possible to make reasonable inferences about the types of host involved (Figure 10). For example, several trematode groups that exploit mammals and especially birds as definitive hosts cycle through *Biomphalaria* snails. For instance, *Patagifer sp.*, and *Petasiger sp.* were recovered from *Biomphalaria* and wild birds and *E. caproni* which is known to use mammals and birds as definitive hosts (Richard and Brygoo 1978).

As we noted above for *C. sukari* and *S. mansoni*, it is unusual for two trematode species to stably and persistently co-infect the same individual snail. Furthermore, among the trematodes potentially vying for a particular species of snail host, the outcomes of co-infections are often predictable such that it is possible to work out a dominance hierarchy among the interacting parasite

species. Figure 11 shows such a hierarchy for the trematodes colonizing *B. pfeifferi* at Asao Stream, the first worked out for human schistosome-transmitting snails in an endemic setting. The hierarchy is worked out in three ways: 1) experimental co-infections of lab-reared snails as noted above (Figure 7); 2) by exposing field-derived *B. pfeifferi* naturally shedding one type of cercariae to miracidia of a second to see if the second species takes over the snail; and 3) observing field-collected *B. pfeifferi* shedding cercariae of one species to see if they switch over naturally to shedding cercariae of a second species. Several features are noteworthy about the hierarchy. First, note the relative positions of *C. sukari* and *S. mansoni* and that the former is dominant to the latter as noted above. Second, *S. mansoni* is not the top competitor, but occupies an intermediate position. Thirdly, echinostome species are dominant. Echinostomes are well-known for their ability to produce rediae that attack and kill the intramolluscan larvae of other trematode species (Lim and Hyneman 1972; Lie 1973; Rashed 2002; Hechinger et al., 2011). We have identified 17 echinostome species transmitted among East African snails, with 7 able to infect *Biomphalaria* (Laidemitt personal communications). The degree of dominance and associated predatory tendencies of the rediae of the different species remain to be ascertained. Given the ability of echinostome rediae to attack and kill the sporocysts of *S. mansoni*, there has been an understandable interest in using them for biological control of schistosomiasis, a topic discussed at length by others (Bayer 1954; Lim and Heyneman 1972; Banes et al., 1974; Pointier and Jourdane 2000). Another intriguing feature is that there is a certain universality to

these hierarchies, often with trematodes producing xiphidiocercariae as most subordinate, and echinostome trematode as dominants, typically but not always with schistosome lying in the middle. One final consideration regarding the hierarchy is that it does not inevitably lead to echinostomes being the most prevalent infections (Figure 11). The input of echinostome eggs, as from avian hosts, into the snail habitat is most probably small in comparison to egg input rates achieved by trematodes of cattle and humans. Consequently, on their own, they may not be able to attain a high prevalence even if they can displace all other species in co-infections.

Most trematode infection in *Biomphalaria* must be viewed as a potential impediment to *S. mansoni* (unless facilitation of *S. mansoni* occurs). This is true if: 1) *S. mansoni* is the first to colonize the snail which is then colonized by a dominant species; 2) *S. mansoni* colonizes a snail already occupied by a dominant – it will then fail; or 3) *S. mansoni* colonizes a snail already occupied by a subordinate species – it will then have to displace the subordinate before its own cercariae are produced. Snails with double infections run higher risks of mortality than uninfected or single infection snails (Lafferty et al., 1994). Consequently, from this perspective, the availability of diverse sets of vertebrate hosts to support a large variety of *Biomphalaria*-infecting trematodes can be viewed as a beneficial effect of biodiversity in diminishing the force of *S. mansoni* transmission.

An additional way that the presence of diverse echinostome and other trematode infections may interfere with *S. mansoni* transmission could involve

the finding that echinostome cercariae released from one snail frequently colonize the excretory systems of other snails, thereby gaining access to the pericardium where they encyst to form metacercariae (Estaban et al., 2010).The cercariae produced in one snail may end up encysting in many different individual snails, as revealed by examination of field snails (Figure 12A). Although the effects of metacercariae are often discounted since they are not proliferative, they may influence *S. mansoni* infections and transmission in subtle ways: 1) provoking higher levels or mortality (Kuris and Warren 1980), 2) dissipating resources in the form of expensive hemocyte encapsulation reactions around metacercariae (Figure 12B); 3) altering a snail's risk of being ingested by a definitive host (Mouritsen and Poulin 2003) and 4) they may be less attractive to *S. mansoni* miracidia than snails lacking metacercariae. If these effects were proven to be substantial, then the impact of other trematode species on *S. mansoni* transmission would be even greater than dictated by the rates of cercariae-producing infections observed.

Four habitat types for transmission, each with its own ecological considerations: *S. mansoni* is favored in its transmission in the Lake Victoria basin because *Biomphalaria* snails can colonize multiple habitats, including ephemeral streams that might remain dry for months, more permanent streams that may be subject to dramatic flooding and drying, the shore of the lake including vast areas of papyrus swamps and associated marshlands, as well as deeper offshore waters of the lake. These habitat types differ in their permanence and biotic complexity.

Ephemeral streams are ubiquitous in East Africa and our studies of one such stream (Kasabong Stream) revealed that it can be without standing water for as long as 10 months, yet *B. pfeifferi* can quickly recolonize to support transmission of *S. mansoni* and *C. sukari*. Several studies of *B. pfeifferi* have shown this snail to be a strong preferential self-fertilizing species (Charbonnel et al., 2000; Tian-Bi et al., 2008), generally considered to be an adaptation favoring life in ephemeral habitats subjected to drying, flooding and requiring rapid colonization ability. *B. pfeifferi* from Kasabong Stream similarly has the characteristics associated with a predominant selfer (Lelo personal communications). Our studies show that the diversity of snail species occupying this habitat is relatively low and the diversity of trematode species colonizing even the dominant species *B. pfeifferi* is low (Table 1). However, the prevalence of infection in *B. pfeifferi* of the few trematode species present can be high (Table 1 and Figure 5A). Trematodes transmitted by rarer hosts than humans or cattle may lack the opportunity or time to colonize snails in such habitats before drying occurs and transmission ceases.

A somewhat different picture emerges from examination of a perennial stream habitat. One such stream (Asao Stream) underwent a massive flood at the end of 2012 prior to the onset of our survey studies and has maintained a steady flow of water since that time, with some expected seasonal fluctuations. *B. pfeifferi* is the dominant freshwater snail species present in the stream, but several additional snail species are also present (Table 1). Likewise, the number of trematode species colonizing *B. pfeifferi* is larger than noted for the ephemeral

stream, but once again the trematode fauna is dominated by *C. sukari* and *S. mansoni* (Table 1). As noted with the Kasabong population of *B. pfeifferi*, a deficiency of heterozygotes is again present and is consistent with a predominant pattern of self-fertilization (Lelo personal communication). This pattern is somewhat paradoxical in that species that can undergo self-fertilization are generally considered to suffer greater inherent risk of the negative consequences of parasitism. Indeed, 12.2% (2,439 out of 19,914) of *B. pfeifferi* from Asao have been found infected with trematodes, suffering castration as a consequence. The retention of self-fertilization is perplexing given the relatively prolonged period of stability with attendant colonization by competing snails and harmful parasites. However, as is entirely possible, another strong flooding event comparable to that occurring in 2012 could happen again. In other words, a period of stability enabling parasites to take advantage of self-fertilizing hosts may be quickly offset by a catastrophic flood that strongly favors a self-fertilizing snail species in recolonization of the habitat.

The shoreline habitats of Lake Victoria can be thought of as ecological hotspot in the sense of King et al. (2011), in that the activity of numerous host species are focused there, facilitating completion of the life cycles of parasites like trematodes. It is noteworthy that experimental exposures of *B. sudanica* indicate this species is significantly less compatible with *S. mansoni* than is *B. pfeifferi*, regardless of whether the parasite is derived from sympatric or allopatric sources (Mutuku et al., 2017).Although the levels of the lake rise and fall with the seasons or with weather extremes, the habitats of snails associated with the

margins of the lake can be considered more stable compared to stream environments and thus can support a greater variety of snail species, including some species endemic to the lake or restricted to lacustrine habitats (Table 1). Examination of shoreline habitats indicate a diverse population of snail species and in this case, using *B. sudanica* as the reference point, a diverse guild of trematodes that clearly differs in composition from what is noted in *B. pfeifferi* in stream habitats (Figure 8, Table 1). For instance, in lake habitats, *C. sukari* is absent, and although *S. mansoni* is still present, its prevalence is much lower (0-2.6%), presumably due to high levels of human excrement that pollutes the lake. Furthermore, in keeping with what is readily observed upon sampling at the Powerhouse Beach site, a large variety of aquatic birds including egrets, hammerkops, ibises, storks, terns, and kingfishers are present and seed the water with eggs with the trematode species they support (Figure 8, Table 1), especially including echinostomes and strigeids.

An ecophenotype of *B. sudanica*, customarily referred to as *B. choanomphala*, is found inhabiting the deeper waters of the lake (Zhang et al., 2018). Whereas *B. sudanica* has a larger, flatter shell, the shell of *B. choanomphala* is smaller in diameter, deeper in its construction and has pronounced angulations that make it distinctive. *B. choanomphala* can be recovered from the shoreline but it is most predictably recovered by dredging from deeper water, particularly adjacent to shorelines facing open waters of the lake with sand/mud bottoms. Although legitimate arguments exist as to what name, *sudanica* or *choanomphala*, deserves systematic priority (Zhang et al.,

2018), it is clear that gene flow occurs between the two forms (Bandoni et al., 2000; Standley et al., 2014). *B. choanomphala* can be considered to occupy an ecological coldspot sensu of King et al. (2011 In support of this idea, thus far the prevalence rates of *B. choanomphala* are low (0.15%), and although this taxon is experimentally susceptible to *S. mansoni*, in fact more so than *B. sudanica* (Figure 2), naturally-infected specimens are rarely found. Furthermore, the diversity of trematode species recovered from *B. choanomphala*, based on both our studies and those of others from specimens recovered by dredging, is relatively low (Table 1). *B. choanomphala* occupies deep water refugia and based on the trematodes we recovered from *B. choanomphala* the chances of transmission of most trematodes except the sanguinicolid and spirorchiid blood flukes from fish and turtles, are lower.

Discussion:

Schistosoma mansoni is ubiquitous in sub-Saharan Africa, aided by multiple transmission options, including by the widespread presence of *Biomphalaria* vector snails in diverse aquatic habitats. Additionally, open human defecation and inadequate sanitation insure widespread contamination of freshwater habitats with *S. mansoni* eggs (Nagi et al., 2014). Against this backdrop, we highlight above that pastoralism, biodiversity and habitat stability all have cascading effects that favor the transmission of additional digenetic trematode species, many of which also depend on *Biomphalaria* for their larval development and transmission. Consequently, inevitable competitive interactions result, with a predictable pattern of outcomes in pairwise interactions between species. From

this, an overall dominance hierarchy among the larval trematodes exploiting *Biomphalaria* in west Kenya can be determined. A first among African species that use African *Biomphalaria* as an intermediate host. We found that *S. mansoni* occupies an intermediate position, predictably dominant to some and subordinate to other species of trematodes. As has been well-established elsewhere, rediae-producing echinostome trematodes tend to be dominant though the rediae of different echinostome species vary in their predatory tendencies and consequently their dominance (Lim and Heyneman 1972; Combs 1982; Hechinger et al., 2011; Garcia-Vedrenne et al., 2016). Dominance by no means translates into abundance however. The input of bird-transmitted echinostome eggs into the stream habitats we studied is dwarfed by the input of eggs from large definitive hosts like people, goats or cattle, meaning that the prevalence achieved by *S. mansoni* or by *C. sukari* is much higher than achieved by echinostomes. Nor does *S. mansoni*'s intermediate position in the dominance hierarchy insure that the presence of competitor/predator trematodes will eliminate schistosome infections in snails.

Most noteworthy with respect to reducing the impact of *S. mansoni* is the presence of the rumen fluke of cattle, *C. sukari*. The need for domestic livestock to water regularly, and the shortage of water sources for them means they must regularly visit streams also heavily used by people for bathing, washing and as a source of drinking water. The prodigious production of dung by these animals in and near these streams insures a ready supply of *C. sukari* eggs will reach the habitat, and miracidia derived from them will commonly encounter and penetrate

snails of their preferred host species, *B. pfeifferi*. Several aspects of our results indicate that *C. sukari* has very poor ability to infect *B. pfeifferi* on its own. If *C. sukari* miracidia enter an uninfected snail, the ensuing sporocyst must await (Figure 7B) the later arrival of *S. mansoni* miracidia (and possibly of other subordinate trematode species) if it is to succeed in producing rediae. The presence of developing *S. mansoni* sporocysts in some way insures that *C. sukari* too can develop, an effect that may be mediated by the known ability of *S. mansoni* from experimental studies to interfere with the snail immune system (Bayne 2009; Buddenborg 2017) to enable development of larvae of other trematode species. Alternatively, miracidia of *C. sukari* may enter a snail already infected with *S. mansoni* and in keeping with the dominance hierarchy noted, can displace *S. mansoni* sporocysts, leading to production of *C. sukari* cercariae. As a consequence, nearly all of the *C. sukari* infections present in streams represent snails once infected with *S. mansoni* larvae that have been displaced and eliminated. The presence of amphistome-infected cattle thereby provides a significant protective effect for people in diminishing the number of snails producing *S. mansoni* cercariae. Our model suggests that in the absence of *C. sukari,* the prevalence of *S. mansoni* would double (Figure 9). For example, if the prevalence of *S. mansoni* is 10%, it would increase to 20% for some time in the absence of *C. sukari* (Figure 9A). An artificial increase of *C. sukari* into the system would also reduce the number of *S. mansoni* cercarial output (Figure 9B). Not only does *C. sukari* influence the number of *B. pfeifferi* infected with *S. mansoni*, but the presence of *S. mansoni* facilitates transmission of a cattle

parasite that would not be successfully transmitted otherwise. It is interesting that the interaction we have noted between *S. mansoni* and *C. sukari* favors the amphistome, but in other instances, including in Kenya, the presence of another amphistome species may facilitate infections with the cattle schistosome *Schistosoma bovis* (Southgate et al., 1989). Schistosomes and amphistomes, possibly because of their intermediate positions in the dominance hierarchy, clearly engage in interesting interactions that seem to favor the colonization of a snail species that might otherwise not easily be infected.

One of the questions posed by the facilitation effect of *S. mansoni* exploited by *C. sukari* is why the latter species should be dependent on the first for its infection of snails. Other amphistome species have not shown such obvious dependency on a facilitation effect. It seems possible that many *C. sukari* infections in snails may never proceed past the initial sporocyst stage if help is not forthcoming by *S. mansoni* sporocysts. One possible explanation is that the colonization of *B. pfeifferi* by *C. sukari* represents a host shift in progress. Our phylogenetic studies of East African amphistomes indicate that few amphistome species colonize *Biomphalaria* species, whereas colonization of other related planorbid snails, particularly in the genus *Bulinus*, is common (Laidemitt et al., 2017). This pattern is consistent with the observation that *Bulinus* has a long evolutionary history in Africa and southwest Asia, whereas *Biomphalaria* snails arrived in Africa no longer than 2 million years ago (Woodruff and Mulvey 1997; DeJong et al 2001; Morgan et al., 2002). The consistent presence of an abundant snail like *B. pfeifferi*, particularly if it can be

immunocompromised by the presence of a trematode like *S. mansoni*, creates an opportunity for an amphistome parasite that normally might develop in a related snail species. The frequent deposition of eggs of the amphistome in *B. pfeifferi* habitats thanks to regular use by cattle of streams would further favor this possibility. In support of the concept of an incipient host-switching event, several phylogenetic studies of schistosomes and other trematodes suggest that host switching with respect to the snail host has occurred repeatedly (Brant and Loker 2013).

The facilitation effect we observed for *C. sukari* represents another example on a growing list of how pastoralism interfaces with human disease ecology. With respect to trematodes, a group of schistosomes distinct from *S. mansoni* includes species like *S. haematobium* that causes urinary schistosmiasis in humans and several closely related species like *S. bovis*, *S. mattheei* and *S. curassoni* that infect domestic ruminants (Webster et al., 2013). Increased application of molecular genotyping techniques to these schistosomes indicate that hybridization is common among them, facilitated by their common use of *Bulinus* snails and of the same aquatic habitats, enabling cercariae of these closely-related taxa to be acquired by both human and ruminants. The use of aquatic habitats by sheep and cows leads to transmission of the liver flukes *Fasciola hepatica* and *F. gigantica* by lymnaeid snails (Correa et al., 2010). These parasites are now also commonly found in humans in many different countries, transmitted to them by ingestion of metacercariae encysted in vegetation (WHO 2015). As another kind of example, two of humanity's most

striking tapeworm species, *Taenia solium* and *T. saginata*, are acquired by ingestion of poorly-cooked pork and beef, respectively, once again pointing out how predictable occurrences of behavior involving humans and their domestic animals has favored the evolution of parasite lineages with distinctive properties (Ito and Budke 2014).

Habitat permanence and the influence it has on shaping biotic diversity and breeding systems of vector snails are also of importance in gaining a general underlying understanding of the impact of other trematodes on *S. mansoni* transmission. Ephemeral habitats support relatively low diversity of snail and trematode species, but trematode egg input from common definitive hosts like cattle and humans can still be high, creating conditions leading to strong interactions between amphistomes and *S. mansoni* noted. Ephemeral or more perennial stream environments both favor habitation by a colonizing specialist, the predominately selfing *B. pfeifferi*, a species found to be highly susceptible to *S. mansoni* across Africa, regardless of whether the parasite is of sympatric or allopatric origin, or whether the *S. mansoni* is derived from an area where it is customarily transmitted by other *Biomphalaria* species (Frandsen 1979; Charbonnel et al., 2000; Mutuku et al., 2017).

In more permanent streams, *B. pfeifferi'*s preference for selfing seems particularly maladaptive given the enhanced opportunities for colonization by many additional trematode species. The presence of a different, preferentially cross-fertilizing species, *B. sudanica* along the more permanent shoreline of the lake make sense in light of the steady pressure imposed by trematodes being

inputted into shoreline habitats by definitive hosts, especially birds. Enhanced levels of resistance to trematode infection might be expected and that is what is seen (15.4% at Kasabong and 12.2% at Asao in *B. pfeifferi*, 10.9% in *B. sudanica* in the lake). Susceptibility of *B. sudanica* to *S. mansoni* is lower than for *B. pfeifferi,* regardless of the origin of the parasite and *S. mansoni* derived from the lake is more infective to *B. pfeifferi* than it is to sympatric *B. sudanica* (Mutuku et al., 2017), suggestive of the evolution of greater infectivity in a coevolutionary hotspot. By contrast, the overall infection rates for *B. choanomphala* dredged from deeper water (0.15%) a coevolutionary coldspot, are much lower than for *B. pfeifferi* or *B. sudanica*, even though this taxon seems more inherently compatible to *S. mansoni* than *B. sudanica*.

In conclusion, we note that in an area of hyperendemic transmission of *S. mansoni* in and around Lake Victoria, this predominantly human parasite experiences a broad variety of interactions with diverse definitive and intermediate hosts that favor its transmission. However, its transmission is modulated by a number of biological realities that relate to animal husbandry, presence of diverse populations of vertebrate species and conditions of habitat permanence that in turn influence not only the likelihood of *S. mansoni* infections becoming established in their snail vectors, but the likelihood that those infections are eventually compromised by other trematode species whose transmission is favored by the presence of domestic or wild vertebrate hosts and habitat permanence. The results add to a framework that permits more realistic models to be constructed which along with field studies lead to a better

understanding of the many factors dictating the abundance of this and other endemic neglected tropical diseases.

METHODS

Field surveys and parasite assessment: To measure transmission patterns of trematode parasites in their snail hosts, we sampled 6 sites along a 200 m stretch of Asao Stream (-0.31810, 35.00690) in western Kenya every other month from January 2014-January 2018. We also sampled 6 sites along a 200 m stretch at Kasabong Stream (-0.15190, 34.33550) and one shoreline site at Lake Victoria (-0.09410, 34.70760) from Jan 2015-Jan 2018. Sampling in the deepwater habitat (-0.085133, 34.071583) in Lake Victoria was done four times between Apr 2015 – May 2017. At the stream and shoreline sites, two people sampled for 15 minutes using a long-handled triangular mesh scoop, scooping the sides of rocks, plants, and the bottom of the substrate to collect snails. We used a metal mesh dredge to collect *B. choanomphala* from 0.5 to 10 m deep approximately 10 m from the shoreline of Lake Victoria. Water velocity and pH (Hanna Instruments® pH/Conductivity/TDS High-Range Tester) measurements were also recorded. Air temperature and rainfall data were collected from the Kisumu, Kenya airport weather station. Snails were then transported back to the lab and following cleaning and sorting were individually placed into 12-well tissue culture plates with 3 ml of aged tap water. The plates were then placed in natural light for 2 hr to induce the release of cercariae (“shedding”). Snails were identified using the key of Brown and Kristensen (1989), and cercariae were provisionally identified using the keys of Frandsen and Christensen (1984), and

Schell (1985). Some snails found to be infected with trematodes were used for experimental exposures to determine which trematodes were dominant or subordinate to *S. mansoni* (see below). Uninfected snails were placed into 20 L plastic aquaria and fed red leaf lettuce and then re-shed 3 weeks post-collection to determine if they had harbored prepatent trematode infections (incompletely developed infections) at the time of collection. Cercariae were saved in 95% ethanol for later molecular analyses.

Because cryptic species commonly exist among trematodes, we employed molecular markers to more precisely differentiate species. We used nuclear markers (28S or ITS) and mitochondrial markers (*cox*1 or *nad*1) to differentiate species. The choice of molecular markers used depended upon the number of GenBank records already available for each trematode superfamily. For example, there was a greater diversity of GenBank records for ITS than the 28S gene for amphistomes. Genomic DNA was extracted from 1-6 cercariae using the Qiagen MicroKit (Qiagen, Valencia CA). Primers and details of PCR cycles were as reported in Morgan et al. (1998) to amplify the *nad*1 gene, Tkach et al. (2016) to amplify the 28S gene and Laidemitt et al. (2017) to amplify the *cox*1 gene. PCR products were purified using ExoSap-IT® (Affymetrix, Santa Clara, CA). Both strands were sequenced using an Applied Biosystems 3100 automated sequencer and BigDye terminator cycle sequencing kit Version 3.1 (Applied Biosystems, Foster City, CA). Sequences were aligned by eye and Maximum Likelihood (ML) phylogenetic analyses were run in Mega 7 (Kumar et al., 2016) to determine species or at least genus into which the samples fell.

Experimental exposures to examine interactions between *S. mansoni* and the amphistome *Calicophoron sukari*:

An experiment was set up to examine the interactions between *S. mansoni* and the common amphistome we provisionally identified as *C. sukari* (Dinnik and Dinnik 1957). Juvenile 2-4 mm lab-reared snails were used in the experimental exposures. They were F1 snails derived from uninfected adult *B. pfeifferi* obtained from Asao stream. Snails were fed red leaf lettuce three times a week. *S. mansoni* eggs were collected from 5 primary school children (see ethics statement below) and *C. sukari* eggs were harvested from cow dung samples collected along the banks of Asao stream. Eggs from both sources were isolated by the techniques reported in Mutuku et al. (2014). After the cattle dung was sieved, half of the retained egg-rich material was placed into the refrigerator as a source of *C. sukari* eggs for later parts of the same experiment, and the other half was placed in plastic containers and aerated in the dark for 14-16 days. Unlike *S. mansoni* eggs that are already embryonated when retrieved from feces, amphistome eggs take approximately 14-16 days to embryonate before hatching.

Fifty or sixty snails were then assigned to each of the following six treatment groups: 1) sham controls (no parasite exposure); 2) snails individually exposed to 5 *S. mansoni* miracidia; 3) snails individually exposed to 5 *C. sukari* miracidia; 4) snails individually exposed to 5 *S. mansoni* miracidia and two weeks later to 5 *C. sukari* miracidia; 5) snails first individually exposed to 5 miracida of *C. sukari* then two weeks later to 5 miracidia each of *S. mansoni*; 6) snails simultaneously exposed to 5 *C. sukari* and 5 *S. mansoni* miracidia. All snails

were exposed to miracidia within 2 hours of hatching. Snails were kept in aerated 20 L tanks and fed red leaf lettuce and shrimp pellets three times a week. Water was changed once a week. Snails were individually placed into 12- well cell culture plates to determine if they were shedding cercariae starting at three weeks post-exposure. This basic experimental protocol was repeated three times, in Nov 2014, Nov 2016 and Mar 2017. Experimental exposures were analyzed by the variation in parasite infection success using non-parametric Kruskal Wallis analysis of variance (ANOVA) of *S. mansoni* and *C. sukari* by exposure type (single parasite, and mixed species co-exposure), followed by pair-wise comparisons. Infection success of each parasite was determined as the proportion of *B. pfeifferi* shedding cercariae.

Experimental exposures to test for dominance among trematodes: To determine which trematodes were more likely to be dominant against *S. mansoni,* two different methods were used to develop the trematode hierarchy. The first method used field-derived *B. pfeifferi* found to be shedding one species of cercariae. These were kept in aquaria and were shed twice a week until the snail died. A "natural takeover" was recorded if the snail ceased shedding one species of cercariae and switched over to shedding a different species of cercariae (for example, *B. pfeifferi* was first shedding *S. mansoni* and later began shedding *C. sukari* cercariae). The second method was to obtain field-derived *B. pfeifferi* shedding one type of cercariae, or to experimentally expose lab-reared *B. pfeifferi* to a particular species of trematode, and then once the snails were shedding cercariae they were re-exposed to a different species of trematode.

These snails were isolated and shed twice a week starting three weeks post second exposure.

Histology: Preparation of field-derived and lab-reared exposed snails for histology were done by placing the snail in Railliet-Henry's fluid for at least 48 hours. The shell of the snail was carefully peeled away from the soft parts of the snails and the body of the snail was placed into 10% neutral buffered formalin. The snails were sent to TriCore Reference Laboratories in Albuquerque, New Mexico for hematoxylin and eosin staining and sectioning.

Mathematical Modelling Methods: A deterministic model framework was developed, formally described by a system of ordinary differential equations, which allowed us to evaluate the impact of *C. sukari* presence and transmission intensity on *S. mansoni* prevalence in *B. pfeifferi a*nd total *S. mansoni* cercarial production. The model addresses the reduction in growth rate, mortality and fecundity associated with *S. mansoni* and *C. sukari* infections and the timing of each trematode infection on consequent *S. mansoni* or *C. sukari* cercarial output. The equilibrium levels of *S. mansoni* and *C. sukari* infection are calibrated to field surveys which identify the proportion of *B. pfeifferi* shedding each trematode. This field survey data was then coupled with experimental laboratory data to calculate the total proportion of *B. pfeifferi* infected with each trematode species, including those *B. pfeifferi* who experience infection, but do not successfully shed trematode cercariae. The impact of varying levels of *C. sukari* miracidial input into the *S. mansoni* transmission system is then evaluated

with respect to the prevalence of *S. mansoni* infection in *B. pfeifferi* and the overall *S. mansoni* cercarial output of the snail population.

Ethical Approval: Human subjects were enrolled into our study to provide fecal samples containing *Schistosoma mansoni* eggs. We used those eggs to do our experimental exposures and to develop our hierarchy. Samples were collected and pooled from five primary school children from Obuon primary school near Asao Stream, Kenya (00°19'01" S, 035°00'22" E). Consent forms were given and signed by the children's parents. The KEMRI Ethics Review Committee (SSC No. 2373) and the UNM Institution Review Board (IRB 821021–1) approved all aspects of this project involving human subjects. All children tested and found positive for *S. mansoni* were treated with praziquantel following standard protocols. Details of recruitment and participation of human subjects for fecal collection are described in Mutuku et al. 2014. This project done with the approval of Kenya's National Commission for Science, Technology, and Innovation (permit number NACOSTI/P/15/9609/4270), National Environment Management Authority (NEMA/AGR/46/2014) and cercariae and snails were exported with the approval of the Kenya Wildlife Service permit number 0004754.

Acknowledgements: We thank Eric Lelo, Joesph Kinuthia, Geoffery Maina and Ibrahim Mwangi for assistance with collection of field samples and Dr. Jennifer Rudgers for her statistical expertise. Technical assistance at the University of New Mexico Molecular Biology Facility was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P30GM110907. We gratefully acknowledge the following agencies for

their financial support: The National Institute of Health (NIH) grant RO1 R37AI101438, and the Bill and Melinda Gates Foundation for the Grand Challenges Explorations Initiative grant. The content for this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This paper was published with the approval of the Director of KEMRI.

References:

Bayer, F.A.H., 1954. Larval trematodes found in some fresh-water snails: A suggested biological method of bilharzia control. Transactions of the Royal Society of Tropical Medicine and Hygiene 48, 414-418.

Brant, S.V., Loker, E.S., 2013. Discovery-based studies of schistosome diversity stimulate new hypotheses about parasite biology. Trends Parasitol 29, 449-459.

Brown, D.S. & Kristensen, T.K.,1989. A field guide to African freshwater snails, southern African species. Danish Bilharziasis Laboratory Publication number 383.

Buddenborg, S.K., Bu, L., Zhang, S.M., Schilkey, F.D., Mkoji, G.M., Loker, E.S., 2017. Transcriptomic responses of *Biomphalaria pfeifferi* to *Schistosoma mansoni*: Investigation of a neglected African snail that supports more *S. mansoni* transmission than any other snail species. PLoS Negl Trop Dis 11, e0005984.

Catalano, S., Sène, M., Diouf, N.D., Fall, C.B., Borlase, A., Léger, E., Bâ, K., Webster, J.P., 2018. Rodents as natural hosts of zoonotic *Schistosoma* species and hybrids: An epidemiological and evolutionary perspective from west Africa. J Infect Dis, jiy029-jiy029.

Charbonnel, N., Angers, B., Razatavonjizay, R., Bremond, P., Jarne, P., 2000. Microsatellite variation in the freshwater snail *Biomphalaria pfeifferi*. Mol Ecol 9, 1006-1007.

Combes, C., 1982. Trematodes: antagonism between species and sterilizing effects on snails in biological control. Parasitology 84, 151-175.

Correa, A.C., Escobar, J.S., Durand, P., Renaud, F., David, P., Jarne, P., Pointier, J.-P., Hurtrez-Boussès, S., 2010. Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of fascioliasis. BMC Evolutionary Biology 10, 381-381.

DeJong, R.J., Morgan, J.A., Paraense, W.L., Pointier, J.P., Amarista, M., Ayeh-Kumi, P.F., Babiker, A., Barbosa, C.S., Bremond, P., Pedro Canese, A., de Souza, C.P., Dominguez, C., File, S., Gutierrez, A., Incani, R.N., Kawano, T., Kazibwe, F., Kpikpi, J., Lwambo, N.J., Mimpfoundi, R., Njiokou, F., Noel Poda, J., Sene, M., Velasquez, L.E., Yong, M., Adema, C.M., Hofkin, B.V., Mkoji, G.M., Loker, E.S., 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni*. Mol Biol Evol 18, 2225-2239.

Dinnik, J.A., Dinnik, N.N., 1957. Development of *Paramphistomum sukari* Dinnik, 1954 (Trematoda: Paramphistomidae) in a snail host. Parasitology 47: 209-216

Garcia-Vedrenne, A.E., Quintana, A.C., DeRogatis, A.M., Martyn, K., Kuris, A.M., Hechinger, R.F., 2016. Social organization in parasitic flatworms--four additional echinostomoid trematodes have a soldier caste and one does not. J Parasitol 102, 11-20.

Estaban, JG, Munoz-Antoli, C., Trelis, M., Toledo, R., 2010. Effects of nonschistosome larval trematodes on Biomphalaria snails. *In Biomphalaria snails*

and larval trematodes. (ed. Toledo, R., and Fried, B.) pp. 132-134. Springer New York Dordrecht Heidelberg London

Frandsen, F., 1979. Studies of the relationship between *Schistosoma* and their intermediate hosts. III. The genus *Biomphalaria* and *Schistosoma mansoni* from Egypt, Kenya, Sudan, Uganda, West Indies (St. Lucia) and Zaire (two different strains: Katanga and Kinshasa). Journal of Helminthology 53, 321-348.

Frandsen, F., Christensen, N.O., 1984. An introductory guide to the identification of cercariae from African freshwater snails with special reference to cercariae of trematode species of medical and veterinary importance. Acta Trop 41, 181-202.

Gouvras, A.N., Allan, F., Kinung'hi, S., Rabone, M., Emery, A., Angelo, T., Pennance, T., Webster, B., Nagai, H., Rollinson, D., 2017. Longitudinal survey on the distribution of *Biomphalaria sudanica* and *B. choanomophala* in Mwanza region, on the shores of Lake Victoria, Tanzania: implications for schistosomiasis transmission and control. Parasit Vectors 10, 316.

Hanelt, B., Mwangi, I.N., Kinuthia, J.M., Maina, G.M., Agola, L.E., Mutuku, M.W., Steinauer, M.L., Agwanda, B.R., Kigo, L., Mungai, B.N., Loker, E.S., Mkoji, G.M., 2010. Schistosomes of small mammals from the Lake Victoria Basin, Kenya: new species, familiar species, and implications for schistosomiasis control. Parasitology 137, 1109-1118.

Hechinger, R.F., Wood, A.C., Kuris, A.M., 2011. Social organization in a flatworm: trematode parasites form soldier and reproductive castes. Proc Biol Sci 278, 656-665.

Ito, A., Budke, C.M., 2014. Culinary delights and travel? A review of zoonotic cestodiases and metacestodiases. Travel Med Infect Dis 12, 582-591.

Johnson, P.T., Preston, D.L., Hoverman, J.T., LaFonte, B.E., 2013. Host and parasite diversity jointly control disease risk in complex communities. Proc Natl Acad Sci U S A 110, 16916-16921.

Keesing, F., Belden, L.K., Daszak, P., Dobson, A., Harvell, C.D., Holt, R.D., Hudson, P., Jolles, A., Jones, K.E., Mitchell, C.E., Myers, S.S., Bogich, T., Ostfeld, R.S., 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468, 647-652.

King, K.C., Jokela, J., Lively, C.M., 2011. Parasites, sex, and clonal diversity in natural snail populations. Evolution 65, 1474-1481.

Kuris, A.M., Warren, J., 1980. Echinostome cercarial penetration and metacercarial encystment as mortality factors for a second intermediate H=host, *Biomphalaria glabrata*. J Parasitol 66, 630-635.

Kuris, A.M., Lafferty, K.D., 1994. Community Structure: Larval Trematodes in Snail Hosts. Annual Review of Ecology and Systematics 25, 189-217.

Lafferty, K.D., Sammond, D.T., Kuris, A.M., 1994. Analysis of Larval Trematode Communities. Ecology 75, 2275-2285.

Laidemitt, M.R., Zawadzki, E.T., Brant, S.V., Mutuku, M.W., Mkoji, G.M., Loker, E.S., 2017. Loads of trematodes: discovering hidden diversity of paramphistomoids in Kenyan ruminants. Parasitology 144, 131-147.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.

Lie, K.J., 1973. Larval trematode antagonism: Principles and possible application as a control method. Experimental Parasitology 33, 343-349.

Lim, H.K., Heyneman, D., 1972. Intramolluscan inter-trematode antagonism: a review of factors influencing the host-parasite system and its possible role in biological control. Adv Parasitol 10, 191-268.

Magendantz, M., 1972. The biology of *Biomphalaria* choanomphala and *B. sudanica* in relation to their role in the transmission of *Schistosoma mansoni* in Lake Victoria at Mwanza, Tanzania. Bull World Health Organ 47, 331-341.

Moravec, F., Barus, V., Rysavy, B., Yousif, F., 1974. Observations on the development of two echinostomes, *Echinoparyphium recurvatum* and *Echinostoma revolutum*, the antagonists of human schistosomes in Egypt. Folia Parasitol 21, 107-126.

Morgan, J.A., Blair, D., 1998. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). Parasitology 116: 289-297.

Morgan, J.A., DeJong, R.J., Jung, Y., Khallaayoune, K., Kock, S., Mkoji, G.M., Loker, E.S., 2002. A phylogeny of planorbid snails, with implications for the evolution of *Schistosoma* parasites. Mol Phylogenet Evol 25, 477-488.

Mouritsen, K.N., Poulin, R., 2003. Parasite-induced trophic facilitation exploited

by a non-host predator: a manipulator's nightmare. Int J Parasitol 33, 1043-1050.

Mutuku, M.W., Dweni, C.K., Mwangi, M., Kinuthia, J.M., Mwangi, I.N., Maina, G.M., Agola, L.E., Zhang, S.M., Maranga, R., Loker, E.S., Mkoji, G.M., 2014. Field-derived *Schistosoma mansoni* and *Biomphalaria pfeifferi* in Kenya: a compatible association characterized by lack of strong local adaptation, and presence of some snails able to persistently produce cercariae for over a year. Parasit Vectors 7, 533.

Mutuku, M.W., Lu, L., Otiato, F.O., Mwangi, I.N., Kinuthia, J.M., Maina, G.M., Laidemitt, M.R., Lelo, E.A., Ochanda, H., Loker, E.S., Mkoji, G.M., 2017. A comparison of kenyan *Biomphalaria pfeifferi* and *B. Sudanica* as vectors for *Schistosoma mansoni*, including a discussion of the need to better understand the effects of snail breeding systems on transmission. J Parasitol 103, 669-676.

Pointier, J.P., Jourdane, J., 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. Acta Trop 77, 53-60.

Rashed, A.A., 2002. Biological studies on the snail intermediate hosts of schistosomiasis with a special emphasis on using larval echinostomes as biocontrol agent against larval schistosomes and snails. J Egypt Soc Parasitol 32, 775-784.

Richard, J., Brygoo, E.R., 1978. Life cycle of the trematode *Echinostoma caproni* Richard, 1964 (Echinostomatoidea) (author's transl). Ann Parasitol Hum Comp 53, 265-275.

Schell, S. C., 1985. Handbook of trematodes of North America north of Mexico. University Press of Idaho, Moscow, Idaho

Southgate, V.R., Brown, D.S., Warlow, A., Knowles, R.J., Jones, A., 1989. The influence of *Calicophoron microbothrium* on the susceptibility of *Bulinus tropicus* to *Schistosoma bovis*. Parasitol Res 75, 381-391.

Standley, C.J., Goodacre, S.L., Wade, C.M., Stothard, J.R., 2014. The population genetic structure of *Biomphalaria choanomphala* in Lake Victoria, East Africa: implications for schistosomiasis transmission. Parasit Vectors 7, 524.

Standley, C.J., Wade, C.M., Stothard, J.R., 2011. A fresh insight into transmission of schistosomiasis: a misleading tale of *Biomphalaria* in Lake Victoria. PLoS One 6, e26563.

Suzan, G., Marce, E., Giermakowski, J.T., Mills, J.N., Ceballos, G., Ostfeld, R.S., Armien, B., Pascale, J.M., Yates, T.L., 2009. Experimental evidence for reduced rodent diversity causing increased hantavirus prevalence. PLoS One 4, e5461.

Tian-Bi, Y.N., N'Goran, E.K., N'Guetta, S.P., Matthys, B., Sangare, A., Jarne, P., 2008. Prior selfing and the selfing syndrome in animals: an experimental approach in the freshwater snail *Biomphalaria pfeifferi*. Genet Res (Camb) 90, 61-72.

Tkach, V.V., Kudlai, O., Kostadinova, A., 2016. Molecular phylogeny and systematics of the Echinostomatoidea Looss, 1899 (Platyhelminthes: Digenea). Int J Parasitol 46, 171-185.

Warren, K.S., Mahmoud, A.A., Cummings, P., Murphy, D.J., Houser, H.B., 1974. Schistosomiasis *mansoni* in Yemeni in California: duration of infection, presence of disease, therapeutic management. Am J Trop Med Hyg 23, 902-909.

Webster, B.L., Diaw, O.T., Seye, M.M., Webster, J.P., Rollinson, D., 2013. Introgressive Hybridization of *Schistosoma haematobium* group species in senegal: species barrier break down between ruminant and human schistosomes. PLOS Neglected Tropical Diseases 7, e2110.

Wiegand, R.E., Mwinzi, P.N.M., Montgomery, S.P., Chan, Y.L., Andiego, K., Omedo, M., Muchiri, G., Ogutu, M.O., Rawago, F., Odiere, M.R., Karanja, D.M.S., Secor, W.E., 2017. A persistent hotspot of *Schistosoma mansoni* infection in a five-year randomized trial of praziquantel preventative chemotherapy strategies. The Journal of Infectious Diseases 216, 1425-1433.

World Health Organization. 2017. Schistosomiasis and soil transmitted helminthiases: number of people treated in 2016. WHO weekly epidemiological record 92, 749-760

World Health Organization. 2015. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015.

Wood, C.L., Lafferty, K.D., DeLeo, G., Young, H.S., Hudson, P.J., Kuris, A.M., 2014. Does biodiversity protect humans against infectious disease? Ecology 95, 817-832.

Woodhall, D.M., Wiegand, R.E., Wellman, M., Matey, E., Abudho, B., Karanja,

D.M., Mwinzi, P.M., Montgomery, S.P., Secor, W.E., 2013. Use of geospatial modeling to predict *Schistosoma mansoni* prevalence in Nyanza Province, Kenya. PLoS One 8, e71635.

Woodruff, D.S., Mulvey, M., 1997. Neotropical schistosomiasis: African affinities of the host snail *Biomphalaria glabrata* (Gastropoda: Planorbidae). Biological Journal of the Linnean Society 60, 505-516.

TABLES AND FIGURES

Table 1. The four different habitats we surveyed, the intermediate hosts for *S. mansoni* found in each, the number of other trematode species that use the respective *Biomphalaria* species, and the number of co-occurring snail species.

Habitat	*Biomphalaria* Species	Number of Other Trematode Species	Number of Other Snail Species
Kasabong Stream	*B. pfeifferi*	6	6
Asao Stream	*B. pfeifferi*	19	9
Lake Victoria- Shoreline	*B. sudanica*	21	12
Lake Victoria- Deepwater	*B. choanomphala*	4	6

Figure 1. A) Typical diagram of the *Schistosoma mansoni* life cycle in western Kenya. B) A more realistic life cycle of *S. mansoni*. In western Kenya; there are multiple species of both mammalian and molluscan intermediate hosts in different habitats, all supporting the transmission *S. mansoni*.

Figure 2. Compatibility of the three different snail vectors for *S. mansoni*. 60 laboratory-bred *B. sudanica*, *B. choanomphala*, and *B. pfeifferi* were individually exposed to 5 *S. mansoni* miracidia. Reported here is the peak infection prevalence.

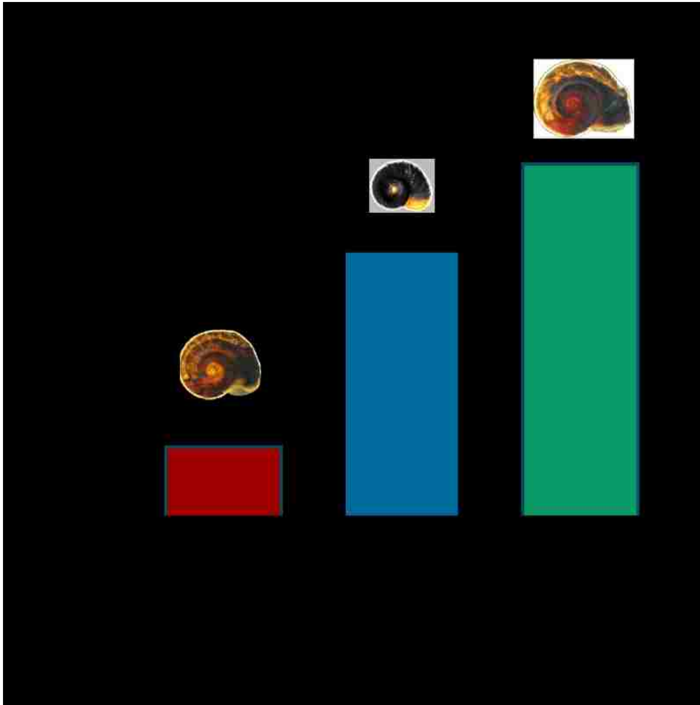


Figure 3. A herd of cattle at Asao Stream in western Kenya, watering alongside local women washing their clothes.

Figure 4. A graphical depiction of the life cycle of *Calicophoron sukari*. Adults reside in the rumen of domestic ruminants and lay eggs which are passed into the environment. Eggs hatch in water into miracidia which seek out the intermediate host, *Biomphalaria pfeifferi*. Miracidia transform into sporocysts which give rise to mother rediae, which in turn give rise to daughter rediae. Rediae produce cercariae, which emerge from the snail and encyst on vegetation and become metacercariae. Metacercariae are consumed by the definitive host (ruminant) and excyst in the intestine to become juveniles which mature into adults in the rumen.

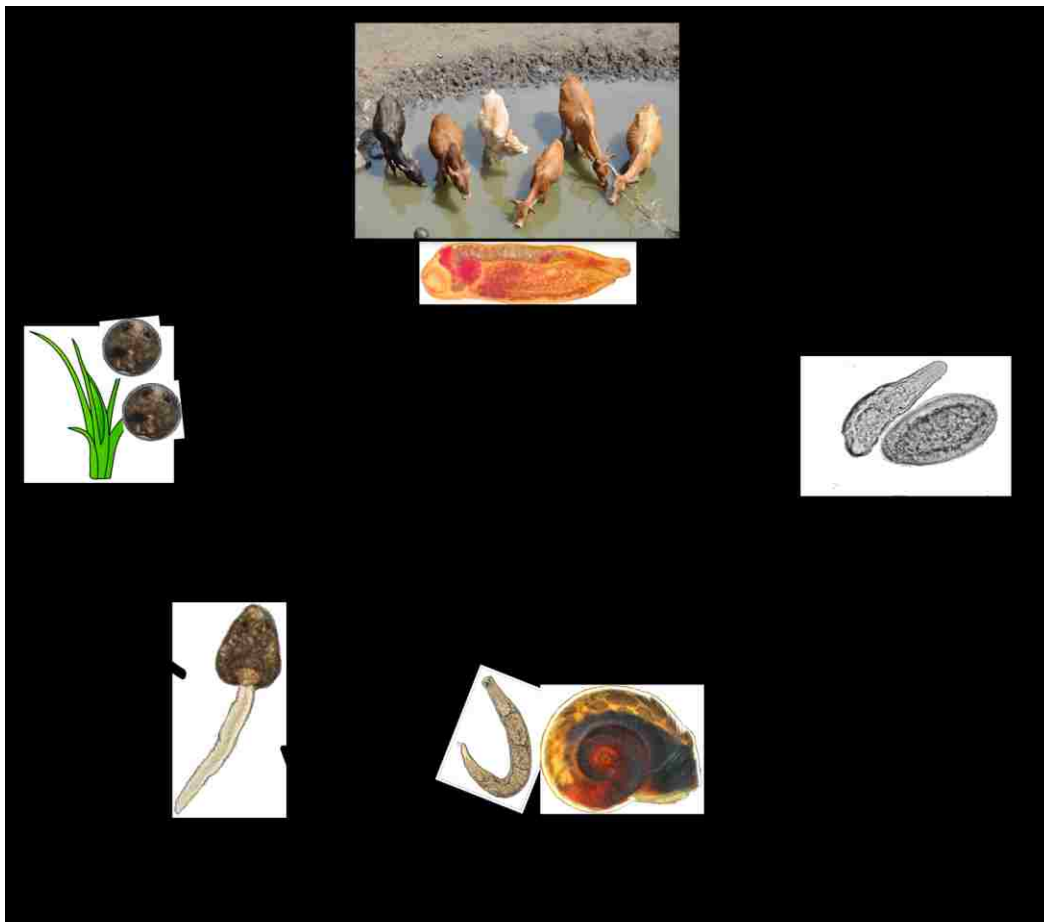


Figure 5. A) Prevalence of *S. mansoni*, *C. sukari*, and other trematodes (combined) at Kasabong and Asao Stream during the bi-monthly surveys. B) The proportion of each major group of trematodes comprising the communities at Kasabong and Asao Stream. Numbers are total trematodes found over total number of *B. pfeifferi* collected. C) The number of observed double infections of *S. mansoni* and *C. sukari* at Asao Stream. Dual infections were observed less than what would be expected by chance (p= < 0.0001). D) Pearson correlation of *S. mansoni* prevalence vs. *C. sukari* prevalence, which are positively correlated (p = 0.0009).

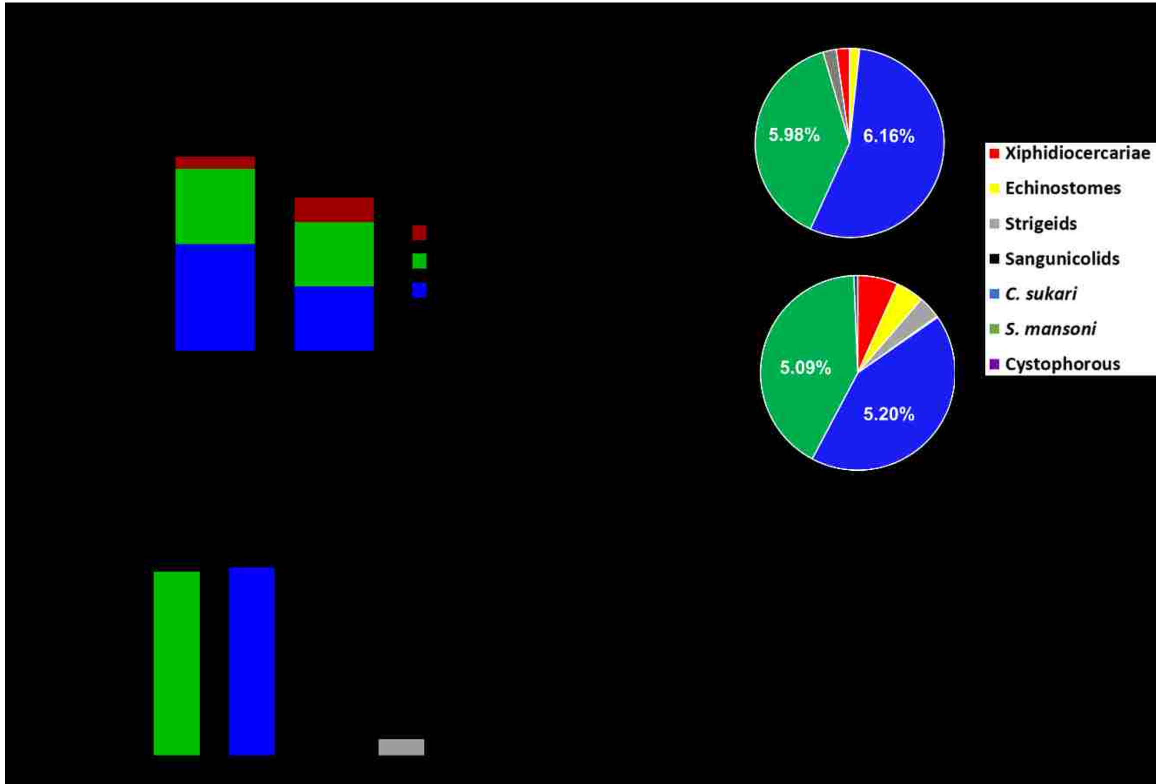


Figure 6. 100 field-derived non-shedding *B. pfeifferi* were put into two treatment groups (50 snails each), unexposed controls or 5 *S. mansoni* miracidia exposed. Compared to unexposed controls, significantly more *B. pfeifferi* shed *C. sukari* ($p < 0.001$).

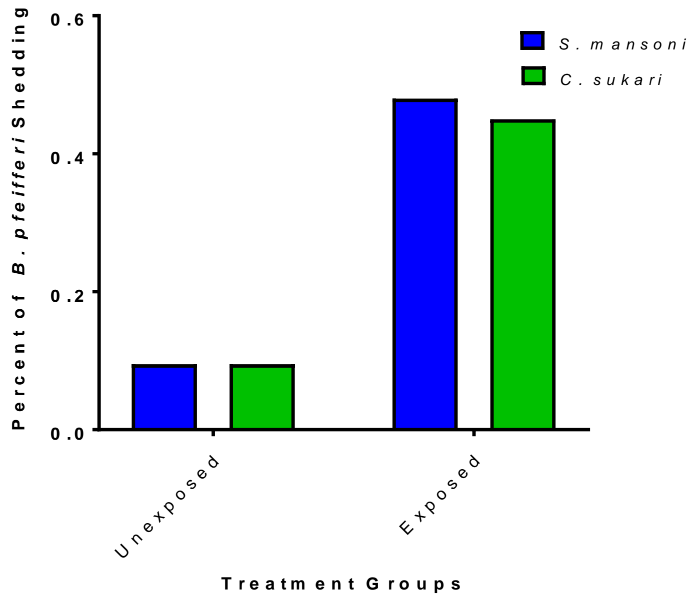


Figure 7. A) Exposure of lab-reared *B. pfeifferi* to *S. mansoni, C. sukari* or to both (single species controls, simultaneously, or first one species then two weeks later the other). Exposures to either species were with 5 miracidia/snail, 50 or 60 snails were used for each of 5 treatments for 3 separate experiments (total of 850 snails used). Separate ANOVAs were done for *S. mansoni* and *C. sukari* (each involving comparison of four groups), followed by pairwise comparisons. B) Hematoxylin and esosin stained section of a lab-reared *B. pfeifferi* exposed to *C. sukari* only. The arrow is pointing to a miracidium embedded in the muscular tissue of foot of snail.

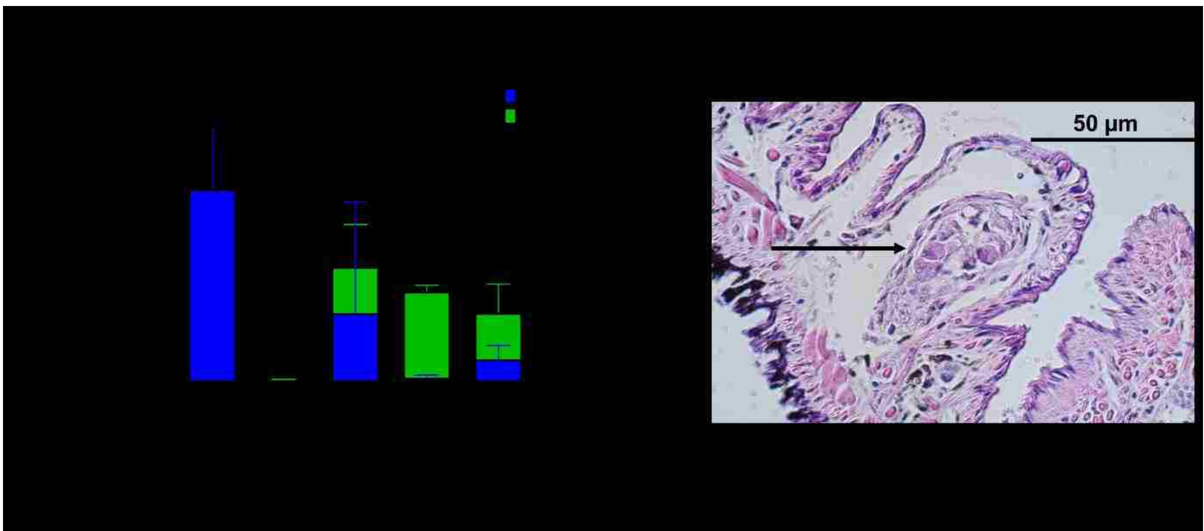


Figure 8. Trematode species that use *B. pfeifferi* and/or *B. sudanica* as first intermediate hosts in western Kenya. Red arrows indicate those trematodes we found from both snail species, and black arrows indicate those we found in one or the other of the snail species. Colored cercariae were dyed with Lugol's solution. Names of species were provisional based on GenBank records.

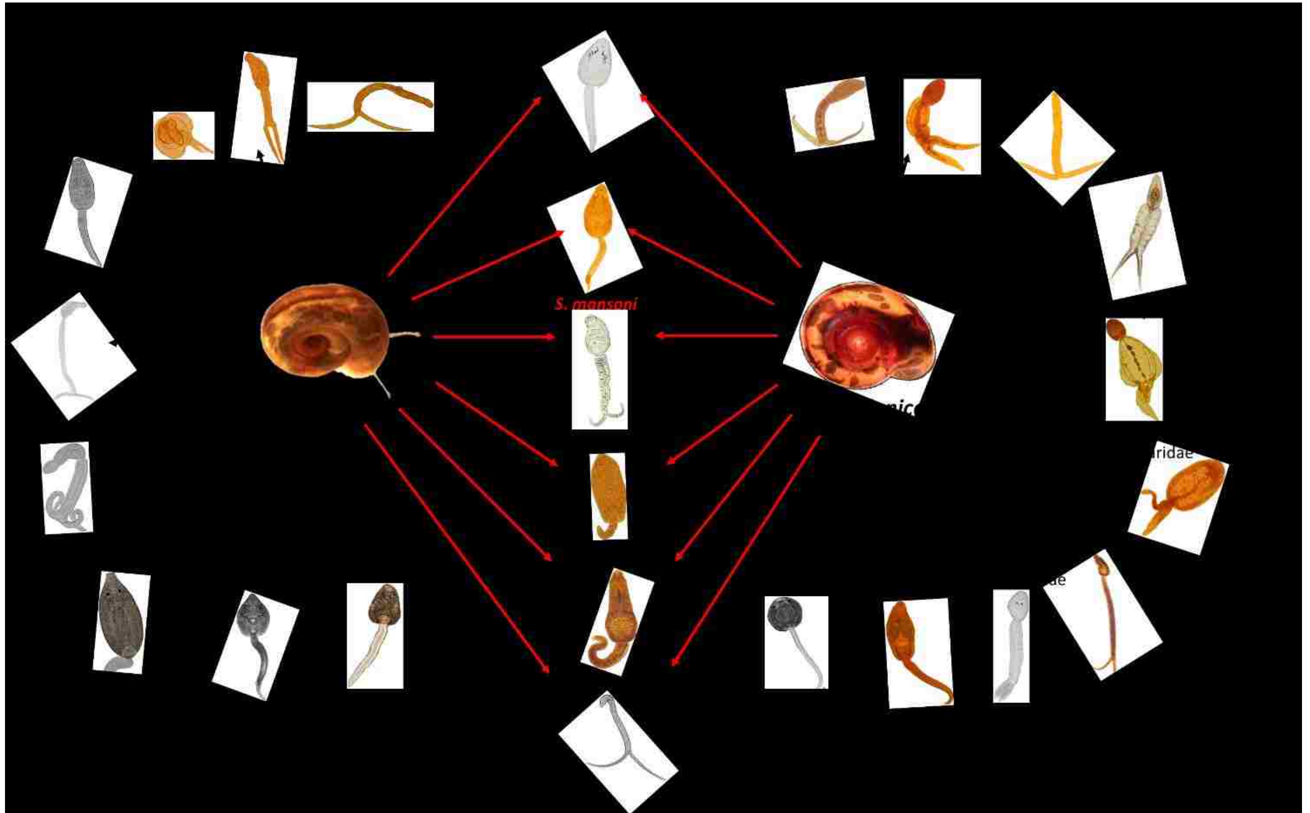


Figure 9. A) The proportion of *B. pfeifferi* infected with *S. mansoni* increases after release from competition with *C. sukari.* This increase holds under low, moderate and high *S. mansoni* infection regimes. After an initial increase in *S. mansoni* infection the prevalence reaches an equilibrium higher than the levels present with the inclusion of *C. sukari* in the system. B) With increases in *S. mansoni* miracidial input the output of *S. mansoni* cercariae also increases. Intensification in the input of *C. sukari* can diminish the impact of increasing *S. mansoni* miracidial input and reduces the output of *S. mansoni* cercariae.

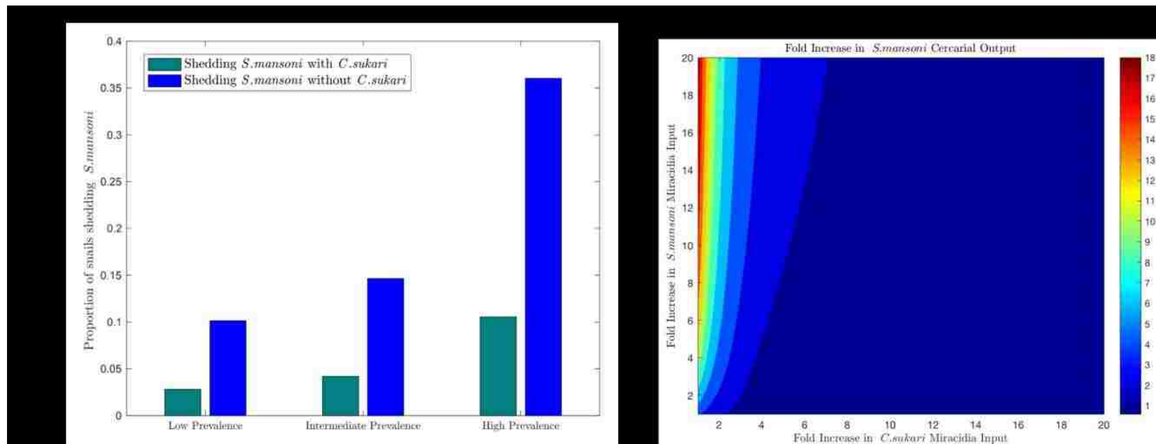


Figure 10. Examples of trematode life cycles that, along with *S. mansoni,* use Kenyan *Biomphalaria* as first intermediate host, exemplifying the diversity of additional hosts they require to complete their life cycles.

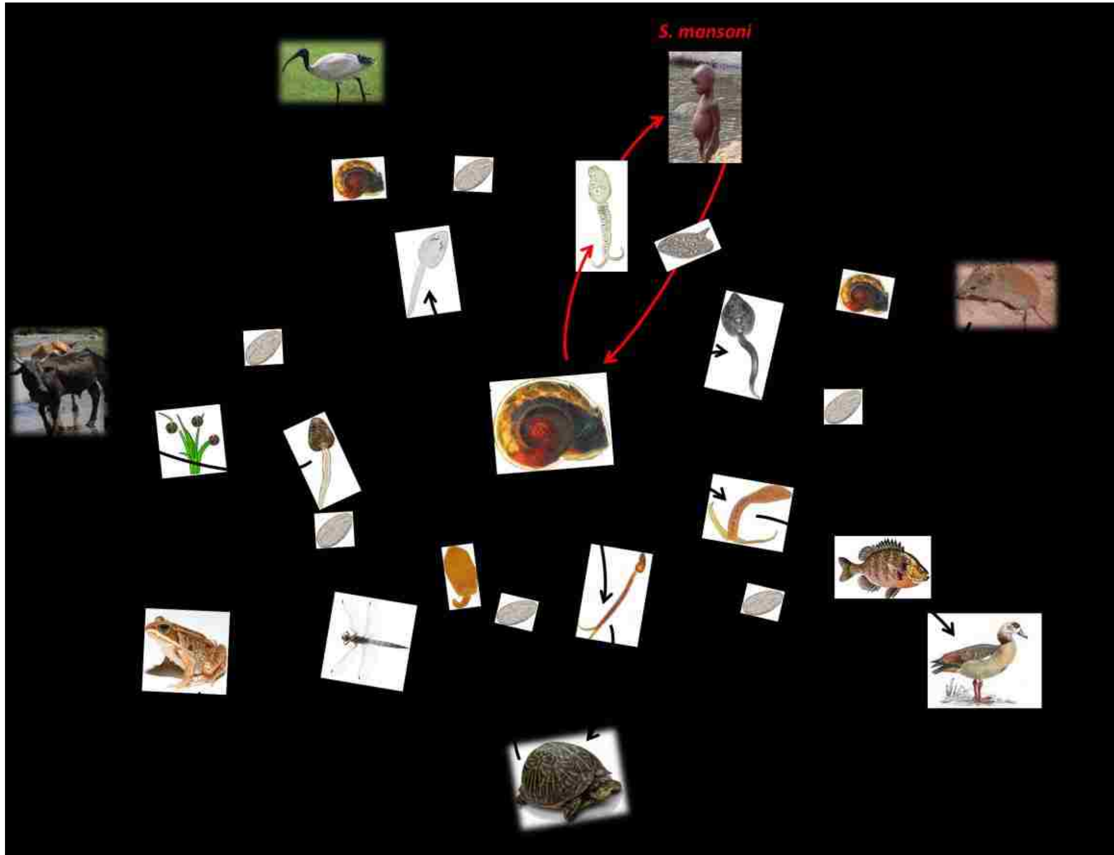


Figure 11. Hierarchy among trematodes from *B. pfeifferi* at Asao Stream. At the top are shown overall prevalence values for each of the groups of trematodes to indicate the most dominant species are not inevitably the most common (see text). The numbers adjacent to arrows indicate observed “take-over” events, of two kinds: 1) natural, in orange, where a snail from the stream was brought to the lab and observed to quit shedding cercariae of one type, and to begin shedding cercariae of another type; and 2) experimental, in blue, where a snail from the stream shedding one type of cercariae was experimentally exposed to miracidia of another trematode species, followed by production of cercariae of the latter species.

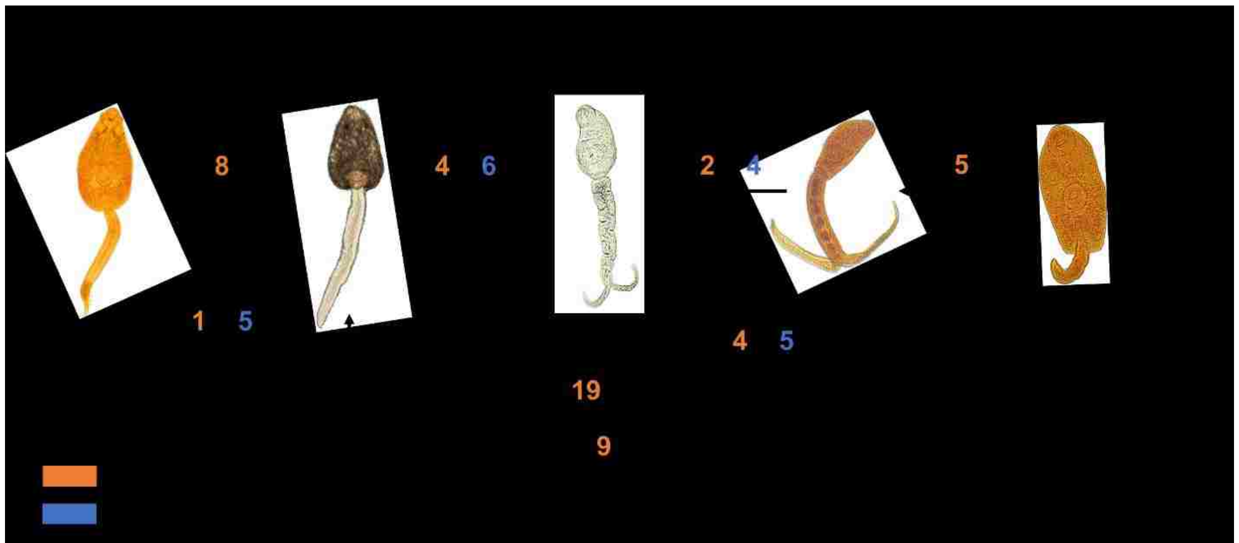
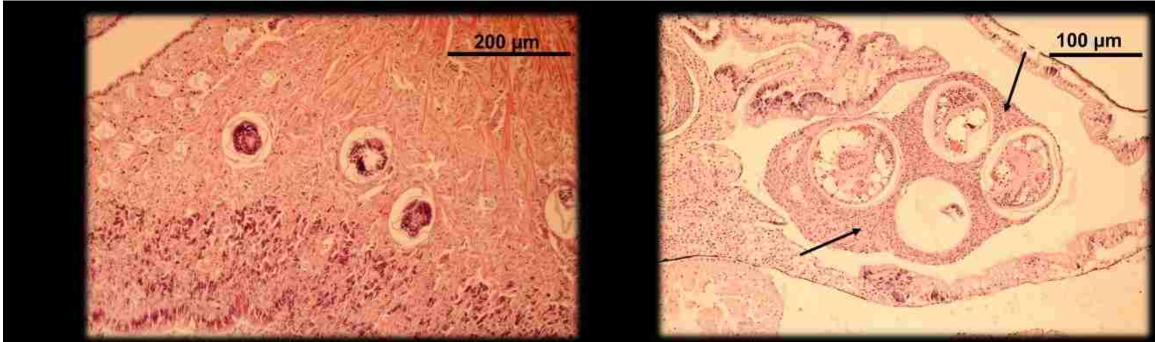


Figure 12. A) Hematoxylin and eosin stained sections of field-derived *B. pfeifferi* A) infected with echinostome metacercariae lodged in dense muscular tissue of foot of snail, and B) hemocyte-encapsulated echinostome metacercariae lodged in the pericardium (black arrows).



CONCLUSIONS

Human schistosomiasis is ubiquitous in sub-Saharan Africa, with over 180 million people infected (WHO 2017). Chemotherapy based control programs play an essential role in contributing to the elimination of human schistosomiasis; however, it has been difficult to sustainably reduce the number of infected individuals because the rates of reinfections are high, the intermediate snail hosts are difficult to control, and the drug praziquantel only targets mature adult worms. Given this situation there is an increasing consensus that chemotherapy needs to be supplemented by other means if interruption of transmission is to be achieved. Therefore, we sought out to characterize the inherent snail and trematode diversity at *S. mansoni* transmission sites, and to test if certain trematodes that use the same obligatory snail host, *Biomphalaria* could disrupt *S. mansoni's* development.

We were motivated to understand interspecific interactions of other trematodes against *S. mansoni* because it has been suggested that trematodes which are more dominant than *S. mansoni* could be used to supplement control programs (Bayer 1954; Lim and Heyneman 1972; Moravec et al., 1974; Pointier and Jourdane 2000). Therefore, we determined how many other trematodes use *Biomphalaria* as an intermediate host in western Kenya. Combining morphology, molecular markers and phylogenetic analyses we found over 20 different species of trematodes that use *Biomphalaria* as an intermediate host. Two groups of trematodes that were prominent are the amphistomes and echinostomes. These two groups of trematodes develop rediae within their intermediate hosts and the

rediae of some echinostome species have been shown to consume *S. mansoni* sporocysts (Lim and Hyneman 1972). We found that 2 different species of amphistomes and 6 different species of echinostomes use *B. pfeifferi* as an intermediate host. We developed a dominance hierarchy among the trematodes we that use *B. pfeifferi* as an intermediate host. We found that echinostomes are the most dominant trematode and can outcompete amphistomes, *S. mansoni,* strigeids and xiphidiocercariae. We also found that an amphistome (*C. sukari*) was more dominant than *S. mansoni*, and *S. mansoni* takes an intermediate position in the hierarchy. The dominance of echinostomes did not translate to be the most abundant trematode we collected in our bi-monthly surveys. In fact, *C. sukari* (the amphistome) and *S. mansoni* were the most common trematodes collected. The input of bird-transmitted echinostome eggs into the stream habitats we studied is likely dwarfed by the input of eggs from large definitive hosts like people and domestic ruminants, meaning that the prevalence achieved by *S. mansoni* or by *C. sukari* is much higher than achieved by echinostomes. Since these two trematodes were commonly transmitted at Asao Stream and Kasabong Stream we did experimental exposures to understand their interspecific interactions.

From our experimental results we found that *C. sukari* cannot infect *B. pfeifferi* on its own, and in fact relies on *S. mansoni* (and potentially other subordinate trematodes) for its own development. Our results also showed that *C. sukari* could prevent *S. mansoni* from developing and taking hold in the snail. Consequently, nearly all of the *C. sukari* infections present in streams represent

snails once infected with *S. mansoni* larvae that have been displaced and eliminated. The presence of amphistome-infected cattle thereby provides a significant protective effect for people in diminishing the number of snails producing *S. mansoni* cercariae. This was further confirmed with our mathematical model that showed if we were to take away *C. sukari* from the system there is the large competitive release and the proportion of *B. pfeifferi* infected with *S. mansoni* increases three-fold.

This dissertation highlighted the realities of human schistosomiasis transmission and how the diversity of other trematodes present in streams and lakes in Kenya can constrain the transmission dynamics of the human parasite, *S. mansoni*. We found that *S. mansoni* transmission is influenced by a number of biological realities that relate to animal husbandry, presence of diverse populations of vertebrate species and conditions of habitat permanence that in turn influence not only the likelihood of *S. mansoni* infections becoming established in their snail vectors, but the likelihood that those infections are eventually compromised by other trematode species whose transmission is favored by the presence of domestic or wild vertebrate hosts and habitat permanence. Our results suggest that certain aspects of biodiversity need to be closely studied to understand the interactions of biodiversity and human disease. We highlighted that multiple mammalian hosts and snail vectors can increase transmission, but on the other hand the diversity of other trematodes that use those same vectors can constrain *S. mansoni* transmission. Our study fits in with the recent and ongoing studies that have showcased how biodiversity can either

increase or decrease disease transmission and within this dissertation we emphasize that certain aspects of parasite biology can provide different biological realities all which relate to human disease transmission.

References:

Bayer, F.A.H., 1954. Larval trematodes found in some fresh-water snails: A suggested biological method of bilharzia control. Transactions of the Royal Society of Tropical Medicine and Hygiene 48, 414-418.

Lim, H.K., Heyneman, D., 1972. Intramolluscan inter-trematode antagonism: a review of factors influencing the host-parasite system and its possible role in biological control. Adv Parasitol 10, 191-268.

Moravec, F., Barus, V., Rysavy, B., Yousif, F., 1974. Observations on the development of two echinostomes, *Echinoparyphium recurvatum* and *Echinostoma revolutum*, the antagonists of human schistosomes in Egypt. Folia Parasitol 21, 107-126.

Pointier, J.P., Jourdane, J., 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. Acta Trop 77, 53-60.

World Health Organization. 2017. Schistosomiasis and soil transmitted helminthiases: number of people treated in 2016. WHO weekly epidemiological record 92, 749-760