Ecology, systematics and evolutionary biology of frog blood parasites in northern KwaZulu-Natal

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Disclaimer

This PhD is disclaimed for purposes of Zoological Nomenclature in accordance with the International Code of Zoological Nomenclature, Fourth Edition Articles 8.2 and 8.3 (ICZN 1999). No new names or nomenclatural changes are available from statements in this PhD thesis.

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Over the last four years, I have isolated myself from the "real world". The consequences of these actions may have lead to the majority of my friends thinking it was merely to remain a student for as long as possible, travel the world, go "camping", go on "game drives", go caving, skip weddings, and enjoy good Belgian beers. If only it were that simple… I would now like to take the opportunity to clear my conscience and guilt (at least that's what I tell myself) and thank the many family and friends for the sacrifices you have made and the neglect you put up with.

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- I am prepared to go anywhere, provided it be forward. – David Livingstone -

SUMMARY

Blood parasites have been recorded in a variety of vertebrate and invertebrate hosts, inhabiting both aquatic and terrestrial environments. Until this study, only a few blood parasite surveys had been carried out on frogs in sub-Saharan Africa. Thus information on the diversity of these parasites remained limited. To increase our knowledge of frog blood parasites, a large multi-approach study on the diversity, evolutionary biology, and ecology of frog blood parasites was undertaken. The majority of the fieldwork took place in northern KwaZulu-Natal (KZN), South Africa, focussing specifically on the area adjacent to the Phongolo River and its associated floodplain. However, samples also included those collected from frogs in the southern regions of the Kruger National Park, South Africa, and from frogs in Belgium. The latter was fortuitous, as Europe is the type locality for many frog blood parasite species and genera. These samples provided essential data for phylogenetic comparisons between the African and European species.

Presently this is the largest multi-species, generic and family amphibian blood parasite survey to be completed, including a total of 643 anurans of 38 species, 20 genera and 13 families. The study was divided into three main components for the collection, analysis and reporting of data. The first component was to determine the frog blood parasite diversity, the second to determine phylogenetic relationships in conjunction with the former component, and lastly the ecological and host-vector-parasite relationships. Blood samples were drawn from the femoral artery of each frog and thin blood smears prepared for screening and morphometrics; the remaining blood fixed in 70% molecular grade EtOH for later molecular analysis. Giemsa stained blood smears were screened microscopically for the presence of any blood inhabiting organisms. Positive infections were then further analysed according to the aims of the respective chapters. Analyses included both morphological and molecular aspects. Morphology was used for the description and identification of species, and molecular analyses were used to assist with the morphology-based descriptions, as well as to allow for phylogenetic relationship comparisons of the blood parasites with one another.

In the present study, three new species of *Hepatozoon* were described from hyperoliid frogs (*Afrixalus fornasini*, *Hyperolius argus*, and *Hyperolius marmoratus*), namely *Hepatozoon involucrum* Netherlands, Cook & Smit, 2018; *Hepatozoon tenuis* Netherlands, Cook & Smit, 2018; and *Hepatozoon thori* Netherlands, Cook & Smit, 2018. Phylogenetic relationships show that species of *Hepatozoon* isolated from African frogs form as a monophyletic group, separate from the species of *Hepatozoon* isolated from European and North American frogs.

Two species of *Dactylosoma* Labbé, 1894, were found parasitising three species of frogs namely, *Ptychadena anchietae* and *Sclerophrys gutturalis* from South Africa, as well as *Pelophylax lessonae* from Belgium. Based on morphometrics and molecular findings a new dactylosomatid, *Dactylosoma* sp. 1, is described form *Pty*. *anchietae* and *Scl*. *gutturalis*. The species of *Dactylosoma* isolated from *Pel. lessonae* conforms morphologically with *Dactylosoma splendens* Labbé 1894, thus placing in question the validity of *D. splendens* synonymy with *D. ranarum* (Kruse, 1890). Phylogenetic analysis shows species of anuran *Dactylosoma* as a monophyletic group, separate from the other haemogregarine groups.

Five species of frogs from South Africa and two from Belgium were found parasitised with haemococcidia. Based on morphological, morphometric and molecular findings *Lankesterella minima* (Chaussat 1850) is redescribed from *Pelophylax* kl. *esculentus* (Linnaeus, 1758) and *Pel. lessonae* (Camerano, 1882) from Belgium. Additionally, two new species of *Lankesterella* were described, namely *Lankesterella* sp. 1 in *Pel. lessonae* from Belgium, and *Lankesterella* sp. 2 in *Afr. delicates* and *Afr. fornasini* from South Africa. Furthermore, a new genus of haemococcidia, with a new species combination, is described from *Phr. mababiensis*, *Pty. anchietae*, and *Pyx. edulis* from South Africa; as well as a new species, haemococcidia sp. 2, described from *Afr. fornasini* from South Africa. This is the first study to provide molecular data for species of haemococcidia from African and European anurans.

A new species of amphibian filarial nematode (Onchocercidae: Waltonellinae) was described from the toads *Scl*. *gutturalis* and *Scl. garmani*. The life history of this nematode was elucidated from its natural mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *Uranotaenia* (*Pfc.*) *montana*. All stages of development were characterised using morphological and molecular methods. This study is the first to elucidate the life history of an amphibian filarial nematode from southern Africa, and provide data on its phylogenetic placement within the Onchocercidae.

In addition to the taxonomic and phylogenetic perspective of this study, this study also aimed at exploring the potential of frog blood parasites as indicators of environmental health. For this, blood parasites infecting grass frogs (*Ptychadena* Boulenger, 1917) from the Phongolo River system in South Africa were used as a case study. In general, findings indicate that frogs from more impacted sites harboured more blood parasites than from less-impacted sites.

In summary, this study explored the efficacy of a large multi-species, multi-approach survey on the diversity of frog blood parasites from northern KwaZulu-Natal, South Africa. Based on the results several new species of frog blood parasites from different taxa were discovered and described, greatly contributing to knowledge and species records on the overall diversity of frog blood parasites from South Africa. Furthermore, this study provides

the first molecular data for species of *Dactylosoma* and *Lankesterella* for frogs from Africa, as well as the first molecular data for a filarial nematode for frogs from South Africa. The phylogenetic relationships of species of *Hepatozoon*, *Dactylosoma*, *Lankesterella*, and the filarial nematode were also characterised based on comparisons to other available molecular data. From an ecological perspective, blood parasites from this study adhere to several criteria of what is considered a good indicator and thus demonstrate potential as indicators for healthy ecosystems and intact food webs. The results of this study establish a foundation for future research into the blood parasite biodiversity in northern KZN, an area that this study has highlighted as not only rich in anuran diversity, but also rich in anuran blood parasite diversity. Furthermore, this study provides a baseline for future taxonomic and ecological studies on these parasite groups, not only in South Africa but globally as well.

Keywords: Anurans; amphibians; Haemoparasites; frog blood parasites; *Hepatozoon*; *Dactylosoma*; Haemococcidia; *Trypanosoma*; filarial nematode; phylogenetics; parasite ecology; life cycle

OPSOMMING

Bloedparasiete word in ‘n verskeidenheid van gewerwelde en ongewerwelde gashere binne beide akwatiese en terrestriële omgewings aangetref. Tot en met die uitvoering van hierdie studie was slegs ‘n paar opnames van die bloedparasiete van paddas in sub-Sahara Afrika uitgevoer. Dus was inligting oor die diversiteit van hierdie parasiete beperk. Ten einde ons kennis van paddabloedparasiete te vermeerder, is ‘n grootskaalse multi-benaderingstudie van die diversiteit, evolusionêre biologie en ekologie van paddabloedparasiete aangepak. Die meerderheid van die veldwerk het in noordelike KwaZulu-Natal (KZN), Suid-Afrika, plaasgevind, met ‘n spesifieke fokus op die area teenaan die Phongolo-rivier en die geassosieerde vloedlaagte. Daarbenewens het die bestudeerde eksemplare ook dié ingesluit wat vanaf paddas in die suidelike gedeelte van die Kruger-wildtuin, Suid-Afrika, en België bekom is. Die laasgenoemde was ‘n gelukskoot, synde Europa die tieplokaliteit van verskeie paddabloedparasietspesies en -genera is. Dit het die filogenetiese vergelyking van Afrika- en Europese spesies bemiddel.

Tot op hede is hierdie die grootste multi-spesies, -genus en -familie amfibiese bloedparasietopname wat nóg voltooi is, met ‘n somtotaal van 643 paddas wat bestudeer is, verteenwoordigend van 38 spesies, 20 genera en 13 families. Die studie is in drie hoofafdelings verdeel vir die versameling, verwerking en verslaggewing van die data. Die eerste afdeling is gewy aan die bepaling van die paddabloedparasietdiversiteit, die tweede, tesame met die eerste komponent, aan die bepaling van die filogenetiese verwantskappe en die derde aan die ekologiese en gasheer-draer-parasiet-verwantskappe. Bloedmonsters is vanaf die femorale slagaar van elke padda bekom en dun bloedsmere is voorberei vir die parasietopnames en morfometrie. Die oorblywende bloed is in 70% molekulêre graad EtOH gefikseer vir latere molekulêre analises. Giemsa-gekleurde bloedsmere is onder die mikroskoop deurgegaan vir die teenwoordigheid van enige bloedbewoners. Positiewe infeksies is dan verder verwerk luidens die doelstellings van die onderskeie hoofstukke. Analises het beide morfologiese en molekulêre aspekte ingesluit. Morfologie het die beskrywing en identifikasie van spesies moontlik gemaak, terwyl molekulêre analises bykomend tot die morfologiese data in spesiebeskrywings gebruik is en ook om filogenetiese vergelykings tussen die bloedparasietspesies te tref.

In hierdie studie is drie nuwe *Hepatozoon*-spesies vanuit paddas in die familie Hyperoliidae beskryf (*Afrixalus fornasini*, *Hyperolius argus* en *Hyperolius marmoratus*), naamlik *Hepatozoon involucrum* Netherlands, Cook & Smit, 2018; *Hepatozoon tenuis* Netherlands, Cook & Smit, 2018; en *Hepatozoon thori* Netherlands, Cook & Smit, 2018. Filogenetiese verwantskappe dui aan dat *Hepatozoon* vanuit Afrika-paddas ‘n monofiletiese

groep vorm wat afgesonder is van *Hepatozoon* vanuit paddas van Europa en Noord-Amerika.

Twee spesies van *Dactylosoma* Labbé, 1894, is waargeneem as parasiete van drie paddaspesies, naamlik, *Ptychadena anchietae* en *Sclerophrys gutturalis* van Suid-Afrika, sowel as *Pelophylax lessonae* van België. Volgens morfometrie en molekulêre bevindinge is ‘n nuwe lid van die Dactylosomatidae, *Dactylosoma* sp. 1, vanuit *Pty*. *anchietae* en *Scl*. *gutturalis* beskryf. Die spesie van *Dactylosoma* vanuit *Pel. lessonae* stem morfologies met *Dactylosoma splendens* Labbé, 1894, ooreen, wat die geldigheid van *D. splendens* se gelykbeduidenis met *D. ranarum* Kruse, 1890, in twyfel trek. Filogenetiese analise toon die paddaparasiterende spesies van *Dactylosoma* as ‘n monofiletiese groep, afgesonder van die ander hemogregariene groepe.

Vyf paddaspesies van Suid-Afrika en twee van België word geparasiteer deur hemokoksidieërs. Gebaseer op morfologiese, morfometriese en molekulêre bevindinge is *Lankesterella minima* Chaussat, 1850, herbeskryf van *Pelophylax* kl. *esculentus* Linnaeus, 1758, en *Pel. lessonae* Camerano, 1882, van België. Daarbenewens is twee nuwe *Lankesterella*-spesies beskryf, naamlik *Lankesterella* sp. 1 in *Pel. lessonae* van België, en *Lankesterella* sp. 2 in *Afr. delicates* en *Afr. fornasini* van Suid-Afrika. Boonop is ‘n nuwe genus van hemokoksidieërs, met ‘n nuwe spesiekombinasie, beskryf van *Phr. mababiensis*, *Pty. anchietae*, en *Pyx. edulis* van Suid-Afrika; tesame met ‘n nuwe spesie, hemokoksidieër sp. 2, beskryf vanuit *Afr. fornasini* van Suid-Afrika. Hierdie is die eerste studie om molekulêre data van hemokoksidieërs van Afrika- en Europese paddas beskikbaar te maak.

‘n Nuwe spesie amfibiese filariale rondewurm (Onchocercidae: Waltonellinae) is van die skurwepaddas *Scl*. *gutturalis* en *Scl. garmani* beskryf. Die lewenssiklus van hierdie nematood is met behulp van sy natuurlike draers, die muskiete *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* en *Uranotaenia* (*Pfc.*) *montana*, aan die lig gebring. Alle ontwikkelingstadia is deur middel van morfologiese en molekulêre metodes geëien. Hierdie studie is die eerste om die lewenssiklus van ‘n Suid-Afrikaanse amfibiese filariale rondewurm toe te lig en om data beskikbaar te maak oor sy filogenetiese posisie binne die Onchocercidae.

Bykomend tot die taksonomiese en filogenetiese aspek van hierdie studie is die paddabloedparasiete se nut as potensiële aanwysers van omgewingsgesondheid ondersoek. Hiervoor is bloedparasiete vanaf graspaddas (*Ptychadena* Boulenger, 1917) van die Phongola-riviersisteem in Suid-Afrika gebruik as ‘n gevallestudie. Die bevindinge dui in die breë aan dat paddas van meer versteurde habitatte meer bloedparasiete huisves as hulle eweknieë in minder versteurde habitatte.

Kortom, hierdie studie het die wesenlikheid ondersoek van ‘n grootskaalse multi-spesie, multi-benadering opname van die diversiteit van paddabloedparasiete van noordelike KwaZulu-Natal, Suid-Afrika. Verskeie nuwe spesies van paddabloedparasiete is

ontdek en beskryf wat heelwat kennis toevoeg tot die bestaande spesielys van paddabloedparasiete in Suid-Afrika. Daarby verskaf hierdie studie die eerste molekulêre data vir *Dactylosoma*- en *Lankesterella*-spesies van paddas vanaf Afrika, sowel as die eerste molekulêre data vir ‘n filariale nematood van paddas vanaf Afrika. Die filogenetiese verwantskappe van spesies van *Hepatozoon*, *Dactylosoma*, *Lankesterella* en die filariale nematood is ook bereken deur middel van vergelykings met ander beskikbare molekulêre data. Vanuit ‘n ekologiese perspektief wil dit blyk asof bloedparasiete wat hier aangetref is voldoen aan verskeie kriteria wat hulle goeie biologiese aanwysers sal maak, wat ‘n potensiële toepassing as aanwysers van gesonde ekosisteme en ongeskonde voedselnetwerke demonstreer. Die verkrygde resultate lê die grondslag vir toekomstige studies van die bloedparasietdiversiteit in die noordelike KwaZulu-Natal, ‘n area wat deur hierdie studie uitgelig word as nie net ryk aan paddas nie, maar ook aan hul bloedparasiete. Verder lê hierdie studie die fondasie vir toekomstige taksonomiese en ekologiese studies van bloedparasiete in Suid-Afrika en wêreldwyd.

PREFACE

This thesis follows the article format style as prescribed by North-West University. Therefore, articles published or unpublished are presented in a unified format. As an additional requirement by the North-West University, Table A details the contributions of authors for each article/manuscript and provides consent for use as part of this thesis.

The following Chapters were included in this work:

Chapter 1 – Article 1 (published): Monophyly of the genus *Hepatozoon* (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa. Parasitology (2018) 145, 1039–1050.

Chapter 2 – Article 2 (unpublished): A review of the Dactylosomatidae (Apicomplexa: Adeleorina: Dactylosomatidae), with the description of a new species of *Dactylosoma* sp. 1 and comments on the synonymy of *D. splendens* Labbé 1894 infecting frogs.

Chapter 3 – Article 3 (unpublished): Anuran Haemococcidia: description of two new species of Lankesterella (Apicomplexa: Lankesterellidae), and a new species of haemococcicida (Apicomplexa: Eimeriorina) in a new genus.

Chapter 4 – Article 4 (unpublished): The description, life history, and experimental transmission of the filarial nematode onchocercid n. gen. n. sp. 1 (Nematoda: Onchocercidae), parasitising the toad *Sclerophrys gutturalis* (Amphibia: Bufonidae) and the mosquito vectors *Uranotaenia* (Pseudoficalbia) *mashonaensis* and *U*. (*Pfc*.) *montana* (Insecta: Culicidae).

Chapter 5 – Article 5 (unpublished): Positive parasitology: spatial heterogeneity in parasite community composition suggests frog blood parasites as potential indicators of environmental health.

Table A: Contributions of authors and consent for use.

Author	Article / Chapter	Contribution	Consent
EC Netherlands	Chapter 1 - 5	Responsible for study design, field sampling, molecular work, data analysis, and drafting the manuscript. Serve as the first author for articles 1 – 4 and joint first author on article 5.	
NJ Smit	Chapter 1 - 5	Supervised the design and execution of the study. Also provided intellectual input on data analyses, and writing of articles and thesis.	
LH Du Preez	Chapter 1 - 5	Supervised the design and execution of the study. Also provided support in the field, intellectual input on data analyses, and writing of articles and thesis.	
L Brendonck	Chapter 1 - 5	Supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	
MPM Vanhove	Chapter 1 - 5	Supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	
CA Cook	Chapter 1 - 5	As assistant promoter, supervised the design and execution of the study. Also provided support in the field, intellectual input on data analyses, and writing of articles and thesis.	
SP Lawton	Chapter 3	As co-author, provided support in intellectual input on data analyses, and writing of article.	S. P. Lawton
R Svitin	Chapter 4	As co-author, supervised the design and execution of the study. Also provided support in field and experimental work, intellectual input on data analyses, and writing of article.	
J Snoeks	Chapter 5	As joint first author, responsible for field sampling, data analysis, and drafting the manuscript.	
V Wepener	Chapter 5	As co-author, provided support in intellectual input on data analyses, and writing of article.	

LIST OF ABBREVIATIONS

A:

AIC	Akaike Information Criterion
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance

B

BCI	Body Condition Index
BI	Bayesian inference
BIC	Bayesian information criterion
BLAST	Basic Local Alignment Search Tool

C

CDC	Centre for Disease Control
CO_2	Carbon dioxide
COI	Cytochrome *c* oxidase subunit I
CV	Coefficient of variation

D

DDT	Dichlorodiphenyltrichloroethane

E

ESC	Evolutionary species concept

K

KNC	Kwa Nyamazane Conservancy
KZN	KwaZulu-Natal

L

LSID	Life Science Identifier

M

MCMC	Markov Chain Monte Carlo
ML	Maximum likelihood
MS222	Tricaine methanesulfonate
Mm	Micrometres

N

NBSAP	National Biodiversity Strategy and Action Plan
NEMBA	National Environmental Management Biodiversity
NGS	Next-Generation Sequencing
NGR	Ndumo Game Reserve
NMB	National Museum, Bloemfontein
Nt	Nucleotides

O

OCPs	Organochlorine pesticides
OsO_4	Osmium tetroxide

P

PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PV	Parasitophorous vacuole

S

SD	Standard deviation
SEM	Scanning electron microscopy

T

TSC	Taxonomic species concept

X

XXX	To be added, after formal publication

GENERAL INTRODUCTION

GI

Introduction to the Amphibia

Amphibians are an extremely ancient group of ectothermic vertebrates that evolved approximately 350 million years ago (Dodd, 2010). Although amphibian fossil records are rarely complete, thus limiting information on the origin or extinction of certain species, they appear to maintain certain characters across orders and families over time (Carroll, 2009). The first fossil records resembling amphibians were discovered almost 150 years ago (Dawson and Owen, 1862; Carroll, 2009). These organisms dominated the Earth early in their history, giving rise to all other terrestrial vertebrates (Carroll, 2009). Living amphibians (Lissamphibia) represented by three orders: Gymnophiona (caecilians), Caudata (salamanders and newts), and Anura (frogs), are descendants of the first terrestrial vertebrates, lobe-finned fishes (Vitt and Caldwell, 2013). Due to this expansion, amphibians have successfully inhabited most terrestrial and aquatic environments, resulting in an astonishing variety of physiological, morphological, behavioural, and ecological characteristics (Dodd, 2010).

As amphibians developed, transformed and adapted to life as terrestrial or semi-terrestrial organisms, they were confronted with major evolutionary pressures (Hanken et al., 1997). External fertilisation, a basic trait of early amphibians, restricted them to maintain an amphibious lifestyle, as freshwater was required for reproduction (Vitt and Caldwell, 2013). Another constraint for early amphibians was their rapid dehydration in dry environments. Due to these demands, amphibians (the adult and larval/tadpole forms), have been forced to either adapt to occupy a smaller niche, tolerating a narrower range of habitat conditions (specialist species) or modify certain morphological features to be able to survive a number of physical environmental elements (generalist species) (Hanken et al., 1997; Vitt and Caldwell, 2013; Klaus and Noss, 2016).

Some of the major adaptations of modern amphibians include the evolution of breeding behaviour, allowing some taxa independence from standing water for breeding (Vitt

and Caldwell, 2013). For example, internal fertilisation, although a relatively rare phenomenon in anurans, takes place in most salamanders and all caecilians. Another example is the terrestrial breeding of rain frogs (Brevicipitidae Bonaparte, 1850), which undergo their entire larval development and metamorphosis in an underground nest. Moisture is presumably provided by a layer of eggless jelly capsules (Minter et al., 2004). Also, the arboreal foam nest frogs of the genera *Chiromantis* Peters, 1854 (Rhacophoridae Hoffman, 1932) create a foam nest in which they lay their eggs. This strategy has several advantages. Firstly, eggs undergo polyandrous fertilisation ensuring genetic diversity; secondly, eggs are kept safe from aquatic predators; and thirdly, once they hatch, tadpoles remain and develop for a few days in the nest before dropping into the water below (Minter et al., 2004; Du Preez and Carruthers, 2017).

Other evolutionary traits of amphibians include adaptations that permit existence in extreme habitats. In some frogs, water loss is reduced through modifications or the use of glands or other structures in the skin. For example, wax tree frogs (species of *Phyllomedusa* Wagler, 1830) secrete lipids from specialised skin glands, which they spread across their bodies using their arms and legs. Subsequently, this secretion becomes impermeable reducing the loss of water (Vitt and Caldwell, 2013). Other examples include the use of chromatophores arranged in layers in the dermis of species of *Chiromantis* and *Hyperolius* Rapp, 1842 (Hyperoliidae Laurent, 1943) (Vitt and Caldwell, 2013). These chromatophores are used to reflect light thereby lowering radiation absorption, and reducing the rate of water loss (Du Preez and Carruthers, 2017). Alternatively, certain species such as *Pyxicephalus* Tschudi, 1838 (Pyxicephalidae Bonaparte, 1850) can survive extended dry seasons by burrowing underground, producing an encased parchment-like cocoon consisting of layers of cornified skin that reduces water loss (Du Preez and Carruthers, 2017).

Another extreme example is the tolerance some species of frogs have to freezing. A select few species (*Pseudacris crucifer* (Wied-Neuwied, 1838), *Hyla versicolor* LeConte, 1825, *Pseudacris triseriata* (Wied, 1838), *Rana sylvatica* LeConte, 1825) have developed adaptations for surviving extracellular freezing, during which most of their extracellular fluid is converted to ice. During this time their life processes such as breathing, a beating heart, and the flow of blood, are suspended (Vitt and Caldwell, 2013). These few examples, provide us with an idea of how these organisms have spread and adapted, and are possibly the reason why amphibians are such a diverse group spread across the globe, except for Antarctica and some isolated islands.

Box 1: *'Frogs' and 'Toads', is there a difference?*

According to Du Preez and Carruthers (2017), for centuries the name 'toad' was applied to the European species of the genus *Bufo* Laurenti, 1768 while 'frog' was the common term for other Anura, mostly of the genus *Rana* Linnaeus, 1758.

Toads generally have shorter legs and rough skin and frogs long legs and smooth skin. These two groups were originally regarded the same as reptiles, lizards and snakes. As scientific taxonomy progressed during the 18th and 19th centuries, frogs and toads were reclassified as two individual families of amphibians. Toads are but one of the frog families, Bufonidae Gray, 1825 along with Ranidae Batsch, 1796, Ptychadenidae Dubois, 1987, Pipidae Gray, 1825, and others. It is therefore incorrect to separate frogs from toads. All toads are frogs, but not all frogs are toads.

Amphibian diversity globally

Amphibians are a diverse group comprising a large portion of the world's vertebrate fauna (Frost et al., 2006). There are currently 7947 amphibian species (Dec 11, 2018), of which the caecilians (Gymnophiona) comprise 210, the salamanders and newts (Caudate) 724, and the frogs (Anura) 7013 (Frost, 2018). Caecilians occur in tropical areas around the globe, except for Madagascar and Oceania. They are mostly fossorial, living in moist soils usually adjacent to water bodies, with a few fully aquatic species. The biology of these secretive earthworm-like amphibians is largely unknown, with our current knowledge only being obtained from observations made during capture or from museum specimens (Vitt and Caldwell, 2013). Salamanders and newts, the tailed amphibians, are mostly restricted to temperate and subtropical areas of the northern Hemisphere, incorporating North America, Europe, Asia and North Africa (Vitt and Caldwell, 2013; Sparreboom, 2014). All tailed amphibians are 'salamanders' and 'newts' are just a subset of the group. Therefore, as with regards to frogs and toads, all newts are salamanders, but not every salamander is a newt (Sparreboom, 2014). However, there is not a clear definition that can separate the two from one another, and the literature is not consistent in its usage of the terms. Most consider the salamander with a semi-aquatic lifestyle as a 'newt'. Terrestrial salamanders generally require moist, typically forested habitats, whereas aquatic salamanders occur in pools, spring seepages, streams, lakes and rivers (Vitt and Caldwell, 2013; Sparreboom, 2014). Anurans, the most diverse group, are distributed across numerous aquatic and terrestrial habitats, excluding estuarine and marine environments. They are found on most major islands and all continents except for Antarctica. Their highest diversity is in moist tropical areas; however, they do also occur in arid or cold-temperate localities (Vitt and Caldwell,

2013). In recent years there was a turning point for scientific efforts and an increase in knowledge on amphibians as the most threatened vertebrate class. This was a result of the discovery of the amphibian-killing fungus, chytridiomycosis (O'Hanlon et al., 2018). Although other factors, such as habitat loss, pollution, and climate change, were recognised as contributing threats before the discovery of chytridiomycosis, they also continue to threaten amphibians globally (Beebee and Griffiths, 2005; Stuart et al., 2008). Paradoxically these efforts, with the use of modern molecular and bioacoustic techniques, and increased scientific surveys in remote areas of the world, have led to the awareness of biodiversity with new species being discovered and described frequently. Since 1985, the total number of recognised species has increased by over 60% (Frost et al., 2006; Stuart et al., 2008; Du Preez and Carruthers, 2017; AmphibiaWeb, 2018).

Anurans diversity in South Africa

The only amphibian order present in southern Africa is the Anura, comprising 13 families, with a total of 169 known species (Du Preez and Carruthers, 2017). South Africa contains three out of the world's 34 terrestrial biodiversity hotspots, namely the Cape Floristic Region, the Succulent Karoo and the Maputaland–Pondoland–Albany region (Biggs et al., 2006; Di Minin et al., 2013). Furthermore, with 124 species, South Africa has approximately 2% of the world's anurans (Du Preez and Carruthers, 2017; AmphibiaWeb, 2018), and accommodates almost 80% of the species from sub-Saharan Africa on 0.8% of the total African landmass (Drinkrow and Cherry, 1995; Mokhatla et al., 2012). Additionally, South Africa demonstrates a high level of endemism, with 43% of anuran species and one family endemic (Drinkrow and Cherry, 1995; Minter, 2011). As a result, South Africa boasts unique and rich amphibian biodiversity. The country has two humid, sub-tropical regions, namely the Maputaland and the coastal area in the province of KwaZulu-Natal (KZN). These areas are transition zones between tropical and temperate climates, characterised by high anuran species richness (Drinkrow and Cherry, 1995; Minter, 2011). KwaZulu-Natal, known for its high diversity of anurans, contains a total of 70 species, of which 10% are endemic to the province (Du Preez and Carruthers, 2017; Phaka et al., 2017).

Well-adapted host

Most anurans follow a biphasic life cycle, in which two stages are present, an aquatic larval stage and a terrestrial or semi-terrestrial adult stage (Hanken et al., 1997; Todd, 2007). The transition from the larval stage to the adult stage is known as metamorphosis, a process involving a relatively brief period of morphological and physiological restructuring. According to Carroll (2009) this biphasic life history has long provided biologists with a model for

evolutionary studies between ancestral fish and fully terrestrial vertebrates. On the other hand, the life history traits of anurans provide parasites with the opportunity to easily infect these hosts or to be transmitted from one individual host to the next. In other words, anurans serve as good hosts for various parasite species, also making them an interesting model for parasitological studies (Todd, 2007). However, despite the increase of studies on amphibians for conservation efforts, research on their parasites has not followed the same route and thus the known diversity of parasites is most likely only a fraction of what is actually present.

Box 2: *What is a parasite?*

Before we can understand what parasites do and the effects they have on their host species or surrounding ecosystem, we need to know what a parasite is? Poulin (2011) defines parasites as *"organisms that have an obligate physical association with a host, and that must obtain a critical resource, usually nutrients, from the host resulting in a fitness cost to the latter."* Parasites are therefore organisms that live in or on other organisms (the host) and feed off them, at the expense of the host.

Like predation, parasitism is a consumer-resource interaction (Getz, 2011) however, parasites, with the exception of parasitoids, are typically much smaller than their hosts and highly dependent on their hosts for transmission and survival (Cizauskas et al., 2017). Parasitism is a kind of symbiosis, a close and persistent long-term biological interaction between the parasite and its host (Martin and Schwab, 2013). Unlike commensalism and mutualism, the parasitic relationship comes at a cost, albeit in some cases minimalistic and often non-fatal. There are six major parasitic strategies, namely castrators, directly transmitted parasites (contact), trophically transmitted parasites (consumption), vector-transmitted parasites, parasitoids, and micropredators (Poulin and Randhawa, 2015). Parasitism is estimated to be the most abundant consumption strategy by animals, and it is possible that for each free-living animal there is at least one species of parasite (Poulin, 1999; Cizauskas et al., 2017).

Parasitism is a common consumer strategy, far outnumbering other major evolutionary shift life history strategies (Poulin and Randhawa, 2015). For an organism to successfully parasitise a host, it must achieve transmission to a particular host, be able to survive its invasion event within the host, and effectively exploit the host's resources to ensure the host's survival is not compromised (Poulin and Randhawa, 2015).

According to Todd (2007), amphibians are highly suitable hosts for parasites as most parasites rely on aquatic infective stages for transmission and reproduction, much in the

same way that amphibians depend on extended exposure to aquatic systems for reproduction and their life history. This in turn increases their host-parasite contact rates. The larval and metamorphosing stages of amphibians also have compromised immune systems, increasing susceptibility to parasites (Todd, 2007). Furthermore the parasites of frogs and other ectotherms have co-evolved over a long period of time and although these parasites are still true parasites per definition, they seldom inflict adverse effects on their hosts, as compared to endotherm hosts (Landau, 1973; Davies and Johnston, 2000).

In a recent review on the diversity of helminth parasites found in South American anurans, data on 225 parasites and 156 anurans from 19 host families was compiled (Campião et al., 2015). The information gathered in that study revealed interesting data on host-parasite relationships. They found a positive correlation between the commonly studied hosts, and host body size (large-bodied hosts may be easier to colonise), compared to hosts with the richest parasite faunas (Campião et al., 2015). Based on these findings, it is clear anurans host a great diversity of helminth parasites (see Fig. 1). However, helminths are certainly not the only parasites of anurans. According to Mitchell (2007) anurans host all major animal parasite groups, including protozoans, mono- and digenetic trematodes, cestodes, nematodes, acantocephalans, parasitic arthropods and molluscs, and leeches. Some amphibians are more suited to certain parasite taxa, probably due to their behaviour, life history and host-parasite interactions over time. Du Preez and Kok (1992), found the frog genus *Ptychadena* Boulenger, 1917 a highly susceptible host for flatworm polystomatid (Monogenea) parasites, based on the ability of this taxon to successfully utilize a variety of habitat types, its extended breeding season, and the fact that it breeds only in stagnant or slow-flowing water.

Anurans play a central role in the food web (Hirai and Matsui, 1999), both as predators and prey, forming a key part in stabilising ecosystem communities (Duellman and Trueb, 1994; Semlitsch and Bodie, 2003). Studies have shown that a high correlation exists between the relative abundance of prey in the area and the frequency of these items in the gut contents of anurans in that same area (Hirai and Matsui, 1999). As prey, anurans (adults and larvae) are an important source of protein for numerous invertebrates, reptiles, birds, mammals, and other amphibian species (Martínez et al., 2004; Minter et al., 2004; Du Preez and Carruthers, 2017). Thus, they can serve as an intermediate or paratenic (transport) host in the life cycle of several parasite taxa.

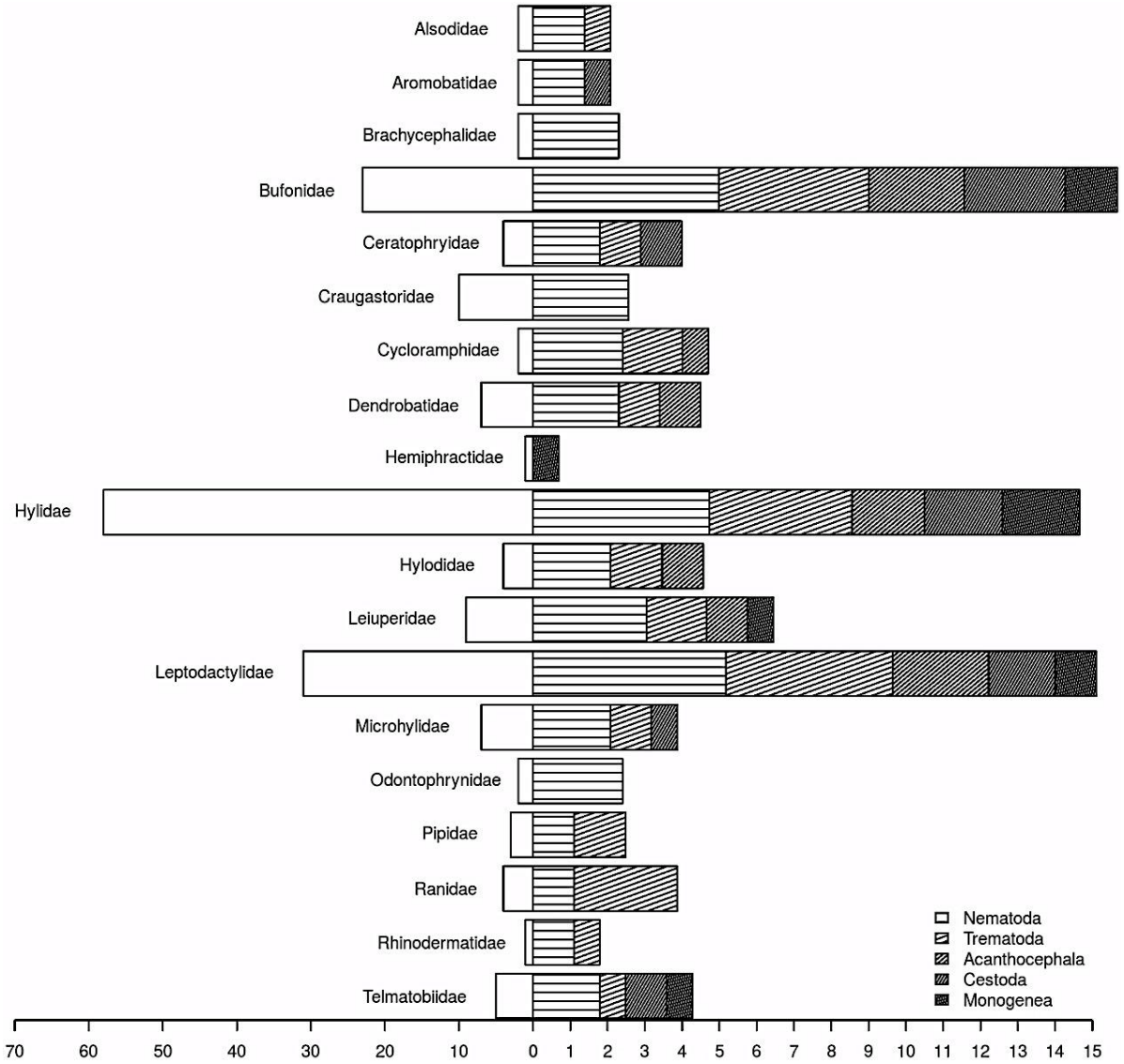


Fig. 1. Barplot of helminth parasite species reported on different South American anuran families. White bars show anuran species surveyed, patterned bars show the number of helminth parasites reported for each host family. *Adapted from Campião et al. (2015), with permission.*

Parasite strategies

Strategies, particularly animal survival strategies, are aimed at staying alive. Survival strategies are long-term investments in a particular way of life, to either enhance persistence or to achieve a more efficient and less strenuous life history (Jochim, 2013). Thus, taking into consideration that any consumers within an ecosystem represent a concentrated source of energy from that particular system, the consumption of that organism would be the most efficient way to obtain those concentrated resources (Duffy, 2002; Navarro et al., 2011; Poulin, 2011). Evolutionary selective pressures seem to favour organisms with the ability to consume other organisms. So if a small animal is able to feed off a larger one, then that

equals a greater gain of resources (Poulin, 2011), which seems so be an effective strategy resembling the life history of most parasite taxa.

According to Poulin (2011), some parasitic taxa appear to have only undergone a single evolutionary transition from a free-living organism to parasitic one. Thus, they have derived from the same ancestral lineage and are a monophyletic group. However polyphyletic groups may be representatives of several independent transitions from a free-living to a parasitic life strategy (Poulin, 2011; Poulin and Randhawa, 2015). Tracing or determining the numbers of these independent transitions to a parasitic mode of life is not always so straightforward. Some parasitic lineages may have gone extinct leaving a missing link and other transitions to parasitism may have been reversed, eliminating all traces of earlier transitions (Siddall et al., 1993; Poulin, 2011). Even though there are many independent origins of parasitism, extant parasites fall within a limited and distinct set of strategies that represent convergent evolution. Convergent evolution is the process by which organisms that are not closely related evolve similar traits adapted to their environment or ecological niches independently (Ogura et al., 2004). This process is similar to parallel evolution, however, the latter process involves closely related organisms that have independently evolve similar adaptations in similar environments (Nosil et al., 2002). There are six parasite strategies that the vast majority of parasites fit into, these are summarised in Fig. 2. Parasitoids utilise a single host that dies in the process as a result of the development of the parasite (see Fig. 3A) (Poulin, 2011). Braconid wasps that use caterpillar hosts for their larvae are well-known examples of parasitoids (Poulin, 2011; Poulin and Randhawa, 2015). Parasitic castrators do not kill their host, but rather stunt the reproductive cycle of the host using it for their reproduction (see Fig. 3B). This strategy shares certain characteristics with parasitoids (Kuris, 1974). Examples include entoniscid isopods in their crustacean hosts and the intramolluscan stages of some trematodes (Poulin, 2011; Poulin and Randhawa, 2015). Directly transmitted parasites infect only one individual host in their lifetime, with pathological effects caused by these parasites ranging from moderate to lethal (see Fig. 3C) (Poulin, 2011). This is a commonly used strategy utilised by endo- and ectoparasites such as nematodes, monogeneans, cymothoid isopods, and even unicellular microsporidians and intestinal coccidia (Poulin, 2011; Poulin and Randhawa, 2015).

Trophically transmitted parasites infect at least two hosts, and are transmitted from an intermediate host (could be an invertebrate or vertebrate) to their definitive host (normally a vertebrate), through predation of the former by the latter (see Fig. 3D) (Poulin, 2011). This strategy includes almost all trematodes and cestodes, numerous nematodes and many taxa of protists (e.g. species of *Toxoplasma* Nicolle and Manceaux, 1909 and *Sarcocystis* Lankester, 1882) (Poulin and Randhawa, 2015).

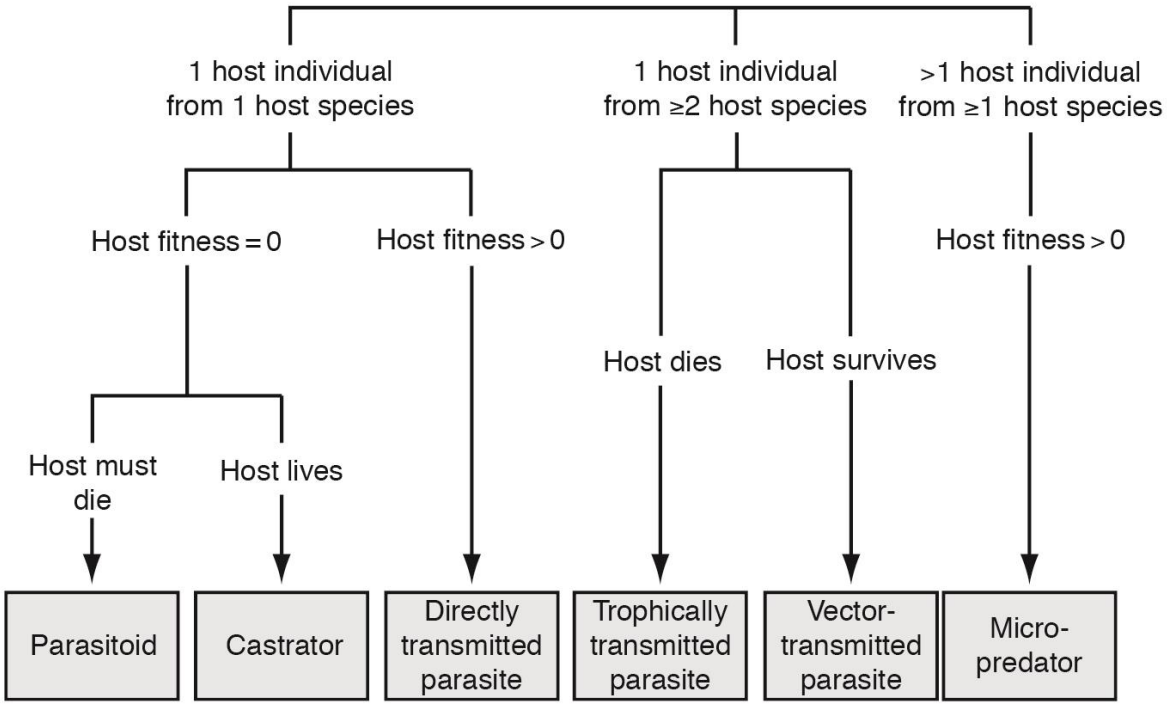


Fig. 2. Illustration of the six parasitic strategies for the vast majority of known parasite taxa. The first division is based on the number of hosts used by one full parasite generation; subsequent divisions are based on fitness impacts on hosts. *Sourced from Poulin (2011), with permission.*

Vector-transmitted parasites infect two hosts to complete a single generation in their life cycle. Usually a blood-sucking micropredator acts as the vector between two vertebrates hosts (see Fig. 3E) (Poulin, 2011). These parasites are usually small including filarial nematodes, as well as many protozoans (*Plasmodium* Marchiafava & Celli, 1885, *Babesia* Starcovici, 1893, *Leishmania* Borovsky 1898, *Trypanosoma* Gruby, 1843, etc.), and numerous bacteria and viruses (Poulin, 2011; Poulin and Randhawa, 2015). Micropredators are well-known parasites that feed on multiple host individuals for selected periods of time, which may belong to one or multiple species depending on host specificity (see Fig. 3F) (Poulin, 2011). Micropredators include leeches, gnathiid isopods, hematophagous or bloodsucking dipteran insects (mosquitoes, sand flies, etc.), ticks, and even vampire bats. Although the direct impact of these parasites on the host fitness is generally minimal, in many cases these parasites act as vectors of virulent pathogens (Poulin and Randhawa, 2015).

These strategies have survived selective evolutionary pressures, and while other parasite strategies may have occurred, it would seem that if they did not adapt to align with these strategies they went extinct (Poulin and Randhawa, 2015). Several aspects associated with parasite transmission of unrelated taxa also share a number of

characteristics, such as long-lived environmentally resistant spores or eggs, to the mechanisms used to penetrate the host's skin. This is suggestive of convergent evolution. However, it is the entire life cycle process, and not any particular transmission event, that is linked to the different parasitic strategies (Poulin, 2011).

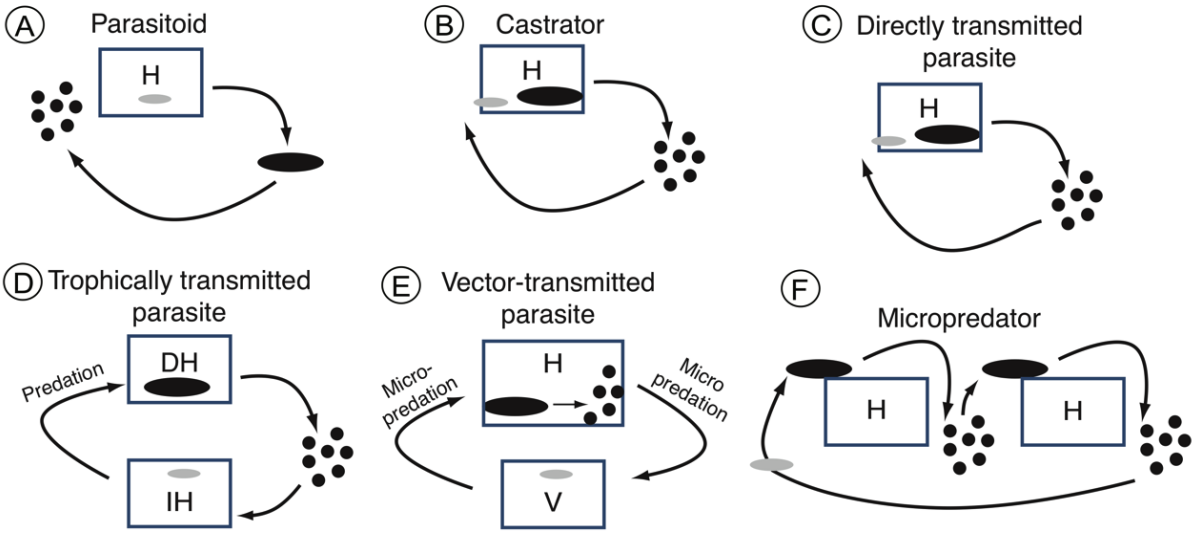


Fig. 3. Characteristic life cycle patterns of the six parasitic strategies that are meant to represent the most typical patterns. In each cycle (A-F), the developmental sequence proceeds clockwise through a single generation; adult parasites are indicated by black ellipses, juvenile stages by smaller grey ellipses, and eggs or other stages of development by clusters of black dots. H, host; DH, definitive host; IH, intermediate host; V, vector. *Adapted from Poulin (2011), with permission.*

The role parasites play and their importance

Parasites are usually considered as harmful and carry negative connotations, especially with regards to human and livestock health (Marcogliese, 2004; Netherlands et al., 2015). However, parasitism is an effective survival strategy and parasites play a crucial part in any functional and healthy ecosystem (Marcogliese and Cone, 1997; Hudson et al., 2006; Lafferty et al., 2008; Netherlands et al., 2015). Parasites often go unnoticed. However, based on their numbers, they make up a great proportion of the planet's biodiversity (Poulin, 1999; Dobson et al., 2008). Furthermore, due to the effects parasites exert on their host's fecundity and survival, they also indirectly influence the ecosystem in which the host lives (Combes, 1996). They also profoundly impact the biodiversity of an ecosystem by influencing aspects such as host competition, migration, speciation and stability (Combes, 1996).

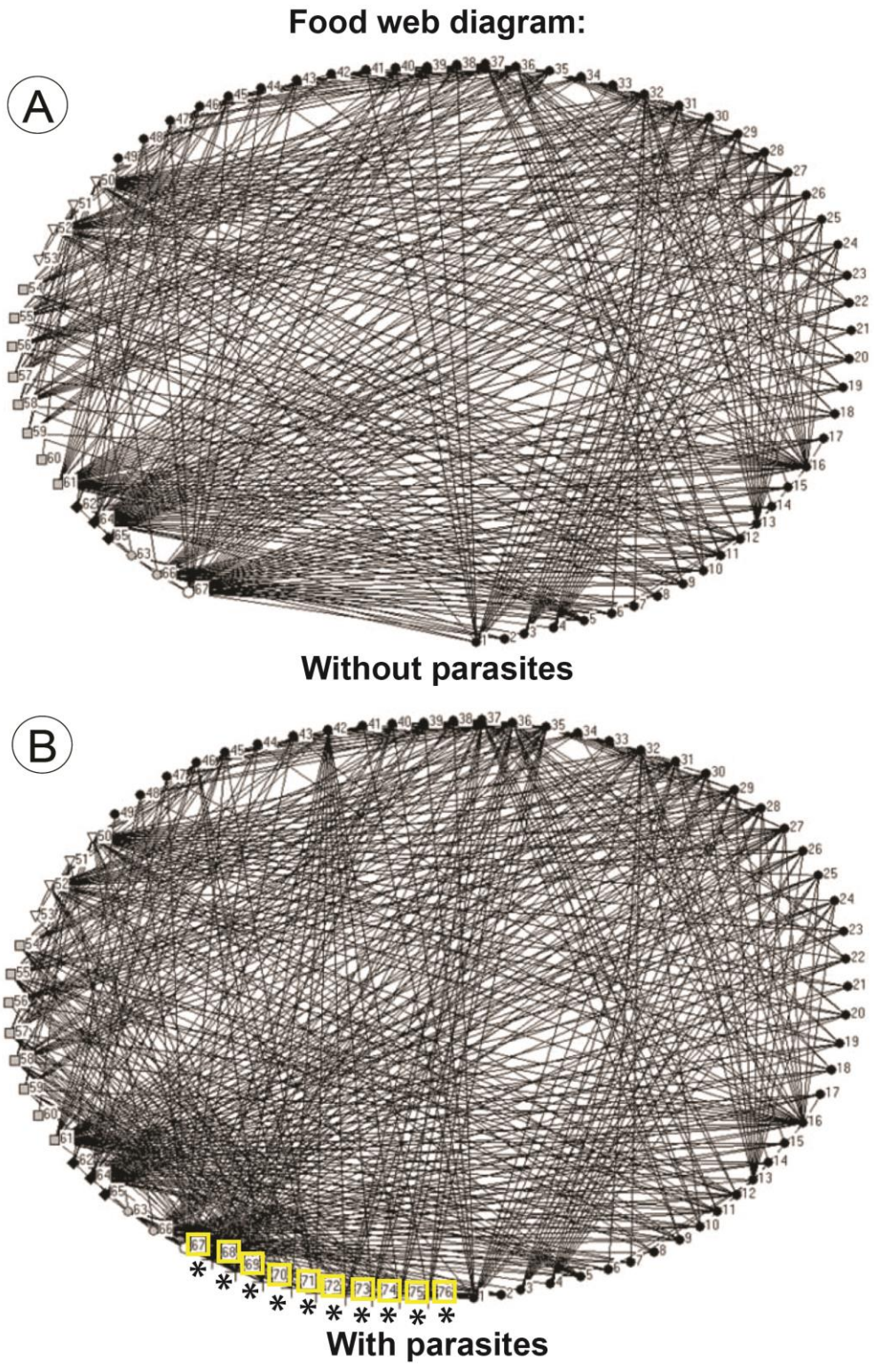


Fig. 4. Food web diagrams with (A) and without (B) the inclusion of helminth parasites (trematodes, nematodes and acanthocephalans). Numbers indicate species, symbols delineate major groups, yellow blocks or asterisk (*) indicate parasites included. (B) Already shows increased complexity in the food web structure with only helminth parasites included. *Adapted from Thompson et al. (2005), with permission.*

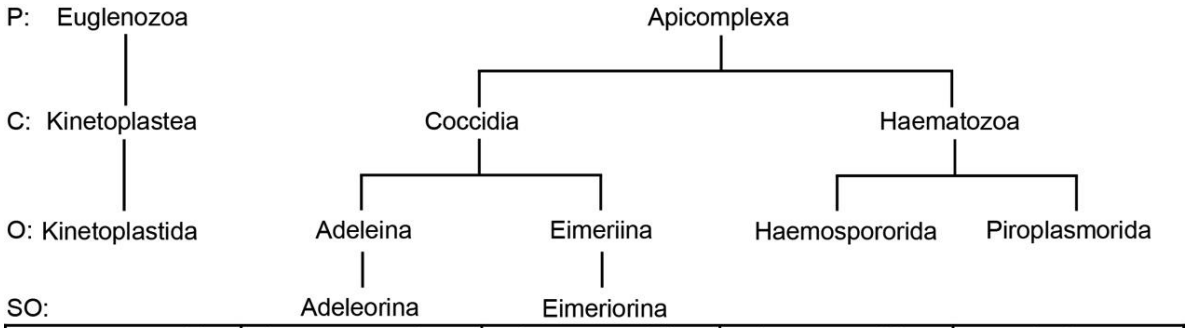
According to Lafferty et al. (2008), parasites have the potential to uniquely alter food-web topology, affecting stability, interaction strength and energy flow (see Fig 4). Thus, to better understand the stability, diversity and complexity of an ecosystem, it is important to understand the influence that parasites may have on the structure, dynamics and function of that ecosystem (Lafferty et al., 2008). Additionally, parasites reflect their host species' environmental interactions, revealing feeding behaviour, geographical ranges and social systems (Dobson et al., 2008). In a stable and healthy natural ecosystem, parasites and their hosts often have had the opportunity to co-evolve, and often parasites may cause hardly any pathogenic effects in a healthy host animal. If however, this well-established and balanced co-existence is disturbed by, for example, habitat destruction or the indiscriminate anthropogenic translocation of host animals between habitats, pathogenic effects may become apparent resulting in the destabilization of the host population (Combes, 1996).

Introduction to blood parasites

Blood parasites have been recorded in a variety of vertebrate and invertebrate hosts and vectors, ranging from aquatic to terrestrial habitats (Davies and Johnston, 2000). Protozoan blood parasites are the most commonly reported, divided into the intracellular apicomplexan parasites and the extracellular euglenozoan flagellates (Lee et al., 2000; O'Donoghue, 2017) (see Fig. 5).

However, it is important to note that these are not the only blood parasite groups that occur. Filarial nematodes make use of a microfilariid blood dwelling stage for transmission to the invertebrate vectors, and *Schistosoma* Weinland, 1858 (Trematoda: Schistosomatidae), *Sanguinicola* Plehn, 1905 (Trematoda: Aporocotylidae) and *Spirorchis* MacCallum, 1919 (Trematoda: Spirorchiidae) blood flukes are also examples blood parasite trematodes, which parasitize the blood capillaries in their hosts. The blood dwelling parasitic groups are diverse and spread across very different supergroups, namely Alveolates, Excavates and Opisthokonta (that include animals) (Fig. 6).

The phylum Apicomplexa is a large and diverse group of unicellular protists (Ogura et al., 2004; Morrison, 2009). This group of obligatory intracellular parasites are entirely parasitic and are characterised by the presence of an apical complex (Portman and Šlapeta, 2014). This structure is defined as a collection of cytoskeletal elements and secretory systems used to gain access to host cells with great ease (Morrison, 2009; Portman and Šlapeta, 2014). Dinoflagellates and ciliates are now recognised as close relatives of the Apicomplexa, forming the superphylum Alveolata (Gould et al., 2006; Moore et al., 2008; Morrison, 2009).



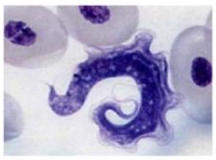
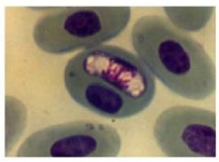
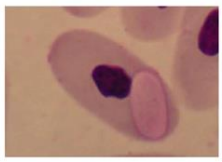
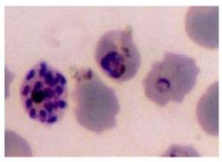
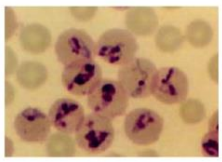
trypanosomatids	haemogregarines	haemococcidia	haemosporidia	prioplasms
				
extracellular blood stages	intracellular blood stages			
	nucleate and anucleate red blood cells, sometimes white			
asexual (mastigote fission)	sexual recombination [gamogony with syngamy (fusion of gametes)]			
	syzygy (gamont alignment)	no syzygy		syzygy? 'ray bodies'
amphibians, reptiles, fish, mammals, birds	amphibians, reptiles, fish, mammals, birds	amphibians, reptiles, birds	reptiles, mammals, birds	reptiles, mammals, birds
dipterans, bugs, leeches	dipterans, ticks, leeches	dipterans, ticks, leeches	dipterans	ticks
Injected contaminative	Ingested (injected)		Injected	

Fig 5. Key characteristics of the five protozoan blood parasite assemblages. *Sourced from O'Donoghue (2017), with permission under a Creative Commons Attribution License (CC BY).*

The apicomplexans are divided into four blood parasitic groups, namely haemogregarines (adeleorinid coccidia), haemococcidia (eimeriorinid coccidia), haemosporidia (pleomorphic haematozoa), and piroplasms (pyriform haematozoa) (Barta, 2000; Davies and Johnston, 2000). Apicomplexans undergo asexual and sexual reproduction, the latter involving the formation of gametes, which subsequently fuse (syngamy), with paired alignment (syzygy) for the haemogregarines and piroplasms, or without paired alignment (syzygy) for the haemococcidia and haemosporidia (Barta, 2000; Valkiūnas, 2004; Karadjian et al., 2015; O'Donoghue, 2017). These four groups have been recorded in all vertebrate classes, namely in amphibians, birds, fishes, reptiles and mammals, and are transmitted by a broad range of haematophagous invertebrate vectors (Davies and Johnston, 2000). Vectors include arthropods (primarily mosquitoes and ticks) and leeches for haemogregarines and haemococcidia; dipterans such as mosquitoes, biting midges and hippoboscid louse flies for the haemosporidia; and ticks for the piroplasms (Barta, 1991; Desser, 1993; Barta, 2000; O'Donoghue, 2017). Typically sexual reproduction occurs in the definitive host and asexual

development occurs in the intermediate host. A host in which no parasite development occurs is a paratenic or mechanical transport host or vector (Desser, 1993; O'Donoghue, 2017).

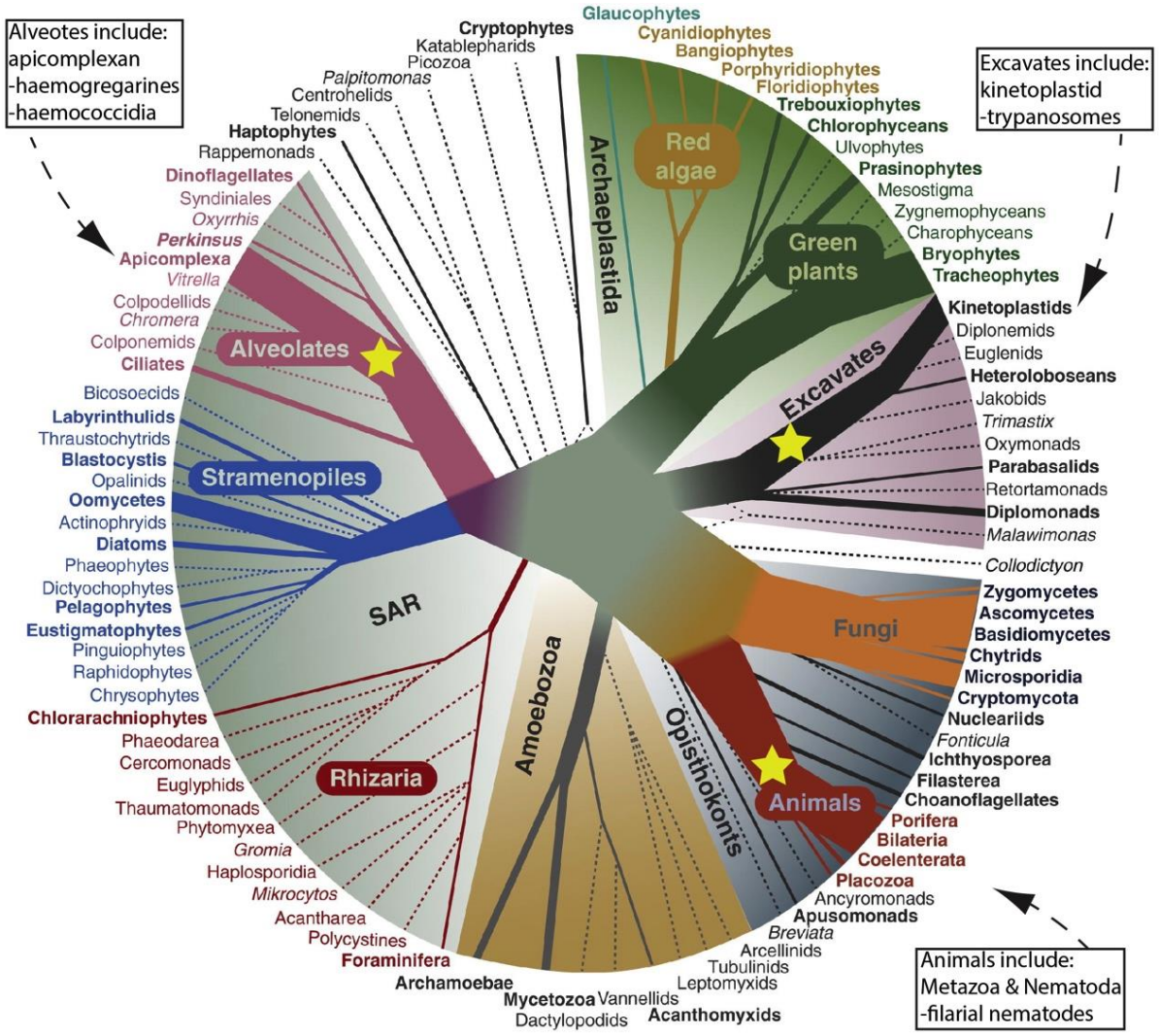


Fig. 6. Evolutionary relationships among eukaryotes. The scheme shows the supergroups SAR (stramenopiles, alveolates, Rhizaria), Amoebozoa, Opisthokonta, Excavates, and Archaeplastida. Tree derived from a consensus of phylogenetic evidence (mostly phylogenomics) and morphological characteristics. The thickness of each branch represents the number of full genome sequences available in that group and its descendants. Dashed lines denote no genome is available. Stars indicate the placement of the different blood parasite taxa found parasitising anurans in this study within the supergroups. *Adapted from Burki and Keeling (2014), with permission.*

The phylum Euglenozoa can be classified into eight classes, 18 orders and 31 families (Cavalier-Smith, 2016). Most Euglenozoa are either heterotrophic, phagotrophic or more rarely osmotrophic, symbiotrophic or parasitic (Cavalier-Smith, 2016). Kinetoplastid protozoans from part of this diverse phylum, and are characterised by the presence of a kinetoplast, which is the derived trait for the group (d'Avila-Levy et al., 2015). Included in the kinetoplastids are the trypanosomatids, one of the most successful groups of parasites on earth. They include several organisms of medical and economic importance (Simpson et al., 2004). The parasitic flagellates include the trypanosomatids and have also been recorded in all vertebrate classes.

These five protozoan blood parasites groups are transmitted via three basic methods; (1) inoculative (parasites injected by vector along with salivary secretions); (2) contaminative (stercorarian transmission, where the vector releases parasites in the wound; (3) or consumptive (the infected host or vector is consumed by the subsequent vertebrate host, similar to predator-prey transmission) (Desser, 1993; Smith, 1996; O'Donoghue, 2017).

Nematodes or roundworms constitute the phylum Nematoda Rudolphi, 1808. This is a large and diverse group that inhabit a broad range of habitats (Lee, 2002). They are generally described as small thread-like and cylindrical, non-segmented animals (Gibbons, 2002). Nematodes possess a simple and basic body plan that consists of an outer cylinder (the body wall) and an inner cylinder (the digestive system), which are separated by a pseudocoelomic body cavity containing its organs (e.g. reproductive tract) (Decraemer et al., 2014). Most nematodes exhibit sexual dimorphism. The male reproductive system (cloaca) opening to the outside of the body and the female system (vulva) opening via a pore found in the ventral body wall (Gibbons, 2002). Nematodes have a secretory-excretory system and a complex nervous system but no circulatory system (Gibbons, 2002). Although most nematoeds are free-living in terrestrial soils and aquatic sediments, generally nematodes are known from the pathogenic species of humans, animals and agriculture (Moens et al., 2013). Millions of people are infected by parasitic nematodes, and the diseases they cause collectively lead to mortality severe morbidity, blindness, anemia, disfigurement of major organs and limbs, and others (Jasmer et al., 2003). The suborder Filarioidea is a superfamily of specialised parasitic nematodes known as filarial worms or filariae (singular "filaria"). Filarial nematodes from the family Onchocercidae Leiper, 1911 (Spirurida) are coelom or tissue dwelling worms, known for causing filariasis (Bain, 2002). As mentioned above, although these worms are not “true” blood parasites they make use of a microfilaria blood dwelling larval stage for transmission to its invertebrate vector.

Anuran blood parasites

From the five groups of protozoan blood parasites mentioned above, the adeleorinid coccidia or haemogregarines, the eimeriorinid coccidia or haemococcidia, and the kinetoplastid trypanosomatids are known to infect anuran hosts (Davies and Johnston, 2000; O'Donoghue, 2017). Other blood parasites commonly reported from anurans are microfilarial nematodes (Bain et al., 2013).

Anurans' apicomplexan parasites include species from *Babesiosoma* Jakowska and Nigrelli, 1956; *Dactylosoma* Labbé, 1894; *Hemolivia* Petit, Landau, Baccam and Lainson, 1990; and *Hepatozoon* Miller, 1908 all from within the suborder Adeleorina Léger, 1911. The haemococcidian genera recorded parasitising anurans from the suborder Eimeriorina Léger, 1911 are *Lankesterella* Labbé, 1899 and *Schellackia* Reichenow, 1919.

For the haemogregarines, species of *Hepatozoon* (Hepatozoidae Wenyon, 1926) are the most commonly reported from anurans with 48 recognised species globally, of which 19 have been reported from African hosts (Smith, 1996; Netherlands et al., 2014a; Netherlands et al., 2018). In South Africa, the second species identified from an anuran host, *Hepatozoon ixoxo* Netherlands, Cook and Smit 2014 was described more than 100 years ago following the description of the first species, *Hepatozoon theileri* (Laveran, 1905). The genus is characterised by gamonts in erythrocytes or leucocytes of the vertebrate host, and formation of large, polysporocystic oocysts within the wall of the haematophagous invertebrate host. The sporocysts each contain four to 16 sporozoites. The parasite is transmitted when an infected vector, containing fully sporulated oocysts, is ingested by the vertebrate host (Fig 7) (Barta, 2000).

The species within *Dactylosoma* and *Babesiosoma* (Dactylosomatidae Jakowska and Nigrelli in 1955), are parasites of anurans and fishes. Currently there are five recognised species of *Dactylosoma*, two from fish hosts, and three species described from anuran hosts. *Dactylosoma ranarum* is the most common and apparently a cosmopolitan species reported in several anuran species from Europe, Central and South America, and from Africa (Barta, 1991). This species was also apparently reported from the Guttural Toad *Sclerophrys gutturalis* (Power, 1927) in South Africa (Fantham et al., 1942).To date, no species of *Babesiosoma* have been reported from African anurans. Dactylosomatid genera are distinguished by the replicative potential throughout the life cycle (between six and 16 merozoites for *Dactylosoma* and four for *Babesiosoma*). The life cycle for only one species is known, *Babesiosoma stableri* Schmittner and McGhee, 1961. Intraerythrocytic merogony produces four merozoites by simultaneous exogenous budding from each cruciform meront. After being ingested in a blood meal, gamonts associate in syzygy within the definitive host, which is suspected to be a leech for all species of *Babesiosoma* (Fig 8) (Barta, 2000).

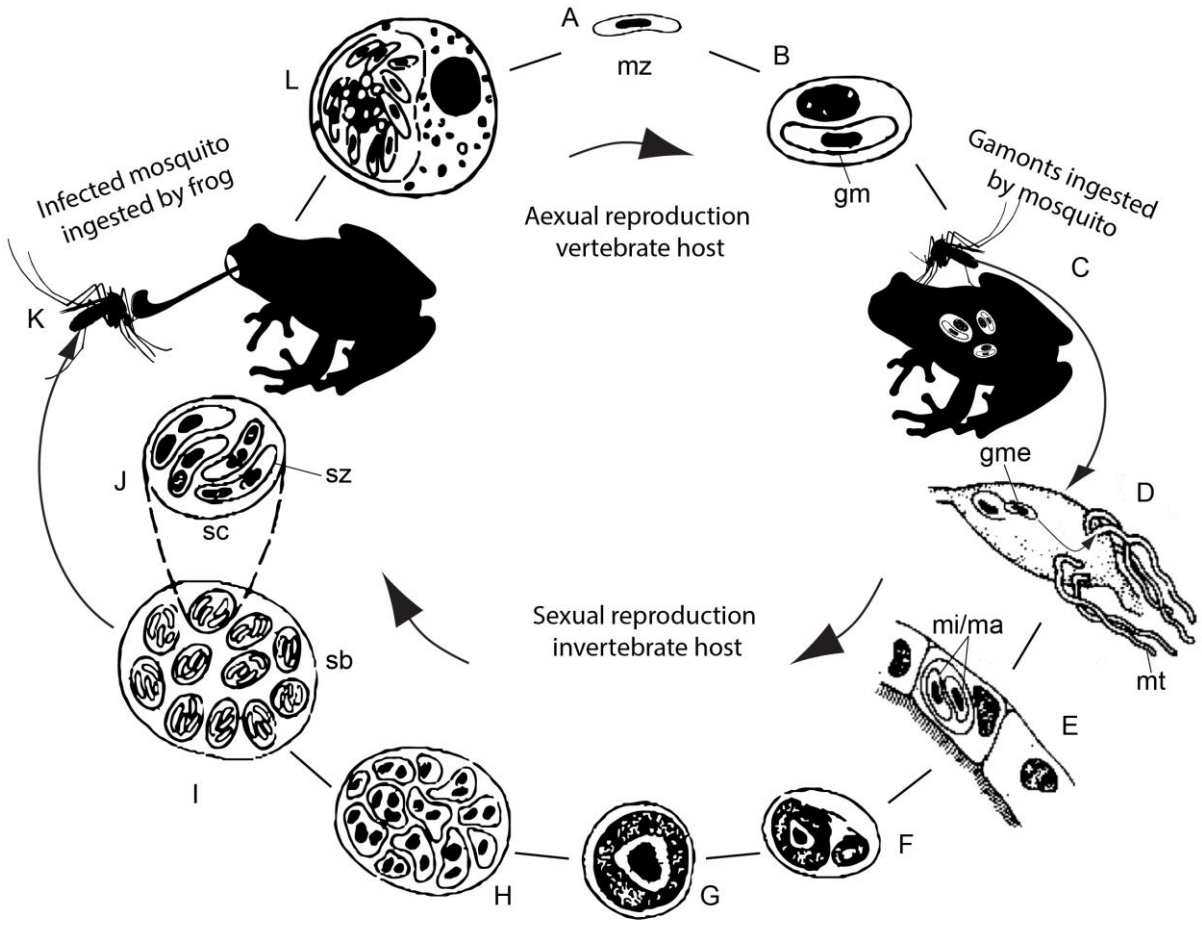


Fig. 7. Summary of the life-cycle of *Hepatozoon catesbianae* in its bullfrog and mosquito hosts. (A) Merozoites (mz) released from hepatic meronts enter erythrocytes. (B) Merozoites transform into erythrocytic gamonts (gm). (C) Mosquitoes feeding on infected frogs ingest erythrocytic gamonts. (D) Gamonts escape (gme) from erythrocytes in gut of mosquito and enter malpighian tubules (mt). (E) Micro- and macrogamonts (mi/ma) come to lie within a common parasitophorous vacuole in tubule cells. (F) Gametogenesis ensues with the formation of 2 biflagellate microgametes, 1 of which fertilizes the macrogamete. (G) The zygote expands into a sphericalo oocyst. (H) Oocysts undergo segmentation to form sporoblasts. (I) Sporoblasts (sb) transform into sporocysts (sc). (J) Each sporocyst contains 4 sporozoites (sz). (K) Frogs are infected by ingesting mosquitoes containing sporocysts. (L) Sporozoites enter hepatic parenchymal cells where they develop into meronts. (Diagram not to scale). *Adapted from Desser et al. (1995), with permission.*

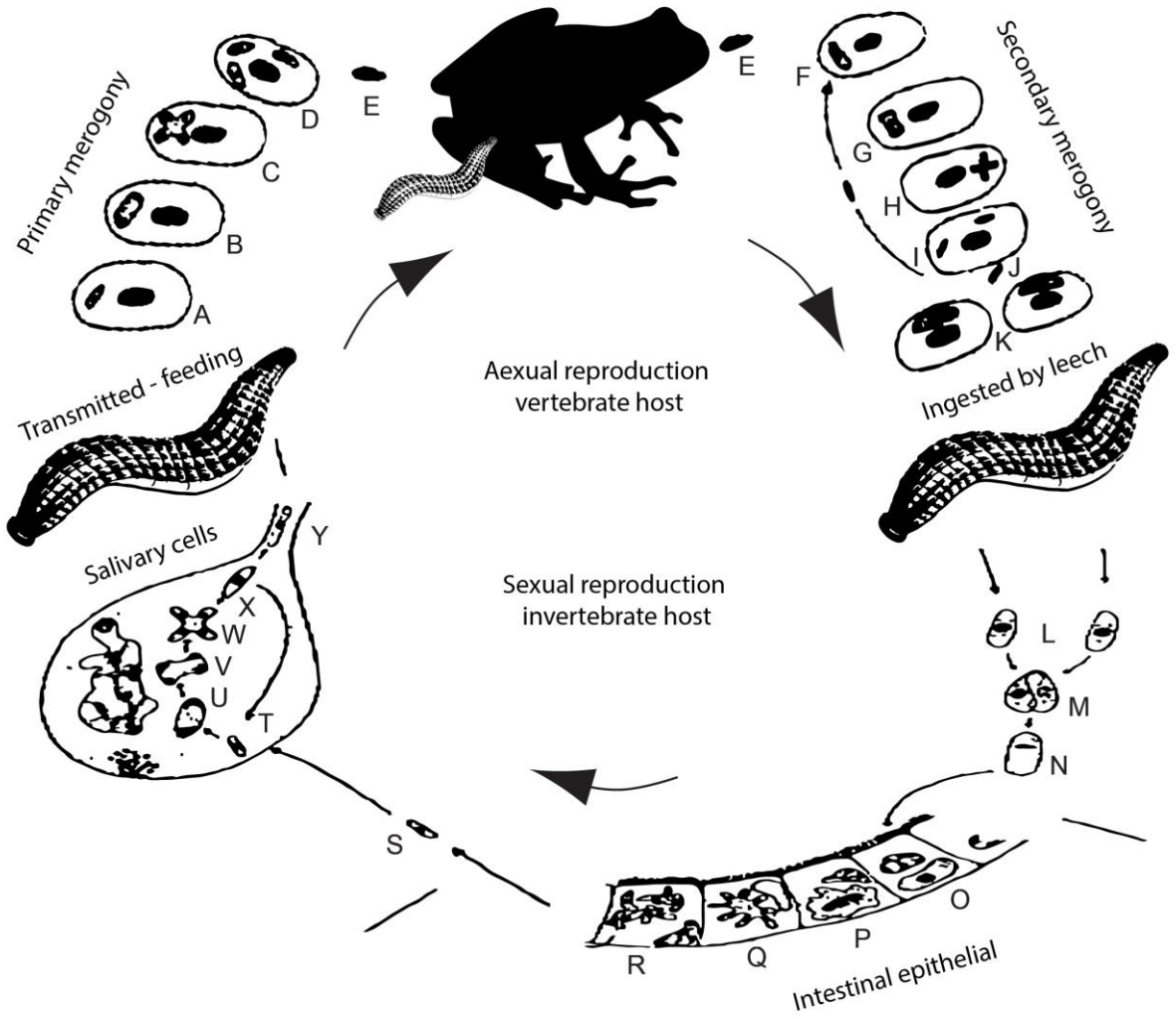


Fig. 8. Summary of the life-cycle of *Babesiosoma stableri* in frogs (*Rana* spp.) and the leech *Desserobdella picta*. Primary merogony (A-E); Merozoites resulting from primary merogony (E) penetrate other erythrocytes and may either repeat a cycle of primary merogonic replication (A-E) or initiate secondary merogony (F-J). These merozoites may either repeat a cycle of secondary merogonic replication (F-J) or mature into gamonts (K). When ingested by a leech, gamonts are freed from the frog erythrocytes (L) and associates in syzygy (M) within the blood meal in the crop of the leech. The paired gamonts mature into gametes and fuse forming an ookinete (N), which penetrates intestinal epithelial cells to initiate sporogony (O-R). Mature sporozoites (R) leave the intestinal epithelium (S) and make their way to the salivary cells of the leech (T). Therein, they undergo merogonic replication similar to that which occurs in the frog erythrocytes (T-X). The resulting merozoites (X) may repeat a cycle of merogonic replication (T-X) or enter the ductules of the salivary cells (Y). *Adapted from Barta (1991), with permission.*

The haemococcidia are heteroxenous parasites, with the entire replication process of their life cycle occurring in the tissues of the vertebrate host (Nöller, 1912, 1920; Desser, 1993). Transmission occurs via a paratenic haematophagous invertebrate host, during feeding or via ingestion of infected vectors (Nöller, 1913; Desser, 1993) (Fig 9). The taxonomy of amphibian haemococcidia, as in other intracellular blood parasite groups, has had an uncertain history. According to Hintze (1902), the first report on a haemococcidia in the blood of an anuran was by Chaussat (1850). The genera *Lankesterella*, *Schellackia* and *Lainsonia* Landau, 1973 are regarded as true haemococcidia (Telford, 1993; Paperna et al., 2009), with species of *Lankesterella* mostly reported from anuran hosts. Currently there are nine species from anurans recognised globally, of which three are from Africa.

Trypanosoma (Euglenozoa: Kinetoplastea: Trypanosomatidae Doflein 1901) comprises a large group of flagellate extracellular blood parasites (Su et al., 2014). The first anuran trypanosome was discovered in 1842, in the European frog *Pelophylax* kl. *esculentus* (Linnaeus, 1758). It was at first classified as *Amoeba rotatoria* (Mayer, 1843). After the re-examination and a fairly complete description of a similar species by Gruby (1843), it was designated the name *Trypanosoma sanguinis* Gruby, 1843, the type species of the genus *Trypanosoma* (Gruby, 1843; Laveran and Mesnil, 1907; Ferreira et al., 2007). In Africa, most of the recorded *Trypanosoma* species from anurans were recorded early in the 20th century. In total there are 13 species of *Trypanosoma* that have been reported from African anurans, with *T. nelspruitense* Laveran, 1904, the only species described from South Africa (Laveran, 1904; Bardsley and Harmsen, 1973). Trypanosomes form extracellular developmental stages in host blood plasma and undergo asexual reproduction by binary fission. The vectors of trypanosomatids include dipterans (mosquitoes, biting midges and hippoboscid louse flies), triatomine bugs and leeches, in which they also undergo asexual reproduction by binary fission (Fig 10) (Feng and Chung, 1940; Desser et al., 1973; O'Donoghue, 2017). In the gut of the vector, forms present may be amastigotes, spheromastigotes, epimastigotes, or metacyclic trypomastigotes, depending on the species of trypanosome (Telford, 2009). The trypomastigotes ingested with a blood meal transform into epimastigotes, which divide by binary fission and become infective metacyclic trypanosomes (Fig 10). In dipteran vectors (mosquitoes or biting midges) ingested trypomastigotes first become amastigotes, develop into spheromastigotes, and finally become epimastigotes, the apparent infective stage. These forms remain in the end-gut of the vector. The parasite is then transmitted through ingestion by the vertebrate host (Fig 10) (Feng and Chung, 1940; Desser et al., 1973; Telford, 2009).

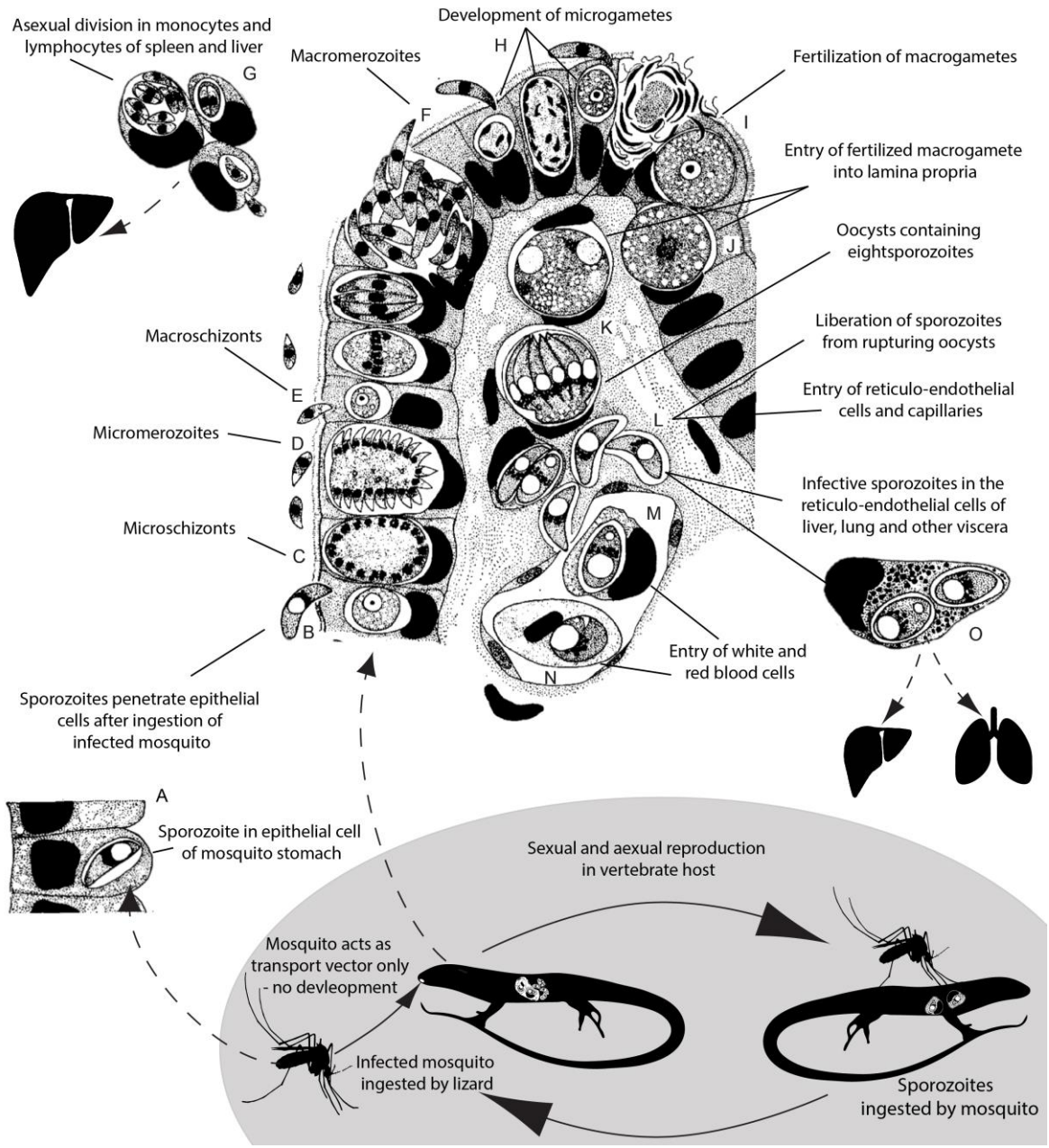


Fig. 9. Representation of the haemococcidian life-cycle of *Schellackia landauae*. (A) Sporozoite in epithelial cell of mosquito stomach. (B) Sporozoites penetrate epithelial cells of small intestine of lizard after ingestion of infected vector. (C, D) Development of microschizonts and micromerozoites. (E, F) Development of macroschizonts and macromerozoites. (G) Asexual division in monocytes and lymphocytes of spleen and liver, apparently by endodyogeny, producing groups of tachyzoites, and probably initiated by micromerozoites. (H, I) Development of microgametes and fertilization of macrogametes in epithelial cells of small intestine. (J, K) Entry of fertilized macrogamete into lamina propria and development of oocysts containing eight sporozoites. (L) Liberation of sporozoites from rupturing oocysts: entry of reticulo-endothelial cells and penetration of capillaries. (M, N) Entry of white and red cells of peripheral blood: note rolled-up form in erythrocyte. (O) Infective, diapausing sporozoites in the reticulo-endothelial cells of liver, lung and other viscera. *Adapted from Lainson et al. (1976), with permission.*

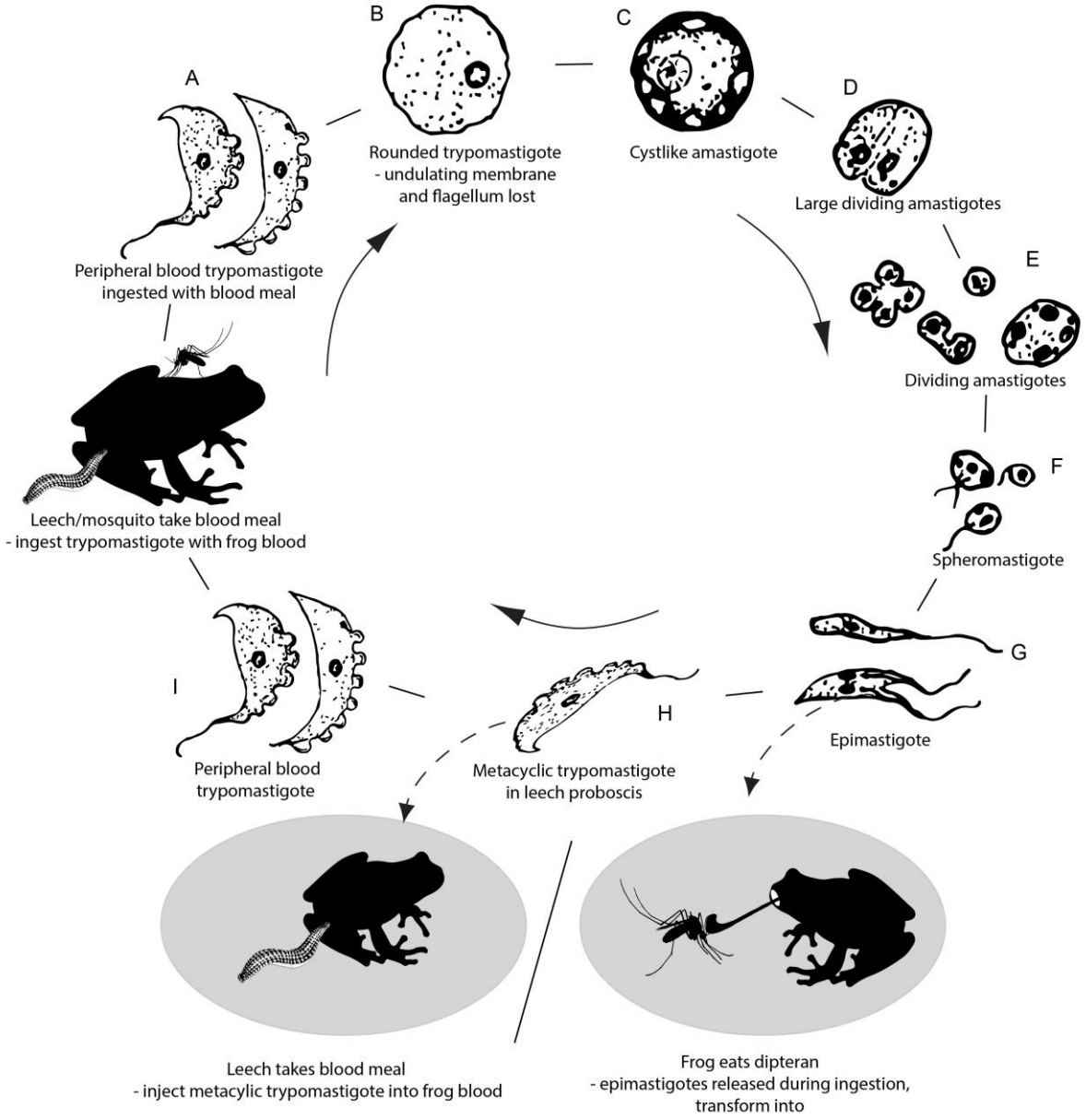


Fig. 10. Diagrammatic representation of the life cycle of frog trypanosome in leech or dipteran vector. (A) Peripheral blood trypomastigote multiply by binary fission in (leech crop or dipteran mid-gut) of the invertebrate vector. (B) Rounded trypomastigote form without undulating membrane or flagellum. (C) Spherical, cystlike amastigote with large vacuoles. (D) Large dividing amastigote from leech crop or dipteran mid-gut. (E) Dividing amastigotes from leech crop or dipteran mid-gut. (F) Mono- and biflagellate spheromastigotes from leech crop or dipteran end-gut. (G) Spheromastigotes transform into epimastigotes, in leech crop or dipteran end-gut. In dipteran, epimastigotes are apparently the infective stage released upon ingestion of the invertebrate by vertebrate host. (H) In leech crop epimastigote transform into infective metacyclic trypomastigote, which can then be injected into peripheral blood of vertebrate host by its leech vector. *Adapted from Desser et al. (1973), with permission.*

In leeches trypomastigotes divide by binary fission and transform into epimastigotes in the leech crop or stomach. Epimastigotes transform into infective metacyclic trypomastigotes and migrate to the proboscis, and are transmitted to the vertebrate host with its next blood meal (Fig 10) (Martin and Desser, 1991).

According to O'Donoghue (2017), the evolution of blood protozoans has not yet been elucidated with clear evidence, however there are several possible scenarios. One is that free-living protists that adapted to a monoxenous or single-host parasitic life history, subsequently adopted blood-borne transmission to become heteroxenous or two-host parasites (Lee et al., 2000). This could have occurred in vertebrate hosts from intestinal parasites i.e. haemococcidia and haemosporidia (Garnham, 1966) or from invertebrates that adapted micropredator parasitic strategies, subsequently allowing these protozoans to adapt and become vector-transmitted parasites (Hoare, 1972). Even based on fossil records these evolutionary theories can not be clearly estimated as ancestors of their current vertebrate and invertebrate host groups overlap considerably, providing no logical sequence to suggest the process of these groups host parasite evolution (O'Donoghue, 2017).

Anuran filarial nematodes are restricted to two small subfamilies (Icosiellinae Anderson, 1958 and Waltonellinae Bain & Prod'Hon, 1974) of the filariae that currently comprise six genera and 41 recognised species. A recent study on the phylogenetic relationships of filarial nematodes showed that several taxa parasitising amphibians (species of *Ochoterenella* Caballero, 1944 and *Icosiella* Seurat, 1917) and reptiles (species of *Foleyella* Seurat (1917), *Madathamugadia* Chabaud, Anderson & Brygoo, 1959*,* and *Oswaldofilaria* Travassos, 1933) clustered together, forming a sister clade to all other taxa evaluated within the Onchocercidae. However, the life histories of only five anuran filarial nematodes from the Waltonellinae, an ancestral group based on molecular phylogenetic studies, have previously been elucidated (Fig 11). Furthermore, data on the natural vectors (*in situ*) and parasite transmission is limited. Excluding Madagascar, only one species has been described in Africa from the Waltonellinae, namely *Foleyellides duboisi* (Gedoelst, 1916) from in the Democratic Republic of Congo.

Amphibians are regard as the most threatened vertebrate class. For this reason knowing the diversity and ecology of amphibians has become increasingly more important (Stuart et al., 2008). Amphibian conservation is essential as they play a vital role in the food web, are indicator species, keep insect populations at bay, and are an important tool for research and education. However, studying amphibian parasites can provide a more comprehensive overview of the ecology, diversity and conservation status of the host (Marcogliese, 2004).

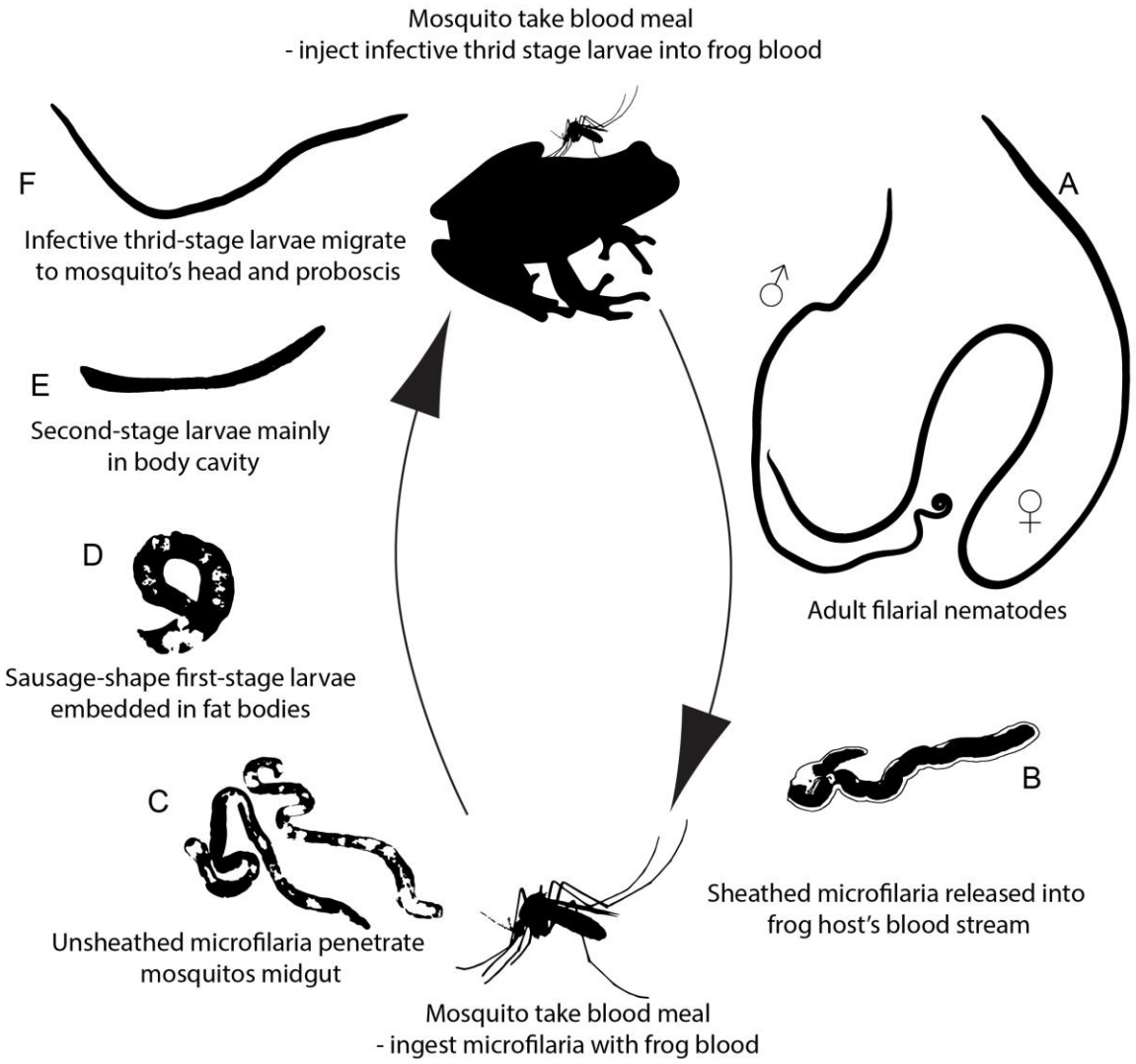


Fig 11. Graphical representation of the life cycle of frog filarial nematode in vertebrate frog host and invertebrate mosquito vector. (A) Male and female adult stages. (B) Sheathed microfilaria in the peripheral blood of frog host. (C) Represents unsheathed microfilaria in the blood meal of the mosquito. (D) Sausage-shaped first-stage larvae. (E) Second-stage larvae, observed from between six and 14 dpi. (F) Third-stage infective larvae. Images not drawn to scale.

Parasites influence food web structures in an ecosystem, as well as are drivers of speciation. To understand the role parasites play, one needs to also study the parasites host's biology and behaviour. Thus, knowledge obtained from studying parasites has broader implications contributing to our basic understanding of the way the biological world functions. Blood parasites of ectotherms have co-evolved over millions of years with their vertebrate hosts and invertebrate vectors, forming balanced relationships that do not produce the same disease severity seen caused by their close relatives in their mammalian and avian hosts (Davies and Johnston, 2000). Human-infecting parasites are often popular

research subjects. Studies on medically relevant parasites are often more individual-oriented, whereas ecological research on frog blood parasites can provide the biomedical studies with complementary information regarding the ecology of parasites, with comparable life cycles. For example, haemogregarines (hepatozoonosis), trypanosomes and filarial nematodes are parasites of human or economic importance with similar life histories in the different groups. Thus, gaining insights from anuran blood parasites can not only provide information of the natural world, but also data on impacts when designing policies to mitigate human parasite outbreaks and epidemics (Marcogliese, 2004). However, before we can begin to assess the evolutionary relationships in these parasites, we need to determine and document their biodiversity, characterize and determine their phylogenetic relationships, determine possible vectors, as well as elucidate their life cycles.

What is a species?

Species concepts also known or referred to as, the species problem, are among the most debated issues in biology (Mayden, 1997; Zachos, 2016). The question of what a species is and if something like a species actually exists may be more a philosophical question than a biological one. Nonetheless the concept of a species remains an important and fundamental aspect of biology (Mayr, 1957; Zachos, 2016). Taxonomy is the practice and science of naming of organisms and classifying them into similar groups. Classification is part of human nature and was used by the earliest humans to recognise useful or harmful plants and animals (Vitt and Caldwell, 2013). Carl Linnaeus, in the tenth edition of *Systema Naturae* in 1758, was the first to provide a hierarchical classification system, consistently using a two-part name (a binomial of genus and species). Although useful, Linnean taxonomy presents a false impression about relationships of taxa, implying that taxonomic categories (genera, orders, classes, etc.) provide information about similarity and that this similarity reflects evolutionary history (Vitt and Caldwell, 2013). Where as evolutionary taxonomy rests on the assumption that species in a genus share characteristics with a common origin (homology), with a different evolutionary history, that contradicts “Linnean” categories (Vitt and Caldwell, 2013). The *International Code of Zoological Nomenclature, Fourth Edition* (the Code), follows Linnean classification and does not reflect evolutionary history. In order to classify a species into this structured system a taxonomist should adopted a species concept and provide species limitations to define a particular taxa (Aldhebiani, 2018). According to (Zachos, 2016) species concepts define the species category, while the entities that fall within this category are the species taxa. There are roughly 30 species concepts in the literature. The most common, the Biological Species Concept, defined as all individuals that

produce fertile offspring. However, this concept is limiting, e.g. exclusion of non-sexually reproducing organisms (Mayden, 1997).

Box 3: *The evolutionary and taxonomic species concepts*

The evolutionary species concept (ESC) is defined as species that are "…ancestor-descendant lineages that evolve separately from other such lineages and have their own evolutionary tendencies and historical fate (Zachos, 2016). Although first defined by Simpson (1951), the definition of the ESC was been further developed and improved to accommodate all known types of biologically equivalent diversity (Wiley, 1978; Frost and Kluge, 1994; Mayden, 1997; Wiley and Mayden, 2000). According to Mayden (1997), the ESC is not an operational concept, but rather a lineage concept that is non-relational, using attributes and patterns of species to determine their decent. It includes or accommodates uniparental species, ancestral species, and hybrid species, with no threshold for the traits required to define the existence of a species (Mayden, 1997).

The taxonomic species concept (TSC) is defined as a species that consists of: "...all the specimens which are, or would be, considered by a particular taxonomist to be members of a single kind as shown by the evidence of the assumption that they are as alike as their offspring or their hereditary relatives within a few generations" (Blackwelder, 1967). According to Mayden (1997) practice of the TSC, mostly used by taxonomists, is non-dimensional, treating species as classes rather than a lineage. In the past this concept was mainly based on morphological traits in the delineation of species, primarily since other character bases were traditionally not available to taxonomists. However for the modern taxonomist character-based limitations are less of a reality. Although morphological attributes will probably remain the most used characters in deciphering taxonomic diversity, modern methods to obtain additional characters (ecology, proteins, behaviour, sequences, etc.) should also be used in the delineation of taxa, with the ultimate aim of following a total evidence based approach (using as much data available as possible i.e. morphology, phylogenetic relationships, and ecology, etc.).

For the purpose of classifying completely different groups of organisms, that does not necessarily follow the same traits i.e. pleomorphic protozoan flagellates that multiply through binary fission as compared to certain sexually reproductive or morphologically distinctive nematodes. A species concept needs to be adopted that considers biological traits that are consistently present overtime, yet offer clear boundaries to distinguishable one "group" or "species" from another. Being a concept, it cannot be observed directly, thus these phenomena observed in nature are referred to as "species", because they conform in their attributes to one of these concepts or to a mixture of several concepts (Mayden, 1997). The Evolutionary Species Concept (ESC) is applicable because everything currently understood about descent, speciation, and species are compatible with the ESC, and for this reason it was adopted to be used throughout this PhD thesis as a primary species concept. Although,

the ESC is the most theoretically significant concept, it requires a secondary concept for support from more operational beliefs of biological diversity. The combination of primary and secondary concepts form a more structured system displaying both their operational and theoretical inter-relationships (Mayden, 1997). To support the primary ESC in the context of this thesis and for the organisms focused on, the Taxonomic Species Concept (TSC) was selected as a supplementary secondary concept.

Problem statement

Amphibians have always been important subjects for biological research. In the 19th century with improved microscopes and methods, and the curiosity of researchers during this time led to the discovery of some of the first blood parasite taxa. The discovery of the first intraerythrocytic blood parasite was when Chaussat (1850) reported on an unidentified parasite in the blood of frogs from Europe. Later Vulpian (1854) and Lankester (1871), report on similar frog blood parasites. These findings together with the discovery of malarial parasites by Laveran (1881) is said to have laid the foundation of our understanding of the Apicomplexa today (Jakowska and Nigrelli, 1955; Levine, 1971; Barta, 1991). The first frog trypanosome was discovered in 1842, which later lead to the erection of the *Trypanosoma* by Gruby (1843). However, with the discovery of human-infecting blood parasites and due to their severity and symptomatic nature, these parasite taxa have received precedence in research focus over the last century. Mainly in efforts to understand their biology, how they were transmitted, and potential cures. In contrast asymptomatic blood parasites, particularly those of ectotherms have not received this attention with knowledge on their diversity, ecology, effects on the host, and modes of transmission limited. To understand the evolutionary history of these groups information on different taxa is required, regardless of their human impact. In doing so we may better understand how to mitigate disease-causing parasites.

Research on ectotherms blood parasites from South Africa, strongly suggests that besides reviewing our identification of new and previously described species, phylogenetic relationships between the genera of these parasites should be revised (Cook et al., 2014; Netherlands, 2014; Netherlands et al., 2014b; Cook et al., 2016; Cook et al., 2018). Thus baseline data is required as a reference from which to work. However, accurate identification of these blood parasites using only morphology is often impossible, requiring molecular techniques to confirm species taxonomy. Molecular data on most anuran blood parasites is lacking, with minimal data available from the African continent. Prior to this study five

sequences were available from two species of *Hepatozoon* (Netherlands et al., 2014a; Netherlands et al., 2014b)and two sequences from one species of *Trypanosoma* of African anurans (Martin et al., 2002). Phylogenetic relationships will possibly provide insight into the evolution, distribution, and transmission of these parasites. In addition, it will also provide more accurate systematic placement for some of these parasite species and genera. To date, no frog blood parasite life cycle has been elucidated in Africa. To answer deeper ecological and evolutionary questions, data on the vectors are needed. Also, the effects of anthropogenic impacts on these organisms and their community structures, is extremely limited.

Aims of the study

The main aim of this study was to establish species diversity, determine potential vectors, elucidate life cycles, refine molecular techniques pertaining to blood parasites and provide a genetic and evolutionary perspective of anuran blood parasites from southern Africa. In addition, a secondary aim of the study was to provide a template for future ecological studies, and assess the potential of anuran haemoparasites as indicators of ecosystem health. In addition to these main aims each chapter will have its own focussed aim and objectives.

Main objectives of the study

To achieve the main aims, the following objectives were formulated.

1. Determine the taxonomic and phylogenetic status of new and undescribed blood parasites infecting frogs, describing them morphologically and molecularly.
2. Establish the phylogenetic relationships between frog blood parasites (haemogregarines, haemococcidians, and filarial nematodes) sampled during this study and other closely related groups.
3. Elucidate the life history (in terms of host and vector relationships) of an amphibian blood parasite using microfilariae as a case study.
4. Compare the ecology and biodiversity of blood parasites of protected areas to anthropogenically impacted areas with regard to frogs belonging to *Ptychadena* as the model host.

Outline of thesis

Following this brief introduction (general introduction), the thesis is divided into five core chapters on the various blood parasite taxa or on their ecology. These five core chapters are a compilation of separate manuscripts, comprising an abstract, introduction, materials and methods, results, discussion and references. These chapters are intended for submission to various selected peer-reviewed journals, with some already published or submitted. The core chapters are followed by a final summative discussion (general discussion).

In **Chapter 1**, the aim was to establish which hyperoliid frog species in northern KZN, are parasitised with haemogregarines of the genus *Hepatozoon*. This was done to ascertain the species richness of these parasites in hyperoliid frog hosts, and if any of the haemogregarines found were previously described or reported species. Based on morphological and molecular findings three new species of *Hepatozoon* are described. Furthermore molecular analyses show anuran *Hepatozoon* species to be a separate monophyletic group, with species isolated from African hosts forming a monophyletic clade within this cluster.

Chapter 2 focussed on haemogregarines from the Dactylosomatidae. In this chapter the aim was to review the current knowledge on dactylosomatid parasites, as well as determine their diversity and phylogenetic relationships in anurans from South Africa and Belgium. This is the first study to characterise a species of *Dactylosoma* in detail, based on morphological and molecular data. Furthermore the possibility of blood feeding invertebrates (other than leeches) being the vectors of these dactylosomatids is explored. Dactylosomatid parasites observed in anurans collected from South Africa and Belgium, were morphologically and phylogeneticlly compared to each other. Based on morphological and molecular findings a new species of *Dactylosoma* is described.

Chapter 3 aimed to establish the diversity, prevalence and phylogenetic placement of anuran haemococcidia from north-eastern South Africa and Belgium. These haemococcidia are heteroxenous, with the entire developmental process of their life cycle occurring in the vertebrate host. There are currently nine recognised species of *Lankesterella* from anuran hosts, with three of these species described being from Africa. In this chapter, based on morphological and molecular findings, *Lankesterella minima* is reassessed and a new lankesterellid species is described from Belgium. Also, two new haemococcidia species from South Africa, are identified, with one designated to a new genus.

In **Chapter 4** a different taxonomic group, namely filarial nematodes is explored. The aim of this chapter was to determine the prevalence, distribution, taxonomic placement, and elucidate the life history of any filarial nematodes from the Bufonidae in northern KwaZulu-

Natal. These are not obligatory blood parasites, as only a single phase of their life cycle is restricted to their host's blood, namely the microfilariae larval stage. In this chapter a framework is provided from which a full taxonomic species description. That includes both morphological and molecular data, as well elucidating the life history of a new filarial nematode genus and species, parasitising its definitive toad host and mosquito vectors.

The aim of **Chapter 5** was to investigate whether frog blood parasites may be used as bioindicators for the condition of an ecosystem. To achieve this, the parasite load, prevalence, and richness of three blood parasite genera, *Dactylosoma*, *Trypanosoma* and *Hepatozoon*, between grass frogs (*Ptychadena* spp.) from the Phongolo River system in KZN is compared from anthropogenically impacted versus less impacted sites, as well as from the dry versus the wet seasons. Findings, based on several criteria of what is considered a good bioindicator, show frog blood parasites can be potential bioindicators.

In the **General discussion**, the results are summarised with a brief overview and discussed according to the main aims and objectives of the study.

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CHAPTER 1

Monophyly of the genus *Hepatozoon* (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa

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Abstract

Haemogregarines (Apicomplexa: Adeleiorina) are a diverse group of haemoparasites reported from almost all vertebrate classes. The most commonly recorded haemogregarines to parasitise anurans are species of *Hepatozoon* Miller, 1908. To date 16 species of *Hepatozoon* have been described from anurans in Africa, with only a single species, *Hepatozoon hyperolli* (Hoare, 1932), infecting a member of the Hyperoliidae. Furthermore, only two species of *Hepatozoon* are known from South African anurans, namely *Hepatozoon theileri* (Laveran, 1905) and *Hepatozoon ixoxo* Netherlands, Cook and Smit, 2014, from *Amietia delalandii* (syn. *Amietia quecketti*) and three species of *Sclerophrys* respectively. Blood samples were collected from a total of 225 individuals representing nine hyperoliid species from several localities throughout northern KwaZulu-Natal, South Africa. Twenty frogs from three species were found positive for haemogregarines, namely *Afrixalus fornasini* (6/14), *Hyperolius argus* (2/39), and *Hyperolius marmoratus* (12/74). Based on morphological characteristics, morphometrics, and molecular findings three new haemogregarine species, *Hepatozoon involucrum* Netherlands, Cook and Smit 2018, *Hepatozoon tenuis* Netherlands, Cook and Smit 2018 and *Hepatozoon thori* Netherlands, Cook and Smit 2018, are described from hyperoliid hosts. Furthermore molecular analyses show anuran *Hepatozoon* species to be a separate monophyletic group, with species isolated from African hosts forming a monophyletic clade within this cluster.

Keywords: *Afrixalus*, amphibia, apicomplexan, blood parasite, haemogregarine, Hyperoliidae, *Hyperolius*, morphology, phylogenetic analysis.

Introduction

Haemogregarines (Apicomplexa: Adeleiorina) are heteroxenous, intraerythrocytic or intraleucocytic parasites, infecting a broad range of vertebrate intermediate hosts including amphibians, reptiles, fishes, birds and mammals. These parasites are possibly transmitted by an equal diversity of haematophagous invertebrate definitive hosts or vectors, such as dipteran insects, ticks, mites, leeches, and even gnathiid isopods (see Smith, 1996; Davies and Johnston, 2000; Curtis et al., 2013). Haemogregarines are currently divided into four families (Barta et al., 2012), namely Dactylosomatidae Jakowska and Nigrelli, 1955, Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908, and Karyolysidae Labbé, 1894.

Within the Hepatozoidae, *Hepatozoon* Miller, 1908 is characterised by the presence of gamonts in erythrocytes or leucocytes, with no merogonic division occurring in the peripheral blood of the vertebrate host. Furthermore, *Hepatozoon* species are characterised by the pairing (syzygy) of gamonts in the definitive invertebrate host or vector following a blood meal. These paired gamonts then penetrate the gut wall and enter the haemocoel where sporogonic development and ultimately the formation of large oocysts occur. These thick-walled oocysts (also known as large multisporocystic oocysts) contain sporocysts with sporozoites, the infective stages of the parasite, which emerge upon the ingestion by the intermediate vertebrate host and give rise to merogonic stages in the liver (Desser et al., 1995; Smith, 1996; Barta, 2000).

Hepatozoon species are the most commonly reported haemogregarines to parasitise anurans. Currently, there are 45 recognised species from anurans globally, with 16 of these described from African hosts (see Smith, 1996; Netherlands et al., 2014a; Netherlands et al., 2014b). According to Netherlands et al. (2014a), the majority of these species (12/16) were described from the Bufonidae, namely *H. aegyptia* (Mohammed and Mansour, 1963), *H. assiuticus* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. boueti* (França, 1925), *H. faiyumensis* (Mansour and Mohammed, 1966), *H. francai* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. froilanoi* (França, 1925), *H. ixoxo* Netherlands, Cook and Smit, 2014, *H. lavieri* (Tuzet and Grjebine, 1957), *H. magni* (Hassan, 1992), *H. moloensis* (Hoare, 1920), *H. pestanae* (França, 1910), and *H. tunisiensis* (Nicolle, 1904). Two species were described from the Ptychadenidae, namely *H. epuluensis* (van den Berghe, 1942), and *H. neireti* (Laveran, 1905), and only a single species from the Pyxicephalidae and Hyperoliidae, namely *H. theileri* (Laveran, 1905), and *H. hyperolli* (Hoare, 1932) respectively. Apart from *H. hyperolli,* which was described from an unidentified *Hyperolius* species in Uganda (Hoare, 1932), the only other *Hepatozoon* species reported from the Hyperoliidae are two unnamed species reported in *Hyperolius marmoratus* and *Hyperolius puncticulatus*, from northern KwaZulu-Natal (KZN), South Africa (Netherlands et al., 2015) and Amani, Tanzania (Ball,

1967), respectively. In South Africa, only two *Hepatozoon* species are known from anurans, namely *H. theileri* and *H. ixoxo*, from the pyxicephalid *Amietia delalandii* (syn. *Amietia quecketti*) and three *Sclerophrys* species (Bufonidae) respectively, namely *Sclerophrys pusilla* (syn. *Amietophrynus maculatus*), *Sclerophrys* (syn. *Amietophrynus*) *garmani* and *Sclerophrys* (syn. *Amietophrynus*) *gutturalis*.

Over the past decade several phylogenetic studies on adeleorinid parasites, using 18S rDNA sequences, have provided useful insight into the evolutionary relationships of this group, as well as better capability to distinguish between species. However, because the 18S rRNA nuclear gene is a relatively conserved marker, it shows certain nodes to be unresolved (Barta et al., 2012; Maia et al., 2012; Haklová-Kočíková et al., 2014; Cook et al., 2016). In an effort to resolve these polytomies, a new genus *Bartazoon* Karadjian, Chavatte and Landau, 2015, was proposed for species previously regarded as belonging to *Hepatozoon* parasitising reptiles, amphibians, marsupials, birds and rodents, and was proposed to be transmitted solely by biting insects (Karadjian et al., 2015). However, the suggested life history of certain species within the proposed genus such as *Hepatozoon fitzsimonsi* Dias, 1953 do not conform to the recommended characteristic defining *Bartazoon* (see Cook et al., 2014; Karadjian et al., 2015). Also as pointed out by Maia et al. (2016a), it is possible that *Hepatozoon perniciosum* Miller, 1908, the type species of the genus *Hepatozoon*, may in fact form part of the newly proposed genus *Bartazoon*, as most other rodent haemogregarine species do. Furthermore, increased work on the phylogenetic relationships of the haemogregarines continues to identify new genetic lineages, showing that *Bartazoon* is not a well-supported monophyletic group (Maia et al., 2016a; Tomé et al., 2016). Thus, to revise the deeper taxonomy (family and genus level) of haemogregarines based on their phylogenetic affinities and life histories, more studies using faster-evolving markers such as mitochondrial genes (e.g. Leveille et al., 2014), elucidating life cycles, and building larger datasets are necessary. Therefore, as suggested and used by Maia et al. (2016b) we will continue to refer to species parasitising anuran hosts as species of *Hepatozoon* and not *Bartazoon*.

Prior to the study of Netherlands et al. (2014a) all the African anuran *Hepatozoon* species descriptions, ranging from the early 1900s till the late 1970s, were solely based on the morphology of the peripheral blood gamont stages. Unfortunately many of these descriptions were scantily illustrated and incomplete, with almost 60% of the species described from the same host (*Sclerophrys regularis*) and in more or less the same geographical area (see Netherlands et al., 2014a; Netherlands et al., 2014b). Thus many of these species may later need to be synonymised once more advanced and standardised methods are used to characterise these haemogregarines. In South Africa only five studies on amphibian haemogregarines have been carried out (Laveran, 1905; Fantham et al.,

1942; Netherlands et al., 2014a; Netherlands et al., 2014b; Netherlands et al., 2015). From these only a single study was a multispecies haemoparasite survey across different anuran families (Netherlands et al., 2015), and although in that study several different haemogregarines were observed in anurans, only one hyperoliid species, *Hyp. marmoratus* (as mentioned above) contained a *Hepatozoon* species, which was not identified to species level.

Thus the objectives of the current study were 1) to establish which hyperoliid frog species in northern KZN, South Africa, contain haemogregarines. 2) to determine the species diversity of the haemogregarine parasites observed. 3) to ascertain if any of the haemogregarines found were previously described or reported species and 4) to compare any parasites characterised in the current study with available molecular data for anuran haemogregarines in order to determine their phylogenetic relationships.

Materials and Methods

Frog collection and study area

A total of 225 individuals representing nine hyperoliid species, were collected from several localities throughout northern KZN, South Africa (Fig. 1), following the collection methods described in Netherlands et al. (2015). Frogs were identified using Du Preez and Carruthers (2009). After processing all specimens were released at site of capture. This study received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5).

Processing of samples and light microscopy screening

Blood (> 0.1 ml) was taken from each frog via cardiac or femoral venipuncture and thin blood smears prepared on clean glass slides, air-dried, fixed and stained using Giemsa-stain (FLUKA, Sigma-Aldrich, Steinheim, Germany). The remaining blood was preserved in 70% ethanol for molecular work (ratio 1:15). Stained blood smears were screened at 1000× and images captured and measured using the imaging software NIS Elements Ver. 4 as described by Netherlands et al. (2015). Fifty mature gamonts were measured per *Hepatozoon* species. Measurements comprised the parasite's length (including recurved tail when present) and width within its parasitophorous vacuole (PV), and the parasite's nucleus length and width. Measurements of the PV length and width, and the length from mid nucleus to both anterior and posterior end of the parasite were also taken. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear, following previous methods (see Cook et al., 2015b).

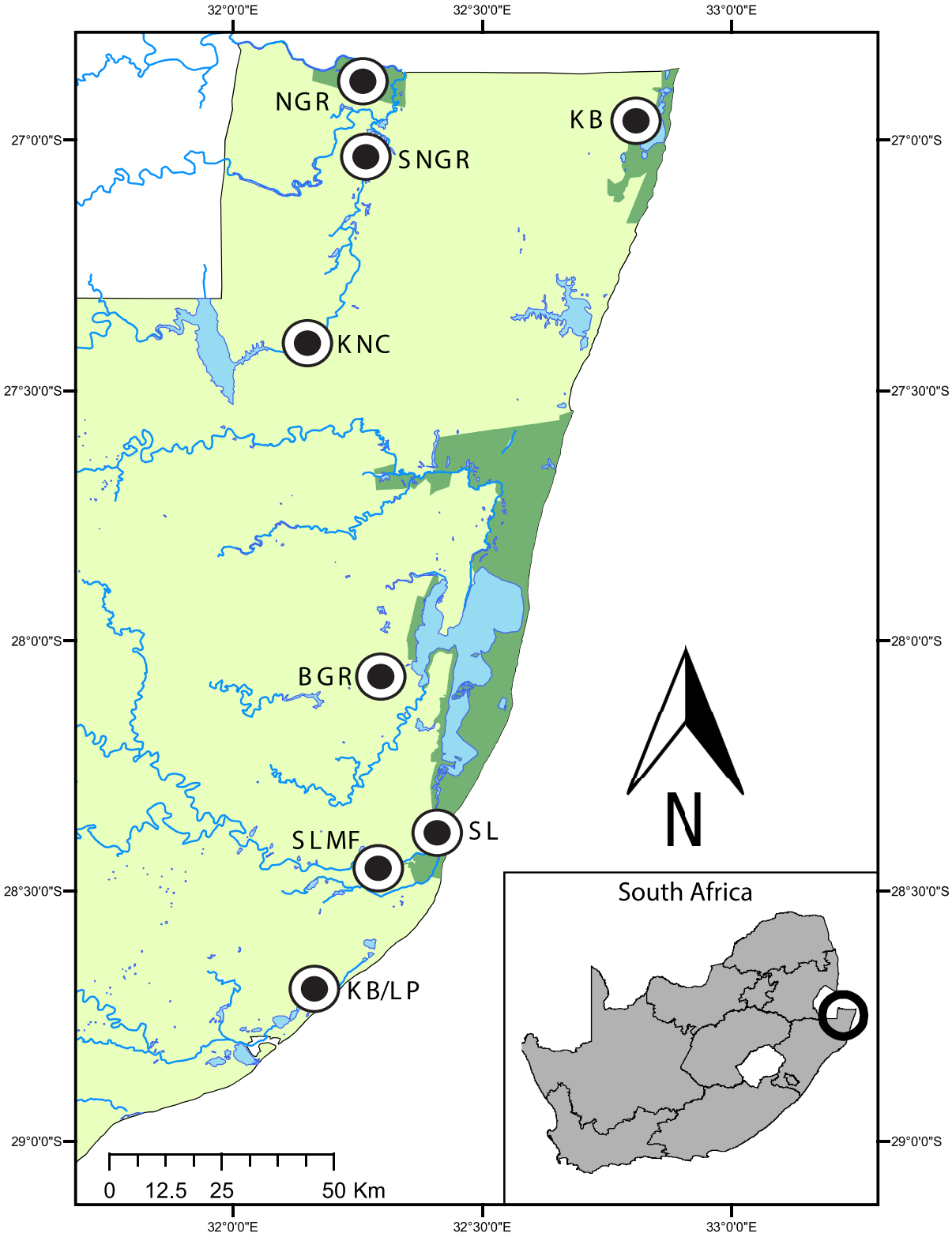

Fig. 1. Map of the sampling localities in northern KZN, South Africa. Ndumo Game Reserve (NGR) 26°52′00″S, 32°15′00″E, the area directly surrounding the NGR (SNGR) 27°00′13″S, 32°16′50″E, Kwa Nyamazane Conservancy (KNC) 27°23′35″S, 32°08′41″E, Bonamanzi Game Reserve (BGR) 28°03′25″S 32°17′42″E, Kosi Bay (KB) 26°57′16″S 32°48′07″E, KwaMbonambi/Langepan (KB/LP) 28°39′43″S 32°10′06″E, St. Lucia (SL) 28°23′10″S 32°24′29″E and St. Lucia Monzi Farm (SLMF) 28°26′56″S 32°17′18″E.

DNA extraction, PCR amplification, and phylogenetic analyses

Ethanol-preserved blood samples from parasitised frog specimens (*n* = 10) were used for molecular work. Two additional blood samples of *A. delalandii* parasitised with *H. theileri* and *S. pusilla* parasitised with *H. ixoxo* from a previous study (Netherlands et al., 2014a) were added to obtain longer comparative sequences as compared to the previous study by Netherlands et al. (2014a). Genomic DNA of haemogregarine species were extracted from the blood samples using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). Once extracted, DNA was used for polymerase chain reaction (PCR) amplification. The PCR reactions targeted two fragments of approximately 940 nt and 1400 nt of the 18S rRNA gene. The 18S rRNA gene sequences were amplified using a combination of two primer sets based on previous studies of haemogregarines belonging to *Karyolysus* Labbé, 1894, *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Hepatozoon* (Ujvari et al., 2004; Criado-Fornelio et al., 2006; Cook et al., 2015a, 2016). The first fragment was amplified using HAM-F (5′-GCCAGTAGTCATATGCTTGTC-3′) and HepR900 (5′-CAAATCTAAGAATTTCACCTCTGAC-3′) (see Ujvari *et al.* 2004; Criado-Fornelio *et al.* 2006), and the second fragment HepF300 (5′-GTTTCTGACCTATCAGCTTTCGACG-3′) and 2868 (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (see Medlin et al., 1988; Ujvari et al., 2004). Conditions for PCR were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles, entailing a 95 °C denaturation for 30 s, annealing at 61 °C for 30 s with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min. PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (final concentration: 2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$), 1.25 µl (10 µM) of each of the primer sets mentioned above, and at least 25 ng DNA. The final reaction volume was made up with PCR-grade nuclease free water (Thermo Scientific). Reactions were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK). Resulting amplicons were visualized under ultraviolet light on a 1% agarose gel stained with gel red using a Bio-Rad GelDoc™ XR+ imaging system (Bio-Rad, Hemel Hempstead, UK). PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions. Resultant sequences were assembled, and chromatogram-based contigs were generated and trimmed using Geneious R9.1 (http://www.geneious.com, (Kearse et al., 2012). Sequence and species identity was verified against previously published sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Sequences obtained in the current

study were deposited in the NCBI GenBank database under the following accession numbers [GenBank: MG041591–MG041605].

For comparison, all 18S rDNA sequences of anuran haemogregarines, longer than 1500 nucleotides (nt) (comprising species of *Hepatozoon*, *Hemolivia, Babesiosoma* and *Dactylosoma*) as well as *Hepatozoon sipedon* Smith, Desser and Martin, 1994, [GenBank: JN181157] from the snake *Nerodia sipedon sipedon*, were downloaded from GenBank and aligned to the sequences generated in the current study. *Hepatozoon sipedon* was selected as it was shown by Barta et al. (2012) to be sister to *H. catesbianae* (Stebbins, 1904) and *H. clamatae* (Stebbins, 1905), at that point the only two species of *Hepatozoon* of frogs for which 18S rDNA sequences were available. Furthermore, *H. sipedon* first makes use of a frog intermediate host in which tissue development occurs before transmission to its second intermediate snake host (see Smith et al., 1994). Thus all species included in the analysis have an anuran host in their life cycle. Although there are other sequences available from a *Hepatozoon* species characterized from the anurans *Pelophylax perezi* [GenBank: KF733812] and *Leptodactylus chaquensis* [GenBank: JX987775], from the Azores in the North Atlantic Ocean, and Pantanal, Brazil respectively, they were not added to our analysis because these concerned shorter fragments (see Harris et al., 2013; Leal et al., 2015). *Babesiosoma stableri* Schmittner and McGhee, 1961 [GenBank: HQ224961] and *Dactylosoma ranarum* Lankester, 1871 [GenBank: HQ224957; HQ224958] were chosen as the outgroup, as they were shown by Barta et al. (2012) to belong to a sister group to our current ingroup. Sequences were aligned using the MUSCLE alignment tool (Edgar, 2004) under the default settings and implemented in Geneious R9.1. The alignment consisted of 14 sequences with a 1,497 nt conserved region selected using the Gblocks 0.91b server (Castresana, 2000). To infer phylogenetic relationships both Bayesian inference (BI) and Maximum likelihood (ML) methods were used. The BI analysis was performed using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001) and the ML analysis was preformed using RAxML Ver. 7.2.8. (Stamatakis, 2014) both implemented from within Geneious R9.1. Prior to the analyses a model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion (AIC) using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). The model with the best AIC score was the Transitional model (Posada, 2003) with estimates of invariable sites and a discrete Gamma distribution (TVM+I+Γ). However, this model was substituted by the General Time Reversible (Tavaré, 1986) model (GTR+I+Γ) in MrBayes and in RAxML, as this was the next model available with the best AIC score. For the BI analysis the Markov Chain Monte Carlo (MCMC) algorithm was run for 10 million generations, sampling every 100 generations, and using the default parameters. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. Results were visualised in Trace (implemented from within

Geneious R9.1), to assess convergence and the burn-in period. For the ML analysis nodal support was assessed using 1000 rapid bootstrap inferences. Model-corrected (TVM+I+Γ) genetic distances were calculated in PAUP version 4.0a152 (Swofford, 2002) with the assumed proportion of invariable sites = 0.598 and the gamma shape parameter = 0.775.

Results

A total of 225 individuals representing nine species from the family Hyperoliidae, namely *Afrixalus aureus* (*n* = 18)*, Afrixalus delicatus* (*n* = 13), *Afrixalus fornasini* (*n* = 14), *Hyperolius argus* (*n* = 39), *Hyperolius marmoratus* (*n* = 74), *Hyperolius tuberlinguis* (*n* = 38), *Hyperolius pusillus* (*n* = 14), *Kassina senegalensis* (*n* = 9), and *Phylctimantis* (syn. *Kassina*) *maculatus* (*n* = 6) were collected and screened for haemogregarines. Twenty frogs (8.9%) from three species were found positive for haemogregarines, specifically *A. fornasini* (6/14), *Hyp. argus* (2/39), and *Hyp. marmoratus* (12/74) (see Fig. 2A–C). Based on peripheral blood stages, the haemogregarines of the current study conform to the genus *Hepatozoon*. Although possible meront stages were observed in the peripheral blood for one species, these were rare and no merogonic division was detected. Furthermore, these haemogregarines did not compare to the closely related genus *Hemolivia*, as no schizogony or cyst formation in the erythrocytes of the hosts were observed.

Fig. 2. Three frog species found positive for haemogregarines. (A) *Afrixalus fornasini*, (B) *Hyperolius argus*, and (C) *Hyperolius marmoratus*.

Species descriptions

Phylum: Apicomplexa Levine, 1970
Class: Conoidasida Levine, 1988
Order: Eucoccidiorida Léger & Duboscq, 1910
Suborder: Adeleorina Léger, 1911
Family: Hepatozoidae Wenyon, 1926
Genus: *Hepatozoon* Miller, 1908

***Hepatozoon involucrum* Netherlands, Cook and Smit 2018**

Type-host: Hyperolius marmoratus Rapp, 1842 (Anura: Hyperoliidae).

Vector: Unknown.

Type-locality: The specimens were collected in the Kwa Nyamazane Conservancy (KNC), KwaZulu-Natal, South Africa (27°23′35″S, 32°08′41″E).

Other localities: St. Lucia on Monzi Farm, KwaZulu-Natal, South Africa (28°26′56″S 32°17′18″E).

Type-material: Hapantotype, 1 × blood smear from *Hyp. marmoratus* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P 467; parahapantotype, 1 × blood smear from *Hyp. marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein (NMB), South Africa, under accession number NMB P 468.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041591–MG041594.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:F73407D7-1E08-4C3C-B066-889058B77C4C. The LSID for the new name *Hepatozoon involucrum* Netherlands, Cook and Smit 2018 is urn:lsid:zoobank.org:act:A43D46E8-5C9F-4405-8907-94D7B02EAEA7.

Etymology: The species epithet is derived from the Latin word *involucrum* meaning envelope or sheath, and is based on the prominent parasitophorous vacuole encircling the gamont.

Description

Trophozoites: rare, occurring singularly within erythrocytes, oval to rounded, measuring 12.2–12.5 (12.3 ± 0.2) µm long by 4.8–5.7 (4.2 ± 0.6) µm wide (*n* = 2) with finely vacuolated cytoplasm staining whitish-pink (Fig. 3A–B), note lysis of the host cell nucleus (Fig. 3B). Nucleus containing loosely arranged chromatin, staining pink, measuring 3.7–5.2 (4.5 ± 1.0) µm long by 3.2–4.9 (4.0 ± 1.2) µm wide (*n* = 2). Mid nucleus position measuring 5.8–7.4 (6.6 ± 1.2) µm to anterior, and 5.4–5.6 (5.5 ± 0.1) µm to posterior.

Meronts: rare, irregular in shape, often with a foamy cytoplasm, staining whitish-blue to purple (Fig. 3C–D), and measuring 9.5 µm long by 8.8 µm wide (*n* = 1). Nucleus containing loosely arranged chromatin, staining pink to purple, measuring 6.8 µm long by 3.7 µm wide (*n* = 1).

Immature gamonts: elongated with small-recurved tail, within a vaguely visible parasitophorous vacuole (PV), cytoplasm staining whitish-purple, causing displacement of the host cell nucleus (Fig. 3E). Parasite (including recurved tail) measuring 16.4–23.0 (19.8 ± 1.8) µm long by 4.4–5.7 (5.1 ± 0.4) µm wide (*n* = 10), PV measuring 14.2–18.4 (15.6 ± 1.3)

µm long by 5.2–9.1 (6.5 ± 1.5) µm wide (*n* = 10). Nucleus rounded, usually situated in the posterior half of the parasite, loosely arranged chromatin, staining purple, and measuring 3.0–7.0 (5.4 ± 1.4) µm long by 2.6–5.6 (3.8 ± 0.9) µm wide (*n* = 10). Mid nucleus position measuring 10.0–13.7 (11.7 ± 1.4) µm to anterior side, and 6.6–11.1 (8.6 ± 1.6) µm to posterior side (n = 10).

Mature gamonts: elongated and oval, encased in a large PV (Fig. 3F−I); often recurved at both the anterior and posterior poles, and in some cases a clear recurved tail is visible (Fig. 3G arrowhead); infrequent extracellular or free moving gamont (Fig. 3F), as well as single erythrocytes parasitised by two gamonts (Fig. 3I); gamonts cause noticeable displacement of the host cell nucleus. Parasite (including recurved tail) measuring 18.7–25.9 (21.8 ± 1.5) µm long by 4.0–6.3 (5.1 ± 0.5) µm wide (*n* = 50), PV measuring 16.5–20.9 (18.3 ± 1.0) µm long by 6.3–10.8 (8.3 ± 1.1) µm wide (*n* = 50). Nucleus elongated or loosely arranged, usually situated in the posterior half of the parasite, loose chromatin strands often visible, staining purely-pink, and measuring 4.8–8.9 (6.4 ± 0.9) µm long by 2.2–4.2 (3.2 ± 0.4) µm wide (*n* = 50). Mid nucleus position measuring 8.4–19.9 (13.8 ± 1.8) µm to anterior side, and 5.4–11.6 (8.2 ± 1.4) µm to posterior side (*n* = 50). Parasitaemia of all infected individuals (*n* = 7) in percentage (%) was 1.0–30.0 (8.0 ± 2.0).

Remarks

Based on the morphology and morphometrics of peripheral blood stages in *Hyp. marmoratus*, *H. involucrum* does not conform morphologically to any of the 16 currently recognised *Hepatozoon* species in African anurans. The only other named species infecting a member of the Hyperoliidae, is *H. hyperolii*, and can be distinguished from *H. involucrum* based on the shape of the former parasite's gamont. The gamont of *H. hyperolii* is cylindrical with rounded ends and a long recurved tail folded onto itself in the absence of a prominent PV (see Fig. 6A–C). In contrast the gamont of *H. involucrum* has an elongated and encased gamont, which is often recurved at both the anterior and posterior poles. The mean length and width of *H. involucrum*, which includes the parasite's PV, is 18.3 µm long by 8.3 µm wide. Although these mean length measurements do overlap with several species namely, *H. faiyumensis*, *H. francai*, *H. moloensis* and *H. neireti*, the mean width in combination with the length of these species do not conform. Overall the gamont measurements of *H. involucrum* compare closest to those of *H. moloensis* (18.8 µm long by 7.8 µm wide), which was described from an unidentified *Sclerophrys* species in Molo, Kenya (see Hoare, 1920). However, the oval shape, recurved tail and absence of a PV in *H. moloensis* are distinctive and distinguishable from *H. involucrum* as described above. Similarly, these distinctive

characteristics of *H. involucrum* n. sp, which differentiate it from *H. moloensis*, also differentiate it from other African anuran species of *Hepatozoon*.

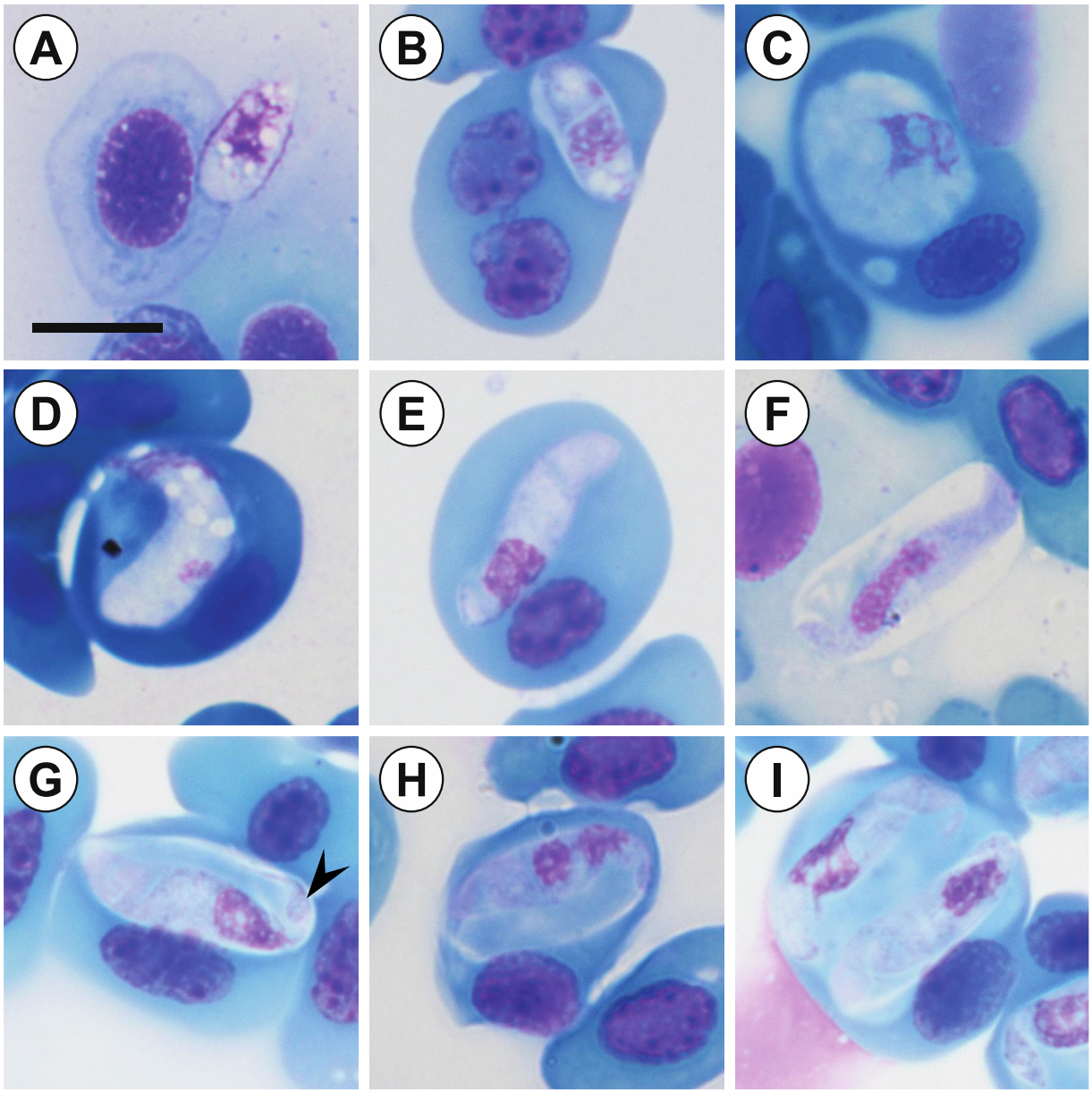

Fig. 3. *Hepatozoon involucrum* in the reed frog *Hyperolius marmoratus*. (A–B) Trophozoite. (C) Possible meront stage. (D) Possible vacuolated meront stage. (E) Immature gamont stage. (F) Extracellular or free gamont. (G, arrowhead) Mature gamont displaying a recurved tail. (H) Mature gamont, note the expanding parasite nucleus and large parasitophorous vacuole. (I) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P 467 & 468). Scale bar: 10 µm.

In South Africa a *Hepatozoon* species corresponding morphologically to *H. involucrum* was reported from the same host and area in an anuran biodiversity blood

parasite survey by Netherlands et al. (2015), however this parasite was not formally described or named (see Netherlands et al., 2015), Fig. 2D).

Globally the species that conforms most closely to *H. involucrum* is *Hepatozoon nucleobisecans* (Shortt, 1916) described from the Indian toad *Duttaphrynus melanostictus* (syn. *Bufo melanostictus*). Although the reported gamont length (18.3 µm long) of *H. nucleobisecans*, including the PV, equals the mean length of *H. involucrum*, the width (4.8 µm wide) is almost half. Furthermore the gamont of *H. nucleobisecans* is not recurved at both the anterior and posterior poles within the PV (see Shortt 1916).

***Hepatozoon tenuis* Netherlands, Cook and Smit 2018**

Type-host: Afrixalus fornasini (Bianconi, 1849) (Anura: Hyperoliidae).

Other hosts: Hyperolius argus; *Hyperolius marmoratus.*

Vector: Unknown.

Type-locality: The specimens were collected in St. Lucia on Monzi Farm, KwaZulu-Natal, South Africa (28°26′56″S 32°17′18″E).

Other localities: Kwambonambi/Langepan , KwaZulu-Natal, South Africa (28°39′43″S 32°10′06″E).

Type-material: Hapantotype, 1 × blood smear from *A. fornasini* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P 469; parahapantotypes, 1 × blood smear from *A. fornasini*, and *Hyperolius marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein (NMB), South Africa, under accession numbers NMB P 470 and NMB P 471, respectively.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041595–MG041599.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:F73407D7-1E08-4C3C-B066-889058B77C4C. The LSID for the new name *Hepatozoon tenuis* Netherlands, Cook and Smit is urn:lsid:zoobank.org:act:AD607D8B-D43D-49C6-8139-2782306FE2F5.

Etymology: The species epithet is derived from the Latin word *tenuis*, which means thin or slender. This refers to the long slender shape of the gamont.

Description

Mature gamonts: slender and elongated, with a pinkish-white staining cytoplasm, within a close-fitting parasitophorous vacuole visible on the concave side of the gamont (Fig. 4A−C); in some cases a recurved tail is visible (Fig. 4A and D arrowhead); also an occasional extracellular or free moving gamont, (Fig. 3E arrow), as well as a single erythrocyte

parasitised by two gamonts (Fig. 4F); gamonts cause obvious displacement of the host cell nucleus. Parasites (including recurved tail when visible) measuring 11.2–16.8 (13.9 ± 1.6) µm long by 3.7– 6.7 (4.8 ± 0.6) µm wide (*n* = 50), PV measuring 17.8–20.7 (19.4 ± 0.8) µm long by 5.0–7.5 (6.7 ± 0.4) µm wide (*n* = 50). Nucleus elongated and neatly arranged, usually situated in the posterior half of the parasite, loose chromatin staining purely-pink, and measuring 2.1–5.2 (3.9 ± 0.6) µm long by 1.6–4.9 (10.8 ± 0.9) µm wide (*n* = 50). Mid nucleus position measuring 4.8–9.4 (6.7 ± 1.1) µm to anterior, and 4.6–10.1 (7.2 ± 1.2) µm to posterior (*n* = 50). Parasitaemia of all infected individuals (*n* = 9) calculated in percentage (%) was 1.0–35.0 (6.0 ± 2.0), two (*Hyp. argus* and *Hyp. marmoratus*) of the nine infected individuals contained mixed infections the parasite described below.

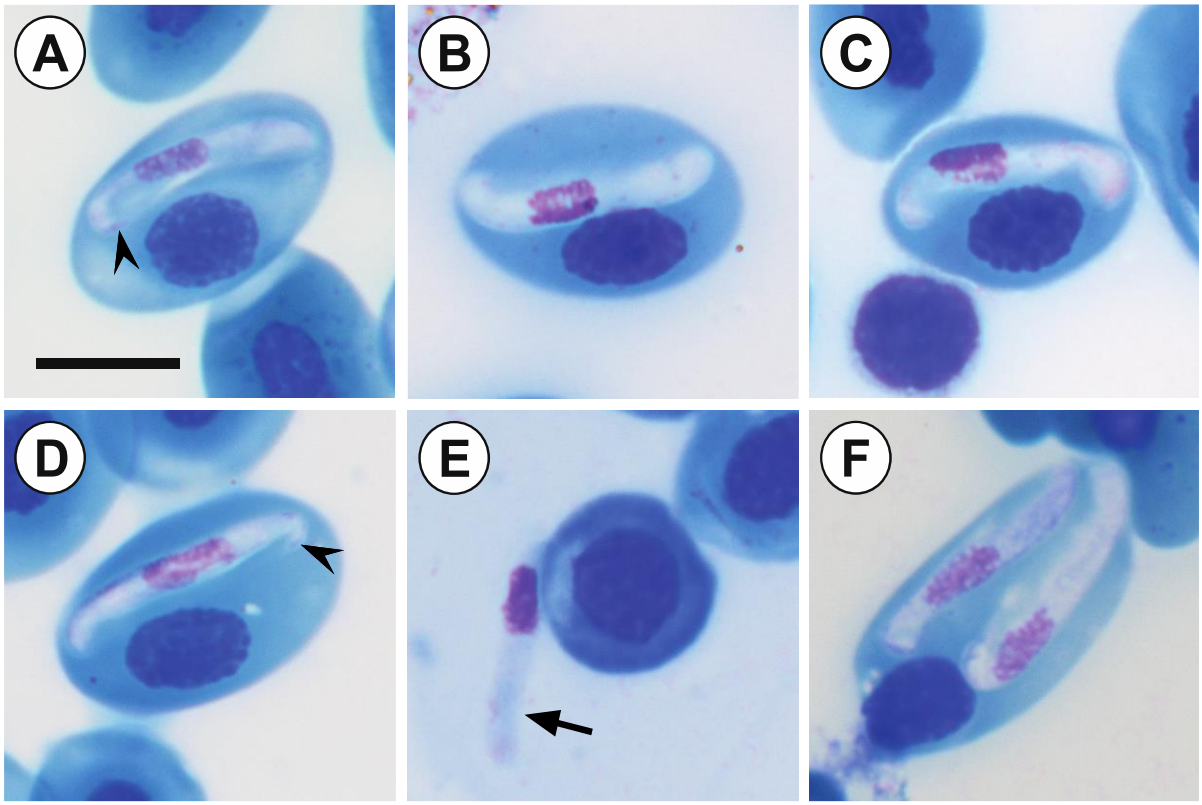

Fig. 4. *Hepatozoon tenuis* mature gamonts parasitising erythrocytes in the leaf folding frog *Afrixalus fornasini* (A–C) and the reed frogs *Hyperolius marmoratus* (D) and Hyperolius argus (E–F). (A–C) Close-fitting parasitophorous vacuole, visible on the concave side of the gamont. (A and D, arrowhead) Gamont with a recurved tail. (E, arrow) Extracellular or free gamont. (F) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P 469–471). Scale bar: 10 µm.

Remarks

Hepatozoon tenuis parasitising *A. fornasini*, *Hyp. argus* and, *Hyp. marmoratus*, can be distinguished from *H. involucrum*, based on the difference in gamont morphometrics. Morphologically, gamonts have an overall similar appearance to *H. involucrum*, however, gamonts of *H. involucrum* measure a mean of 21.8 µm long by 5.1 µm wide (*n* = 50) (PV not included) and a mean of 18.3 µm long by 8.3 µm wide (*n* = 50) (PV included), as compared to gamonts of *H. tenuis* measuring a mean of 13.9 µm long by 4.8 µm wide (*n* = 50) (PV not included) and a mean of 19.4 µm long by 6.7 µm wide (*n* = 50) (PV included). This slender looking parasite can be distinguished from other anuran *Hepatozoon* species based on the marginally visible PV, as well as often being recurved at both the anterior and posterior poles within the PV.

***Hepatozoon thori* Netherlands, Cook and Smit 2018**

Type-host: Hyperolius marmoratus Rapp, 1842 (Anura: Hyperoliidae).

Other hosts: Hyperolius argus; *Hyperolius puncticulatus*.

Vector: Unknown.

Type-locality: The specimens were collected in the Kwa Nyamazane Conservancy (KNC), KZN, South Africa (27°23′35″S, 32°08′41″E).

Other localities: Kwambonambi/Langepan, KZN, South Africa (28°39′43″S 32°10′06″E); Amani, Tanzania.

Type-material: Hapantotype, 1 × blood smear from *Hyp. marmoratus* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P 472; parahapantotype, 1 × blood smear from *Hyp. marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein, South Africa, under accession number NMB P 473.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041600–MG041603.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:F73407D7-1E08-4C3C-B066-889058B77C4C. The LSID for the new name *Hepatozoon thori* Netherlands, Cook and Smit is urn:lsid:zoobank.org:act:00CD84D9-D6A8-4B41-A048-DFD0DBF4B045.

Etymology: The species epithet is derived from Norse mythology after the hammer-wielding god Thor. This is based on the hammer-like shape of the gamont.

Description

Immature gamonts: rare, elongated without a visible parasitophorous vacuole (PV), cytoplasm staining whitish-purple, measured 18.7 µm long by 5.5 µm wide (*n* = 1), causing displacement of the host cell nucleus and found parasitising a single erythrocyte together with a mature gamont (Fig. 5A arrow). Nucleus rounded, situated in the posterior half of the parasite, loosely arranged chromatin, staining purple, and measuring 8.1 µm long by 2.7 µm wide (*n* = 1). Mid nucleus position measured 8.9 µm to anterior side, and 9.8 µm to posterior side (*n* = 1).

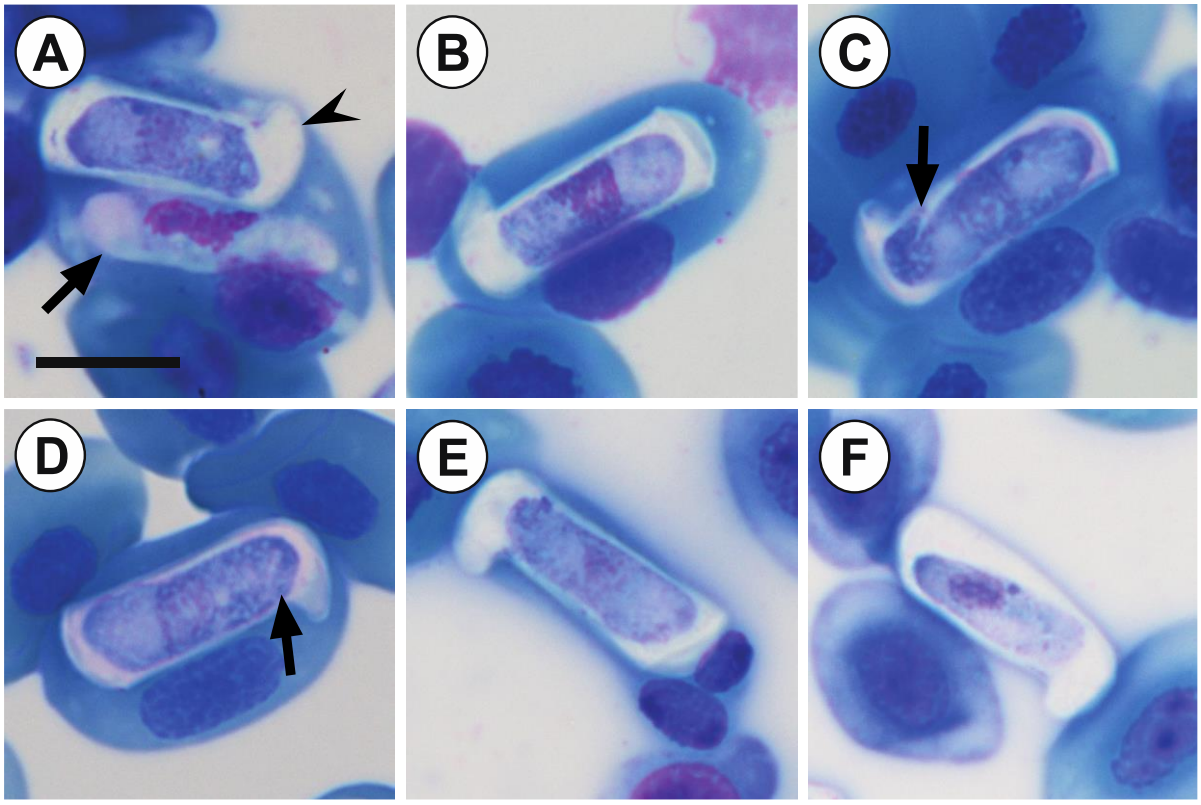

Fig. 5. *Hepatozoon thori* gamonts parasitising erythrocytes in the reed frogs *Hyperolius marmoratus* (A–C) and *Hyperolius argus* (D–F). (A) Double infection of a single erythrocyte, with an immature (arrow) and mature (arrowhead) gamont. (B–F) Prominent hammer-like or boot-shaped parasitophorous vacuole, allowing only a certain portion of the gamont to be visible. (C and D, arrow) Gamont displaying a short recurved tail. (E) Gamont causing the host cell nucleus to lyse. (F) Extracellular or free gamont. All images captured from the deposited slides (NMB P 472 & 473). Scale bar: 10 µm.

Mature gamonts: elongated, causing displacement of the host cell nucleus. Encased in a prominent hammer-like or boot-shaped PV, with a pseudopodial-like projection (Fig. 5A−F); occasionally a short recurved tail is visible (Fig. 5C−D arrow); mature gamonts cause

the host cell nucleus to lyse (Fig. 5E); extracellular or free moving gamont, possibly probing to enter new host cell (Fig. 5F). Parasite measuring 11.2–16.8 (13.9 ± 1.6) µm long by 3.7–6.7 (4.8 ± 0.6) µm wide (*n* = 50), with the PV measuring 17.8–20.7 (19.4 ± 0.8) µm long by 5.0–7.5 (6.7 ± 0.4) µm wide (*n* = 50). Parasites, including the recurved tail (see Fig. 5C−D arrow), measuring 19.1–21.7 (20,4 ± 1.1) µm long (*n* = 5). Nucleus elongated or loosely arranged, usually situated in the posterior half of the parasite, loose chromatin strands often visible, staining purely-pink, and measuring 2.1–5.2 (3.9 ± 0.6) µm long by 1.6–4.9 (10.8 ± 0.9) µm wide

(*n* = 50). Mid nucleus position measured 4.8–9.4 (6.7 ± 1.1) µm to anterior, and 4.6–10.1 (7.2 ± 1.2) µm to posterior (*n* = 50). Parasitaemia of all infected individuals (*n* = 6) in percentage (%) was 1.0–21.0 (3.0 ± 2.0), two (*Hyp. argus* and *Hyp. marmoratus*) of the six infected individuals contained mixed infections with *H. tenuis*.

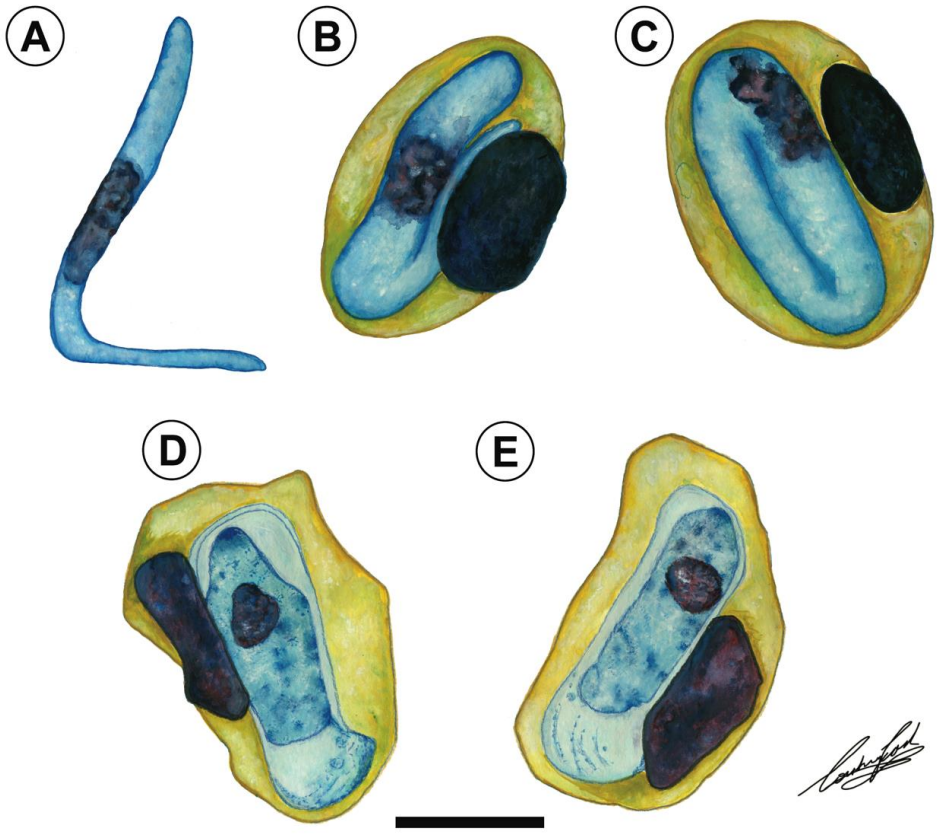

Fig. 6. Illustrations of haemogregarine blood parasites in African hyperoliids. (A–C) *Hepatozoon hyperolii* Hoare 1932, described from an unidentified *Hyperolius* species in Uganda. Redrawn and adapted from Hoare (1932). (D–E) Unnamed *Hepatozoon* species reported in *Hyperolius puncticulatus*, from Amani, Tanzania. Redrawn and adapted from Ball (1967). Scale bar: 10µm.

Remarks

Hepatozoon thori parasitising *Hyp. argus* and *Hyp. marmoratus* can be distinguished from *H. involucrum*, *H. tenuis*, and other anuran *Hepatozoon* species based on the distinctive shape of the hammer-like or boot-shaped PV that has a pseudopodial-like projection. The mean length and width of the parasite measures 13.9 µm long by 4.8 µm wide (PV not included) and 19.4 µm long by 6.7 µm wide (*n* = 50) (PV included). Based on the size and shape, the only other haemogregarine *H. thori* conforms closest to is an unnamed *Hepatozoon* species (see Fig 6D–E), measuring a mean of 14.1 µm long by 4.8 µm wide (PV not included) and 20.8 µm long by 6.7 µm wide (PV included). This unnamed species was reported in *Hyperolius puncticulatus*, from Amani, Tanzania (see Ball, 1967) (see below).

Phylogenetic analysis

Amplicons of between 1640 and 1701 nt were derived from *H. involucrum*, *H. tenuis*, and *H. thori* from the blood of *A. fornasini, Hyp. argus* and *Hyp. marmoratus*. Additionally, sequences of *H. ixoxo* and *H. theileri*, were amplified from the blood collected in a previous study (Netherlands et al., 2014a) from *S. pusilla* and *A. delalandii,* respectively. The details of sequences used in the analyses are presented in Table 1.

Based on 1,497 nt sequence comparisons of the 18S rRNA gene (see Table 2), the interspecific divergence (model-corrected genetic distance) between *H. involucrum* and its closest relative *H. tenuis* was 1.0 %. *Hepatozoon involucrum* and *H. thori* had an interspecific divergence of 2.0 %, and *H. tenuis* and *H. thori* differed by 1.8 %. The interspecific divergence between the *Hepatozoon* species parasitising anuran hosts and *Hepatozoon sipedon* Smith, Desser and Martin, 1994 [GenBank: JN181157] was between 7.7–10.6 %. The intergeneric divergence between the *Hepatozoon* species parasitising anuran hosts, and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1989 [GenBank: KP881349], *B. stableri* [GenBank: HQ224961] and *D. ranarum* [GenBank: HQ224957; HQ224958] were between 4.9–5.8 %, 8.8–9.6 % and 8.5–9.7 %, respectively (Table 2).

Table 1: List of the sequence (18S rDNA) information used in the current study. The table includes the GenBank accession number, species, host species and the reference study

Accession No.	Species	Host	Reference
MG041591	*Hepatozoon involucrum*	*Hyperolius marmoratus*	Current study
MG041596	*Hepatozoon tenuis*	*Afrixlus fornasini*	Current study
MG041598	*Hepatozoon tenuis*	*Hyperolius argus*	Current study
MG041599	*Hepatozoon tenuis*	*Hyperolius marmoratus*	Current study
MG041600	*Hepatozoon thori*	*Hyperolius argus*	Current study
MG041601	*Hepatozoon thori*	*Hyperolius marmoratus*	Current study
MG041605	*Hepatozoon theileri*	*Ametia delalandii*	Current study
MG041604	*Hepatozoon ixoxo*	*Sclerophrys pusilla*	Current study
HQ224962	*Hepatozoon* cf. *clamatae*	*Rana clamitans*	Barta *et al.* (2012)
HQ224963	*Hepatozoon* cf. *clamatae*	*Rana clamitans*	Barta *et al.* (2012)
HQ224954	*Hepatozoon* cf. *catesbianae*	*Rana catesbeiana*	Barta *et al.* (2012)
HQ224960	*Hepatozoon magna*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)
JN181157	*Hepatozoon sipedon*	*Nerodia sipedon sipedon*	Barta *et al.* (2012)
KP881349	*Hemolivia stellata*	*Amblyomma rotondatum* ex *Rhinella marina*	Karadjian *et al.* (2015)
HQ224961	*Babesiosoma stableri*	*Rana septentrionalis*	Barta *et al.* (2012)
HQ224957	*Dactylosoma ranarum*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)
HQ224958	*Dactylosoma ranarum*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)

Table 2. Estimates of divergence between partial 18S rDNA sequences from the haemogregarine species used in the current study. Distance matrix showing ranges for the model-corrected genetic distances between the sequences. Alignment length 1,497 nt. Genetic distances shown as percentage (%)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. MG041591 *H. involucrum*															
2. MG041596 *H. tenuis*	1,0														
3. MG041598 *H. tenuis*	1,0	0,2													
4. MG041601 *H. thori*	2,0	1,8	1,9												
5. MG041605 *H. theileri*	2,1	1,8	1,9	2,1											
6. MG041604 *H. ixoxo*	1,7	1,4	1,4	1,5	1,4										
7. HQ224962 *H.* cf. *clamatae*	2,5	2,2	2,3	2,2	2,4	1,6									
8. HQ224963 *H.* cf. *clamatae*	2,6	2,4	2,5	2,4	2,6	1,7	0,1								
9. HQ224954 *H.* cf. *catesbianae*	2,5	2,2	2,3	2,2	2,4	1,6	0,1	0,3							
10. HQ224960 *H. magna*	2,2	1,7	1,8	1,9	2,1	1,6	1,8	2,0	1,8						
11. JN181157 *H. sipedon*	10,1	9,9	10,0	10,4	10,1	9,6	10,2	10,5	10,2	9,3					
12. KP881349 *Hemolivia stellata*	5,7	5,3	5,2	5,6	5,2	5,5	5,6	5,8	5,6	4,9	7,7				
13. HQ224961 *B. stableri*	9,0	9,0	9,2	9,4	8,9	9,6	9,1	9,3	9,3	9,0	10,6	5,0			
14. HQ224957 *D. ranarum*	9,1	9,1	9,3	9,5	9,0	9,7	9,2	9,4	9,4	9,1	10,7	5,1	0,6		
15. HQ224958 *D. ranarum*	8,7	8,6	8,8	9,0	8,5	9,3	8,8	9,0	9,0	8,6	10,3	4,7	0,3	0,0	

For the phylogenetic analyses the topologies of both the BI and ML trees were similar. The analyses showed *Hemolivia stellata* [GenBank: KP881349] as a well-supported sister taxon to the *Hepatozoon* species cluster, with *H. sipedon* [GenBank: JN181157] shown to be a sister species to a well-supported monophyletic clade comprising *Hepatozoon* species isolated from anuran hosts. The *Hepatozoon* species isolated from African and North American anurans formed two well-supported monophyletic clades, respectively, and were separate from the European species *H. magna* [GenBank: HQ224960]. The African *Hepatozoon* clade represents a polytomy with *H. involucrum* and *H. tenuis*, forming a well-supported monophyletic clade and *H. ixoxo* and *H. theileri*, forming a poorly-supported clade, nested within this polytomy and separate to *H. thori*.

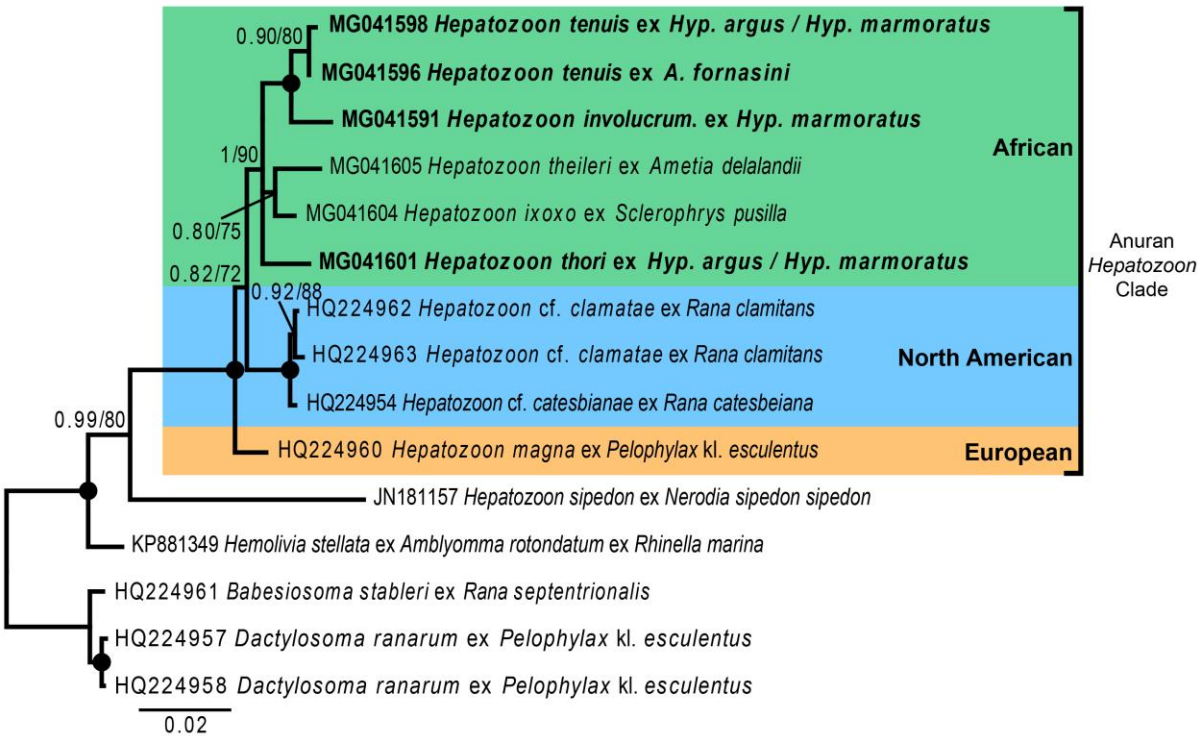

Fig. 7. Consensus phylogram of anuran haemogregarines based on 18S rDNA sequences. Tree topologies for both Bayesian inference (BI) and Maximum Likelihood (ML) analyses were similar (represented on the BI tree), showing the phylogenetic relationships for *H. involucrum*, *H. tenuis*, and *H. thori* (represented in bold), compared to other species of anuran *Hepatozoon* (with the exception of *Hepatozoon sipedon*), *Hemolivia*, and three species from the Dactylosomatidae as outgroup. Clades that neither produced 0.80 posterior probability (BI) or 70 bootstrap (ML) nodal support values were collapsed. Black circles represent 100% support for both BI/ML. The scale bar represents 0.02 nucleotide substitutions pre site.

Discussion

In the present study, we screened the peripheral blood of 225 individual frogs from nine species within the Hyperoliidae. Six species (*A. aureus*, *A. delicates*, *Hyp. tuberlinguis*, *Hyp. pusillus*, *K. senegalensis* and *P. maculatus*), totalling 205 specimens were found negative for haemogregarine parasites. Only 20 frogs from three species were found positive, namely *A. fornasini* (6/14), *Hyp. argus* (2/39), and *Hyp. marmoratus* (12/74).

Morphological and molecular data indicate that the haemogregarines parasitising these hosts represent three distinct species of *Hepatozoon*, herein described as *H. involucrum* parasitising *Hyp. marmoratus*; *H. tenuis*, parasitising *A. fornasini*, *Hyp. argus* and *Hyp. marmoratus*; and *H. thori* parasitising *Hyp. argus* and *Hyp. marmoratus*. Mature gamonts of *H. involucrum* are characterised by the prominent parasitophorous vacuole (PV) encircling the large gamont, as well as the recurved ends of both poles of the gamont. When compared to *H. tenuis*, the overall appearance and characteristics are similar, except for a difference in size of the gamont and PV. The interspecific divergence between these two species is 1.0 %. This has been shown in several studies to correspond to species-level differences in haemogregarines and for the slow evolving 18S rRNA marker (see Barta et al., 2012; Cook et al., 2015a; Borges-Nojosa et al., 2017). *Hepatozoon thori* can be distinguished from both *H. involucrum* and *H. tenuis* based on the distinctive hammer-like shape of the gamont's PV. The interspecific divergence between *H. thori*, *H. involucrum* and *H. tenuis* was 2.0 % and 1.8 % respectively.

The only other named species of *Hepatozoon* infecting a member of the Hyperoliidae is *H. hyperolii* described in an unidentified *Hyperolius* species by Hoare (1932), this parasite being vermicular in shape and folding over on itself within its host erythrocyte (see Fig 6A–C) and therefore does not conform to any of the *Hepatozoon* species of the present study. However, Ball (1967) reported a second, but unnamed species in *Hyperolius puncticulatus* from Amani, Tanzania, and this species conforms both in size and shape to *H. thori* (see Fig 6D–E). In the current study, we propose that these two species are the same, despite parasitising different hosts and possibly being geographically isolated. However, to confirm this, molecular data for this species from Amani, Tanzania is required.

In our phylogenetic analysis, *Hepatozoon* species isolated from anuran hosts formed a well-supported monophyly, separate to other closely related species of *Hepatozoon*. Furthermore, the African clade formed a monophyly, with *H. thori* separate from the other species within this clade. *Hepatozoon involucrum* and *H. tenuis* form a well-supported monophyletic clade nested within the larger African clade. With an interspecific divergence of 1.0 % (model-corrected distance), these two species are closely related, which concurs with their close morphological resemblance. *Hepatozoon ixoxo* and *H. theileri* form a less

well-supported (0.80/75) monophyletic group. The BI statistical information for the bipartitions of this group showed that apart from the 80 % probability support, only 13 % included *H. thori* as part of this clade and 9 % showed *H. theileri* formed a clade with *H. involucrum* and *H. tenuis*, thus explaining the low support of this group. Furthermore, *H. ixoxo* and *H. theileri* differ considerably in morphological structure (see Conradie *et al.* 2017), and if compared to the phylogenetic and morphological relationship of *H. involucrum* and *H. tenuis* (as mentioned above), the former two species are not expected to be sister species. This underlines the importance of increased taxon sampling for these parasites, as the addition of more species to this dataset could result in better-supported clades and the polotomy of the African clade could be resolved. Additionally, faster-evolving markers (e.g. mtDNA) may further explain the biogeography and evolutionary history of these species globally. However, to date, only one haemogregarine, *H. catesbianae* isolated from the frog *Rana catesbeiana* has mtDNA sequence data available (see Leveille et al., 2014). Although these markers (mtDNA) may be complementary in providing an evolutionary perspective among these parasite groups, a lot more data is required if we want to use similar sized datasets such as those available for 18S rDNA sequences for haemogregarines, especially in terms of vertebrate host diversity (amphibians, reptiles, fishes, birds and mammals) and geographical distribution.

This study highlights the importance of screening anurans from different families and genera in an effort to increase the known biodiversity of these parasites and types of hosts they infect. This study also shows the significance of providing detailed descriptions or reports of species, localities and host records, as we were able to link a species reported by Ball (1967) with *H. thori* in the current study based on the morphological details he provided. However, although morphological details are important, the use of them in combination with molecular tools provides a richer dataset with which to work, allowing us to infer historical relationships. Furthermore, if molecular data was available for all the currently recognised species of *Hepatozoon*, those with close morphological characteristics could be correctly distinguished. This stresses the importance of using both of these techniques in combination when describing species, and where possible to provide molecular data for already described species. Future research should, when possible, include faster evolving genes, identification of possible definitive hosts or vectors and life cycle studies.

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CHAPTER 2

A review of the Dactylosomatidae (Apicomplexa: Adeleorina: Dactylosomatidae), with the description of a new species of *Dactylosoma* sp. 1 and comments on the synonymy of *D. splendens* Labbé 1894 infecting frogs

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Abstract

Haemogregarine (Apicomplexa: Adeleiorina) blood parasites are commonly reported from anuran hosts. Dactylosomatidae (Jakowska and Nigrelli, 1955) is a small group of haemogregarines comprising the genera *Dactylosoma* Labbé, 1894 and *Babesiosoma* Jakowska and Nigrelli, 1956. Currently the genera *Dactylosoma* and *Babesiosoma* contain five recognised species of each. In the current study, a total of 643 anurans, comprising 38 species, 20 genera and 13 families were collected and their blood screened for the presence of dactylosomatid parasites, in South Africa (n = 618) and Belgium (n = 25). Three anuran species were found infected namely, *Ptychadena anchietae* (Bocage, 1868) and *Sclerophrys gutturalis* (Power, 1927) from South Africa, and *Pelophylax lessonae* (Camerano, 1882) from Belgium. Based on morphological characteristics, morphometrics and molecular findings a new dactylosomatid, *Dactylosoma* sp. 1 is described form *Pty*. *anchietae* and *Scl*. *gutturalis*. The species of *Dactylosoma* isolated from *Pel. lessonae* conforms morphologically with *Dactylosoma splendens* Labbé 1894, thus questioning the validity of *D. splendens* from synonymy with *D. ranarum* (Kruse, 1890). Phylogenetic analysis shows species of anuran *Dactylosoma* as a monophyletic group separate from the other haemogregarine groups. Additionally, the mosquitoes *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* Theobald, 1901 and *U*. (*Pfc.*) *montana* Ingram and De Meillon, 1927 were observed feeding on *Scl. gutturalis in situ* and their role as potential vectors for *Dactylosoma* sp. 1 is discussed. This study is the first to describe a dactylosomatid parasite based on morphological and molecular data as well as to provide evidence of a dipteran as a potential vector for these parasites.

Keywords: Anuran, Blood parasites, frog, haemogregarine, PCR, vectors, *Uranotaenia* sp., morphology

Introduction

Intracellular haemogregarine blood parasites are a diverse group of adeleorine coccidia (Apicomplexa: Adeleiorina). This group is currently divided into four families, namely Dactylosomatidae Jakowska and Nigrelli, 1955, Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908, and Karyolysidae Labbé, 1894. The discovery of the first intraerythrocytic blood parasite was by Chaussat (1850) who reported on an unidentified parasite in the blood of frogs from Europe. Later Vulpian (1854) and Lankester (1871) provided information on similar frog blood parasites. These findings, together with the discovery of malarial parasites by Laveran (1881), are considered to have laid the foundation of our understanding of the Apicomplexa today (see Jakowska and Nigrelli, 1955; Levine, 1971; Barta, 1991). The Dactylosomatidae is a small group of apicomplexan haemogregarines that currently comprises the genera *Dactylosoma* Labbé 1894 and *Babesiosoma* Jakowska and Nigrelli, 1956. The family was initially named Dactylosomidae, but Becker (1970) later emended the name to Dactylosomatidae. According to Barta (1991), the taxonomic affinities of members of this family have been uncertain since their original discovery. They were often placed within genera now considered *incertae sedis* (Barta, 1991). This uncertain taxonomic placement may be the reason why until recently information on the biology of these parasites, beyond light microscopic observations in the vertebrate host, was non-existent (Boulard et al., 1982; Barta and Desser, 1986; Barta, 1989, 1991). To date, the life cycles and transmission mechanisms of only two species have been elucidated, namely, *Babesiosoma mariae* (Hoare, 1930) from a fish host and leech vector, and *Babesiosoma stableri* Schmittner and McGhee, 1961 from a frog host and leech vector (see Barta and Desser, 1989; Negm-Eldin, 1998). Light microscopy constitutes the majority of observations on dactylosomatids and still plays an important role in documenting the development and parasitaemia of the parasite in its host. Members of this family have to date been recorded only in organisms with prolonged water contact, namely amphibian or fish hosts and leech vectors.

The hypothesis for this study was that anurans from South Africa would be parasitised by a higher diversity of dactylosomatid parasites as compared to anurans from Europe. This hypothesis based on the diversity of other anuran haemogregarines recently found in anurans from South Africa (Netherlands et al., 2018).

The aim of this study is to review our current knowledge on dactylosomatid parasites and provide a foundation for future taxonomic work. This will be achieved through the following objectives: (1) detailed literature review on current knowledge of this group; (2) providing a framework for the description of species, based on morphological and molecular

data; and (3) determining the species diversity and phylogenetic relationships of the dactylosomatid parasites observed in anurans from South Africa and Belgium.

Morphological and ultrastructural classification

Prior to ultrastructural studies (Boulard et al., 1982; Barta and Desser, 1986; Barta, 1991), dactylosomatids were considered closely related to the Haemosporida and Piroplasmida (Levine, 1971, 1984). Similarly, the piroplasm *Anthemosoma garnhami* Landau, Boulard and Housin, 1969 was first seen as part of the Dactylosomatidae (see Landau et al., 1969; Vivier and Petitprez, 1969; Levine, 1971; Boulard et al., 1982). Levine (1971) also included *Haemohormidium* Henry, 1910 and *Sauroplasma* du Toit, 1937 in this group. However, following the first ultrastructural study on *Dactylosoma ranarum* (Kruse, 1890) by Boulard et al. (1982), it was clear that species of *Dactylosoma* posses typical apicomplexan structures, and do not belong to the piroplasms. Dactylosomatids rather fit within the Coccidia and were subsequently moved to the order Eucoccidiorida (syn. Eucoccidiida) by Boulard et al. (1982), where they have remained (Barta, 1991). However, the genus *Babesiosoma* was still considered as a junior synonym of the genus *Haemohormidium* (see Levine, 1971), and was later transferred to the Haemohormidiidae Levine, 1984 a family erected by Levine (1984) for the “piroplasm-like” parasites *Haemohormidium* and *Sauroplasma*. Later, Barta and Desser (1986) showed *B. stableri* to have similar ultrastructural features to *D. ranarum* as reported by Boulard et al. (1982), such as a parasitophorous vacuole, complete apical complex and a trilaminate pellicle. The shape of the merozoites with a large Golgi body associated with the nucleus and lipid inclusions with their conoid shape, size, distribution of the micronemes and rhopteries are also similar to *D. ranarum*, with the main difference being the number of merozoites produced (see Barta and Desser, 1986). Based on these observations *Babesiosoma* was resurrected and transferred back to the Dactylosomatidae from the Haemohormidiidae (see Barta and Desser, 1986; Barta, 1991).

Phylogenetic placement

Barta (1989) completed a phylogenetic analysis of the class Sporozoea using phenotypic characters to infer evolutionary relationships. That study showed the piroplasms and adeleorine or adeleid coccidia to form a monophyletic group, separate from the eimeriid coccidia and haemosporidia. These findings showed that members of Dactylosomatidae exhibit characteristics similar between some adeleid blood parasites and piroplasms, such as similar development and replication within intestinal epithelial cells of its definitive invertebrate vector instead of in the haemocoel (Barta, 1989). Furthermore, certain characters of dactylosomatids seem to be shared between various distantly and closely

related blood inhabiting Apicomplexa. For example, intraerythrocytic development or asexual reproduction (exogenic merogony and binary fission) is a feature shared with various highly derived haemogregarines (*Haemogregarina* Danilewsky, 1885 (*sensu lato*) and *Cyrillia* Lainson, 1981), some piroplasms and all species of the Haemosporidia (see Smith et al., 2000). These findings were supported by related studies focused at phenotypic characters of haemogregarines (Siddall, 1995; Smith and Desser, 1997; Smith et al., 2000). The analysis of these characters grouped species that shared definitive hosts, implying that the definitive hosts of apicomplexan parasites may be an informative character as originally suggested by Barta (1989). O'Donoghue (2017) proposed that the environment in which the hosts occur could also play a role in these patterns, e.g. the parasites of terrestrial versus aquatic hosts and vectors forming monophyletic clades.

Although most molecular phylogenetic studies tend to support relationships inferred from morphological or biological life history data, the evolutionary relationships between apicomplexans are far from simple (O'Donoghue, 2017). A recent molecular phylogenetic study by Barta et al. (2012) on adeleorine coccidia using 18S rDNA sequences, showed that monoxenous adeleorine coccidia and heteroxenous haemogregarines (Hepatozoidae, Karyolysidae, Haemogregarinidae, and Dactylosomatidae) form a highly supported monophyletic group, separate from the Haemosporidia and Piroplasmida. However, the Haemosporidia and Piroplasmida appear to have closer affinities based on morphological or life history data, such as the shared developmental feature of syzygy (paring of gamonts) in the definitive host with the adeleorine coccidia (and haemogregarines) rather than with the enteric eucoccidia (O'Donoghue, 2017). On the other hand, species from the Haemogregarinidae and Dactylosomatidae that undergo sporogonic development within their definitive host or vector without the formation of a resistant oocyst structure, were shown to cluster together (Barta et al., 2012). These groups may have lost the need for such resistant oocysts as are transmitted via the bite of their vectors (Hayes et al., 2006; Barta et al., 2012), compared to the other haemogregarine families that are transmitted via ingestion of the definitive host. It is clear that additional taxon sampling, life cycle elucidation and molecular phylogenetic analysis using multiple or faster evolving genes may provide better support for these scenarios.

Dactylosoma

Members of *Dactylosoma* have undergone a fair number of taxonomic disputes, with *D*. *ranarum* being the first described species of the genus. Although Lankester (1871) is often given credit for the discovery of *D. ranarum* (see Saunders, 1960; Levine, 1971; Barta et al., 1987; Barta, 1991; Davies and Johnston, 2000), it is clear from the original

descriptions (Lankester, 1871, 1882) that he was working with a species of *Lankesterella* Labbé 1899 (initially known as "*Drepandidium*" erected by Lankester (1871, 1882), however, this name had already been used by Ehrenberg (1861) for a ciliate). Nöller (1913) clarified that the first description of *D. ranarum* was by Kruse (1890) who believed he was describing additional developmental stages of the "*Drepandidium*" parasite that in fact was *Lankesterella minima* (Chaussat, 1850). Kruse (1890) suggested that the parasite should be grouped with the haemogregarines, proposing the name *Haemogregarina ranarum* (Kruse, 1890) if the generic name "*Drepandidium*" was to fall away. Celli and San Felice (1891) also regarded it as *H. ranarum*, and Grassi and Feletti (1892) as a malarial parasite, due to its resemblance to human malaria. However, Labbé (1894) recognised differences within these genera, subsequently erecting *Dactylosoma* based on: (1) different elongated and amoeboid body forms; (2) areolar structure, vesicular nucleus and hyaline appearance of the protoplasm; (3) lack of pigment, but presence of retractile granules; (4) minimal effect on the host cell and nucleus; and (5) sporulation that gives rise to between 5 - 12 sporozoites grouped in a rosette- or fan-like appearance. Labbé (1894) named the species he placed in this newly erected genus, *Dactylosoma splendens* Labbé 1894, however, according to Wenyon (1926) who supported the designation of Nöller (1913), Kruse (1890) had first used the species name "*ranarum*" for this species, thus the parasite was synonymised to *Dactylosoma ranarum* (Kruse 1890).

Currently there are five recognised species of *Dactylosoma*, two of which infect fish hosts, namely *Dactylosoma Iethrinorum* Saunders, 1960, and *Dactylosoma salvelini* Fantham, Porter and Richardson, 1942 (see Table 1). The remaining three species are described from anuran hosts. The first, *Dactylosoma ranarum*, is the most common and apparently a cosmopolitan species reported in several anuran species. According to Barta (1991) this species has been recorded from *Pelophylax* kl. *esculentus* (Linnaeus, 1758) in France (Laveran, 1899), the Caucasus (Finkelstein, 1908), and Corsica (Boulard et al., 1982) and other countries within Europe (França, 1908; Nöller, 1913). Furthermore, this species has also been recorded from Central and South America in *Rhinella marina* (Linnaeus, 1758) (see Walton, 1946) and in an unidentified toad from Pará, Brazil (Durham, 1902). Additionally, there have been reports in Africa from *Pel*. kl. *esculentus* in Constantine, Algeria (Billet, 1904), *Pelophylax saharicus* (Boulenger in Hartert, 1913) from Morocco (Seabra-Babo et al., 2015), and *Pelophylax ridibundus* (Pallas, 1771) (syn. *R. ridibunda*) from North Africa (Walton, 1947, 1949), all included within the Palearctic realm. From the Ethiopian realm, hosts include *Hyperolius* sp. (syn. *Rappia marmorata*), *Hylarana galamensis* (Dumeril and Bibron, 1841) (syn. *R. galemensis*), *Ptychadena oxyrhynchus* (Smith, 1849) (syn. *R. oxyrhynchus*), *Ptychadena submascareniensis* (Guibé and Lamotte, 1953) (syn. *R. mascareniensis*) and *Sclerophrys regularis* (Reuss, 1833) (syn.

Amietophrynus regularis) from the Gambia (Dutton et al., 1907). It is clear from the illustrations provided, that the latter hosts were infected with several different blood parasites, all grouped as "*Drepandidia*" (Dutton et al., 1907; Walton, 1948). *Hylarana albolabris* (Hallowell, 1856) (syn. *Rana albolabris*) is reported as a host for *D. ranarum* from the current Democratic Republic of the Congo (Schwetz, 1930; Walton, 1947) in Central Africa, and *Scl*. *gutturalis* (syn. *Scl. regularis*) in South Africa (Fantham et al., 1942). The giant bullfrog, *Pyxicephalus adspersus* Tschudi, 1838 is also reported to be infected with *D. ranarum* from an unspecified location possibly in sub-Saharan Africa (Walton, 1947). Lastly, there are also reports from *Sylvirana guentheri* (Boulenger, 1882) from Tonkin, northern Vietnam (Mathis and Léger, 1911) and from Yung Foh Lee, Yang Ming Shan, Taiwan (Manwell, 1964). The type host and type locality for this species are *Pel.* kl. *esculentus*, possibly from Naples, Italy (Kruse, 1890). The second species, *Dactylosoma sylvatica* Fantham, Porter and Richardson, 1942 was described from the wood frog *Rana sylvatica* (LeConte, 1825) collected in the Province of Quebec, Canada. The third species is *Dactylosoma taiwanensis* Manwell, 1964, described from the alpine cricket frog, *Fejervarya limnocharis* (Gravenhorst, 1829) (syn. *Rana limnocharis*) in Hualien, Hua Lien Hsien, Taiwan (see Table 1). In addition to the summary of reported species of *Dactylosoma* mentioned above, Netherlands et al. (2015) reported on an unidentified species of *Dactylosoma* in *Ptychadena anchietae* (Bocage, 1868) from northern KwaZulu-Natal, South Africa.

Species previously regarded as members of *Dactylosoma* are *Babesiosoma mariae* (Hoare, 1930) and *Babesiosoma jahni* (Nigrelli, 1929). Two enigmatic species previously assigned to the genus *Dactylosoma*, *Dactylosoma tritonis* (Fantham, 1905) and *D. amaniae* (Awerinzew, 1914) are more likely inclusions of rickettsial organisms according to Levine (1988) and Barta (1991). *Dactylosoma clariae* (Haiba, 1962) (syn. *Cytauxzoon clariae* and *Haemohormidium clariae*) and *Dactylosoma tilapiae* Imam, Marzouk, Hassan, Derhall and Itman, 1985 were considered by Negm-Eldin (1998) to not contain sufficient proof to document the validity of these previously regarded species (Smit et al., 2003). Species of *Dactylosoma* are characterised by similar merogonic development as for species of *Babesiosoma*, with the exception of the morphologically distinct primary and secondary meronts and varying number of merozoites produced in these cycles. In summary, *D. ranarum* undergoes primary merogony when a merozoite enters the host frogs' erythrocytes. Within the erythrocytes, the merozoites undergo simultaneous peripheral budding, transforming into a large multinucleate meront producing up to 16 merozoites arranged in the characteristic rosette- or hand-like nature for which the genus was named (see Labbé, 1894; Nöller, 1913; Barta, 1991; Lainson, 2007). Merozoites then separate and penetrate other erythrocytes, either repeating the cycle of primary merogony or initiating secondary

merogony. The cycle is repeated in secondary merogony with meronts producing up to eight merozoites that either repeat secondary merogony or mature into gamonts (see Nöller, 1913; Barta et al., 1987). To date, none of the life cycles of species of *Dactylosoma* have been elucidated. However, Barta (1991) used frogs captured on the island of Corsica, France, to experimentally infect the North American glossiphoniid leech *Desserobdella picta* (Verrill, 1872), natural vector of *B. stableri* (see below) with *D. ranarum*. In his study, although no observations on the development of gametes or zygote formation was made, *D. ranarum* was found to undergo sporogonic development within the intestinal epithelium of this experimental leech host. The oocysts observed appear to be polysporoblastic producing 30 or more sporozoites by a process of exogenous budding directly into the cytoplasm of the epithelial cell (Barta, 1991). Other haematophagous invertebrates have also been considered as potential vectors. Nöller (1913) was the first to experimentally attempt transmission of *D. ranarum* using the glossiphoniid leech *Hemiclepsis margmata* (Müller, 1774) however, this was unsuccessful despite repeated attempts. Boulard et al. (1982) also tested the mosquito *Culicoides nubeculosus* (Meigen, 1830) as the potential vector of *D. ranarum*, but the experiments yielded no results. Although phlebotomine sand flies are the known vectors for different blood parasites of anurans (Feng and Chung, 1940; Desportes, 1942), none thus far have been tested as potential vectors for any species of *Dactylosoma*.

Babesiosoma

More than 60 years after the establishment of *Dacytylosoma*, *Babesiosoma* was erected to incorporate the species that: (1) contain less granular but more vacuolated cytoplasm; (2) have a nucleus similar to species of *Babesia*, without a definite karyosome; (3) reproduce by schizogony or binary fusion; and (4) do not produce more than four merozoites. Barta (1991) pointed out that certain of these characters, that, together, define *Babesiosoma*, are in general hard to unambiguously recognise. For example, (1) the less granular, more vacuolated cytoplasm evidently indicates more amylopectin inclusions; (2) ultrastructurally a definite karyosome is present in the nuclei; and (3) the multiplication through binary fusion is dubious as this was probably confused with mature paired merozoites after merogonic duplication. However, the separation of the two genera is still considered justified as species of *Babesiosoma* produce only four merozoites during each merogonic cycle and double the number of oocyst sporozoites (eight) in the definitive host (Barta, 1991) compared to species of *Dactylosoma* that produce up to 16 merozoites and double the number of oocyst sporozoites (Barta and Desser, 1986; Barta, 1991).

There are currently five recognised species of *Babesiosoma* (see Table 2), three of which are described from fish hosts (Smit et al., 2003), namely *Babesiosoma bettencourti*

(França, 1908), *Babesiosoma mariae* (syn. *Dactylosoma mariae*; *B. hannesi* Paperna, 1981) and *Babesiosoma tetragonis* Becker and Katz, 1965. The remaining two species are described from North American amphibian hosts. *Babesiosoma jahni* (syn. *Dactylosoma jahni*) is the type species of the genus, described from the Eastern newt host *Notophthalmus viridescens* (Rafinesque, 1820). *Babesiosoma stableri* was described from the northern leopard frog *Rana pipiens* Schreber 1782 and was experimentally transmitted to *Anaxyrus americanus* Holbrook, 1836, *A. woodhousii* (Girard, 1854), *A. terrestris* (Bonnaterre, 1789), *Lithobates catesbeiana* (Shaw, 1802), *L. septentrionalis* (Baird, 1854) and *L. clamitans* (Latreille, 1801) (see Table 2).

Species previously regarded as *Babesiosoma*, but formally transferred to other genera are *Haemohormidium aulopi* (Mackerras and Mackerras, 1925), *Haemohormidium ophicephali* (Misra, Haldar and Chakravarty, 1969) (syn. *Babesiosoma hareni*, *Babesiosoma batrachi*, *Dactylosoma striata*, *Dactylosoma notopterae*), and *Haemohormidium rubrimarensis* (Saunders, 1960) (see Siddall et al., 1994; Cook et al., 2015). Enigmatic taxa, removed from the genus *Babesiosoma* are *incertae sedis* (*Babesiosoma*) *anseris* Haiba and El-Shabrawy, 1967, *incertae sedis* (*Babesiosoma*) *gallinanun* Fahmy, Arafa, Mandour, Kalifa and Abdel-Salem, 1979, and *incertae sedis* (*Babesiosoma*) *ptyodactyli* El-Naffer, Abdel-Rahman, and Khalifa 1979 (see Barta, 1991). Furthermore *Babesiosoma aegypta* Mohamed, 1978, *Babesiosoma tilapiae* Imam, Marzouk, Hassan, Derhall and Itman, 1985, and *Babesiosoma aegyptiacus* Eid, Negm-Eldin and Imam, 1991 were considered by Negm-Eldin (1998) to not contain sufficient proof to document the validity of these previously regarded species (Smit et al., 2003).

Babesiosoma species are characterised by merogonic duplication within the vertebrate host and merogonic and sporogonic duplication in the invertebrate host or vector (see Jakowska and Nigrelli, 1956; Barta and Desser, 1986; Barta, 1991). In the vertebrate host, the development comprises primary and secondary merogony, with the exception of *B. mariae*. The latter species was shown by Negm-Eldin (1998) to contain a third merogonic cycle (tertiary merogony). *Babesiosoma stableri* undergoes primary merogony when a merozoite enters a vertebrate frog host's erythrocyte. Within the erythrocytes, the merozoites undergo nuclear division, transforming into a binucleate meront. These meronts then undergo a second nuclear division, ultimately forming the characteristic and tetranucleate cruciform meront (four merozoites arranged in a cross shape) typical of members of *Babesiosoma* (see Barta and Desser, 1986; Barta, 1991). The cruciform meront's merozoites then separate and penetrate other erythrocytes, either repeating the cycle of primary merogony or initiating secondary merogony. The secondary merogonic cycle is completed in the same manner as primary merogony ultimately producing secondary merozoites that either repeat secondary merogony or mature into gamonts (Barta

and Desser, 1986; Barta, 1991). For *B. mariae* in its fish host, the primary and secondary merogonic cycles are shorter compared to *B. stableri* and mature gamonts are produced from tertiary merozoites in the third merogonic cycle (Negm-Eldin, 1998). Mature gamonts are then ingested in the blood meal of the natural leech vector, *Desserobdella picta* and undergo syzygy, ultimately maturing into gametes that fuse to form an ookinete. The ookinete then penetrates intestinal epithelial cells to initiate sporogony. Mature sporozoites then make their way to the salivary glands, where they undergo similar merogonic replication as in the vertebrate host, producing merozoites that will repeat the merogonic cycle or enter the ductules of the salivary glands. When the leech takes its next blood meal, the merozoites are injected into its new vertebrate host (Jakowska and Nigrelli, 1956; Barta and Desser, 1986; Barta, 1991).

Table 1. Recognised *Dactylosoma* species, host records, distribution, and morphometrics.

Species	Host(s)	Distribution	Morphometrics (given in µm). * Measurements obtained from figure scale bar. Primary Merogony:	Secondary Merogony:	References
Dactylosoma sp. 1	*Ptychadena anchietae* (plain grass frog) and *Sclerophrys gutturalis* (guttural toad)	KwaZulu-Natal, South Africa	Trophozoites: 5.3–7.7 × 2.6–4.4 Meronts: 8.3–12.2 × 5.1–8.0 Merozoites: 5.0–6.6 × 1.8–3.2	Meronts: 5.6–8.6 × 4.4–6.9 Merozoites: 4.2–5.5 × 1.8–3.5 Gamonts: 7.8–15.0 × 1.5–3.0	Current study
Dactylosoma sp. (*splendes*)	*Pelophylax lessonae*	Rijmenam, Het Ven, Belgium	Trophozoites: 5.3–7.4 × 2.6–4.9 Meronts: 8.4–16.5 × 7.1–12.6 Merozoites: 3.1–5.0 × 1.9–3.1	Meronts: 5.9–8.9 × 5.8–8.5 Merozoites: 4.1 × 2.3 Gamonts: 7.9–12.1 × 2.2–2.5	Current study
Dactylosoma lethrinorum Saunders, 1960	*Lethrinus nebulosus* (spangled emperor), and *L. lentjan* (Pink ear emperor).	Waters of the Red Sea near Al Ghardaqa, Egypt	Meronts: 8.0 × 10.5. Merozoties: 1.9 × 2.4		Saunders, 1960
Dactylosoma ranarum (Kruse, 1890) syn. *Dactylosoma splendes* Labbé, 1894	*Pelophylax* kl. *esculentus*		Trophozoites: 3.0–4.0 × 1.5–2.0 Meronts: 10–15 × 2.0–3.0 Merozoites: 2.8 × 0.7 Meronts: 7.3 × 4.3 Merozoites: 4.3 × 1.3	Meronts: 9.0 × 4.0. Merozoites: 2.0–3.0 × 1.0–1.5 Gamonts: 5.0–8.0 × 1.5–3.0 Meronts: 4.7 × 3.4 Merozoites: 3.4 × 0.9 Gamonts: 7.0 × 3.4	Kruse, 1890; Barta et al. 1987
Dactylosoma salvelini Fantham, Porter and Richardson, 1942	*Salvelinus fontinalis* (brook trout).	Province of Quebec, Canada		Meronts: 5.8–8.5 × 3.7–7.0 Gamonts: 4.4–7.8 × 1.5–3.0	Fantham et al. 1942
Dactylosoma sylvatica Fantham, Porter and Richardson, 1942	*Rana sylvatica* (wood frog).	Province of Quebec, Canada	Trophozoites: 7.0–8.5 × 6.3–7.6 Meronts: 7.4–11.5 × 7.0–9.3	Trophozoites: 4.4 × 3.0 Meronts: 5.2 × 4.0 Merozoites: 4.4–5.9 × 1.1–2.0 Gamonts: 7.0–12.6 × 1.5–3.0	Fantham et al. 1942
Dactylosoma taiwanensis Manwell, 1964	*Fejervarya limnocharis* (alpine cricket frog)	Hualien, Hua Lien Hsien, Taiwan	*Trophozoites: 3.9 × 7.3	* Meronts: 6.9–7.9 × 5.6–7.3 * Gamonts: 11.8–13.6 × 2.1–2.9	Manwell, 1964

Table 2. Recognised *Babesiosoma* species, host records, distribution, and morphometrics.

Species	Synonym (s)	Host(s)	Distribution	Morphometrics (given in µm)	References
Babesiosoma bettencourti (França, 1908)	*Haemogregarina bettencourti* França, 1908; *Desseria bettencourti* Siddall, 1995	*Anguilla anguilla* (European eel)	In the Ancora, Este, Febros and Olivas river systems near Alcobaça, Portugal	Single meronts: 2.0–6.0 × 0.8 –4.0 Meronts: 4.0–6.0 × 0.5–2.0 (Cruciform) Merozoits: 2.0–3.0 × 0.8–1.5 Gamonts: 3–10 × 1–4	Cruz and Davies, 1998
Babesiosoma mariae (Hoare, 1930)	*Dactylosoma mariae* Hoare, 1930; *Babesiosoma hannesi* Paperna, 1981	*Haplochromis nubilus* (blue victoria mouthbrooder); *H. cinereus*, *H. serranus* and *H*. sp. (Pisces: Teleostei). *Oreochromis esculentus* (singida tilapia); *Serranochromis angusticeps* (thinface largemouth tilapia)	Victoria Nyanza, near Entebbe, Uganda.	Merozoites: 2.8 × 0.9. Trophozoites: 3.8–5.7 × 1.9–5.0. Gamonts: 6.6–8.5 × 1.9–3.5 (Crescent); 5.7–6.6 × 3.8–5.7 (Rounded)	Hoare, 1930
Babesiosoma tetragonis Becker and Katz, 1965 (Holotype US National Museum, No. 60066)	*Haemohormidium tetragonis* (Becker and Katz, 1965) Laird and Bullock, 1969	*Catostomus* sp.	Shasta River, Montague, California, USA.	Trophozoites: 2.2–3.7 × 4.4–6.6 (Elliptical); 3.3–4.8 × 3.8–5.5 (Oval) Meronts: 3.0–3.9 × 5.5–6.7 (Tetranueleate); 5.5–6.9 × 5.8–7.4 (Cruciform); Merozoites: 1.4–2.6 × 2.6–5.3; Gamonts: 3.0–4.8 × 6.1–8.2 (Macro); 1.6–2.8 × 4.9–7.2 (Micro); Extracellular merozoites: 1.5–3.1 × 2.3–3.4;	Becker and Katz, 1965
Babesiosoma jahni (Nigrelli, 1929)	*Haemohormidium jahni* (Nigrelli, 1929) Laird and Bullock, 1969; *D. Jahni* Nigrelli, 1929	*Notophthalmus viridescens* (eastern newt)	Pennsylvania, USA		Nigrelli, 1929; Jakowska and Nigrelli, 1955
Babesiosoma stableri Schmittner and McGhee, 1961	*Haemohormidium stableri* (Schmittner and McGhee, 1961) Laird and Bullock, 1969	*Rana pipiens pipiens* and experimental infection to *Rana catesbeiana, Rana septentrionalis, Rana clamitans, Bufo americanus, Bufo woodhousei, and Bufo terrestis*	Purchased from Steinhilber and Co. Inc., Wisconsin, North America.	Trophozoites: 6.6 × 2.3 Immature binucleate stage: 7.0 × 2.3 Meronts: 7.6 × 2.4 (Binucleate) Meronts: 6.8 × 2.4–3.8 (Tetranueleate) Merozoites: 3.6 × 1.7 Gamonts: 6.1–10.3 × 2.0–2.7	

Materials & Methods

Sample collection:

A total of 643 anurans, belonging to 38 species of 20 genera from 13 families were collected at night using active sampling methods and their blood screened for the presence of dactylosomatid parasites. The majority of the samples were collected from South African anurans (n = 618) with the remaining samples collected from European anurans (n = 25). Anurans were collected from several sampling localities in South Africa and Belgium (see Dataset S1). This study received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5). Ezemvelo KZN Wildlife issued research permits OP 526/2014, OP 839/2014, OP 4374/2015, OP 4092/2016, and OP 4085/2017. Agentschap Natuur & Bos issued the permit (ANB/BL/FF-V17-00091) for collection and sampling of amphibians in Belgium, with strict collection protocol followed as instructed by the permit office. Furthermore, authors (ECN, CAC, and LHdP) responsible for sample collection and processing of specimens have undergone specialised training in ethical handling of aquatic ectotherms (NWU Ectothermic Vertebrates Handling and Ethics).

Light microscopy screening:

Blood was collected and processed following standard methods (Netherlands et al., 2015; Netherlands et al., 2018). Stained blood smears were screened at 1000× using a Nikon Eclipse E100 compound microscope and images of parasite stages captured and measured using the imaging software NIS Elements Ver. 4.60. Trophozoites, primary meronts and merozoites, and secondary meronts, merozoites and gamonts were measured per dactylosomatid species. Measurements comprised the parasite's length and width. Measurements are in micrometres (µm), with the average, standard deviation and range given. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear.

DNA extraction, PCR amplification and phylogenetic analyses:

Ethanol preserved blood samples of individual anurans parasitised with dactylosomatids (n = 11) were used for molecular work. Samples were selected based on a high intensity of infection as well as if individuals were not infected with multiple blood parasites (i.e. *Hepatozoon* sp.). Genomic DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of PCR products were all completed following the methods detailed in

Netherlands et al. (2018). The software package Geneious R11 (http://www.geneious.com (Kearse et al., 2012)) was used to assemble and edit resultant sequence fragments. Species identity was verified against previously published sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Sequences obtained were deposited in the NCBI GenBank database under the accession numbers [GenBank: XXX].

Sequences for species of *Hemolivia* Petit, Landau, Baccam & Lainson, 1990, *Hepatozoon* Miller, 1908, *Karyolysus* Labbé, 1894, *Haemogregarina*, and *Dactylosoma* (parasitising amphibian, avian, reptilian and mammalian hosts) were downloaded from GenBank and aligned to the sequences generated in this study. As in previous studies on haemogregarines *Adelina dimidiate* Schneider, 1875, *Adelina grylli* Butaeva, 1996 [GenBank: DQ096835–DQ096836] and *Klossia helicina* Schneider, 1875 [GenBank: HQ224955], from the suborder Adeleiorina were selected as outgroup. Sequences were aligned using the Clustal W 2.1 alignment tool (Larkin et al., 2007) implemented within Geneious R11. GBlocks was used to remove any alignment gaps and ambiguities (Castresana, 2000; Talavera and Castresana, 2007). The final alignment contained 43 sequences at a length of 1637 nt. A model test was performed to determine the most suitable nucleotide substitution model, according to the Bayesian information criterion (BIC), using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). The model with the best BIC score was the General Time Reversible (Tavaré, 1986) model, with estimates of invariable sites (0.5100), and a discrete gamma distribution (gamma shape 0.7840) (GTR + I + Γ). Phylogenetic relationships were inferred via Bayesian inference (BI) analysis using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001), and Maximum likelihood (ML) analysis using RAxML 7.2.8. (Stamatakis, 2014), implemented in Geneious R11. For the BI analysis, the Markov Chain Monte Carlo (MCMC) algorithm was run for 1 million generations, sampling every 100 generations. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. The Tracer tool (implemented from within Geneious R11) was used to assess convergence and the 'burn-in' period (Rambaut et al., 2018). For the ML analysis, the GTR + I + Γ model was used and nodal support was assessed using 1000 rapid bootstrap inferences. Furthermore model-corrected (GTR + I + Γ) genetic distances for all the sequences used in the phylogenetic analysis were also calculated in PAUP version 4.0a152 (Swofford, 2002).

Vector and life history evaluation:

During anuran sampling, care was taken to search for potential haematophagous invertebrate vectors. When possible, following sampling, screening and releasing of collected individuals, sites that were identified to harbour larger numbers of infected

individuals were revisited. The behaviour of anurans from these localities was documented *in situ*, via sedentary observations with an infrared light (not to disturb the host or vectors). Macrophotography was also used to capture images of any possible haematophagous invertebrates feeding on the anurans. Haematophagous invertebrates observed feeding were collected using an aspirator. Specimens were either fixed in 70% alcohol to be identified later or dissected (to separate relevant organs and body parts) and smeared on a glass slide to screen for possible blood parasite development. If possible, the individual anuran that the haematophagous invertebrate was feeding on, was also collected to be screened for blood parasites. In November 2017 and January 2018, whilst collecting *Scl. gutturalis* (Dataset S1) for a separate study on microfilarial nematodes (unpublished data) in Sodwana Bay, site SB-1 (S27.488591°; E32.664259°), mosquitoes were observed feeding on calling *Scl. gutturalis* and collected using an aspirator. Collected mosquitoes housed in plastic jars (350 ml) with moist cotton wool were transported back to the NWU frog lab, to keep humidity levels up and provide mosquitoes with a source of water when necessary. Mosquitoes were euthanized between 1–7 days post-infection (dpi) with carbon dioxide (CO_2), identified using Jupp (1996) and dissected under a stereomicroscope using modified entomology pins. The mosquito's intestines and fat bodies were subsequently smeared on a glass slide using the same methods for the blood smear preparation.

Results

From 643 anurans screened for dactylosomatids, 71 were found parasitised with haemogregarines conforming morphologically to representatives of *Dactylosoma*, based on the hyaline appearance of the different elongated and amoeboid forms occurring in erythrocytes. Three species were found infected namely, *Ptychadena anchietae* (61/160) and *Sclerophrys gutturalis* (9/73) from South Africa, and *Pelophylax lessonae* (1/14) from Belgium.

Species descriptions:

Phylum: Apicomplexa Levine, 1970
Class: Conoidasida Levine, 1988
Subclass: Coccidiasina Leuckart, 1879
Order: Eucoccidiorida Léger, 1911
Suborder: Adeleina Léger, 1911
Family: Dactylosomatidae (Jakowska and Nigrelli, 1955)
Genus: *Dactylosoma* Labbé, 1894

***Dactylosoma* sp. 1**

Type-host: *Ptychadena anchietae* (Bocage, 1868) (Anura: Ptychadenidae).

Other host: *Sclerophrys gutturalis* (Power, 1927) (Anura: Bufonidae).

Site in host: Peripheral blood.

Vector: Possible vectors (see below): the mosquitoes *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U*. (*Pfc.*) *montana* (Culicinae: Uranotaeniini); and an African phlebotomine, *Grassomyia* sp.

Type-locality: Ndumo Game Reserve, KZN, South Africa (S26.926179°; E32.332416°).

Other localities: Sodwana, KZN, South Africa (S27.488591°, E32.664259°)

Type-material: Hapantotype, 1 × blood smear with a parasitaemia of 5.7% from *Pty. anchietae* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa, under accession number [NMB P XXX]; parahapantotype, 2 × blood smears: 1 × *Pty. anchietae* and 1 × *Scl. gutturalis* with a parasitaemia of 2.0% and 0.2 %, respectively deposited in the Protozoan Collection of the National Museum, Bloemfontein, South Africa, under accession number [NMB P XXX]. Parahapantotype, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

Representative DNA sequences: The 18S rRNA gene sequences have been submitted to the GenBank database under the accession numbers XXX. The model-corrected genetic distance between the species of *Dactylosoma*, ranged between 0.2 – 0.5%.

Description

Two distinct stages of merogony present in erythrocytes and occasionally in thrombocytes and monocytes of infected *Pty. anchietae* (Fig 1A-L) and *Scl. gutturalis* (Fig 2A-L). The first stage characterised by large meronts producing up to approximately 12 merozoites. The second stage meront, seemingly smaller, produces up to six merozoites. The general appearance of the cytoplasm is hyaline yet coarse. Parasitaemia of infected individuals (n = 70) given in percentage (%) was 0.4 ± 1.2 (0.0 – 5.7).

Primary merogony: Young trophozoites (Fig 1A, 2A), rare, observed within erythrocytes, ovoid crescent shape, largely non-stained except peripherally. Nuclei not clearly defined, located at blunt end (broader end of parasite). Condensed chromatin staining deep magenta (Fig 1A arrowhead). Measuring 4.2 ± 0.6 (3.6 – 4.9) long × 2.9 ± 0.8 (1.8 – 3.7) wide (n = 4).

Trophozoites (Fig 1B-D, 2B,C) elongated to oval, usually tapering towards one end, distinct vacuoles frequently present (Fig 1B-D, 2B-C arrow). Small round dense nuclei located at

blunt end, chromatin staining deep magenta (Fig 1B-D and Fig 2B, arrowhead). Trophozoites measure 6.7 ± 2.2 (5.3 – 7.7) long × 3.5 ± 1.2 (2.6 – 4.4) wide (n = 20).

Young primary meronts (Figs 1E, 2E) large ovoid to round shape, causing slight displacement of the host cell nucleus, distinct vacuoles frequently present (Fig 1E arrow). Multinucleate, nuclei division located peripherally, chromatin staining bright deep magenta (Fig 1E arrowhead). Young meronts measure 7.8 ± 0.9 (6.3 – 9.5) long × 5.7 ± 1.3 (4.0 – 8.3) wide (n = 20).

Primary meronts (Fig 1F,G) varying in form, round to crescent shape, causing slight distortion or displacement of the host cell or nucleus, cytoplasm staining whitish-purple. Multinucleate, normally showing more than 10 nuclei located peripherally (Fig 1F,G arrowhead), chromatin staining deep magenta. Meronts measure 9.9 ± 1.2 (8.3 – 12.2) long × 6.9 ± 0.8 (5.1 – 8.0) wide (n = 10).

Primary merozoites (Fig 1H) elongate to ovoid, hyaline cytoplasm staining pinkish-purple. Small round dense nuclei located at blunt end, chromatin staining deep magenta (Fig 1H, arrowhead). Merozoites measure 5.6 ± 0.5 (5.0 – 6.6) long × 2.7 ± 0.4 (1.8 – 3.2) wide (n = 16).

Secondary merogony: Young secondary meront (Fig 2E) irregular ovoid to round shape, cytoplasm staining whitish-purple, causing distortion of the host cell and slight displacement of the host cell nucleus. Multinucleate, nuclei located peripherally, chromatin staining bright pink to dark purple (Fig 2E, arrowhead). Young meronts measure 5.4 ± 0.5 (4.3 – 6.3) long × 4.6 ± 0.6 (3.4 – 5.8) wide (n = 19).

Secondary meronts (Figs 1I, 2F-H) differ in form, ovoid to quadrilateral shape, mature forms have dactylate (hand-like) appearance (Fig 2G,H), cytoplasm staining dark purple. Multinucleate, normally between 4 and 6 nuclei located peripherally (Figs 1I, 2F-H arrowhead), chromatin staining deep magenta. Secondary meronts measure 7.2 ± 0.8 (5.6 – 8.6) long × 5.7 ± 0.9 (4.4 – 6.9) wide (n = 16).

Secondary merozoites (Fig 2G-H, L arrow) elongate to ovoid, hyaline cytoplasm staining purple. Small round dense nuclei located closer to the centre, chromatin staining deep magenta (Fig 2G-H, L arrowhead). Secondary merozoites measure 4.8 ± 0.5 (4.2 – 5.5) long × 2.3 ± 0.5 (1.8 – 3.5) wide (n = 11).

Gamonts (Figs 1J-L, 2I-K) elongate and slender, often tapering to one end. In some cases slight displacement of the host cell nucleus is visible. Immature forms largely non-staining, except peripherally - staining dark purple (Fig 1J). Intracellular mature gamont forms have a hyaline appearance, although nuclei not clearly defined, nucleoplasm conglomeration visible off centre closer to blunt side (Fig 1K). Extracellular forms elongate often with slight curvature, nuclei clearly visible slightly off centre, staining dark purplish-pink. Gamonts measure 10.6 ± 2.5 (7.8 – 15.0) long × 2.2 ± 0.3 (1.5 – 3.0) wide (n = 40).

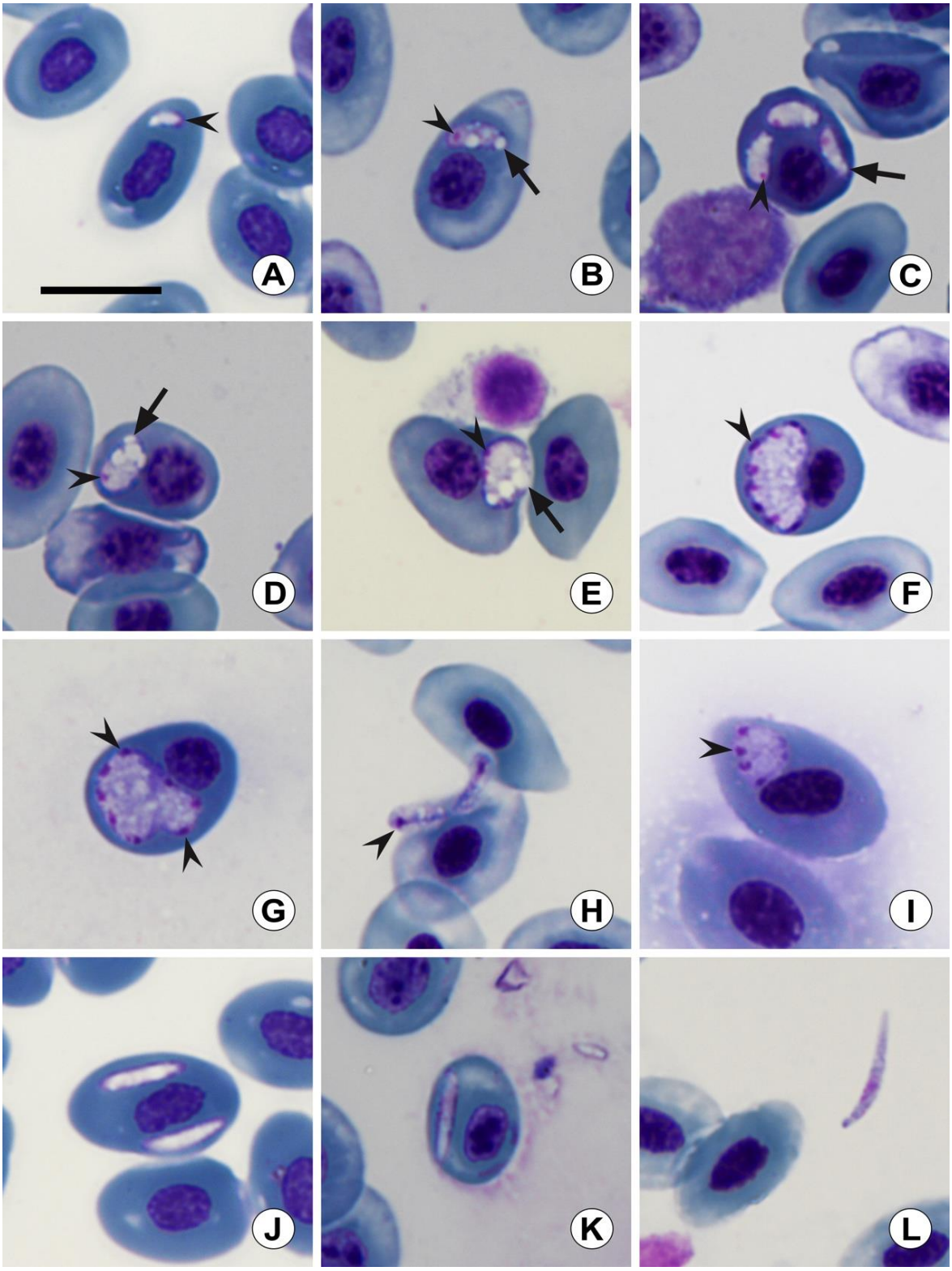

Fig. 1. (A-L). *Dactylosoma* sp. 1 from the grass frog *Ptychadena anchietae*. (A-H) Primary merogony. (A) Young trophozoite. (B-D) Trophozoites. (E) Young meront. (F-G) Primary meronts. (H) Merozoites. (I-L) Secondary merogony. (I) Secondary meront. (J) Immature gamont. (K) Gamont. (L) Extracellular gamont. Arrowheads show condensed chromatin (A-I); arrows show vacuoles (B-E). All images captured from the deposited slides [XXX]. Scale bar 10 µm.

Remarks

Dactylosoma sp. 1 can be characterised by its slender trophozoite and small merozoite stages; the number of primary (up to 14) and secondary (up to six) merozoites produced by meronts; and the vacuolated trophozoite stages. This species can be distinguished from all currently recognised species of *Dactylosoma* from anuran hosts, namely *D. ranarum*, *D. sylvatica*, and *D. taiwanensis*, based on several morphometric comparisons. Morphologically this parasite conforms closest to *D. sylvatica* with measurements overlapping across trophozoite, meront, merozoite and gamont stages. However, only a single, most probably the secondary merogonic cycle is reported, with meronts only producing up to eight merozoites. In comparison the number of merozoites produced by *Dactylosoma* sp. 1 differs in primary and secondary merogony with up to 14 or six merozoites observed in the meronts from the current species, respectively. Although measurements do overlap, *Dactylosoma* sp. 1 seems to possess generally more slender trophozoite stages and smaller merozoite stages. Also, *D. sylvatica* is reported from the wood frog *Rana sylvatica* from Canada. *Dactylosoma ranarum* is apparently a cosmopolitan species recorded from several hosts across Europe, Central and South America, Africa, and Asia. Based on morphometric data from the original description of *D. ranarum*, as well as from a more recent ultrastructural study of this species' morphology, several stages differ in size when compared to *Dactylosoma* sp. 1 (see Table 1). The most notable differences as compared to the relatively smooth to finely vacuolated cytoplasm of *D. sylvatica* and *D. ranarum* are the largely vacuolated trophozoite stages seen in *Dactylosoma* sp. 1 (Figs 1B,D-E; 2B-C arrow). The third species, *D. taiwanensis*, described from the alpine cricket frog *Fejervarya limnocharis* from Taiwan, is similar to *Dactylosoma* sp. 1 in that the trophozoite stages of both species contain vacuoles, the cytoplasm appears to be relatively coarse, and several measurements seem to overlap based on the scale provided. However, *D. taiwanensis*, similar to *D. sylvatica*, has meronts producing up to only eight merozoites. Likewise, the merozoites produced by *D. taiwanensis* are morphologically different to those of *Dactylosoma* sp. 1, the nuclei seem to be larger and more prominent in comparison to the rest of the merozoites.

Based on the comparisons of *Dactylosoma* sp. 1 to the currently recognised species, it is clear these parasites share close or overlapping morphology between species, and since dactylosomatids possess several life stages in the vertebrate host, species can easily be misidentified. For example, gamont stages between the different species have largely comparable morphology and morphometrics. However, after careful review, screening a large number of hosts, comparing the stages of observed in different individuals from different sites and seasons, we suggest *Dactylosoma* sp. 1 is a new species (note – PhD disclaimer).

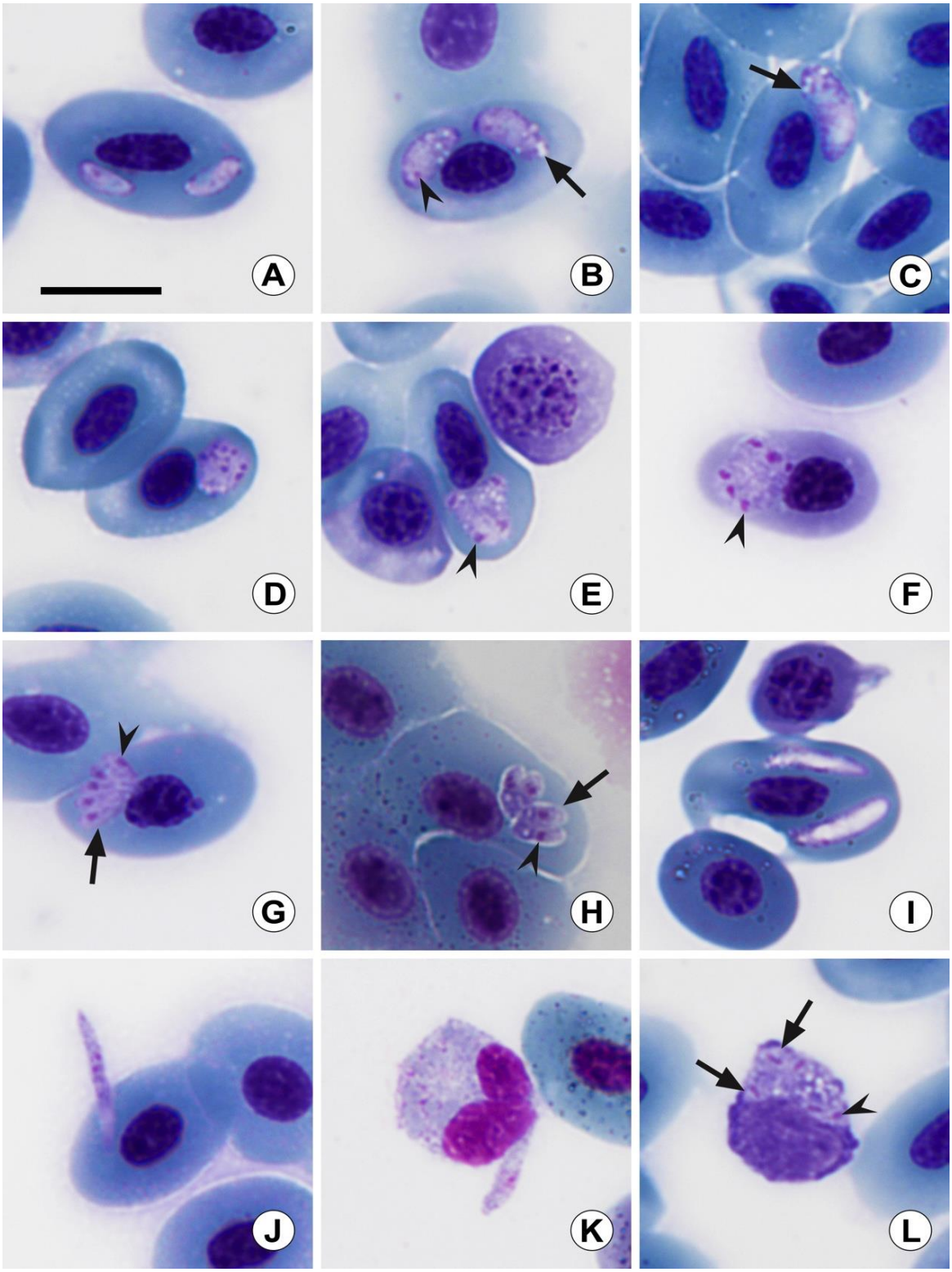

Fig. 2. (A-L). ***Dactylosoma*** **sp. 1 from the guttural toad** ***Sclerophrys gutturalis.*** (A-D) Primary merogony. (A) Young trophozoite. (B-C) Trophozoites. (D) Young meront. (E-L) Secondary merogony. (E) Young secondary meront. (F-H) Secondary meront. (G-H) Secondary merozoites. (I) Gamont. (K) Extracellular gamont. (L) Secondary meront in leukocyte. Arrowheads show condensed chromatin (B,E-H, L); arrows show vacuoles (B-C) and merozoites (G-H, L). All images captured from the deposited slides [XXX]. Scale bar 10 µm.

***Dactylosoma* sp. and comments on suggested resurrection of *Dactylosoma splendens* Labbé 1894 from synonymy with *D. ranarum* (Kruse, 1890).**

(Note this is not a valid nomenclatural change, see PhD disclaimer).

Host: *Pelophylax lessonae* (Camerano, 1882) (Anura: Ranidae).
Other hosts: *Pelophylax* kl. *esculentus* (Linnaeus, 1758) (Anura: Ranidae).
Site in host: Peripheral blood.
Vector: Unknown.
Type-locality: Paris, France (Labbé, 1894)
Other-localities: Rijmenam, Het Ven, Belgium (N51.010440°, E4.593420°).
Voucher material: Hapantotype, deposited in the Protozoan collection of the National Museum, Bloemfontein, South Africa, under accession number [NMB P XXX]. Parahapantotype, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].
Representative DNA sequences: The 18S rRNA gene sequences have been submitted to the GenBank database under the accession numbers XXX. The model-corrected genetic distance between the species of *Dactylosoma*, ranged between 0.2 – 0.5%.

Description

Two distinct stages of merogony present in erythrocytes of *Pel. lessonae* (Fig 3A-L). The first stage characterised by large dactylate meronts producing up to approximately 14 clear merozoites. The second stage meronts, seemingly smaller, although no clear merozoites were observed. The cytoplasm has a hyaline appearance. Parasitaemia of infected individual (n = 1) was 0.3 %.

Primary Merogony: Trophozoites (Fig 3A) elongated to oval, usually tapering towards one end, vacuoles in some cases present (Fig 3A arrow). Small round dense nuclei located at blunt end (Fig 3A arrowhead), chromatin staining deep magenta. Trophozoites measure 6.3 ± 0.9 (5.3 – 7.4) long × 3.8 ± 0.8 (2.6 – 4.9) wide (n = 10).
Young primary meronts (Fig 3B) round to ovoid causing slight displacement of the host cell nucleus. Multinucleate, nuclear division located peripherally, chromatin staining bright pink to dark purple (Fig 3B arrowhead). Young meronts measure 9.4 ± 1.1 (8.0 – 10.9) long × 8.0 ± 0.5 (7.0 – 8.6) wide (n = 7).

Primary meronts (Fig 3C-G) vary in form, round to pear shape, in some cases causing slight distortion or displacement of the host cell or nucleus, cytoplasm staining whitish-purple. Mature forms have dactylate appearance (Fig 3F-G). Multinucleate, normally

showing more than 10 nuclei (Fig 3C-E arrowhead), staining deep magenta. Meronts measure 11.3 ± 2.8 (8.4 – 16.5) long × 9.1 ± 1.9 (7.1 – 12.6) wide (n = 10).
Primary merozoites (Fig 3F-H) elongate to ovoid, hyaline cytoplasm staining white to bluish-purple. Dense nuclei chromatin centrally located, staining deep magenta (Fig 3F-H, arrowhead). Merozoites (Fig 3F-H, arrow) measure 4.0 ± 0.6 (3.1 – 5.0) long × 1.9 ± 0.6 (1.1 – 3.1) wide (n = 20).

Secondary merogony: Young secondary meront (Fig 3I) irregular ovoid shape, cytoplasm staining bluish-purple, occasionally causing displacement of the host cell nucleus. Multinucleate, nuclei located peripherally, staining deep magenta (Fig 3I, arrowhead). Young meronts measure 6.2 ± 1.1 (4.5 – 7.5) long × 4.5 ± 1.5 (2.7 – 7.3) wide (n = 7).
Secondary meronts (Fig 3J) ovoid shape, cytoplasm stains dark bluish-purple. Multinucleate, up to 6 nuclei located peripherally, chromatin staining dark purple (Fig 3J, arrowhead). Secondary meronts measure 7.7 ± 0.9 (5.9 – 8.9) long × 6.6 ± 1.1 (5.8 – 8.5) wide (n = 10).
Secondary merozoites (Fig 3K) rare, short crescent shape, hyaline cytoplasm staining purple. Small round dense nuclei located peripherally, staining deep magenta (Fig 3K arrowhead). Secondary merozoites measure 4.1 long × 2.3 wide (n = 1).

Gamonts (Fig 3L) elongate and slender shape, hyaline appearance, nuclei chromatin visible slightly off centre (Fig 3L arrowhead). Extracellular forms elongate often with slight curvature, nucleoplasm visible slightly off centre, staining dark purplish-pink. Gamonts measure 9.8 ± 1.8 (7.9 – 12.1) long × 2.3 ± 0.1 (2.2 – 2.5) wide (n = 5).

Remarks

Morphologically this species of *Dactylosoma* can be characterised by its round young meront stages, the dactylate appearance of the mature meront stages, and the hyaline appearance of the cytoplasm. Although, the trophozoite and gamont stages of *Dactylosoma* sp. 1 are largely similar in morphology and morphometrics, primary and secondary meront stages are wider (primary meront: 5.1 – 8.0 *vs* 7.1 – 12.6; secondary meront: 4.4 – 6.9 *vs* 5.8 – 8.5) and secondary merozoite stages slightly shorter (4.2 – 5.5 *vs* 4.1) in the *Dactylosoma* sp. from *Pel. lessonae*. This species also conforms closely to *D. sylvatica*, however this species of *Dactylosoma* produces more than eight merozoites in primary merogony and less than eight in secondary merogony. Also the secondary meront stages are wider as compared to *D. sylvatica*. Furthermore this species of *Dactylosoma* possesses several phenotypic characteristics mentioned in the original description of *Dactylosoma splendens*, later synonymised with *D. ranarum*, such as the round shape of the meronts and number of primary and secondary merozoites produced (ranging approximately between 12 and five).

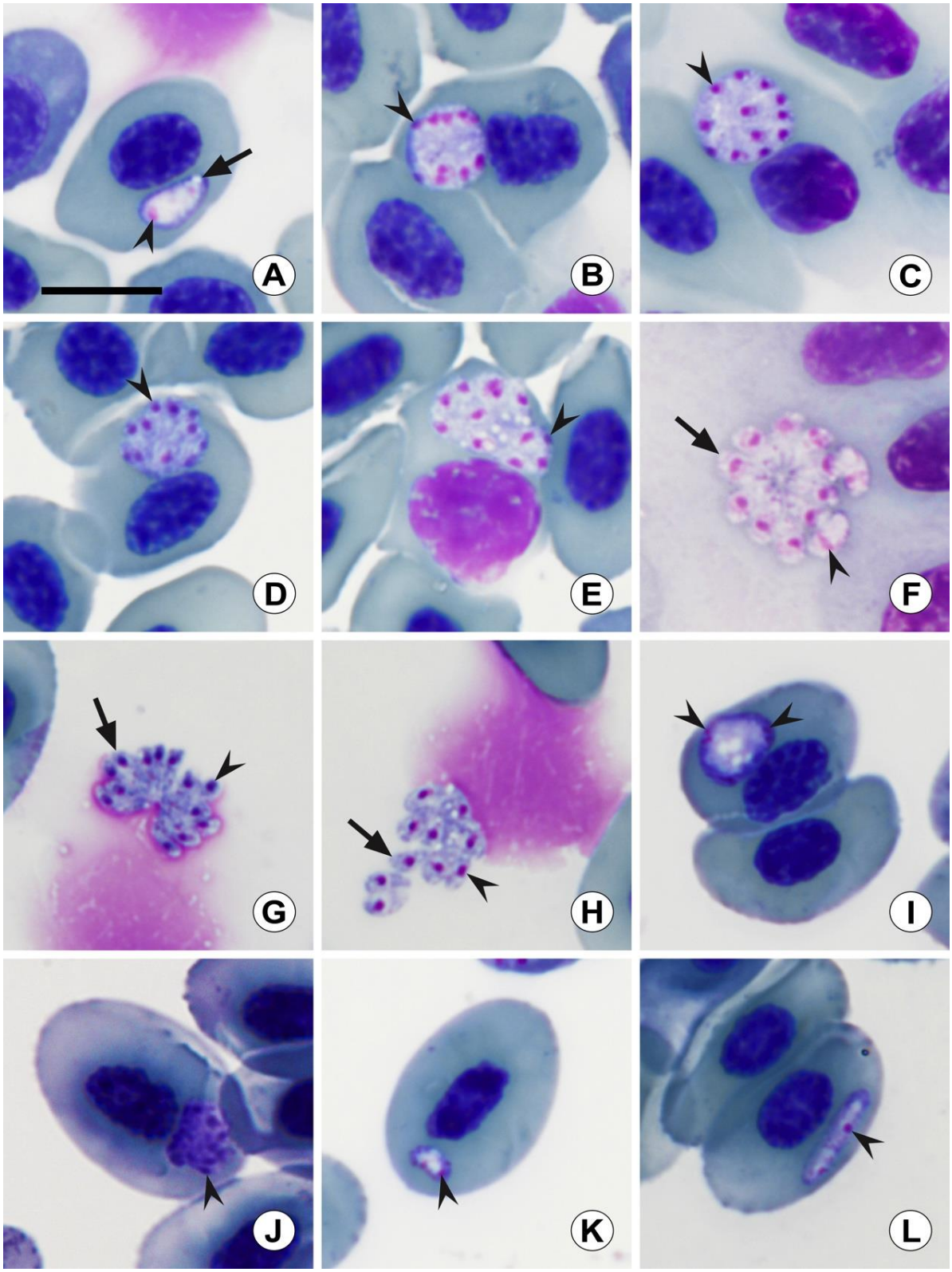

Fig. 3. (A-L). *Dactylosoma* sp. (*splendens*) from *Pelophylax lessonae*. (A-D) Primary merogony. (A) Trophozoite. (B) Young meront. (C-G) Secondary meronts. (F-H) Merozoites, arrows. (I-L) Secondary merogony. (I) Young meront. (J) Meront. (K) Merozoite. (L) Gamont. Arrowheads show condensed chromatin; arrows show vacuoles (A) and merozoites (F-H). All images captured from the deposited slides [XXX]. Scale bar 10 µm.

Although the synonymy of *D. splendens* is currently recognised as valid, this synonymisation happened over 100 years ago when knowledge of the close resemblance between different species was unknown. Unfortunately, no clear measurement data are available from the original description of *D. splendens*, which was described from *Pel.* kl. *esculentus* in Paris, France (Labbé, 1894). However, based on the information of this species of *Dactylosoma* provided above, in terms of morphology and geographical distribution, we suggest this species is conspecific with *D. splendens* and different from *D. ranarum* (However, this PhD is disclaimed for purposes of Zoological Nomenclature in accordance with the International Code of Zoological Nomenclature, Fourth Edition Articles 8.2 and 8.3 (ICZN 1999). No new names or nomenclatural changes are available from statements in this PhD thesis).

Phylogenetic analysis

Amplicons between 1548 and 1736 nt long were isolated from the dactylosomatids collected from *Ptychadena anchietae* (n = 7), *Sclerophrys gutturalis* (n = 3) and *Pelophylax lessonae* (n = 1). At the base of the phylogeny (Fig 4), species of *Dactylosoma* formed a highly supported monophyletic clade sister to a large clade of species of *Haemogregarina, Hemolivia*, *Hepatozoon* and *Karyolysus*. The *Haemogregarina* clade formed a sister group to a large clade comprising species of *Hemolivia*, *Hepatozoon* and *Karyolysus*. Species of intraleucocytic *Hepatozoon* (*Hepatozoon* Clade A) and *Karyolysus* formed a monophyletic clade sister to another monophyletic clade comprising species of *Hemolivia* and species of intraerythrocytic *Hepatozoon* (*Hepatozoon* Clade B). *Dactylosoma* isolated from European anurans (Palearctic realm) and identified as *D. ranarum* and *Dactylosoma* sp. (*splendens*), formed a highly supported clade with *Dactylosoma* sp. 1 from African anurans (Ethiopian realm) as a sister taxon.

Based on 1637 nt sequence comparisons of the 18S rRNA gene (see Table S1), the model-corrected genetic distance between the species of *Dactylosoma*, ranged between 0.2 – 0.5%. The interspecific divergence between *D. ranarum* and *Dactylosoma* sp. 1 from South Africa, isolated from *Pty. anchietae* and *Scl. gutturalis* was 0.5% (Table S1), and 0.2% between *D. ranarum* and *Dactylosoma* sp. (*splendens*). Several other haemogregarines also have an interspecific divergence below 1%, namely *Hepatozoon* cf. *clamatae* [GenBank: HQ224963] and *Hepatozoon* cf. *catesbianae* [GenBank: HQ224954] with 0.3%; *Hepatozoon chinensis* Han, Wu, Dong, Zhu, Li, Zhao, Wu, Pei, Wang, Huang, 2015 [GenBank: KF939620] and *Hepatozoon ayorgbor* Sloboda, Kamler, Bulantová, Votýpka et Modrý, 2007 [GenBank: EF157822] with 0.3%; *Hemolivia parvula* (Dias, 1953) [GenBank: KR069083] and *Hemolivia mauritanica* (Sergent and Sergent, 1904) [GenBank: KF992698] with 0.7% (Table S1).

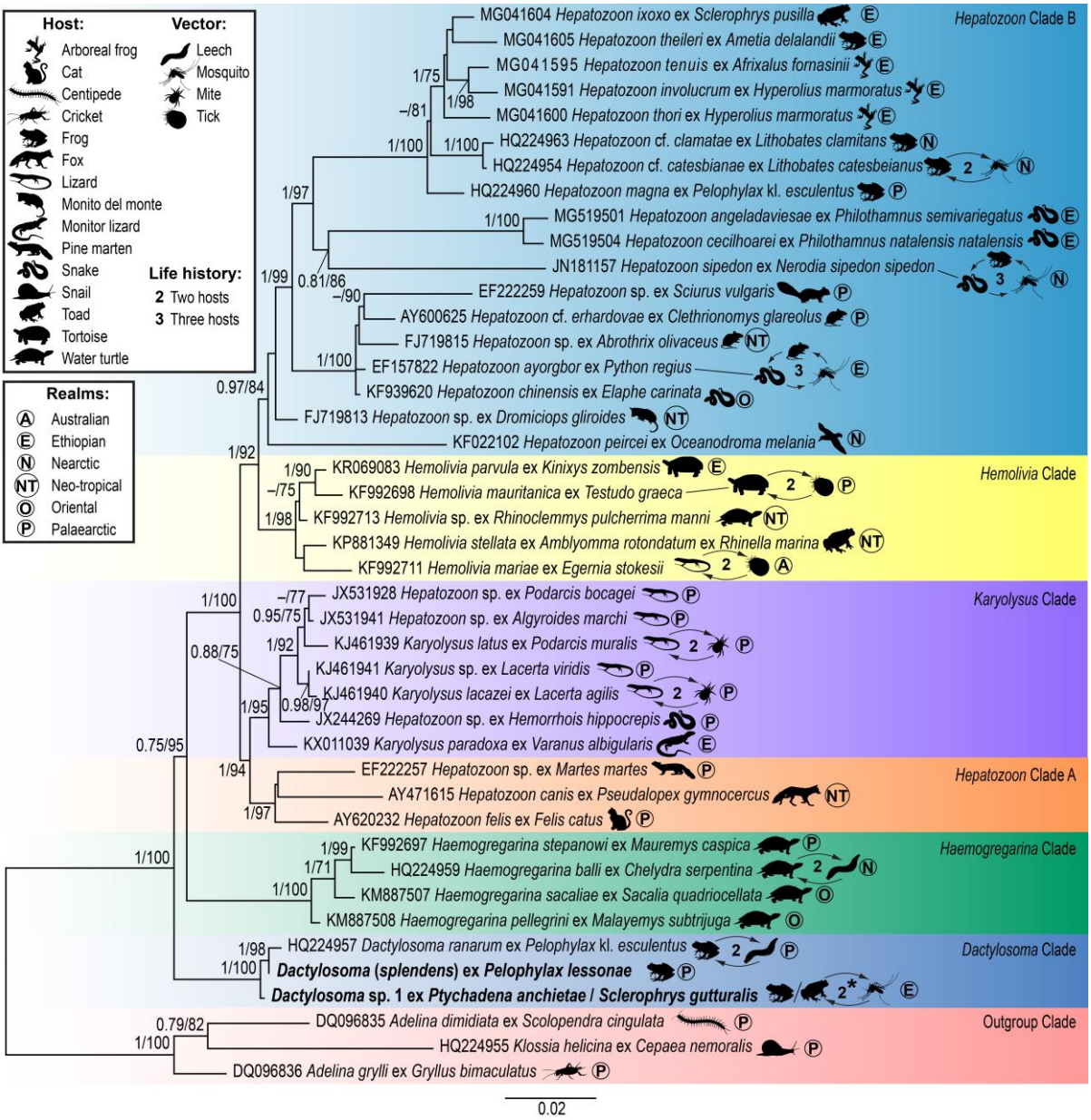

Fig. 4. Consensus phylogram of haemogregarines based on 18S rDNA sequences. Tree topologies for Bayesian inference (BI) and Maximum likelihood (ML) analyses were similar (represented on the ML tree), showing the phylogenetic relationships for *Dactylosoma* sp. 1 and *Dactylosoma* sp. (*splendens*) (represented in bold), compared to other species of *Haemogregarina*, *Hepatozoon*, *Karyolysus*, *Hemolivia*, and *Adelina* and *Klossia* as outgroup. Nodal support values lower than 0.80 posterior probability (BI) or 70 bootstrap (ML) were omitted. The scale bar represents 0.02 nucleotide substitutions per site. The host, geographical distribution (according to the zoogeographical realms), and if known the vector and life history cycle are also provided for the different sequences using symbols and pictograms. Asterisks (*) indicate the proposed life history strategy of *Dactylosoma* sp. 1 based on data from the current study.

Vector and life history evaluation

In the current study only phlebotomine sand flies, identified as species of *Sergentomyia* were observed feeding on *Pty. anchietae* and *Scl. gutturalis*. Mosquitoes identified as *U.* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana* were observed feeding on *Scl. gutturalis in situ* (Fig 5A-D).

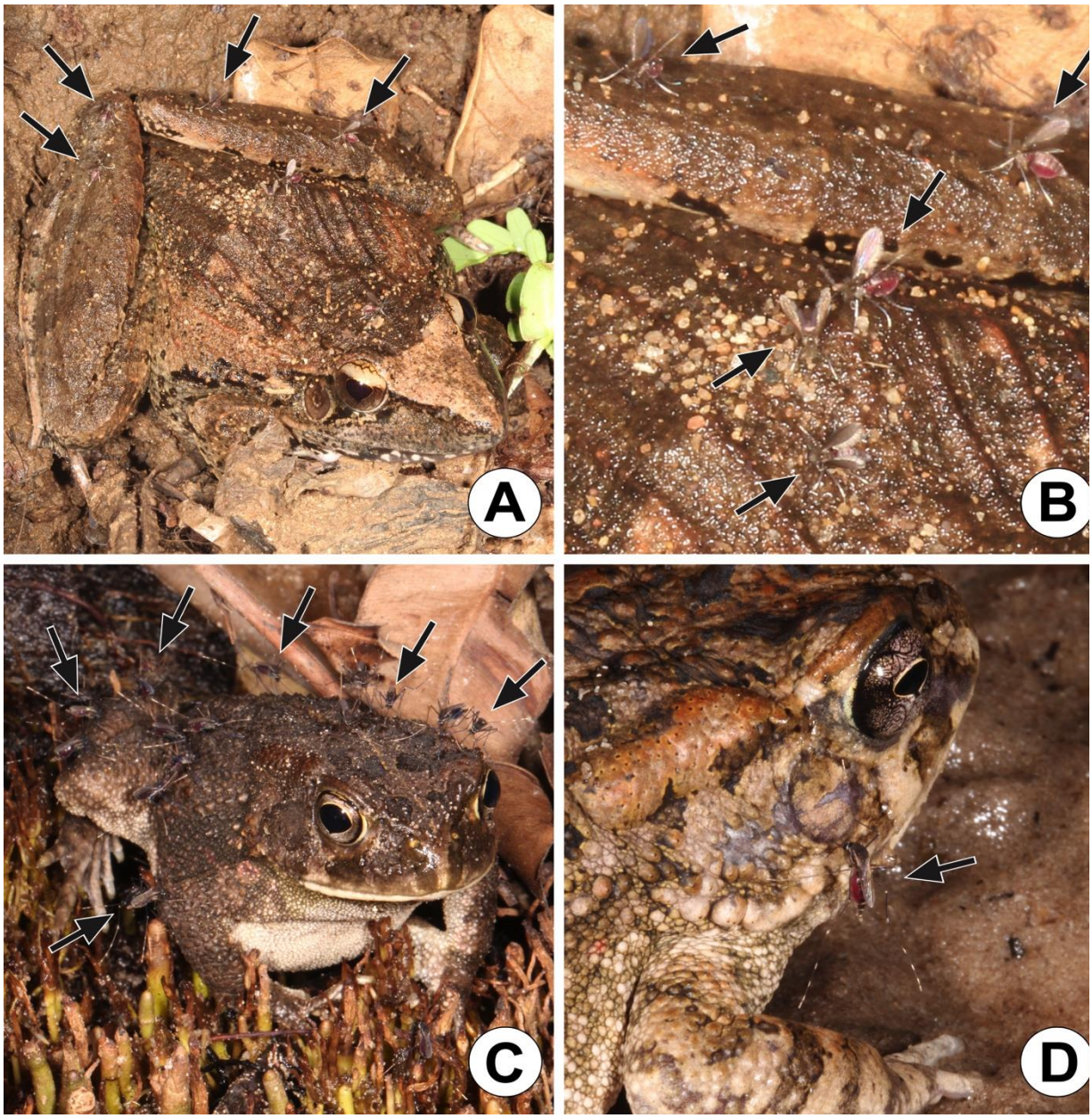

Fig. 5. (A-B). African phlebotomine sand flies (arrows) *Sergentomyia* sp. feeding on *Ptychadena anchietae in situ*. (C-D) Mosquitoes (arrows), *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U*. (*Pfc.*) *montana* feeding on *Sclerophrys gutturalis in situ*.

Unfortunately, although species of *Sergentomyia* were collected from localities with infected *Pty. anchietae*, observations were rare and none were collected from infected hosts. In contrast several *U.* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana* were collected from *Scl. gutturalis* parasitised with *Dactylosoma* sp. 1.

A total of approximately 68 mosquitoes were collected, of which 15 fed on a parasitised *Scl. gutturalis in situ* (parasitaemia 0.2 ± 0.2 (0.0 – 0.5) %). Intraerythrocytic and extracellular stages of *Dactylosoma* sp. 1 were seen in smears made from *Uranotaenia* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana* between 1–7 dpi. After one dpi, several stages were observed in the gut contents of *U.* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana*. Probable intra- and extracellular meront stages of *Dactylosoma* sp. 1 were observed with undigested erythrocytes (Fig 6A-B). Stages were elongated to oval and in most cases vacuoles were present (Fig 6A-B arrow). Nuclei chromatin stained deep magenta (Fig 6B arrowhead).

Within one dpi, free gamonts coupled in syzygy in the gut contents of the mosquitoes (Fig 6C-D). Merging of gametes was visible, with nuclei fusing into large nucleus staining light pink (Fig 6D arrow), condensed chromatin, most likely from the different gametes, staining deep magenta (Fig 6D arrow head). Cytoplasm vacuolated and staining dark purple. Immature ookinetes measure 10.4 ± 0.2 (10.2 – 10.5) long × 7.9 ± 1.3 (7.0 – 8.9) wide (n = 2). Subsequently round ookinetes were formed, with a vacuolated cytoplasm staining dark purple (Fig 6E). Nucleus staining light pink (Fig 6E arrow) with condensed chromatin staining deep magenta (Fig 5E arrow head). Ookinetes measured 11.6 ± 2.9 (8.5 – 15.9) long × 9.5 ± 4.0 (7.6 – 12.4) wide (n = 7), and developed into slightly larger immature oocysts (Fig 6F). Immature oocysts with a vacuolated cytoplasm staining dark purple, clearly visible nucleus staining light pink (Fig 6F arrow). Immature oocysts measure 16.2 long × 15.9 wide (n = 1).

Sporogony leading to the formation of at least six sporozoites was observed in the gut or haemocoel of the mosquito smears from six dpi (Fig 6G). Several free sporozoites were also observed at six dpi (Fig 6G). Free sporozoites elongate and broad; cytoplasm staining light purple with a more or less centrally placed nucleus staining deep magenta (Fig 6H). One or two distinct vacuoles occurring either anterior or posterior to the nucleus, as well as deep-staining granules of various sizes. These sporozoites measure 12.7 ± 1.3 (10.5 – 14.2) long × 1.6 ± 0.2 (1.4 – 2.0) wide (n = 9). Also observed in the mosquitoes' gut or haemocoel were the remains of a probable meront producing roughly 10 immature merozoites (Fig 6J), similar in shape and size to the secondary merogonic merozoites that occur in the peripheral blood of the vertebrate anuran host. Merozoites (Fig 6J arrow) elongate to ovoid, hyaline cytoplasm staining purple. Small round dense nuclei located closer to the centre, chromatin staining dark purple (Fig 6J arrow head). Merozoites measure 5.9 ± 0.2 (5.6 – 6.2) long × 1.5 ± 0.1 (1.4 – 1.8) wide (n = 5).

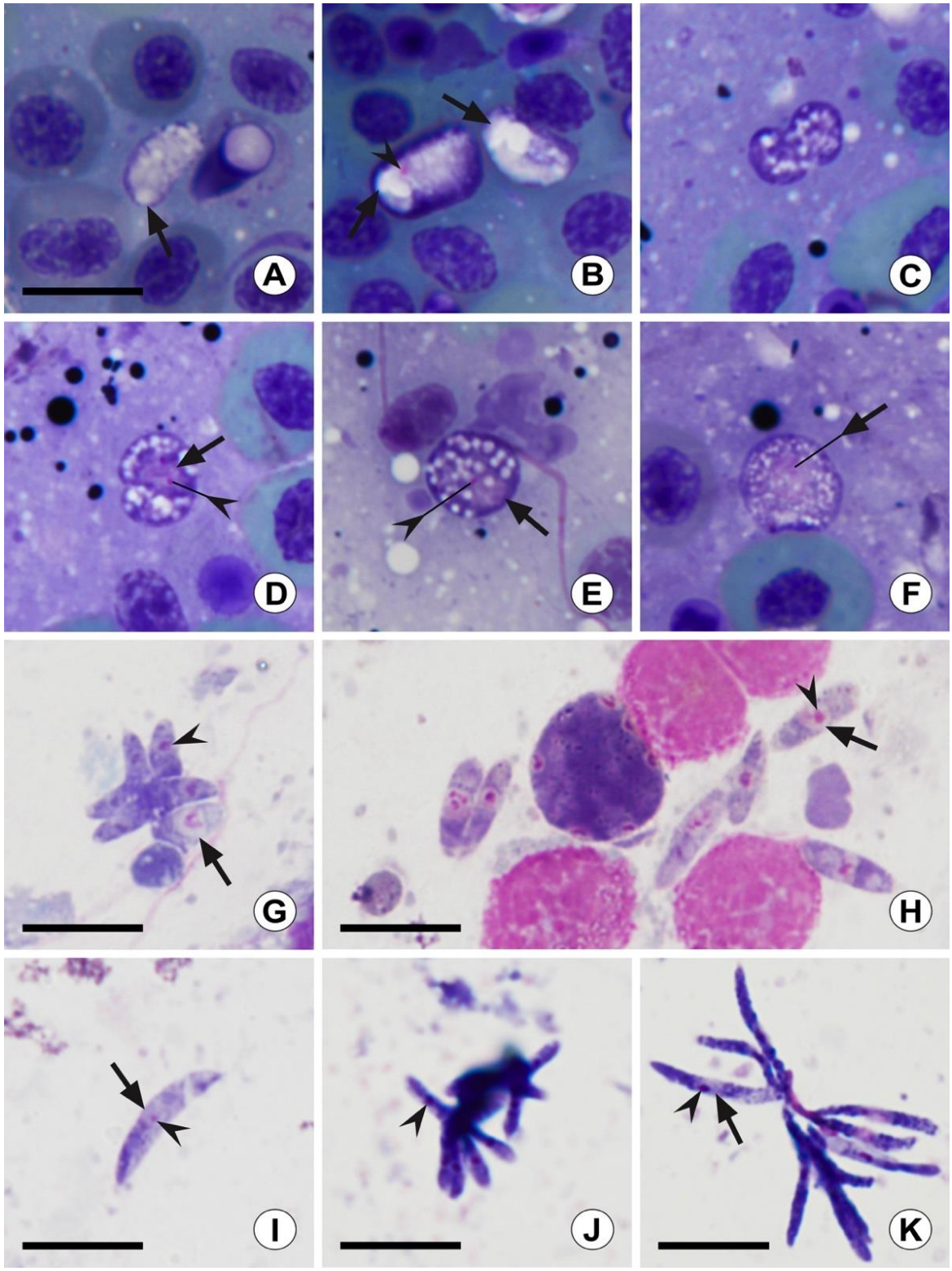

Fig. 6. (A-K). Possible development of *Dactylosoma* sp. 1 in the gut or haemocoel from the mosquitoes *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U.* (*Pfc.*) *montana*, from infected *Sclerophrys gutturalis*. (A) Intracellular meront. (B) Intra- and extracellular meront. (C-D) Merging of gametes. (E) Ookinete. (F) Immature oocyst. (G-I) Free sporozoites. (J) Probable meront producing immature merozoites. (K) Probable meront, producing long and slender mature merozoites. Vacuoles – arrow (A-B); Nucleus – arrow (D-K); Condensed chromatin – arrowhead (B, D-K). Scale bar 10 µm.

In the gut or haemocoel of the mosquitoes, we observed a probable meront, producing approximately 10 long and slender mature merozoites (Fig 6K). Mature merozoites similar in shape and size to extracellular gamont forms that occur in the vertebrate host. These forms are elongate often with slight curvature, cytoplasm granulated staining dark purple. Nucleus clearly visible, slightly off centre, staining light pink (Fig 6K arrow), in some cases condensed chromatin visible staining deep magenta (Fig 6K arrow head). Mature merozoites measure 8.7 ± 3.0 (5.7 – 12.7) long × 3.0 ± 0.5 (2.2 – 3.8) wide (n = 9).

Discussion

In the present study, a total of 643 anurans were collected and screened for the presence of dactylosomatids. Out of 38 species from 20 genera of 13 families, only three species of three genera and three families were found infected. Morphological and molecular data indicate that the dactylosomatid found parasitising *Pty*. *anchietae* and *Scl*. *gutturalis* from South Africa represent a new species, *Dactylosoma* sp. 1.

For the species of *Dactylosoma* found parasitising a single *Pel*. *lessonae* from Belgium, possess several phenotypic characters of the synonymised species *D. splendens*, originally described form *Pel.* kl. *esculentus* from Paris, France (Labbé, 1894). Thus based on the original description of *D. splendens* as well as morphological and geographical data from the current study it is suggested to reconsider the validity of *Dactylosoma splendens* Labbé 1894 from synonymy with *D. ranarum* (note - see PhD disclaimer).

Parasite prevalence and host association

Interestingly *Dactylosoma* sp. 1 species had a prevalence of 38% and 12% in the respective host frog *Pty. anchietae* and toad *Scl. gutturalis* hosts. The distribution range of *Dactylosoma* sp. 1 in *Pty. anchietae* was also larger with infected individuals collected from various localities and habitats, as compared to *Scl. gutturalis* that were found infected only from Sodwana Bay (see Dataset S1). Frogs belonging to *Ptychadena*, are known to harbour a range of different parasites, which could be due to their ability to successfully populate different habitat types, and to their feeding habits and behaviour (Du Preez and Kok, 1992). A higher prevalence of blood parasites is also reported from *Pty. anchietae* as compared to other species within the study area. In the present study, the prevalence of *Dactylosoma* was considerably higher as compared to the results of the anuran blood parasite survey of the same locality by Netherlands et al. (2015) in which 16.7% (13/78) of the *Pty. anchietae* screened were reported infected. These findings could be due to the severe droughts

experienced in South Africa between 2015 and 2017 (Archer et al., 2017), affecting potential water-borne invertebrate vector numbers.

Parasitaemia of genera such as *Dactylosoma* may be maintained or even potentially increase within the host without re-infection (due to their development within the vertebrate host; that is the parasite's ability to multiply asexually in the peripheral blood), thus out-competing representatives of other blood parasite genera such as *Hepatozoon*, for which some species may need re-infection. This may be a possible explanation for the observations made by Netherlands et al. (2015), in which species of *Hepatozoon* were found to be the most prevalent blood parasite at the time, parasitising 39.7% (31/78) of the *Pty. anchietae* screened. This, was in contrast to a more recent survey by Snoeks et al. (in prep) in the same area and on the same host, with species of *Dactylosoma* recorded to have a higher overall prevalence than species of *Hepatozoon*. Indeed, certain haemogregarines such as *Hepatozoon ixoxo* Netherlands, Cook and Smit, 2014, seem to be able to maintain a constant and high parasitaemia over long periods of time without re-infection (Netherlands et al., 2014). According to Conradie et al. (2017), this could be due to the dense capsule surrounding this species. Thus species of *Hepatozoon* which are not encapsulated, like the species parasitising *Pty. anchietae* and *Hepatozoon theileri* (Laveran, 1905), may not be as 'dormant' or 'maintain stable infection levels' as *H. ixoxo* (see Conradie et al., 2017). The longevity of representatives of an infrapopulation of blood parasites, without re-infection, however, needs to be tested experimentally before this can be concluded with any certainty.

Phylogenetic analysis

The 18S rRNA gene is a well-used marker for a variety of apicomplexan parasites with large datasets available for members of the Adeleorina. This gene contains conserved regions for a wide range of organisms, with scattered variable regions providing sufficient evolutionary information to compare close and distant phylogenetic relationships (Medlin et al., 1988). Nonetheless, the 18S rRNA gene has been shown to possess limited resolution between closely related species within the Adeleorina (Barta et al., 2012). Thus, while this gene provides sufficient information on the phylogenetic relationships between different genera it is less informative for species differentiation. On the other hand, mitochondrial DNA has been shown to provide better resolution for species-level diagnostics and molecular phylogenetics of the intestinal eimeriid coccidia (Ogedengbe et al., 2011).

As expected the species of *Dactylosoma* from the present study and *D. ranarum* [GenBank: HQ224957] formed a well-supported monophyly, distinct from the other genera of which sequences were available (Figure 4). These three species can be separated based on specific phenotypic characters and the conservative 18S rRNA marker. Model-corrected

interspecific divergence ranges between 0.2 – 0.5% (Table S1). Although the interspecific divergence value within other haemogregarines (*Haemogregarina*, *Karyolysus*, *Hemolivia*, and *Hepatozoon*) is low (1% and below), based on the sequence comparisons of the conservative 18S rRNA gene, it seems as if low interspecific divergence is more prominent between anuran dactylosomatids. Hence, increased sampling of species of *Dactylosoma*, especially from fish hosts, will be an interesting comparison, confirming if this trend of low interspecific divergence based on the 18S rRNA gene is consistent among all dactylosomatids or only those from anuran hosts. Also apparent from the phylogenetic analysis is the polyphyletic placement of different species from the genus *Hepatozoon.* Apart from in *Hepatozoon* cade A and clade B, species of *Hepatozoon* were also observed in the *Karyolysus* clade. The latter species most likely misidentified as belonging to *Hepatozoon*. A new genus *Bartazoon* Karadjian, Chavatte and Landau 2015, was proposed for species belonging to *Hepatozoon* clade B. The new genus suggested for species transmitted solely by haematophagous insects and in an effort to resolve the polyphyletic placement of the genus *Hepatozoon* (Karadjian et al., 2015). However, as pointed out by Maia et al. (2016), the type species the of the genus *Hepatozoon perniciosum* Miller, 1908 may form part of the newly proposed genus *Bartazoon*, as other rodent haemogregarine species do. Thus until molecular data from type species is available for comparison, it is suggested to rather continue to refer to species from *Hepatozoon* clade B as *Hepatozoon* as opposed to *Bartazoon* (Maia et al., 2016).

Life history and vector identification

Prior to the availability of molecular data for haemogregarines, microscopic observations of peripheral blood stages, ultrastructural studies of different stages, and life cycle data were the primary methods to classify and distinguish between different haemogregarines (Boulard et al., 1982; Barta and Desser, 1986; Barta, 1989, 1991). Life cycle elucidation of members of the Dactylosomatidae including their natural vectors is limited to only two species, *B. mariae* and *B. stableri* (Barta and Desser, 1989; Negm-Eldin, 1998). Thus far the only attempt to elucidate the life cycle of a species of *Dactylosoma* was when Barta (1991) used the natural leech vector of *B. stableri, Des. picta*, to transmit *D. ranarum* from infected frogs from Corsica, France. However, only sporogonic development within the intestinal epithelium of the experimental vector was observed. Due to these observations and the life cycle data available for members of the Dactylosomatidae, these parasites were considered to be transmitted to new hosts only through leech vectors (Barta, 1991; Negm-Eldin, 1998).

In southern Africa three species of leeches are known to be haematophagous on amphibians, namely *Oosthuizobdella stuhlmanni* (Blanchard, 1897), *Marsupiobdella africana*

Goddard & Malan, 1912 and *Hirudo michaelseni* Augener, 1936. Of these *M. africana* is the smallest (12 mm) and is reported to exclusively feed on species of *Xenopus*. However, this species also exhibits a phoretic interaction with freshwater crabs (Badets and Du Preez, 2014). *Oosthuizobdella stuhlmanni* is the second largest (33 mm) species, reported to feed on various anurans. The largest is *H. michaelseni* (65 mm), which is not host specific and feeds on amphibians, but also on mammals and fish, with immature leeches feeding on freshwater snails. In the present study anurans were examined for the presence of leeches, however no leeches were ever observed. Although it is likely that leech species haematophagous on amphibians are found in northern KZN, it would appear that they are not common and do not act as the vector of anuran blood parasites from this area. Considering the proportion of infected anurans (from this area) parasitised with species of *Dactylosoma* and *Trypanosoma* Gruby, 1843 (Netherlands et al., 2015; Snoeks et al., in prep), which are commonly transmitted by leech vectors (Bardsley and Harmsen, 1973; Rhoden and Bolek, 2012), these findings support the use of other possible vectors, such as dipterans in the transmission of these parasites.

Phlebotomine sand flies are other haematophagous invertebrates that are known to transmit blood parasites, and have been reported as the vectors for species of *Trypanosoma*, *Leishmania* (Borovsky 1898) and even microfilarial worms (Feng and Chung, 1940; Desportes, 1942). In Africa, phlebotomines reported to feed on amphibian hosts are species of *Sergentomyia* (Franca and Parrot, 1920) and *Grassomyia* Theodor 1958 (see Krüger, 2015; Kvifte and Wagner, 2017). Species of *Sergentomyia* are the most widespread and diverse group of the Phlebotominae in Africa. Although they are considered to primarily feed on reptiles (Kvifte and Wagner, 2017), in the present study, species of *Sergentomyia* were observed feeding on *Pty. anchietae* and *Scl. gutturalis*. Although none of the species of *Sergentomyia* collected took blood meals from infected hosts, the localities from where these sand flies were collected did have *Pty. anchietae* parasitised with *Dactylosoma* sp. 1. Thus, even though no direct link was made between species of *Sergentomyia* collected in the current study and *Dactylosoma* sp. 1, these dipterans remain a possible vector for this parasite.

Mosquitoes are known to be the vectors of amphibian haemogregarines, particularly for representatives of *Hepatozoon* Miller, 1908 (Desser et al., 1995; Smith, 1996). Even though, no mosquitoes have been reported as potential vectors for representatives of *Dactylosoma*, the mosquito *C. nubeculosus* was experimentally tested by Boulard et al. (1982) as the vector for *D. ranarum* without success. *Uranotaenia* Lynch Arribalzaga 1891 is relatively species-rich with approximately 256 species occurring in nearly all zoogeographical regions (Coetzee, 2017). Several members of the genus are known to feed selectively on amphibians (Cupp et al., 2004), with some species reported to be attracted by

the calls of their host species (Borkent and Belton, 2006; Camp et al., 2018). In the current study several *U.* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana* were collected from a calling *Scl. gutturalis* parasitised with *Dactylosoma* sp. 1 *in situ*. To determine if these mosquito species could serve as potential vectors of this parasite, collected blood fed mosquitoes were kept alive to be systematically screened for any form of development of *Dactylosoma* sp. 1.

In only one dpi, several stages of development, from gamonts coupled in syzygy to possible immature oocysts, were observed in the gut contents of the screened mosquitoes. At six dpi several possible sporozoite and merozoites stages, and even a probable meront were observed in mosquito gut smears. Although this was only a preliminary observation with minimal data, the fact that development of a haemogregarine, most likely *Dactylosoma* sp. 1, was reported in these hosts also suggests that dactylosomatids may not solely be transmitted by leech vectors, thus, contributing to our understanding of the biology of these parasites.

Furthermore, it is interesting that stages of development were only found in the gut contents or haemocoel of infected mosquitoes. This would mean that this species of *Dactylosoma* is possibly transmitted to a new host through ingestion (new host consuming infected vector) rather than through the feeding of an infected vector as expected? This is similar as for *Haemogregarina bigemina* Laveran and Mesnil, 1901, which is probably transmitted via the ingestion of infected gnathiids by fishes, rather than by the feeding of the gnathiid (Davies and Smit, 2001). If this is the case, then the theory that the closely related families Haemogregarinidae and Dactylosomatidae evolved to be transmitted via the bite of their definitive vectors is challenged (see Barta et al., 2012). However, resistant and thick walled oocysts that are present in species of *Hepatozoon* that are transmitted through ingestion were not observed in the examined mosquitoes. Thus, although these mosquitoes are most likely the vectors of this dactylosomatid parasite, based on the development of different stages and the lack of other haematophagous invertebrates found feeding on *Scl. gutturalis* from this locality, more data on the process and site of development is required before any definite conclusions can be made on the life history of *Dactylosoma* sp.1.

Also if mosquitoes are the vectors of *Dactylosoma* sp.1, then its phylogenetic placement with *D. ranarum* (possibly leech transmitted) based on the 18S rRNA gene contradicts the theory that these parasites adapted within their invertebrate hosts to become vector transmitted rather than evolving with the vertebrate host (see Barta et al., 2012; O'Donoghue, 2017).

Concluding remarks

This study highlights the importance of screening and comparing anurans from different genera and even families, in an effort to not only increase the known biodiversity of these parasites, but also the types of hosts that they infect. The importance of using different techniques in the description of new species is also emphasised. The hypothesis that anurans from South Africa would be parasitised by a higher diversity of dactylosomatid parasites as compared to anurans from Europe was not supported. Only *Dactylosoma* sp. 1 was found parasitising anurans from South Africa, where as *Dactylosoma* sp. (*splendens*) and *D. ranarum* are found parasitising European anurans.

Future work should include the elucidation and use of faster evolving markers to help increase resolution between different species of *Dactylosoma*. However, to date only a single haemogregarine has mtDNA sequence data available (Leveille et al., 2014). Furthermore, more rigorous life-history experimental work, testing the role of *U.* (*Pfc.*) *mashonaensis* and *U*. (*Pfc.*) *montana* in the transmission development of *Dactylosoma* sp. 1 to *Scl. gutturalis*, should be done. These studies may even be extended to determining the possibility of phlebotomine sand flies, specifically belonging to *Sergentomyia*, as potential vectors.

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CHAPTER 3

Anuran Haemococcidia: description of two new species of *Lankesterella* (Apicomplexa: Lankesterellidae), and a new species of haemococcicida (Apicomplexa: Eimeriorina) in a new genus.

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Abstract

Haemococcidia (Apicomplexa: Eimeriorina) are heteroxenous intracellular blood parasites. The genera *Lankesterella* Labbé, 1899, *Schellackia* Reichenow, 1919 and *Lainsonia* Landau, 1973 are regarded as true haemococcidia, with the entire replication process of their life cycle occurring in the tissues of the vertebrate host. Transmission occurs via a paratenic or mechanical haematophagous invertebrate host, with sporozoites released during feeding or via ingestion of infected vectors. Of the nine recognised species, four species of *Lankesterella* have been reported or described from African anurans. From a total of 643 individual anurans collected and screened in South Africa and Belgium, 21 (3.3%) individuals belonging to seven species were found parasitised with haemococcidia. Five species of anurans from South Africa and two from Belgium were found parasitised with haemococcidia. Based on morphological, morphometric and molecular findings in the current study we redescribe *Lankesterella minima* (Chaussat 1850) in *Pelophylax* kl. *esculentus* (Linnaeus, 1758) and *Pel. lessonae* (Camerano, 1882) from Belgium. As well as describe two species of *Lankesterella*, *Lankesterella* sp. 1 in *Pel. lessonae* from Belgium, as well as *Lankesterella* sp. 2 in *Afr. delicates* and *Afr. fornasini* from South Africa; and a new genus haemococcidia n. gen., with a new species combination haemococcidia sp. 1 n. gen. n. comb. (*Lankesterella ptychadeni*), described in *Pty. nilotica*, from, Kenya (Paperna and Ogara, 1996), and now from *Pyx. edulis*, *Phr. mababiensis* and *Pty. anchietae* and a new species, haemococcidia sp. 2 in *Afr. fornasini* and from South Africa. Phylogentic analyses of both 18S rDNA and COI gene sequences, confirmed monophyly of species of *Lankesterella* separate from the newly proposed genus of haemococcidia. This is the first study to provide molecular data for species of *Lankesterella* from African and European anurans.

Keyword: Anurans, Belgium, South Africa, new genus, blood parasite

Introduction

Coccidia are intestinal apicomplexans considered to be among the most abundant and diverse groups of parasites known, and are reported to infect a wide range of animal phyla (Duszynski et al., 2007). Most species of eimeriid coccidia follow a homoxenous life cycle characterised by asexual (merogony) and sexual reproduction (gametogony) in the vertebrate host's intestinal tract (Barta et al., 2001). This is followed by formation of sporulated and resistant oocysts (sporogony), either within the host or external environment, that pass through to the next host (Barta et al., 2001). Sporogony includes post-fertilization meiosis and is therefore frequently included in the sexual reproduction of these parasites. In addition, numerous studies report on extra-intestinal development of eimeriids across various hosts (Desser, 1978; Duszynski et al., 1979; Lima, 1979; Hawkins et al., 1984; Parker and Duszynski, 1986; Paperna, 1995; Matsubayashi et al., 2005; Duszynski et al., 2007). Coccidia that are known or have been reported to contain stages in the blood of their host are *Isospora* (Schneider, 1881) syn. *Atoxoplasma* (Garnham, 1950) and some species in the Sarcocystidae Poche, 1913.

The major difference between intestinal coccidia and haemococcidia is the absence of sporocyst formation in the latter group (Telford, 2009). These haemococcidia are heteroxenous, with the entire replication process of their life cycle (merogony, gametogony, and sporogony) occurring in the tissues of the vertebrate host (Nöller, 1912, 1920; Desser, 1993). Transmission occurs via a paratenic or mechanical haematophagous invertebrate host, with sporozoites released during feeding or via ingestion of infected vectors (Nöller, 1913; Desser, 1993). *Lankesterella* Labbé, 1899, *Schellackia* Reichenow, 1919 and *Lainsonia* Landau, 1973 are regarded as "true" haemococcidia (Telford, 1993; Paperna et al., 2009). *Schellackia* is characterised by the presence of a soft-walled oocyst, producing eight naked sporozoites in the intestinal epithelium or lamina propria and reticuloendothelial system (RES) cells of its host. In contrast, representatives of *Lankesterella* produce 32 or more naked sporozoites in RES cells in the host's visceral organs, and undergo three or four cycles of merogony before gamonts are produced. *Lainsonia* species are reported from New World lizard hosts, sharing characteristics between species of *Lankesterella* and *Schellackia*, with oocysts containing eight naked sporozoites found in RES cells of its host's visceral organs. Nöller (1912) was the first to describe this merogonic and sporogonic cycle of a species of *Lankesterella*, and successfully transmitted it through feeding or injecting infected blood or spleen to *Lankesterella*-free frogs (Nöller, 1912). Furthermore, Nöller (1913) and Tse et al. (1986) suggested the respective leeches *Hemiclepsis marginata* (O. F. Müller, 1774) and *Desserobdella picta* (Verrill, 1872) serve as a vector for *Lankesterella minima* (Chaussat 1850). However, Desser et al. (1990) was the first to successfully transmit

a species of *Lankesterella* to tadpoles (*R. catesbeiana* Shaw, 1802) using *D. picta* as the vector.

The taxonomy of amphibian haemococcidia, similar to other intracellular blood parasite groups, has had an uncertain history. According to Hintze (1902), *L*. *minima* was the first observed haemococcidia in the blood of an anuran (Chaussat, 1850). Nöller (1913) agreed with the classification of *L. minima* provided by Hintze (1902) and provided the following synonyms: *Anguillula minima* (Chaussat, 1850); *Drepanidium ranarum* (Lankester, 1871, 1892); "Würmchen" (Gaule, 1880, 1881, 1885); "Cytozoën" (Bütschli, 1882); *Drepanidium ranarum* (Lankester, 1882; Wallerstein, 1882); *Drepanidium princeps* (Labbé, 1894); "Drepanidio piccolo" (Grassi and Feletti, 1892); *Lankesterella ranarum* (Labbé, 1894); *Haemogregarina minima* (Laveran, 1898; Mathis and Léger, 1911).

Including *L. minima*, there are currently nine recognised species of *Lankesterella* globally (see Table 1). Four species of *Lankesterella* have been reported or described from African anurans. The first report of a species of *Lankesterella* morphologically similar to *L. minima* was in the crowned bullfrog, *Hoplobatrachus occipitalis*, in a survey of different blood parasites of anurans from Stanleyville, DR Congo (Schwetz, 1930). Later, *L. bufonis* Mansour and Mohammed 1962, *L. ptychadeni* Paperna and Ogara 1996 and *L. dicroglossi* Paperna and Ogara 1996 were described from *Sclerophrys regularis* in Egypt, and *Ptychadena nilotica* and *Dicroglossus occipitalis* from Kenya, respectively (Mansour and Mohammed, 1962; Paperna and Ogara, 1996; Paperna and Martin, 2001).

Nöller (1920) showed species of *Lankesterella* as a monosporous, polyzootic coccidium, and based on the structure of the oocysts and number of naked sporozoites, erected the family Lankesterellidae Nöller 1920. Later, Grassé (1953) placed the genus *Schellackia* in its own family Schellackiidae Grassé, 1953. However, despite comparisons of fine structural data (Paperna and Ogara, 1996), successive studies regarded the genera *Lankesterella* and *Schellackia* as part of the Lankesterellidae (Upton, 2000; Telford, 2009).
Molecular studies on different blood parasites such as the haemococcidia and haemogregarines have provided useful insights in the evolutionary relationships within and between these apicomplexans, as well as enhanced capabilities to distinguish between species and genera (Megía-Palma et al., 2013; Cook et al., 2015, 2016; Megía-Palma et al., 2017; Netherlands et al., 2018). Barta et al. (2001) obtained the first molecular data for a lankesterellid, with the characterisation of the 18S rDNA gene of *L. minima*.

Table 1 Summary on currently recognised species of *Lankesterella* of anurans globally

Species	Type host	Type locality	Source
L. alencari Costa and Pereira, 1971	*Leptodactylus latrans Chaussat, 1850*	Rio de Janeiro, Brazil	(Costa and Pereira, 1971)
L. bufonis Mansour and Mohammed 1962	*Sclerophrys regularis* (Reuss, 1833)	Giza Province, Egypt	(Mansour and Mohammed, 1962)
L. canadensis Fantham, Porter and Richardson 1942	*Rana catesbeiana* Shaw, 1802	Quebec and Ontario, Canada	(Fantham et al., 1942)
L. dicroglossi Paperna and Ogara 1996	*Dicroglossus occipitalis* Günther, 1858	Lake Baringo, Kenya	(Paperna and Ogara, 1996)
L. hylae Cleland and Johnston 1910	*Litoria caerulea* (White, 1790)	Sydney, Australia	(Cleland and Johnston, 1910)
L. minima (Chaussat 1850) Hintze 1902	*Pelophylax* kl. *esculentus* (Linnaeus, 1758)	Paris, France	(Chaussat, 1850)
L. petiti Lainson and Paperna, 1995	*Rhinella marina* (Linnaeus, 1758)	Belém, Brazil	(Lainson and Paperna, 1995)
L. poeppigii Paperna, Bastien, Chavatte and Landau, 2009	*Rhinella poeppigii* (Tschudi, 1845)	Peru	(Paperna et al., 2009)
L. ptychadeni Paperna and Ogara 1996	*Ptychadena nilotica* (Seetzen, 1855)	Lake Victoria, Kenya	(Paperna and Ogara, 1996)

Subsequent studies on the phylogenetic placement of *Lankesterella* and *Schellackia* species (using 18S rRNA gene sequences) show these genera as distantly related; confirm the occurrence of species of *Lankesterella* in not only anuran hosts, but also lizard, and avian hosts; and also show their close relationship to other eimeriid coccidia (Merino et al., 2006; Biedrzycka et al., 2013; Megía-Palma et al., 2013; Megía-Palma et al., 2014; Megía-Palma et al., 2017; Martínez et al., 2018). Furthermore, Megía-Palma et al. (2014) resurrected the Schellackiidae based on the molecular characterisation (18S rRNA gene) and phylogenetic placement of the type species *Schellackia bolivari* Reichenow 1919.

Megía-Palma et al. (2017) suggested that life history traits such as the type of host, blood cells infected, host species, or the number of refractile bodies are not valid diagnostic characters to differentiate between *Schellackia* and *Lankesterella*. These inconsistencies between phenotypic and genetic characters seem to occur with other eimeriid coccidia (Ogedengbe et al., 2018). In an effort to resolve the paraphyletic eimeriid coccidia, several studies have utilised a multi-gene approach (Ogedengbe et al., 2015; Ogedengbe et al., 2016; Ogedengbe et al., 2018). Ogedengbe et al. (2015) illustrated that nuclear 18S rDNA

sequences are suitable for determining deeper evolutionary events and that mitochondrial COI sequences are better suited for inferring more recent evolutionary events among closely related taxa. However, the combination of these gene regions provides a compact and informative molecular dataset for inferring the evolutionary relationships among these taxa (Ogedengbe et al., 2015). According to Ogedengbe et al. (2018), to eventuelly resolve the paraphyly seen within the coccidia, numerous genera should be erected following criteria established by Vences et al. (2013): well-supported monophyly, clade stability and phenotypic diagnosability. However reliable phenotypic traits that reflect evolutionary history remain an important yet challenging criterion for diagnosability of eimeriid coccidia (Ogedengbe et al., 2018).

The aim of this study was to use morphological and molecular characterisation to establish the diversity, prevalence and phylogenetic placement of anuran haemococcidia from north-eastern South Africa and Belgium. These localities were selected due to the high diversity of anurans found in north-eastern South Africa, and based on the history of haemococcidia and European anurans, Belgium was selected as a central locality from which to collect samples for comparison. To achieve this aim the objectives were (1) to determine which anuran species in South Africa and Belgium are parasitised by haemococcidia; (2) to establish the haemococcidian species diversity observed and (3) to compare any parasites molecularly characterized (18S rDNA and COI sequences) in the current study with available molecular data for anuran haemococcidia and other closely related taxa to determine their phylogenetic relationships and taxonomic placement.

Material and Methods

Sample collection and study area

Anurans were collected from several localities throughout the north-eastern part of South Africa and Belgium from 2014 to 2018 (Dataset S1), the same dataset as in Chapter 2. In South Africa, a total of 618 individuals from 35 species of 19 genera were collected. Specimens were primarily collected at night by hand, placed in individual marked containers with sufficient moisture and ventilation, and transported back to a field workstation. Species were identified using field guides and a mobile identification application on the frogs of southern Africa (Du Preez and Carruthers, 2009, 2017). Note that some of the samples collected or used in previous studies (Netherlands et al., 2018; Netherlands et al., in prep; Snoeks et al., in prep) have also been included in the dataset of the current study for a more comprehensive representation of parasite occurrence, prevalence and host specificity.

Collected specimens were released after taking blood samples, unless indicated otherwise in previous studies. Ezemvelo KwaZulu Natal (KZN) Wildlife issued the sampling and collection permits OP 526/2014, OP 839/2014, OP 4374/2015, OP 4092/2016, and OP 4085/2017.

In Belgium, a total of 25 individuals representing three species were collected. Samples comprised seven *Pelophylax* kl. *esculentus*; four *Pelophylax ridibundus* (Pallas, 1771); and 14 *Pelophylax lessonae* (Camerano, 1882). Species were collected diurnally and processed, photographed, identified with the "Reptielen en Amfibieën van Nederland" mobile identification application and a field guide (van Diepenbeek and Creemers, 2009), and released at site of capture. Agentschap Natuur & Bos issued the permit (ANB/BL/FF-V17-00091) for collection and sampling of amphibians in Belgium, with strict collection protocol followed as instructed by the permit office. This study has received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5).

Processing of samples and light microscopy screening

Blood samples were collected, processed, screened, and parasitaemia calculated as detailed in Netherlands et al. (2018). Micrographs of peripheral blood stage sporozoites were taken and measured using the imaging software NIS Elements Ver. 4.60. Measurements for morphological characterisation and comparison were adapted according to the parasite morphotype or stage and consisted of the length and width of the parasitophorous vacuole (PV) if present; the parasite's length and width when visible (if not covered by a PV); and the parasite's nucleus length and width if measurable. Measurements of the PV length and width were taken across the longest and widest points. Length measurements of the parasites were measured across the middle of the parasite following the bow, if the parasites were curved. Measurements are presented in micrometres (µm), with the average, standard deviation and range given.

DNA extraction, PCR amplification and phylogenetic analyses

Genomic DNA was extracted from ethanol preserved blood samples taken from anurans parasitised with haemococcidia (confirmed through microscopy). The standard protocol for human or animal tissue and cultured cells was used to extract DNA from the samples as detailed in the NucleoSpin® Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Düren, Germany). For some samples no whole blood was collected, thus, the blood from methanol-fixed, Giemsa-stained blood smears taken from parasitised specimens, was scraped onto separate sterile foil strips with an individual sterile scalpel blade. Scrapings were then

collected and transferred to individual sterile 1.5 mL reaction tubes and extracted as mentioned above. Once extracted, DNA was used for polymerase chain reaction (PCR) amplification. The PCR reactions initially targeted a fragment (~600 nt) of the nuclear 18S rRNA gene (a gene widely used in apicomplexan research and thus allowing for comparison with phylogenetically more distant taxa). The 18S rDNA sequence fragments were first amplified using the primer set HepF300 (5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′) and HepR900 (5′-CAA ATC TAA GAA TTT CAC CTC TGA C-3′) sourced from Ujvari et al. (2004). This primer set was used due to variable amplification success across a wide range of apicomplexan taxa. To obtain longer 18S rDNA sequence fragments (~1200 nt) another PCR using HepF300 and ER: (5′-CTT GCG CCT ACT AGG CAT TC-3′) sourced from Kvičerová et al. (2008) was carried out. Cytochrome *c* oxidase subunit I (COI) gene sequence fragments (~480 nt) were amplified using the degenerate primer set 400F (5′-GGD TCA GGT RTT GGT TGG AC-3′) and 500R (5′-CAT RTG RTG DGC CCA WAC-3′) sourced from El-Sherry et al. (2013). For the 18S rDNA HepF300 and HepR900 primer sets PCR conditions followed Cook et al. (2016). For the 18S rDNA HepF300 and HepR900 primer set PCR conditions followed (Cook et al., 2016). For the HepF300 and ER primer set PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, with an annealing temperature of 60 °C for 30 s, and an extension of 72 °C for 2 min; and following the cycles a final extension step of 72 °C for 10 min. Conditions for the COI primer set PCR were: initial denaturation at 96 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, with an annealing temperature of 55 °C for 30 s, and an extension step of 72 °C for 30 s, and a final extension of 72 °C for 10 min. PCR reactions were performed with volumes of 25 µL, using 12.5 µL OneTaq® 2X Master Mix with Standard, 1.25 µL (10 µM) of each of the primer sets mentioned above, and at least 25 ng DNA. The final reaction volume was reached by adding PCR-grade nuclease-free water (Thermo Scientific). An Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA USA) was used for amplification, and the resultant amplicons visualized under ultraviolet light on a 1% agarose gel stained with EZ-Vision® Bluelight DNA dye using an E-BOX CX5 imaging system (Vilber Lourmat Deutschland, Eberhardzell, Germany). PCR products from each sample were sent to a sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions, using the primers used from the initial PCR reactions. The software package Geneious R11 (http://www.geneious.com, ((Kearse et al., 2012)) was used to assemble resultant sequence fragments. Species identity was compared against previously published sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Sequences obtained in the current study were deposited in the NCBI GenBank database under the following accession numbers [GenBank: XXX].

Resultant parasite 18S rDNA sequences generated from the blood of infected individuals were aligned with sequences of a diverse range of apicomplexan parasites within the Eimeriidae Minchin, 1903 and Lankesterellidae, downloaded from the GenBank database (Dataset S2). The tree was rooted with *Toxoplasma gondii* (Nicolle & Manceaux, 1909) [GenBank: L37415] from the Sarcocystidae, as this species was selected as an outgroup in previous phylogenetic studies of the Eimeriidae (Ogedengbe et al., 2011; Megía-Palma et al., 2017). Sequences were aligned using the Clustal W 2.1 alignment tool (Larkin et al., 2007) under the default settings implemented within Geneious R11. The GBlocks server was used to remove any alignment gaps and ambiguities selecting the parameters to allow for smaller final blocks with gap positions (Castresana, 2000; Talavera and Castresana, 2007). The final alignment contained 1518 nt from 112 sequences. A model test was performed to determine the most suitable nucleotide substitution model, according to the Bayesian information criterion (BIC) using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). The model with the best BIC score for the 18S rDNA sequence alignment was the General Time Reversible (Tavaré, 1986) model, with estimates of invariable sites, and a discrete gamma distribution (GTR + I + Γ) in MrBayes. Nucleotide saturation was analysed by plotting the number of transitions and transversions against the corrected genetic distance estimated with a GTR model in the software DAMBE 6.4. (Xia and Lemey, 2009; Xia, 2017). Saturation plots revealed little saturation for the 18S rDNA sequence dataset. Bayesian inference (BI) was used to infer phylogenetic relationships and was preformed using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001) implemented from within Geneious R11. For the BI analysis, the Markov Chain Monte Carlo (MCMC) algorithm was run for 1 million generations, sampling every 100 generations. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. Results were visualized using the Tracer tool (implemented from within Geneious R11), to assess convergence and the burn-in period (Rambaut et al., 2018).

Resultant parasite COI gene sequences and representative sequences downloaded from GenBank were aligned using the Clustal W 2.1 (Dataset S3), as stated above. The GBlocks server was used to remove any alignment gaps and ambiguities. The final alignment contained 758 nt from 61 sequences. *Toxoxplasma gondii* [GenBank: 1HM771689] was chosen as the outgroup, following Ogedengbe et al. (2015). Nucleotide saturation was analysed in the software DAMBE 6.4 as stated above (Xia and Lemey, 2009; Xia, 2017). Saturation plots revealed little saturation for the COI gene sequence dataset. The BI analysis was performed with GTR + I + Γ selected as the model with the best BIC score. However, due to a large number of resultant polytomies, a reversible-jump MCMC algorithm (nst = mixed) was used instead of the GTR model. The MCMC algorithm was run for 1 million generations, sampling every 100 generations. The first 25% of the trees were

discarded as ‘burn-in’ with no ‘burn-in’ samples being retained. Results were visualized in Tracer to assess convergence and the burn-in period (Rambaut et al., 2018).

Sequences from Dataset S2 and S3, were used to construct a concatenated 18S rDNA and COI gene sequence alignment. A partition homogeneity test (1000 replicates - heuristic search) calculated using PAUP version 4.0a163 (Swofford, 2002), was applied to determine the compatibility of the 18S rRNA and COI gene trees based on rates of divergence and branching order. The partition homogeneity test ($P < 0.001$) rejected the combination of the 18S rRNA and COI genes as a compatible dataset and was therefore not analysed. Uncorrected pairwise distances (p-distance) were calculated for sequences from Dataset S2 and S3 in PAUP version 4.0a163 (Swofford, 2002) (see Dataset S2 and S3).

Results

From a total of 643 individual anurans collected and screened, 21 (3.3%) individuals belonging to seven species were found parasitised with haemococcidia (Dataset S1). Two species, *Pel.* kl. *esculentus* and *Pel. lessonae*, from Belgium were found infected with haemococcidia, conforming morphologically to *L. minima*. A second undescribed species of *Lankesterella* (*Lankesterella* sp. 1) was also observed in a single *Pel. lessonae* from Belgium (Table 2). In South Africa, haemococcidia were observed in the peripheral blood of five species of frogs, *Afrixalus delicatus* Pickersgill, 1984, *Afr. fornasini (*Bianconi, 1849), *Phrynobatrachus mababiensis* FitzSimons, 1932*, Ptychadena anchietae* (Bocage, 1868), and *Pyxicephalus edulis* Peters, 1854 (Table 2, Fig. 1). The first haemococcidian observed was a species of *Lankesterella* (*Lankesterella* sp. 2) found infecting *Afr. delicatus* and *Afr. fornasini*. The second, haemococcidian (haemococcidia sp. 1), similar in appearance to sporozoites of a lankesterellid within a greatly expanded PV, was found infecting *Phr. mababiensis, Pty. anchietae*, and *Pyx. edulis*, and a third haemococcidian (haemococcidia sp. 2) were found infecting a single *Afr. fornasini*.

Species classifications and descriptions

Phylum: Apicomplexa Levine, 1970
Class: Conoidasida Levine, 1988
Subclass: Coccidiasina Leuckart, 1879
Order: Eucoccidiorida Léger, 1911
Suborder: Eimeriorina Léger & Duboscq, 1911
Family: Lankesterellidae Nöller, 1920
Genus: *Lankesterella* Labbé, 1899

Table 2 The frog species found infected with haemococcidia, as well as the locality, frogs screened, number infected, morphotype and parasitaemia given in percentage (%).

Frog species	Country	Screened	Infected	Species	Parasitaemia
Pel. kl. *esculentus*	Bel	7	2	*L. minima*	0.1 ± 0.0 (0.0 – 0.1)
Pel. lessonae	Bel	12	2	*L. minima*	0.5 ± 0.3 (0.2 – 1.1)
Pel. lessonae	Bel	12	1	*Lankesterella* sp. 1	0.6
Afr. delicatus	SA	25	5	*Lankesterella* sp. 2	0.1 ± 0.1 (0.0 – 0.3)
Afr. fornasini	SA	20	1	*Lankesterella* sp. 2	0.2
Phr. mababiensis	SA	22	3	Haemococcidia sp. 1	0.1 ± 0.1 (0.0 – 0.1)
Pty. anchietae	SA	160	6	Haemococcidia sp. 1	0.1 ± 0.1 (0.0 – 0.2)
Pyx. edulis	SA	33	5	Haemococcidia sp. 1	0.5 ± 0.2 (0.0 – 0.7)
Afr. fornasini	SA	20	1	Haemococcidia sp. 2	0.2

Abbreviations: Bel = Belgium; SA = South Africa.

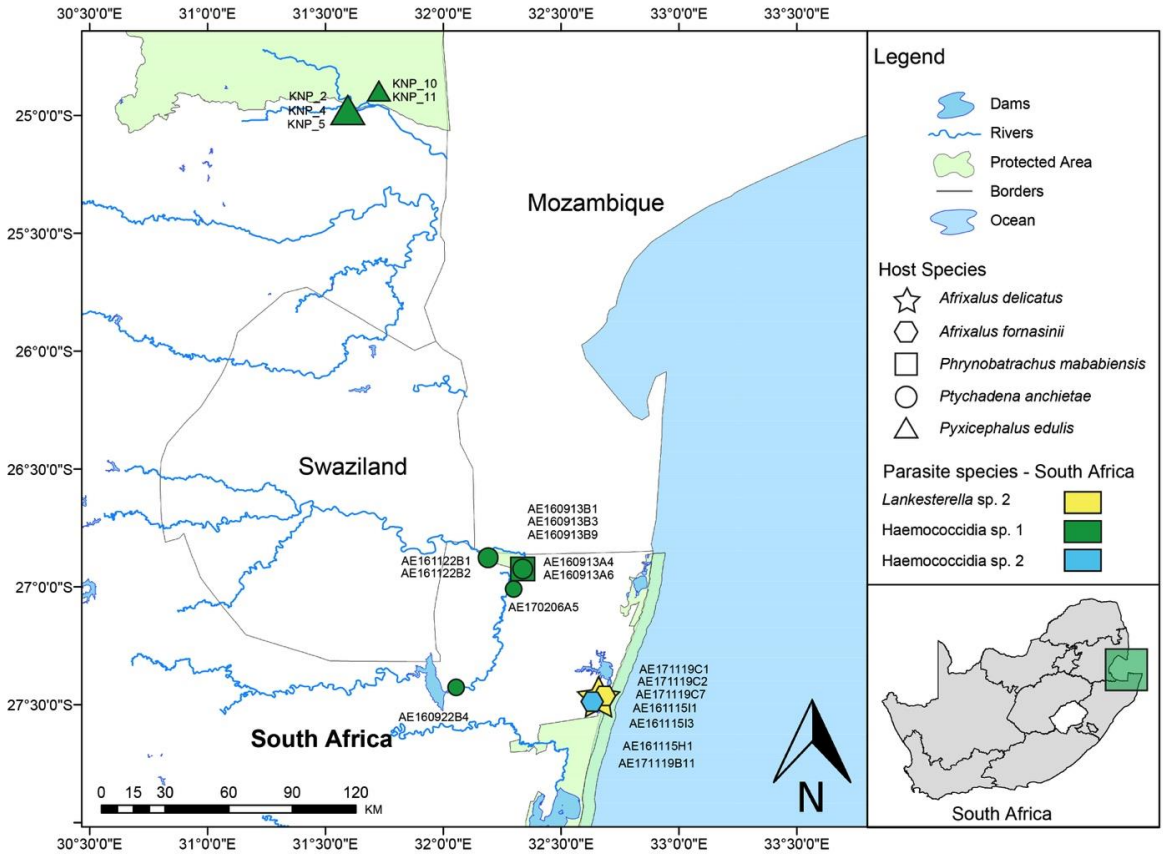

Fig. 1. Map of sampling localities of infected anurans, in northern KZN, and Kruger National Park, South Africa. Shapes indicate the host species sampled, and colour the haemococcidia species observed. Field numbers correspond with the shapes, to indicate the sampling locality of infected individuals.

Redescription of *Lankesterella minima* (Chaussat, 1850) Hintze, 1902

Synonyms: *Anguillula minima* Chaussat, 1850; *Drepanidium ranarum* Lankester, 1871, 1892; "Würmchen" Gaule, 1880, 1881, 1886; "Cytozoën" Bütschli, 1882; *Drepanidium rananrum* (Lankester, 1892) Wallerstein 1882; *Drepanidium princeps* Labbé, 1894; "Drepanidio piccolo" Grassi and Feletti 1892; *Lankesterella ranarum* Labbé, 1899; *Haemogregarina minima* (Laveran, 1898) Mathis and Léger, 1911; *Lankesterella minima* from Ontario, Canada (Barta and Desser, 1984) (see phylogenetic analysis).

Type-host: *Pelophylax* kl. *esculentus* (Linnaeus, 1758) (Anura: Ranidae).
Other host: *Pelophylax lessonae* (Camerano, 1882) (Anura: Ranidae).
Site in host: Peripheral blood.
Vector: *Hemiclepsis marginata* (O. F. Müller, 1774) (Rhynchobdellida: Glossiphoniidae).
Type-locality: Paris, France.
Other-localites: Haacht, Belgium (N50.979434°, E4.659686°); Rijmenam, Het Ven, Belgium (N51.010440°, E4.593420°).
Type-material: Voucher material, deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number [NMB P XXX]. Voucher material, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].
Representative DNA sequences: The 18S rRNA and COI gene sequences have been submitted to the GenBank database under the accession numbers XXX and XXX. The intraspecific divergence (uncorrected p-distance) between *L. minima* and its closest relative for the 18S rDNA and COI gene sequences was 2.3% (MG808274 - *Lankesterella* sp. from *Plectrophenax nivalis*) and 5.2% (*Lankesterella* sp. 2 from *Afr. delicates*), respectively.

Description

Immature sporozoites (Fig. 2B-E): Three morphotypes observed, cytoplasm staining purplish-blue; when visible nucleus staining purple-pink, with loosely arranged chromatin at centre. First immature sporozoite morphotype (Fig. 2B, arrow), slightly curved, in some cases a slight bulge visible in the centre of the sporozoite. Measuring 8.5 ± 0.6 (7.8 – 9.5) µm long × 4.0 ± 0.3 (3.6 – 4.3) µm wide (n = 7). Nucleus when visible measuring 2.3 ± 1.0 (1.6 – 3.4) µm long × 2.3 ± 1.0 (1.6–3.4) µm wide (n = 3). Second immature sporozoite morphotype (Fig. 2C), oval to rounded, measuring 8.5 ± 0.3 (8.1 – 9.0) µm long × 4.7 ± 0.7 (4.0 – 5.6) µm wide (n = 5). No clear nucleus visible, only loosely arranged chromatin. Third immature sporozoite morphotype (Fig. 2C-E, arrow), folded over itself at nucleus, measuring

8.4 ± 2.1 (7.0 – 9.5) µm long × 4.9 ± 0.4 (4.2 – 5.5) µm wide (n = 18). Nucleus when visible measuring 3.2 ± 0.2 (3.1 – 3.3) µm long × 2.4 ± 0.7 (1.8 – 2.9) µm wide (n = 2).

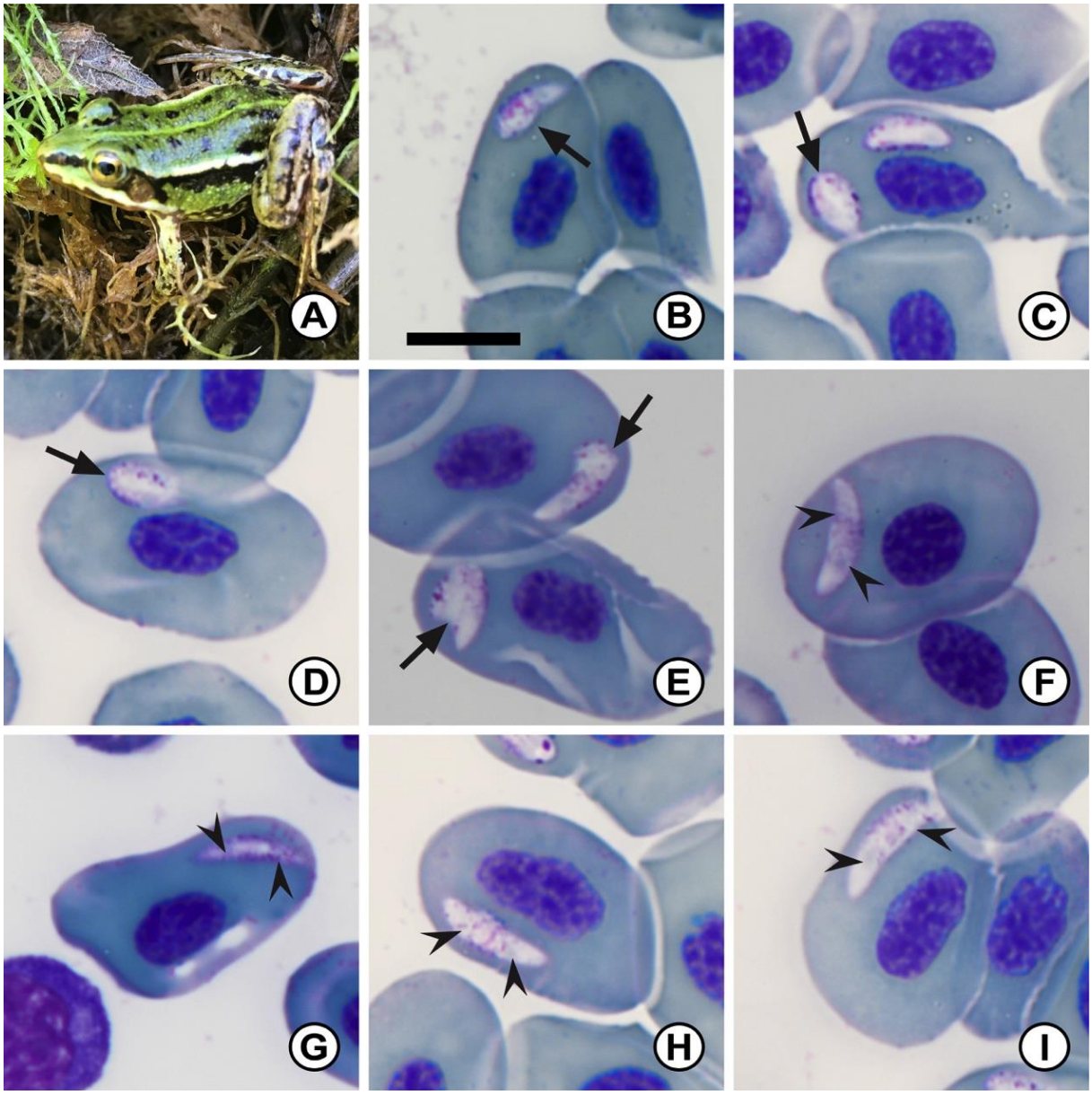

Fig. 2. *Lankesterella minima* (Chaussat 1850) Hintze 1902, observed in the peripheral blood of the type-host *Pelophylax* kl. *esculentus* and (A) *Pelophylax lessonae* from Belgium. (B-E) Immature sporozoites, arrow shows parasite flooded over itself. (C) Double infection of a single erythrocyte. (E) Sporozoites opening into half moon. (F-I) Mature sporozoites, arrowhead showing visible refractile bodies. All images captured from the deposited slides (NMB P XXX and XXX). Scale bar: 10 µm.

Mature sporozoites (Fig. 2F-I): elongated body shape slightly curved; observed in erythrocytes; cytoplasm staining purplish-blue and nucleus staining dark pink to purple, with

loosely arranged chromatin primarily at centre; one or two refractile bodies sometimes visible (Fig. 2F-I, arrow head); and the nucleus chromatin is condensed to the sides of the body (Fig. 2E). Measures 11.1 ± 1.1 (9.4 – 13.0) µm long × 3.6 ± 0.4 (2.7 – 4.6) µm wide (n = 30). Nucleus when visible measures 3.0 ± 0.5 (1.6 – 3.8) µm long × 3.0 ± 0.5 (2.1 – 4.1) µm wide (n = 22).

Remarks

Chaussat (1850) was the first to report on *L. minima* from the blood of *Pel.* kl. *esculentus* referring to it as "*Anguillula minima*" (now *Lankesterella minima*). Based on the scale provided the parasites measure 12 – 13 µm long × 3 – 3.5 µm wide. No clear refractile bodies are visible in the provided drawings. Localities of the collected specimens are not provided; the type locality is most likely in the vicinity of Paris, France. Lankester (1871, 1882) and Labbé (1894), report on apparently the same parasite, under the name "*Drepanidium ranarum*" and "*Drepanidium princeps*", respectively. Subsequently Hintze (1902) reviews all so-called "*Drepanidium*" parasites of frogs and synonymises "*Drepanidium ranarum*" under the name *L. minima*. Nöller (1913) supports Hintze (1902) regarding all these parasites as the same, provides the synonyms and classifies them under the name *Lankesterella minima.* The parasite from the current study conforms closely to the above descriptions, with mature sporozoites measuring 11.1 ± 1.1 (9.4–13.0) µm long × 3.6 ± 0.4 (2.7–4.6) µm wide (n = 30), and although not from the type locality, this parasite is found in various host species, including the type host as reported by Chaussat (1850). Morphologically this parasite conforms close to the mature sporozoites of a *Lankesterella* species considered to be *L. minima* from Ontario, Canada (see Barta and Desser, 1984). However, that species has a clear and compact nucleus with minimal chromatin granules spread across the sporozoites, and a slight concave almost PV like structure to the one side of the parasite. This is in contrast to *L. minima* from the current study, which is closest to the type locality. The *Lankesterella* species from Ontario, Canada, should thus be reassessed, and not referred to that species as *L. minima*.

***Lankesterella* sp. 1**

Type-host: *Pelophylax lessonae* (Camerano, 1882) (Anura: Ranidae).

Site in host: Peripheral blood.

Vector: Unknown.

Type-locality: Rijmenam, Het Ven, Belgium (N51.010440°, E4.593420°).

Type-material: Hapantotype, deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number [NMB P XXX]. Parahapantotype,

deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

Representative DNA sequences: The 18S rRNA and COI gene sequences have been submitted to the GenBank database under the accession numbers XXX and XXX. The intraspecific divergence between *Lankesterella* sp. 1 and its closest relative for the 18S rDNA and COI gene sequences was 2.6% (*Lankesterella* sp. 2) and 4.3% (*Lankesterella* sp. 2), respectively.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub: XXX. The LSID for the new name XXX is urn:lsid:zoobank.org:act:XXX.

Etymology: XXX

Description

Immature sporozoites (Fig. 3B-F): three morphotypes observed; cytoplasm staining bluish-purple, with dark pink chromatin granules spread throughout; nucleus staining light pink to purple centre; one or two refractile bodies visible staining greenish-blue (Fig. 3B-E, arrowhead). First immature sporozoite morphotype (Fig. 2B), oval to round, measuring 7.0 ± 0.6 (6.1 – 7.7) µm long × 4.9 ± 0.7 (3.7 – 5.8) µm wide (n = 8). Nucleus measuring 2.6 ± 0.4 (2.2 – 3.0) µm long × 2.4 ± 0.1 (2.4 – 2.5) µm wide (n = 3). Second immature sporozoite morphotype (Fig. 3C-D, arrow), folded over itself at nucleus into oval, measuring 6.6 ± 0.5 (6.0 – 7.7) µm long × 4.9 ± 0.6 (3.7 – 6.1) µm wide (n = 12). Nucleus measuring 2.8 ± 0.0 (2.8 – 2.9) µm long × 2.7 ± 0.3 (2.5 – 2.9) µm wide (n = 2). Third immature sporozoite morphotype (Fig. 3E-F, arrow), folded over itself at nucleus, opens into a half moon, measuring 7.1 ± 0.6 (5.9 – 7.8) µm long × 4.7 ± 0.9 (3.8 – 6.8) µm wide (n = 9). Nucleus measuring 2.1 ± 1.1 (1.3 – 2.9) µm long × 2.3 ± 0.4 (1.9 – 2.6) µm wide (n = 2).

Mature sporozoites (Fig. 3G-I): short and stout parasite with slight curvature; parasitising erythrocytes; cytoplasm, nucleus and chromatin granules staining the same as immature sporozoites; one or two refractile bodies visible staining greenish-blue (Fig. 3G-I, arrow head). Mature sporozoites measure 8.8 ± 0.8 (7.9 – 11.0) µm long × 3.8 ± 0.5 (2.1 – 3.7) µm wide (n = 26). Nucleus measuring 2.8 ± 0.5 (2.1 – 3.7) µm long × 2.3 ± 0.4 (1.8 – 3.5) µm wide (n = 16).

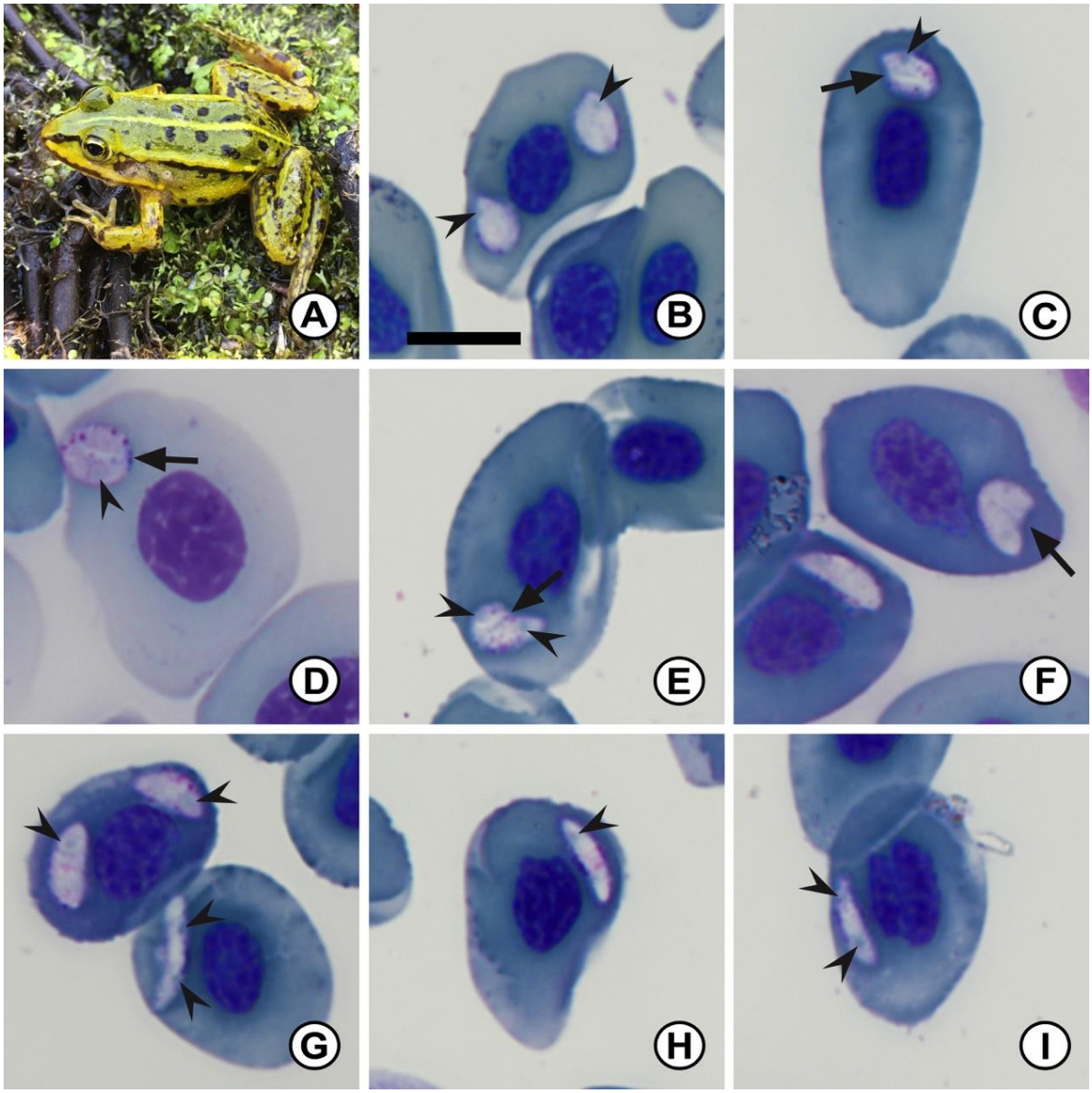

Fig. 3. *Lankesterella* sp. 1 observed in the peripheral blood of the host (A) *Pelophylax lessonae* from Belgium. (B-F) Immature sporozoites, arrowhead showing visible refractile bodies. (B) Double infection of a single erythrocyte. (C-F) Arrow showing sporozoites folded over itself. (E-F) Sporozoites opening into half moon. (G-I) Mature sporozoites, arrowhead showing visible refractile bodies. (G) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P XXX). Scale bar: 10 µm.

Remarks

Lankesterella sp. 1 is characterised by its half-moon shaped immature sporozoites, that are often also folded over itself; short and stout mature sporozoites with one or two refractile bodies visible and staining a greenish-blue. *Lankesterella* sp. 1 is the most similar in size to

the only other species described from Europe, *L. minima*. However, the mature sporozoites of *Lankesterella* sp. 1 are generally shorter as compared to *L. minima*, with less chromatin granules spread across sporozoites. In some cases mature sporozoites of *L. minima* contain a small recurved tail, which is in contrast to *Lankesterella* sp. 1. Morphometrically this parasite conforms closest to the mature sporozoites of *L. poeppigii* Paperna, Bastien, Chavatte and Landau, 2009 from Peru, however, mature sporozoites *Lankesterella* sp. 1 do not taper sharply at the ends, and its nuclear chromatin does not gather at the periphery as in *L. poeppigii.* Based on these characters, *Lankesterella* sp. 1 can be distinguished from all nine currently recognised species of *Lankesterella* from anurans globally, namely *L. alencari* Costa and Pereira, 1971, *L. bufonis* Mansour and Mohammed 1962, *L. canadensis* Fantham, Porter and Richardson 1942, *L. dicroglossi*, *L. hylae* Cleland and Johnston 1910, *L. minima*, *L. petiti* Lainson and Paperna, 1995, *L. poeppigii*, and *L. ptychadeni*. We suggest *Lankesterella* sp. 1 is considered to be a new species.

***Lankesterella* sp. 2**

Type-host: *Afrixalus delicatus* Pickersgill, 1984 (Anura: Hyperoliidae)(Fig. 2A).

Other host: *Afrixalus fornasini* (Bianconi, 1849) (Anura: Hyperoliidae).

Site in host: Peripheral blood.

Vector: Unknown.

Type-locality: Sodwana, KZN, South Africa (S27.488591°, E32.664259°).

Type-material: Hapantotype, 1 × blood smear deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P XXX; parahapantotype, 1 × blood smear deposited in the Protozoan Collection of the National Museum, Bloemfontein, South Africa, under accession number [NMB P XXX]. Parahapantotype, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

Representative DNA sequences: The 18S rRNA and COI gene sequences have been submitted to the GenBank database under the accession numbers XXX and XXX. The intraspecific divergence (uncorrected p-distance) between *Lankesterella* sp. 2 and its closest relative (*Lankesterella* sp. 1) for the 18S rDNA and COI gene sequences was 2.8% and 4.9%, respectively.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub: XXX. The LSID for the new name XXX is urn:lsid:zoobank.org:act:XXX.

Etymology: XXX

Description

Immature sporozoites (Fig. 3B-C): slender, elongated and slightly curved; anterior pole tapered down; cytoplasm granular, staining whitish-purple to pink; nucleus granulated, situated in the anterior half of the parasite; in some cases the immature sporozoites folded over itself at the nucleus (Fig. 2C). Immature stages measure 7.5 ± 0.3 (7.3–7.9) µm long × 4.0 ± 0.8 (2.9–4.9) µm wide (n = 5).

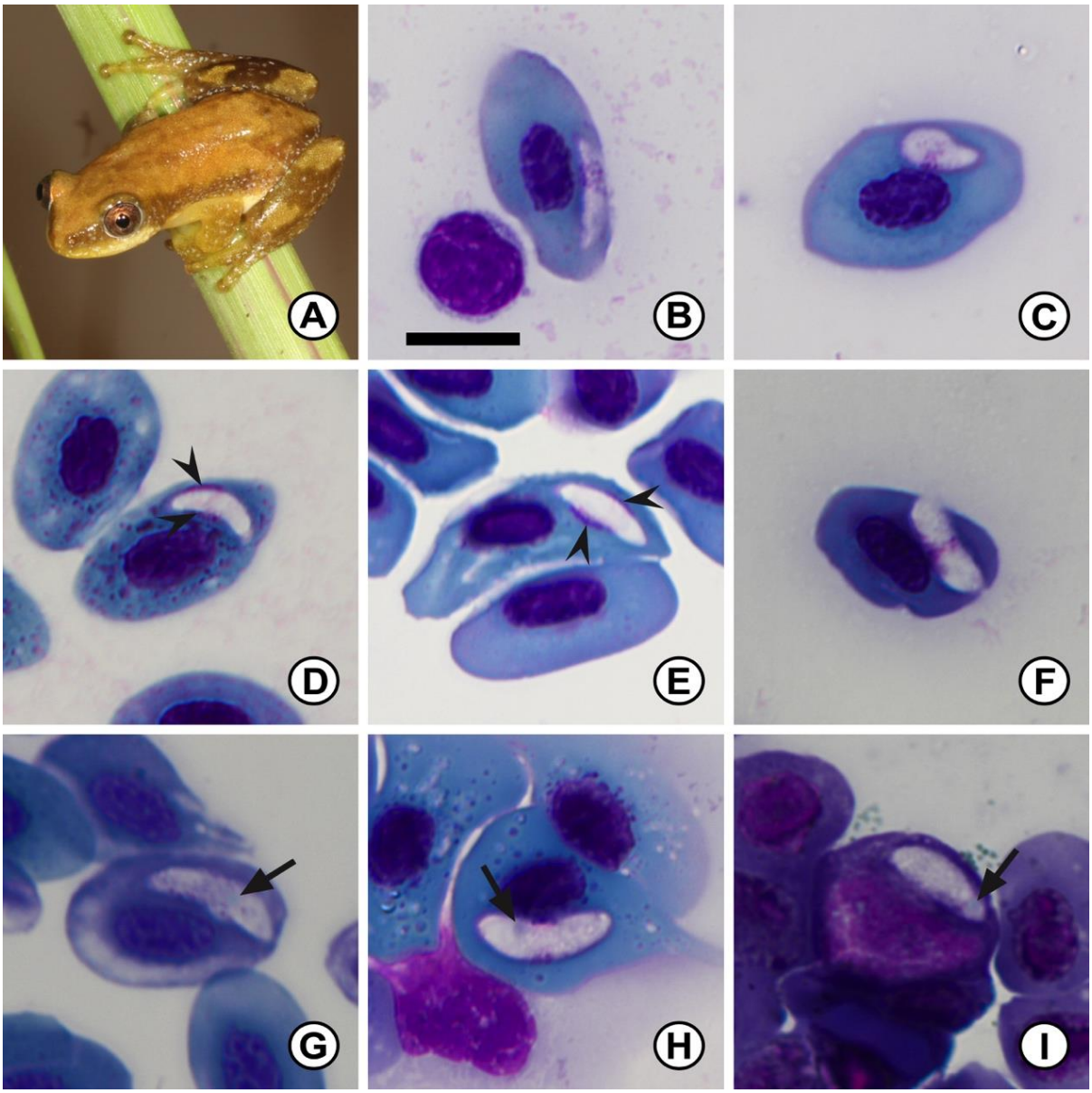

Fig. 4. *Lankesterella* sp. 2, observed in the peripheral blood of (A) *Afrixalus delicates* from KZN, South Africa. (B-C) Immature sporozoites. (C) Sporozoite folded over itself. (D-I) Mature sporozoites. (D-E) Arrowhead showing nucleus chromatin situated on either side of sporozoites walls. (G-I) Arrow showing visible refractile bodies. (I) Parasitising lymphocyte. All images captured from the deposited slides (NMB P XXX and XXX). Scale bar: 10 µm.

Mature sporozoites (Fig. 3D-I): large and variable, elongated, ellipsoid, and curved, tapering towards the anterior pole of the parasite; cytoplasm granular and staining whitish-purple to pink (Fig. 3F); nucleus situated in the anterior half of the parasite; often nucleus chromatin situated on either side of sporozoites walls (Fig. 3D-E, arrow head); one refractile body visible (Fig. 3G-I, arrow); infect erythrocytes (primarily) and lymphocytes (rarely) (Fig. 3I). Mature stages measure 10.9 ± 1.3 (8.4–14.4) µm long × 3.4 ± 0.7 (2.1–5.0) µm wide (n = 30). Nucleus measuring 2.8 ± 0.7 (1.3–4.2) µm long × 2.8 ± 0.8 (1.6–4.3) µm wide (n = 24).

Remarks

Lankesterella sp. 2 is characterised by its large mature sporozoites, with often nucleus chromatin situated on either side of sporozoite walls. Mature sporozoites also taper to one end with one clear refractile body visible. This species can be distinguished from *Lankesterella* sp. 1 based on differences in sporozoites morphometrics, as well as general appearance. *Lankesterella* sp. 2 contains larger sporozoites, a thicker posterior pole tapering towards the anterior pole of the parasite and measures 10.9 (8.4 – 14.4) µm long × 3.4 (2.1 – 5.0) µm wide. This species can also be distinguished from the sporozoites of other African anuran species of *Lankesterella*. *Lankesterella* sp. 2 can be differentiated based on the thickness of sporozoites stages, as compared to *L. bufonis*, measuring 10.3 (6.2 – 12.8) µm long × 1.2 (0.7 – 2.0) µm wide and *L. dicroglossi*, measuring 7.0 – 9.0 µm long × 1.0 – 2.8 µm wide. Morphometrics of *Lankesterella* sp. 2 can be differentiated from *L. ptychadeni*, which measures 4.2 – 7.7 µm long × 1.4 – 2.8 µm wide. Morphologically this parasite conforms closest to the mature sporozoites of *L. minima* from Belgium. However, in contrast to *L. minima* no ellipsoid stages were observed in immature sporozoites, and the arrangement and staining of the nucleus differ for mature sporozoites *Lankesterella* sp. 2.. The general appearance of *L. minima* is elongated and slender. Based on these phenotypic characters and comparisons we are confident that *Lankesterella* sp. 2. is a new species.

Genus: Haemococcidia n. gen.

Type-species: Haemococcidia sp. 1 (*L. ptychadeni*) (Paperna and Ogara 1996) n. comb.

Etymology: XXX

Differential diagnosis

Haemococcidia n. gen.: Enlarged PV, seems to extend away from the crescent shaped parasite lying within; smooth ellipsoid to crescent shape on one side with parasite, asymmetrical semicircle extended PV on opposite; cytoplasm or the PV not always staind,

white vacuole-like appearance, dark purple or eosiniphilic stained border; no evident refractile bodies present; no visible cytopathological effects on the host cell.

Remarks

Differences in the peripheral blood sporozoites are supported by the molecular phylogenetic analyses using both 18S rDNA datasets and the robust partitioned nuclear 18S rRNA and mitochondrial COI gene datasets (see phylogenetic analysis). Based on these findings and to maintain monophyly of species designated to the these genera *Lankesterella* and *Schellackia*, it can be suggested to name the new genus, *Haemovaulata* n. gen., for species of haemococcidia that contain mature intracellular sporozoites encompassed by an expanded PV, present within peripheral blood cells of the vertebrate host. *Lankesterella ptychadeni*, described from *Pty. nilotica* share this feature of their sporozoites encompassed by an expanded PV. Thus, the data from the development of *L. ptychadeni* in the vertebrate hosts organs, which was reported by Paperna and Ogara (1996), explains the distinct features of haemococcidia n. gen., as compared to typical *Lankesterella* and *Schellackia* species. based on the morphological similarities between *L. ptychadeni* and the species from *Phr. mababiensis*, *Pty. Anchietae*, and *Pyx. edulis*. All are considered as conspecific (see description below). The first developmental characteristic that distinguishes haemococcidia n. gen. from the genus *Lankesterella* is oogony and sporogony, which takes place in the gut epithelium and in the lamina propria, similar to species of *Schellackia*. Secondly, and also similar to *Schellackia* species, oocysts yield eight sporozoites that accumulate in the gut mucosal epithelium and in the lamina propria (Paperna and Ogara, 1996). Merogonic replication takes place in the liver; and sporogony, with eight sporozoites seems to also occur in detached endothelial cells in the blood (Paperna and Ogara, 1996). However, these characteristics still need to be confirmed by additional observations or experimental trials as suggested in the original study (Paperna and Ogara, 1996). Furthermore young oocysts lack type-1 and -2 wall-forming bodies of eimeriid macrogamonts distinguishing haemococcidia n. gen. from species of *Schellackia* (Ostrovska and Paperna, 1987; Paperna and Ogara, 1996).

Haemococcidia sp. 1 (n. comb. *L. ptychadeni* (Paperna and Ogara 1996))

Synonym: *Lankesterella ptychadeni* Paperna and Ogara 1996.

Type-host: *Ptychadena nilotica* (Seetzen, 1855) Anura: Ptychadenidae.

Other host: *Phrynobatrachus mababiensis* FitzSimons, 1932, *Ptychadena anchietae* (Bocage, 1868), and *Pyxicephalus edulis* Peters, 1854.

Site in host: Peripheral blood.

Vector: Unknown.

Type-locality: Lake Victoria, Kenya.

Other-localites: Kruger National Park, South Africa. Coordinates: (S24.990610°;E31.602001°); Phongolo River in Ndumo Game reserve, South Africa. Coordinates: (S26.926179°; E32.332416°); Phogola River, South Africa. (S27.020375°;E32.302959°).

Voucher-material: Voucher, 3 × blood smear from *Pyx. edulis*, *Phr. mababiensis* and *Pty. anchietae* respectively, deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number [NMB P XXX, NMB P XXX and NMB P XXX]. Parahapantotype, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

Representative DNA sequences: The 18S rRNA and COI gene sequences have been submitted to the GenBank database under the accession numbers XXX and XXX. The intraspecific divergence (uncorrected p-distance) for the 18S rRNA gene of haemococcidia sp. 1 (n. comb. *L. ptychadeni*), was 0.6 %. For haemococcidia sp. 1 (n. comb. *L. ptychadeni*) and its closest relative for the COI gene the intraspecific divergence was 6.5% (haemococcidia sp. 2), with the intraspecific divergence of haemococcidia sp. 1 (n. comb. *L. ptychadeni*) 0.8 %.

Description

Immature sporozoites (Fig. 5B-C): crescent shaped, irregular on the one side and convex on the opposite; staining a light purplish-pink, with eosiniphilic bands along the sides of the parasite; no visible cytopathological effects on the host cell. Immature intraerythrocytic stages measure 5.6 ± 0.3 (5.1 – 5.9) µm long × 3.1 ± 0.3 (2.7 – 3.5) µm wide (n = 6).

Mature sporozoites (Fig. 4D-F,H-I,K-L): enlarged PV, seems to extend away from the crescent shaped parasite lying within (Fig. 3D-F,I); irregular shape ranging from ellipsoid (Fig. 4D) to smooth crescent shape on one side (parasite) and asymmetrical or unbalanced semicircle (extended PV) on opposite (Fig. 4E-F,I); cytoplasm staining a light purplish-pink, with eosiniphilic stained spots at random; in some cases cytoplasm or the PV did not stain, causing a white vacuole-like appearance with dark purple stained border (Fig. 4H,K-L); no evident refractile bodies present; no visible cytopathological effects on the host cell.

The mean average measurements of the PV across *Pyx. edulis* (Fig. 4A), *Phr. mababiensis* (Fig. 4G) and *Pty. anchietae* (Fig. 4J) measured 6.3 ± 0.8 (4.9 – 8.5) µm long by 3.8 ± 0.8 (2.5 – 6.8) µm wide (n = 64). In *Pyx. edulis* when visible the parasites inside the

PV measured 6.6 ± 0.6 (5.5 – 7.8) µm long by 2.6 ± 0.3 (2.1 – 3.3) µm wide (n = 20). Nucleus measuring 2.0 ± 0.7 (1.3 – 3.4) µm long × 1.3 ± 0.4 (0.8 – 1.9) µm wide (n = 7). In *Phr. mababiensis* when visible the parasites inside the PV measured 6.9 ± 0.4 (6.7– 7.4) µm long by 2.6 ± 0.6 (2.1 – 3.2) µm wide (n = 3). Nucleus measuring 1.7 ± 0.7 (1.3 – 2.6) µm long × 1.0 ± 0.7 (0.6 – 2.2) µm wide (n = 5).

Remarks

Haemococcidia sp. 1 (n. comb. *L. ptychadeni*) is characterised by its enlarged PV extending away from the crescent shaped parasite lying within the PV; smooth crescent shape on one side and asymmetrical extended PV on opposite; in some cases cytoplasm or the PV did not stain, causing a white vacuole-like appearance with dark purple stained border; no evident refractile bodies present; and no visible cytopathological effects on the host cell. This species can be distinguished form *L. alencari*, *Lankesterella* sp. (syn*. L. minima*, Canada), *L. canadensis*, *L. dicroglossi*, *L. hylae*, *Lankesterella* sp. 2, *L. minima*, *L. petiti*, *L. poeppigii*, and *Lankesterella* sp. 1 based on the enlarged PV forming an irregular ellipsoid, extending away from the crescent shaped parasite lying within the PV. Mature sprozoites of haemococcidia sp. 1 (n. comb. *L. ptychadeni*), from the current study measuring 6.6 µm long by 2.6 µm wide, and lying within the extended PV can be distinguished from the sporozoites of *L. bufonis*, that are generally longer and more slender measuring, 10.3 µm long by 1.2 µm wide as compared to haemococcidia sp. 1 (n. comb. *L. ptychadeni*). Morphometric data of haemococcidia sp. 1 (n. comb. *L. ptychadeni*), from the original description overlap with haemococcidia sp. 1 (n. comb. *L. ptychadeni*) in the current study. Staining of the PV was similar to the morphotype observed in *Phr. mababiensis* and *Pty. anchietae*, causing a white vacuole-like appearance with dark purple eosiniphilic stained border.

Haemococcidia sp. 2

Type-host: *Afrixalus fornasini* (Bianconi, 1849) (Anura: Hyperoliidae).

Site in host: Peripheral blood.

Vector: Unknown.

Type-locality: Sodwana, KZN, South Africa (S27.488591°; E32.664259°).

Type-material: Hapantotype, 1 × blood smear deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number [NMB P XXX]. Parahapantotype, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

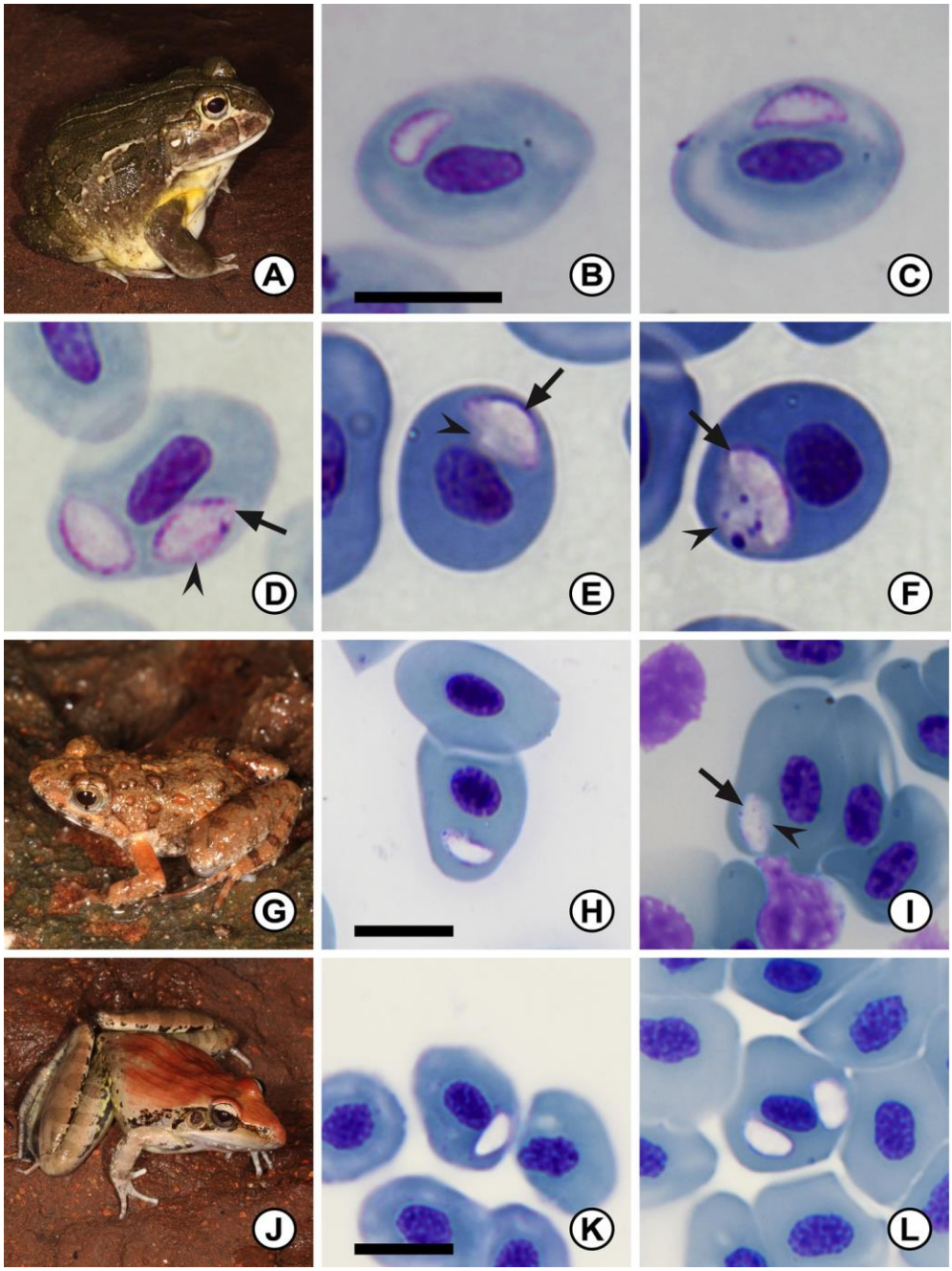

Fig. 6. Haemococcidia sp. 1 (n. comb. *L. ptychadeni* (Paperna and Ogara 1996)) observed in the peripheral blood of (A) *Pyxicephalus edulis* from the Kruger National Park, South Africa. (B-C) Immature sporozoites. (D-F) Mature sporozoites, arrowhead showing enlarged PV, arrow showing the crescent shaped parasite lying with in PV. (D) Double infection of a single erythrocyte. (G) *Phrynobatrachus mababiensis* from KZN, South Africa. (H-I) Mature sporozoites from *Phr. mababiensis*. (H) Cytoplasm or PV not stained, causing a white vacuole-like appearance with dark purple stained boarder. (I) Arrowhead showing PV, arrow showing the crescent shaped parasite lying with in PV. (J) *Ptychadena anchietae* from KZN, South Africa. (K-L) Mature sporozoites from *P. anchietae*, cytoplasm or PV not stained, causing a white vacuole-like appearance with dark purple stained boarder. (L) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P XXX, XXX and XXX). Scale bar: 10 µm.

Representative DNA sequences: The COI gene sequences have been submitted to the GenBank database under the accession numbers XXX and XXX. The intraspecific divergence of COI gene sequences (uncorrected p-distance) between the different species of haemococcidia n. gen. (haemococcidia sp. 2 and haemococcidia sp. 1 n. comb.) was 6.5 – 6.8 %.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:XXX. The LSID for the new name XXX is urn:lsid:zoobank.org:act:XXX.

Etymology: XXX

Description

Immature sporozoites (Fig. 6B-E): mostly petite crescent shaped parasite, with slight PV visible some individuals (Fig. 4D, arrow head); cytoplasm finely granulated staining a light bluish-purple; no visible cytopathological effects on the host cell. Immature stages measure 8.8 2.0 (7.5 – 11.0) µm long × 3.1 ± 0.6 (2.1 – 4.0) µm wide (n = 23). Nucleus measuring 2.0 ± 0.4 (1.3 – 2.7) µm long × 1.9 ± 0.5 (0.9 – 2.5) µm wide (n = 23).

Mature sporozoites (Fig. 5F-I): enlarged PV (Fig. 3F-I, arrow head), seems to extend away from the crescent shaped parasite lying within the PV (Fig. 5F-I, arrow); PV irregular shape ranging from ellipsoid (Fig. 5F), to smooth crescent shape on side with parasite to asymmetrical semicircle (extended PV) on opposite side (Fig. 5G-I); mature forms often found in cells parasitised with *Hepatozoon tenuis* (Fig. 5G-I, asterisk), in certain cells up to four sporozoites visible (Fig. 3I); cytoplasm staining a whitish to purplish-pink, frequently with pink-purple eosiniphilic stained spots at random; PV staining bluish-white (Fig. 5F-I, arrow head); granulated nucleus situated slightly off centre toward tapering end, staining dark pinkish-purple; no refractile body detectible; no visible cytopathological effects on the host cell. Mature forms including PV measured 9.6 ± 0.9 (8.5 – 12.0) µm long by 5.8 ± 0.7 (4.3 – 7.4) µm wide (n = 30). Sporozoites inside PV measured 9.4 ± 0.9 (8.1 – 12.1) µm long by 2.6 ± 0.4 (1.8 – 3.5) µm wide (n = 30). Nucleus measuring 2.2 ± 0.5 (1.5 – 3.4) µm long × 2.0 ± 0.3 (1.5 – 2.5) µm wide (n = 30).

Remarks

Haemococcidia sp. 2 is characterised by a slender crescent shaped parasite lying within an extended PV; PV irregular shape ranging from smooth ellipsoid to crescent shape on side with parasite, to asymmetrical semicircle (extended PV) on opposite side.

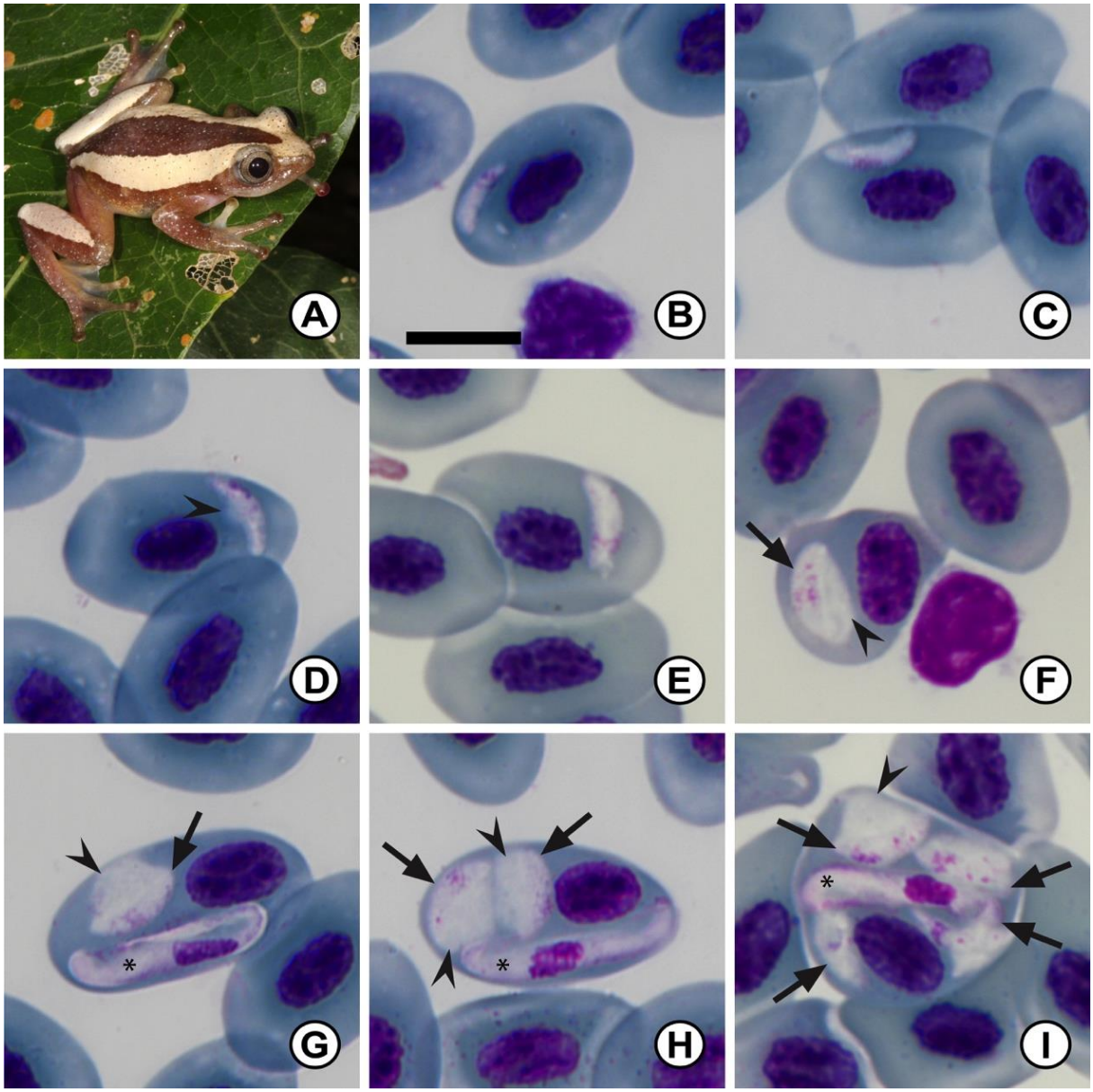

Fig. 5. Haemococcidia sp. 2, observed in the peripheral blood of (A) *Afrixalus fornasini* from KZN, South Africa. (B-E) Immature sporozoites. (D) Arrowhead showing slight PV. (F-I) Mature sporozoites, arrowhead showing enlarged PV, arrow showing the crescent shaped parasite lying with in PV. (G) Infection of a single erythrocyte with *Hepatozoon tenuis*. (H) Double infection of a single erythrocyte with *H. tenuis*. (I) Four visible sporozoites parasitising a single erythrocyte with *H. tenuis*. All images captured from the deposited slides (NMB P XXX). Scale bar: 10 µm.

This species can be distinguished from *L. alencari*, *Lankesterella* sp. (syn. *L. minima*, Canada), *L. bufonis*, *L. canadensis*, *L. dicroglossi*, *L. hylae*, *Lankesterella* sp. 2, *L. minima*, *L. petiti*, *L. poeppigii*, and *Lankesterella* sp. 1 based on the enlarged and extended PV forming away from the crescent shaped parasite lying within the PV. This species can also be distinguished from the sporozoites of haemococcidia sp. 1 (n. comb. *L. ptychadeni*) (see

above) based on morphological differences in length or width of mature sporozoite stages. Although the sporozoite of haemococcidia sp. 2 is most similar to *L. bufonis* (10.3 µm long by 1.2 µm), haemococcidia sp. 2 is wider measuring 9.4 µm long by 2.6 µm wide.

Phylogenetic analysis

Fifteen 18S rDNA and COI gene amplicons of between 577 and 1243 nt (depending on the primer set used), and 428 and 499 nt, respectively, were obtained from haemococcidia parasitising *Afr. delicates*, *Afr. fornasini*, *Phr. mababiensis*, *Pty. anchietae*, and *Pyx. edulis* from South Africa; and *Pel.* kl. *esculentus* and *Pel. lessonae* from Belgium.

In the BI phylogenetic analysis based on 18S rDNA sequences, *Eimeria* Schneider, 1875 along with *Isospora* from the Eimeriidae, are polyphyletic and spread across several clades. The different haemococcidia genera (Fig. 7. highlighted in green) form separate clades nested between clusters from the Eimeriidae. Species of *Schellackia* isolated from chelonian and lizard hosts, formed a monophyly with *Eimeria arnyi* Upton and Oppert, 1991, and *Eimeria ranae* Dobell, 1909. From within the Lankesterellidae three clades of *Lankesterella* species are recognised, clade A, B and C. Clade A comprises isolates from the Desert Iguana *Dipsosaurus dorsalis* (Baird and Girard, 1852) from North America, and is sister to clade B and C. Clade B formed a strongly supported group comprising *Lankesterella* parasites isolated from lizard hosts from North America, the Middle East and the Mediterranean. Lastly, Clade C is a large clade, comprising species of *Lankesterella* isolated from reptilian, amphibian and avian hosts, as well as the sequences characterised from anurans in the present study. Furthermore, this phylogenetic analysis shows a monophyly comprising a previously enigmatic intraerythrocytic haemococcidium, assigned to haemococcidia n. gen. (see description above). Haemococcidia sp. 1 (n. comb. *L. ptychadeni*) isolated from *Pyx. edulis*, *Phr. mababiensis*, and *Pty. anchietae*, represents a polytomy with a clade, consisting of *Eimeria* species from marsupial hosts and the clade of *Schellackia* species isolated from chelonian and lizard hosts, with *E. arnyi*, and *E. ranae*. The haemococcidia sp. 1 (n. comb. *L.*) haplotypes from *Phr. mababiensis*, and *Pty. anchietae* are identical, with an interspecific uncorrected p-distance of 0.6 % to haemococcidia sp. 1 (n. comb. *L. ptychadeni*) isolated from *Pyx. edulis*.

For the COI phylogenetic analysis, as in the 18S rDNA analysis, species of *Eimeria* and *Isospora* are polyphyletic. *Choleoeimeria pogonae* Yang, Brice, Ryan 2016 from the Eimeriidae is situated basal to the entire ingroup. *Isospora amphiboluri* Cannon, 1967 forms a sister taxon to *Caryospora bigenetica* Wacha and Christiansen, 1982.

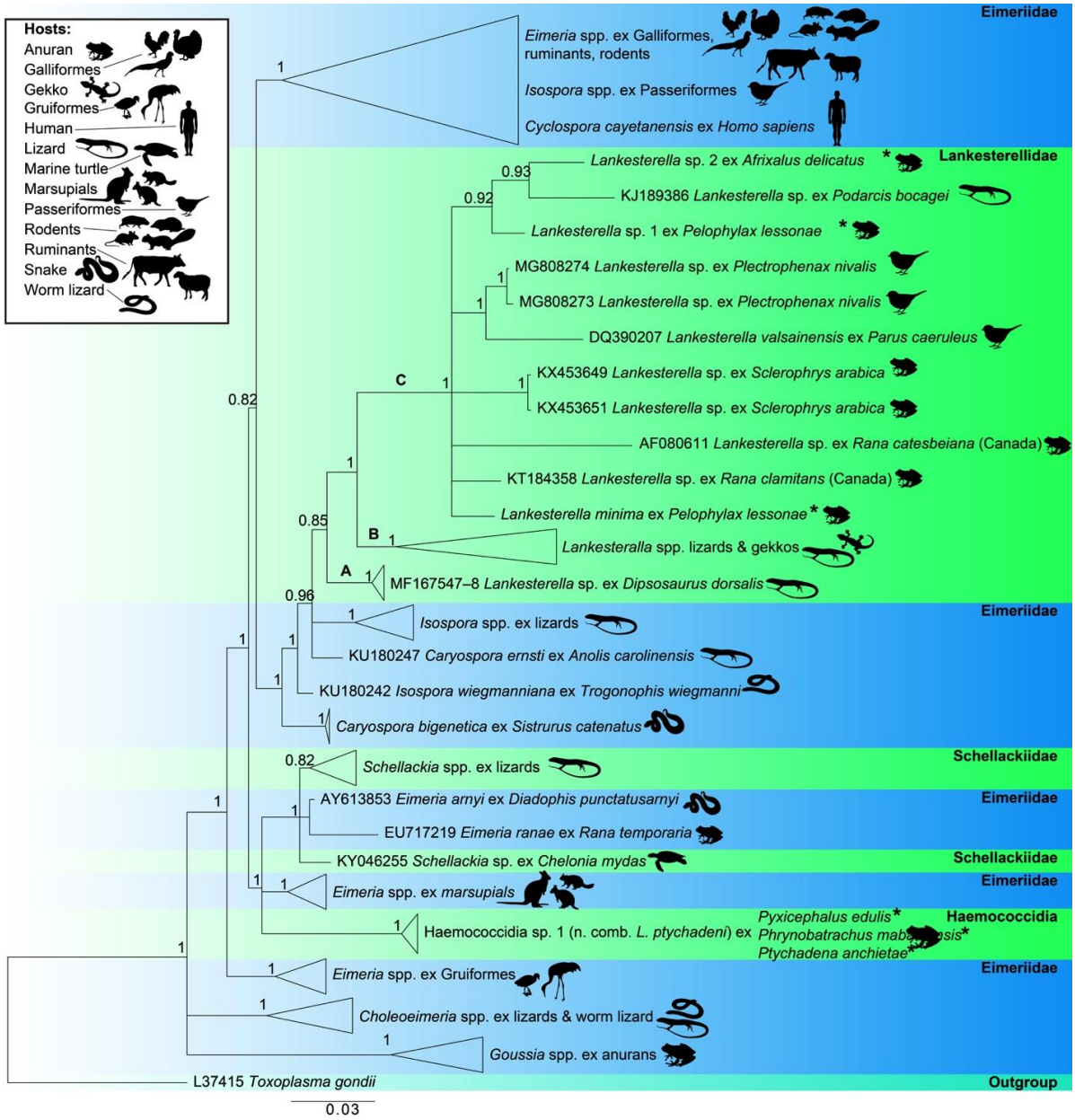

Fig. 7. Bayesian Inference (BI) phylogram based on partial 18S rDNA sequences. Analysis shows the phylogenetic relationships of several taxa from the Eimeriidae, Lankesterellidae, and Schellackiidae. The final dataset contained 1518 nt from 112 sequences, rooted with *Toxoxplasma gondii* [GenBank: L37415] from the Sarcocystidae. Asterisks (*) indicate the sequences obtained in the current study. Only nodal posterior probability support values of >0.75 shown. The scale bar represents 0.03 nucleotide substitutions per site. Pictograms illustrate host species group.

Species of *Lankesterella*, *I. amphiboluri*, and *Car. bigenetica*) form a monophyly that represents a polytomy with haemococcidia n. gen., and a large monophyletic clade of *Isospora* isolated from passeriform hosts, and *Eimeria* species from Galliformes, mammals, and marsupials, with *Cyclospora cayetanensis* Ortega, Gilman & Sterling, 1994.

haemococcidia sp. 2 isolated from *Afr. fornasini* is sister to a monophyletic clade of haemococcidia sp. 1 (n. comb. *L. ptychadeni*) isolated from *Phr. mababiensis*, *Pty. anchietae*, and *Pyx. edulis*. The haplotypes from *Phr. mababiensis*, and *Pty. anchietae* are identical for the COI marker, with an uncorrected p-distance of 0.8 % to haemococcidia sp. 1 (n. comb. *L. ptychadeni*), isolated from *Pyx. edulis*.

A summary of the sequence comparisons of the 18S rRNA gene (Dataset S2) and the intergeneric divergence (uncorrected p-distance) between clades from the taxa of *Lankesterella* and haemococcidia n. gen. compared to all the other major clades can be found in Table 3.

Table 3. Summary of the interspecific divergence (uncorrected P-distance) rages based on the 18S rDNA sequences between the clades of *Lankesterella* and haemococcidia n. gen. and the other clades or taxa

	Lankesterella spp.	Haemococcidia n. gen.
Eimeria ex galliforms	3.7 – 14.0	5.1 – 7.1
Eimeria ex mammals	3.0 – 14.8	4.6 – 8.8
Eimeria ex marsupials	3.0 – 13.4	0.5 – 6.0
Eimeria ex gruiforms	4.3 – 12.8	4.8 – 5.7
Eimeria ex ectotherms	3.6 – 14.3	4.7 – 5.6
Isospora ex passeriforms	2.8 – 13.5	
Haemococcidia n. gen.	4.3 – 13.7	0.0 – 0.6
Cyc. cayetanensis	3.8 – 13.3	5.1 – 5.3
Isospora ex reptiles	2.0 – 11.8	4.4 – 5.4
Caryospora ex reptiles	2.1 – 11.5	4.1 – 4.4
Choleoeimeria ex reptiles	5.3 – 17.5	6.1 – 6.9
Schellackia ex reptiles	3.6 – 14.4	0.2 – 5.8
Goussia	6.9 – 17.4	7.0 – 7.6
Lankesterella	0.0 – 12.5	4.3 – 13.7

A summary of the interspecific divergence (uncorrected P-distance) sequence comparisons of the COI gene (Dataset S3) between clades from the taxa of *Lankesterella* and haemococcidia n. gen. compared to all the other major clades can be found in Table 4.

Table 4. Summary of the interspecific divergence (uncorrected P-distance) rages based on the COI gene between the clades of *Lankesterella* and haemococcidia n. gen., and the other clades or taxa

	Lankesterella spp.	Haemococcidia n. gen.
Eimeria ex galliforms	9.4 – 17.1	1.8 – 17.8
Eimeria ex mammals	8.2 – 17.0	10.2 – 18.8
Eimeria ex marsupials	6.3 – 14.8	8.7 – 13.7
Isospora ex passeriforms	9.0 – 11.2	
Haemococcidia n. gen.	9.2 – 11.4	0.0 % – 6.8
Cyc. cayetanensis	10.1 – 12.0	11.9 – 13.9
Isospora amphiboluri	7.4 – 9.9	10.2 – 10.8
Caryospora bigenetica	7.7 – 9.7	9.3 – 10.9
Eimeria paludosa	13.7 – 14.8	16.9 – 7.7
Choleoeimeria pogonae	20.1 – 21.0	19.1 – 20.9
Lankesterella	1.6 – 6.5	9.2 – 11.4

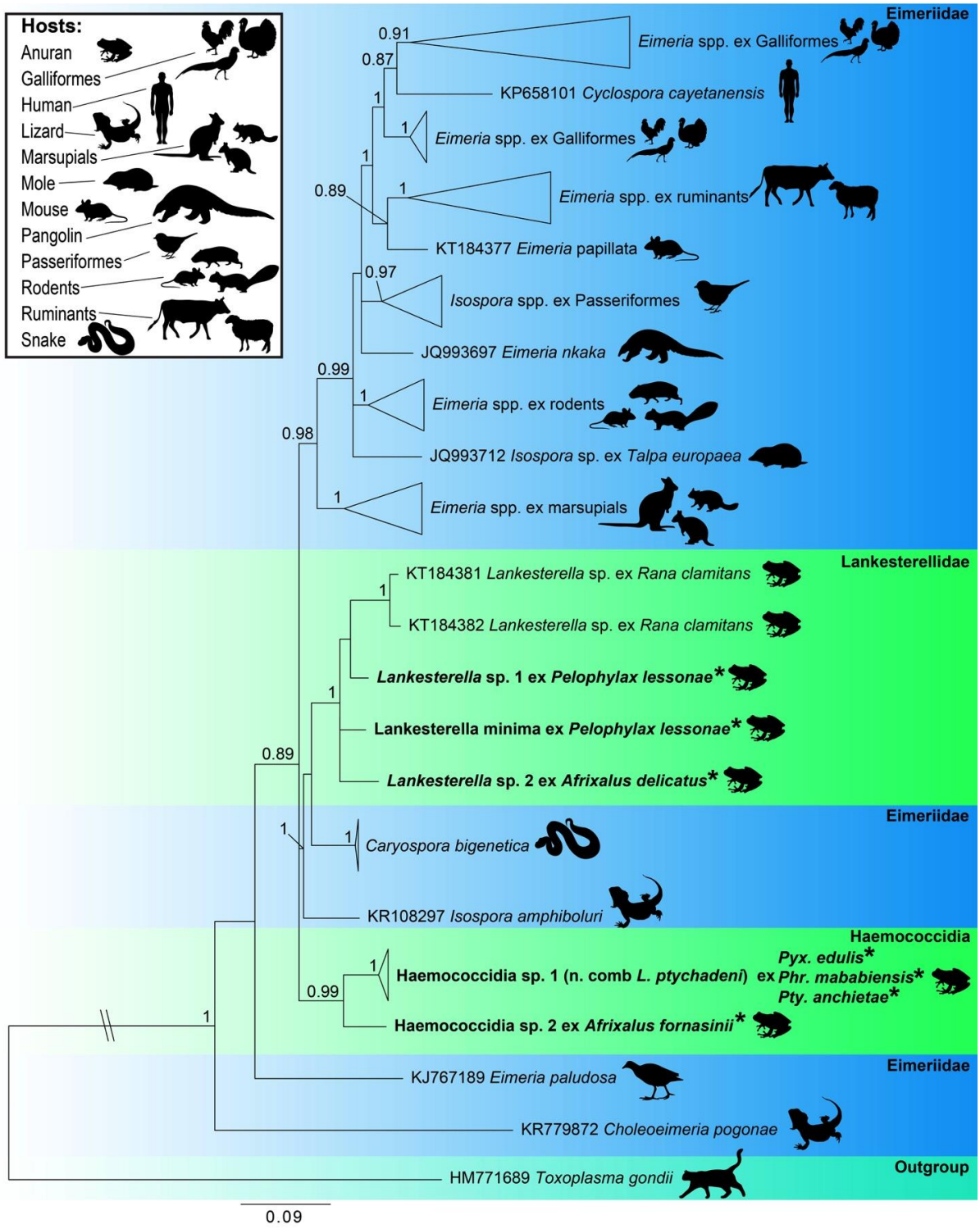

Fig. 8. Bayesian Inference (BI) phylogram based on mitochondrial cytochrome c oxidase subunit I (COI) sequence dataset. The BI analysis was performed with GTR + I + Γ selected as the model with the best BIC score. The final dataset contained 758 nt from 61 sequences, rooted with *Toxoxplasma gondii* [GenBank: HM771689]. Asterisks (*) indicate the sequences obtained in the current study. Only nodal posterior probability support values of >0.75 shown. The scale bar represents 0.09 nucleotide substitutions per site. Pictograms illustrate host species group.

Discussion

The taxonomy of the true haemococcidia has been unstable since they were first discovered. Similar morphological character states have made it challenging to clearly define or distinguish between not only species but also the different haemococcidia genera. Although molecular data has provided new insights assisting in unravelling the complex evolutionary history of these groups (Megía-Palma et al., 2014; Megía-Palma et al., 2017), morphological, especially fine structural data has been the basis on which genera have been defined (Tse et al., 1986; Paperna and Ostroska, 1989; Desser et al., 1990). Only recently have studies started comparing certain morphological characteristics of haemococcidia with supporting molecular data in attempts to find reliable characteristics to distinguish between species and genera (Megía-Palma et al., 2017).

In the current study, attempts were made to determine the haemococcidia biodiversity of South African anurans, comparing the African species to species from Belgium. In total 643 individuals were screened, of which only 21 (3.3%) were found parasitised with haemococcidia based on microscopy screening. Several morphotypes were identified and PCR amplification of fragments of the 18S rRNA and COI genes performed. Based on the molecular analyses, a total of five haemococcidia species were identified in the current study. Following morphological comparison four species were considered undescribed and one conforming to *L. minima* from Europe. Additionally, after careful revision of the currently recognised species of *Lankesterella*, it is clear based on existing morphological and molecular data that a species previously identified as *L. minima* from Algonquin Park, Ontario (Barta and Desser, 1984), is an undescibed species. Furthermore, a species previously recongnised as belonging to *Lankesterella* is designated to a new genus, haemococcidia n. gen., based on phenotypic traits and molecular phylogenetic analyses, namely haemococcidia sp. 1 (n. comb. *L. ptychadeni*).

Phylogenetic analysis of partial 18S rDNA sequences showed different eimeriid coccidia (species of *Eimeria* and *Isospora*) as polyphyletic, this is consistent with several previous studies looking at the relationships between these and closely related taxa (Morrison et al., 2004; Ogedengbe et al., 2011; Megía-Palma et al., 2014; Ogedengbe et al., 2015). Although some genera are polyphyletic, it seems that species isolated from the same or closely related hosts form well-supported monophyletic clades, such as *Eimeria* isolated from Gruiformes, Galliformes, ruminants, rodents, and marsupials, as well as *Isospora* isolated from Passeriformes, and certain lizards. The haemococcidia genera *Schellackia* isolated from lizard hosts, haemococcidia n. gen. from anurans, and *Lankesterella* isolated from reptiles, anurans, and avian host formed separate highly supported monophyletic clades. Surprisingly *E. ranae* [GenBank: EU717219] and *E. arnyi* [GenBank: AY613853]

from frog and snake hosts clustered with representatives of the haemococcidian genus *Schellackia* isolated from lizard hosts and an unidentified species of *Schellackia* [GenBank: KY046255] isolated from the Green Sea Turtle, *Chelonia mydas* from Australia. A study by Megía-Palma et al. (2014), suggested that *E. ranae* might have been misidentified as belonging to the genus *Eimeria* when they actually belong to haemococcidia that contaminated the sample containing the target parasites.

Analysis of 18S rDNA sequences, show lankesterellids from lizard hosts as the most early diverging species of *Lankesterella*, with isolates from the Desert Iguana (*Dipsosaurus dorsalis*) from North America being the most basal lankesterellid of the *Lankesterella* clade. Based on these findings, lankesterellids from anurans and avian hosts seem to have derived from species infecting lizards. However, one *Lankesterella* sp. isolated from the lizard *Podarcis bocagei* (Seoane, 1884) also clustered with the anuran and avian lankesterellid species. Thus, this species could represent one of the links between the taxa from clade B and C.

The close relationship between the anurans and avian lankesterellid species could also be as a result of shared vectors with which host switching occurred. However, Martínez et al. (2018) suggest that mites found feeding on the Snow Bunting *Plectrophenax nivalis* (Linnaeus, 1758) nestlings, which are restricted to the Arctic, are the only suitable vectors as they are less dependent on weather conditions. This is in contrast to anuran lankesterellids, as leeches are the only known vectors of these parasites (Nöller, 1913; Tse et al., 1986; Desser et al., 1990). Alternately, adult Snow Bunting's could have initially acquired infections during migration events to areas where the habitat is more suited for dipteran vectors, subsequently being transmitted to nestlings via the mites. This would mean that vector specificity, for at least certain *Lankesterella* species may be low, however vector specificity has not yet been investigated for any lankesterellids. Low vector specificity could be as a result of the vector serving only as a paratenic host, meaning that in theory, the sporozoites only need to be taken up with the blood meal and survive witin its invertebrate host until it is transmitted to a new vertebrate host. Another avian lankesterellid species, *Lankesterella valsainensis* Martínez et al., 2006 is found parasitising the Blue Tit, *Cyanistes caeruleus* (Linnaeus, 1758) (see Merino et al., 2006; Martínez et al., 2018). This bird species has also been reported to be infected with avian malaria (Ferrer et al., 2012), which is well know to be transmitted by dipteran vectors, such as mosquitoes, biting midges and hippoboscid louse flies (Valkiūnas, 2004). Dipterans such as mosquitoes and biting midges could also serve as the vector for lankesterellid blood parasites to these bird hosts. If these vectors are found to also transmit these parasites in anuran hosts, this could explain the close relationship to anuran lankesterellids. In the current study no leeches were observed or found feeding on any of the host species infected with haemococcidia. Thus, other hematophagous

invertebrates such as mosquitoes and biting midges should not be ruled out as potential vectors for these parasites. It is also common knowledge that a large number of bird species, such as ducks, geese, swans, wading birds, gulls, ravens and hawks prey on amphibians. Furthermore, experimental work has shown that certain anuran *Lankesterella* species can be transmitted through ingestion of infected organs (Paperna and Martin, 2001). Thus avian *Lankesterella* species could have also first been accidentally transmitted through ingestion of infected prey, which over time adapted and established itself as a successful bird parasite.

The intraerythrocytic parasite designated to the genus haemococcidia n. gen. isolated from *Pyx. edulis*, *Phr. mababiensis*, and *Pty. anchietae* formed a well-supported monophyly with *Eimeria* species from marsupial hosts and *Schellackia* species isolated from chelonian and lizard hosts, with *Eimeria arnyi*, and *Eimeria ranae*. This suggests that haemococcidia n. gen. parasites found in the peripheral blood of these anurans are closely related to certain species of *Eimeria*, but also, as could be expected, to the haemococcidian genus *Schellackia*. According to Parker and Duszynski (1986), there are numerous reports of extra-intestinal development within various *Eimeria* species. *Eimeria gruis* Yakimoff and Matschoulsky, 1935 and *E. reichenowi* Yakimoff and Matschoulsky, 1935 have been shown to contain extra-intestinal stages such as in the liver and heart tissue of its host (Parker and Duszynski, 1986). Although there have been no reports of *Eimeria* species containing blood stages, these stages probably use the circulatory system of the host to transport to the various organs and tissues within the host. These reports are possibly similar to the occurrence of haemococcidia sp. 1 (n. comb. *L. ptychadeni*) sporulating oocysts found within detached endothelial cells circulating in the blood of its host as reported by Paperna and Ogara (1996).

The COI dataset also showed several taxa as paraphyletic. *Choleoeimeria pogonae* infecting the western bearded dragon *Pogona minor minor* Loveridge 1933, formed a balsal taxon to the ingroup. *Caryospora bigenetica* isolated from the massasauga rattlesnake *Sistrurus catenatus* (Rafinesque, 1818), species of *Lankesterella* isolated from anurans, and *I. amphiboluri* from the central bearded dragon *Pogona vitticeps* Ahl, 1927 forming a highly supported monophyletic clade. *Lankesterella* species isolated from European, African and North American anurans from a well-supported monophyletic clade, supported by the morphological data of the peripheral blood sporozoites. Although, lankesterellids from anuran hosts form a well-supported monophyly, increased sampling from other hosts, namely avian and reptilian hosts, especially to obtain data for the mitochondrial COI marker, may provide better insights into the evolutionary relationships between lankesterellids of anurans, and avian hosts (Fig. 7, clade C) and those exclusively infecting lizard hosts (Fig. 7, clade A and C).

Several previous studies on phylogenetic relationships of different coccidia show the close affinities between the genera *Isospora* and *Caryospora* (infecting reptiles), and *Lankesterella* (Barta et al., 2001; Megía-Palma et al., 2016; Megía-Palma et al., 2017). It has long been suggested that heteroxenous apicompleaxan parasites arose from monoxenous parasites (Barta et al., 2001), with intermediate hosts forming part of the life cycle of these heteroxenous parasites due to behavioural changes in their host species. Also, based on the fact that vectors are paratenic or mechanical, it is possible that different hematophagous invertebrates transmit these parasites, specifically in cases where a single species, i.e. haemococcidia sp. 1 (n. comb. *L. ptychadeni*), is found infecting several different hosts with different behavioural or ecological habits. For example the african bullfrog *Pyx. edulis*, dwarf puddle frog *Phr. mababiensis*, and plain grass frog *Pty. anchietae*, posses completely different morphological traits, size, and behavioural characteristics, but are all infected with haemococcidia sp. 1 (n. comb. *L. ptychadeni*). This finding further articulates the importance of not basing species descriptions merely on the occurrence of a parasite species in different or new host species.

Phenotypic characters remain essential in the classification and identification of eimeriid coccidia, and although phenotypic and genetic characters are expected to concur, even homologous morphological characters, such as basic oocyst configuration fail to agree with molecular phylogeny (Ogedengbe et al., 2016; Ogedengbe et al., 2018). Eventually, splitting these groups into multiple genera based on the criteria of monophyly, clade stability, and phenotypic diagnosability as stated by Vences et al. (2013) will probably be a true reflection of the variety genera within the coccidia. However, according to Ogedengbe et al. (2018), the ultimate challenge facing the splitting of these groups will be finding suitable morphological characters that reflect evolutionary history. An additional reason for the polyphyletic phylogenetic relationships observed in these groups could be historical inaccuracies in their original identification or classification. This ultimately suggests the need for renaming or re-describing certain taxa, owing to the vast scattering of genera across multiple clades. In a recent molecular phylogenetic study on tissue coccidia by Ogedengbe et al. (2016), this approach was followed and a new genus was erected to re-establish monophyly of certain genera in the Sarcocystidae.

The phylogenetic relationships of the haemococcidia, seem to follow the trend highlighted for the eimeriid coccidia, showing a clear polyphyletic distribution among groups. Although phenotypic traits between these groups are highly similar, with several characteristics overlapping, differences in the mature sporozoite morphology and development, seem to be supported by the molecular phylogeny based on the large dataset of available 18S rDNA sequences. In the current study clear phenotypic differences in the peripheral blood stages of the *Haemovaulata* n. gen. and those of *Lankesterella* and

Schellackia are observed. However, as this study was part of a larger project that focused on opportunistic and non-lethal surveying of anuran blood parasites in premier conservation areas, no anurans infected with this enigmatic haemococcidian, were dissected or screened for additional stages in the life cycle. Nonetheless, based on these findings and to maintain monophyly of species designated to the genera *Lankesterella* and *Schellackia*, it is suggested to designate a new genus, *Haemovaulata* n. gen., for species of haemococcidia that contain mature intracellular sporozoites encompassed by an expanded PV within its hosts peripheral blood cells. Other characteristics of *Haemovaulata* n. gen. are oogony and sporogony that take place in the gut epithelium and in the lamina propria, as well as oocysts that yield eight sporozoites in its vertebrate host (Paperna and Ogara, 1996). Although these findings overlap with the genus *Schellackia*, young oocysts lack type-1 and -2 wall-forming bodies of eimeriid macrogamonts, a characteristic more suited to species of *Lankesterella* (Ostrovska and Paperna, 1987; Paperna and Ogara, 1996). However, these developmental traits still require molecular characterisation to ultimately confirm these findings as part of the genus *Haemovaulata* n. gen.

Thus, in the current study we redescribe for the first time *L. minima* in *Pel.* kl. *esculentus* and *Pel. lessonae* from Belgium; describe *Lankesterella* sp. 2 in *Afr. delicates* and *Afr. fornasini* from Sodwana, KZN, South Africa; *Lankesterella* sp. 1 in *Pel. lessonae* from Rijmenam, Het Ven, Belgium; and a new genus *Haemovaulata* n. gen., with the species haemococcidia sp. 2 in *Afr. fornasini* from Sodwana, KZN, South Africa; and new a species combination haemococcidia sp. 1 (n. comb. *L. ptychadeni*), described in *Pty. nilotica*, from Lake Victoria, Kenya (Paperna and Ogara, 1996), and now from *Pyx. edulis*, *Phr. mababiensis* and *Pty. anchietae* from Kruger National Park, along the Phogola River, and Ndumo Game reserve, South Africa. See Table 3, for an updated list of the currently recognised haemococcidia of anurans.

Future work should, if possible, include the elucidation of the life cycles of the parasites described in the current study, especially for species designated to the genus haemococcidia n. gen., determine possible modes of transmission. Additionally it is important for phylogenetic relationship comparisons to increase the number mitochondrial COI gene sequences of haemococcidia from other hosts, namely avian and reptilian hosts, and from the genus *Schellackia* and *Lainsonia*.

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CHAPTER 4

The description, life history, and experimental transmission of the filarial nematode onchocercid n. gen. n. sp. 1 (Nematoda: Onchocercidae), parasitising the toad *Sclerophrys gutturalis* (Amphibia: Bufonidae) and the mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U*. (*Pfc*.) *montana* (Insecta: Culicidae).

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Abstract

Filarial nematodes of Onchocercidae (Spirurida) are primarily tissue-dwelling, thread-like roundworms known for medical and veterinary disease. These nematodes infect a broad range of hosts, which includes amphibians, reptiles, birds, and mammals. Onchocercids infecting anurans either belong to the subfamily Icosiellinae Anderson (1958) or Waltonellinae Bain and Prod'Hon (1974). They have a complex life cycle, involving different hosts species for development and transmission. Their life cycle includes a definitive vertebrate host in which adult worms release microfilaria into the bloodstream, and an intermediate haematophagous arthropod vector. Data on the biodiversity, ecology and phylogenetic relationships of anuran onchocercids is limited, especially from Africa, with only five species described of which the life cycle of only one has been elucidated. The aim of this study was to determine the biodiversity and ecology of onchocercids infecting anurans of the Bufonidae Gray, 1825 from South Africa. A total of 128 individuals representing four species of bufonid host, namely *Sclerophrys garmani* (Meek, 1897), *Sclerophrys gutturalis* (Power, 1927), *Sclerophrys pusilla* (Mertens, 1937), and *Schismaderma carens* (Smith, 1848), were collected and their blood screened for the presence of microfilaria. Collected *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* Theobald, 1901 and *Uranotaenia* (*Pfc.*) *montana* Ingram and De Meillon, 1927 mosquitoes, enticed to feed on infected toads were progressively dissected, and nematodes extracted and fixed according to the stages of their development. Light and scanning electron microscopy and PCR amplification were used for morphological and molecular characterisation each of these developmental stages. In the present study, we describe and elucidate the life history of a new amphibian filarial nematode, onchocercid n. gen. n. sp. 1. Additionally, we report on the host-seeking behaviour of *U.* (*Pfc.*) *mashonaensis* and *U.* (*Pfc.*) *montana* that respond to the calls of their toad hosts. The complex host-vector relationship and specialised host-seeking behaviour by these mosquitoes may be the reason why mostly male toads were found infected with microfilaria. This study is the first to elucidate the life history of an amphibian filarial nematode from southern Africa.

Keywords: anuran, amphibian, frog, parasite, life cycle, filarial nematode, microfilaria

Introduction

Filarial nematodes from the family Onchocercidae Leiper, 1911 (Spirurida), the major group of the suborder Filarioidea, are long thread-like, coelom or tissue dwelling worms, known for causing medical and veterinary diseases (Bain, 2002). This group is reported infecting a broad range of hosts including amphibians, reptiles, birds and mammals (Lefoulon et al., 2015). These nematodes have a complex life cycle, involving a definitive vertebrate host and invertebrate vector. Within the vertebrate host adult worms release specialised eggs or microfilaria that migrate to or in the host's lymph or bloodstream (Bain, 2002). Subsequently a haematophagous arthropod vector ingests the microfilaria initiating development that continues from the early first-stage larvae up to the infective third-stage larvae, all in the same individual. Once the infective larvae have been deposited on or inoculated into a new definitive host, via the vector's subsequent blood meal, development continues to the fourth-stage juvenile and final adult stage (Bain, 2002; Bain et al., 2013).

Onchocercids of anurans either belong to the subfamily Icosiellinae Anderson (1958) or Waltonellinae Bain and Prod'Hon (1974). Icosiellinae is monogeneric comprising nine species of the genus *Icosiella* Seurat (1917), species are recognised based on the presence of two pairs of cephalic submedian spines, a subterminal anus, and few caudal papillae on a relatively short tail. The Waltonellinae was erected to accommodate anuran "*Foleyella*"-like filariae and currently comprises five genera, namely *Foleyellides* Caballero (1935), *Ochoterenella* Caballero (1944), *Madochotera* Bain and Brunhes (1968), *Paramadochotera* Esslinger (1986b) and *Paraochoterenella* Purnomo and Bangs (1999) (Table 1). Members of this subfamily are characterised by the absence of submedian cephalic spines; the presence of a pair of lateral, parastomal projections; the anus not being in a sub-terminal position; and several pairs of papillae on a somewhat long tail (Souza et al., 2012; Bain et al., 2013).

The most diverse genus of the Waltonellinae is *Ochoterenella,* currently with 16 species reported parasitising bufonid, hylid and leptodactylid hosts from the Neotropical and Indomalayan realms (Bain et al., 2013). Caballero (1944) erected the genus and described the type species *Ochoterenella digiticauda* Caballero, 1944 from the body cavity of the Cane toad *Rhinella marina* (Linnaeus, 1758) in Mexico. According to Esslinger (1986b) this species has been reported in several studies from various anurans throughout the Neotropical region (Brenes and Hollis, 1959; Travassos and de Freitas, 1960; Marinkelle, 1970; Masi Pallares and Maciel, 1974; Vicente and Santos, 1976; Dyer and Altig, 1977; Vicente and Jardim, 1980).

Table 1: Summary of valid species of Waltonellinae, with type host, host family and country of type locality. Type host according to AmphibiaWeb (2018).

Genus	Species and Authority († = type species)	Type host (syn.)	Host family	Country of type locality
Foleyellides Caballero, 1935	*Foleyellides mayenae* Romero-Mayén and León-Règagnon, 2016	*Rana psilonota* (syn. *Lithobates psilonota*)	Ranidae	Mexico
	Foleyellides americana (Walton, 1929)	*Rana pipiens* (syn. *Lithobates pipiens*)	Ranidae	USA
	Foleyellides brachyoptera (Wehr and Causey, 1939)	*Rana sphenocephala* (syn. *Lithobates sphenocephalus*)	Ranidae	USA
	Foleyellides confusa (Schmidt and Kuntz, 1969)	*Fejervarya vittigera* (syn. *Rana limnocharis vittigera*)	Dicroglossidae	Philippines
	Foleyellides dolichoptera (Wehr and Causey, 1939)	*R. sphenocephala* (syn. *L. sphenocephalus*)	Ranidae	USA
	Foleyellides duboisi (Gedoelst, 1916)	*Pelophylax ridibundus** (syn. *Rana esculenta ridibunda*)	Ranidae	DRC
	Foleyellides flexicauda (Schacher and Crans, 1973)	*Rana catesbeiana* (syn. *Lithobates catesbeianus*)	Ranidae	USA
	Foleyellides malayensis (Petit and Yen, 1979)	*Pulchrana glandulosa* (syn. *Rana glandulosa*)	Ranidae	Malaysia
	Foleyellides ranae (Walton, 1929)	*R. catesbeiana* (syn. *L. catesbeianus*)	Ranidae	USA
	Foleyellides rhinellae García-Prieto, Ruiz-Torres, Osorio Sarabia and Merlo-Serna, 2014	*Rhinella marina*	Bufonidae	Mexico
	Foleyellides striatus (Ochoterena & Caballero, 1932) †	*Rana montezumae* (syn. *Lithobates montezumae*)	Ranidae	Mexico

Table 1 continued

Genus	Species and Authority († = type species)	Type host (syn.)	Host family	Country of type locality
Madochotera Bain and Brunhes, 1968	*Madochotera alata* Bain and Brunhes, 1968 †	*Rhacophorus* sp.	Racophoridae	Madagascar
	Madochotera landauae Prod'hon and Bain, 1974	*Rhacophorus* sp.	Racophoridae	Madagascar
	Madochotera pichoni Bain and Brunhes, 1968	*Rhacophorus* sp.	Racophoridae	Madagascar

Table 1. Continued

Ochoterenella Caballero, 1944	*Ochoterenella albareti* (Bain, Kim and Petit, 1979)	*R. marina* (syn. *Bufo marinus*)	Bufonidae	French Guyana
	Ochoterenella caballeroi Esslinger, 1987	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico
	Ochoterenella chiapensis Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico
	Ochoterenella complicata Esslinger, 1989	*R. marina* (syn. *B. marinus*)	Bufonidae	Columbia
	Ochoterenella convoluta (Molin, 1858)	*Leptodactylus pentadactylus*	Leptodactylidae	Brazil
	Ochoterenella digiticaudata Caballero, 1944 †	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico
	Ochoterenella dufourae (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana
	Ochoterenella esslingeri Souza Lima and Bain, 2012	*Bokermannohyla luctuosa*	Hylidae	Brazil
	Ochoterenella figueroai Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana
Genus	Species and Authority († = type species)	Type host (syn.)	Host family	Country of type locality
	Ochoterenella guyanensis (Bain and Prod'Hon, 1974)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana
	Ochoterenella lamothei Esslinger, 1988	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico
	Ochoterenella nanolarvata Esslinger, 1987	*R. marina* (syn. *B. marinus*)	Bufonidae	Mexico
	Ochoterenella oumari (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana
	Ochoterenella royi (Bain, Kim and Petit, 1979)	*R. marina* (syn. *B. marinus*)	Bufonidae	French Guyana
	Ochoterenella scalaris (Travassos, 1929)	*Leptodactylus macrosternum* (syn. *L. ocellatus*)	Leptodactylidae	Brazil
	Ochoterenella vellardi (Travassos, 1929)	*R. marina* (syn. *B. marinus*)	Bufonidae	Brazil
Paramadochotera (Bain and Prod'Hon, 1974)	*Paramadochotera guibei* (Bain and Prod'Hon, 1974) †	*Gephyromantis redimitus* (syn. *Mantidactylus redimitus*)	Mantellidae	Madagascar
Paraochoterenella Purnomo and Bangs, 1999	*Paraochoterenella javanensis* Purnomo and Bangs, 1999†	*Fejervarya cancrivora* (syn. *Rana cancrivora*)	Dicroglossidae	Indonesia

USA = United States of America; DRC = Democratic Republic of the Congo

Later, Esslinger (1986b) redescribed the type species *O. digiticauda,* subsequently redefining the genus, and transferring eight species previously included in *'Waltonella'* (*W. convoluta*, *W. scalaris*, *W. vellardi*, *W. guyanensis*, *W. royi*, *W. dufourae*, *W. oumari*, *W. albareti*) to *Ochoterenella* (see Table 1). Esslinger (1986b) also transferred *Ochoterenella guibei* (Bain & Prod'Hon, 1974) to *Paramadochotera*, and considered *Ochoterenella papuensis* Johnston, 1967 described from a frog in New Guinea as *incertae sedis*. Based on the revision of the subfamily and redefinition of the genus (Esslinger, 1986a, b), a further seven species have been described and designated to *Ochoterenella* (Esslinger, 1987, 1988a, b, 1989; Souza et al., 2012). *Ochoterenella* is characterised by the presence of cuticularized parastomal structures, the lack of lateral and caudal alae, the presence of a distinct buccal capsule, and the presence of bands of longitudinally oriented bosses in mid-body region (Esslinger, 1986a).

Madochotera is a small group of filariae exclusively described from Malagasy rhacophorids. Currently the genus comprises three species namely *M. alata* Bain & Brunhes, 1968, *M. landauae* Prod'hon & Bain, 1974 and *M. pichoni* Bain & Brunhes, 1968. It is characterised by the presence of cuticularized parastomal structures, the presence of lateral alae, the lack of a distinct buccal capsule, the cuticle with sometimes transversely oriented ridges or bosses, and a vulva that is distinctly posterior to the oesophagus (Esslinger, 1986a).

Paramadochotera is monotypic and as mentioned above, was erected to accommodate *P. guibei* described from a banded madagascar frog *Gephyromantis redimitus* (Boulenger, 1889) in Madagascar. *Paramadochotera* is characterised by the presence of cuticularized parastomal structures, the lack of lateral and caudal alae, the presence of a distinct cuticularized buccal capsule, the cuticle of the female with transversely oriented ridges or bosses present on the dorsal and ventral surfaces, and a body that is abruptly attenuated at the extremities (Esslinger, 1986a).

Paraochoterenella is also monotypic and was erected to accommodate *Paraochoterenella javanensis*, described from the mesentery of the crab-eating frog *Fejervarya cancrivora* (Gravenhorst, 1829) in West Java, Indonesia. *Paraochoterenella* is characterised by the presence of cuticularized parastomal structures, the lack of lateral and caudal alae, the presence of a distinct cuticularized buccal capsule, and the presence of scattered (non-oriented) minute bosses on the cuticle of the midbody region (Purnomo and Bangs, 1999).

In a recent study on the phylogenetic relationship of the Onchocercidae, several species of filariid nematodes parasitising amphibians (*Ochoterenella* spp. and *Icosiella neglecta* (Diesing 1851)) and reptiles (*Foleyella candezei* (Fraipont, 1882), *Madathamugadia*

hiepei, Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick & Bain, 2000, *Oswaldofilaria petersi* Bain & Sulahian, 1974 and *Oswaldofilaria chabaudi* Pereira, Souza & Bain, 2010) were included (Lefoulon et al., 2015). This study, based on both nuclear (18S rRNA, 28S rRNA, MyoHC, rbp1) and mitochondrial (COI, 12S rDNA, hsp70) markers, showed Icosiellina, Waltonellinae, and Oswaldofilariinae (onchocercids from crocodilian and squamate hosts), forming a well-supported clade, sister to all the other taxa evaluated within the Onchocercidae. Furthermore, species of *Foleyella* (Dirofilariinae) found infecting lizard hosts, was shown as distantly related to this group (Oswaldofilariinae, Icosiellinae and Waltonellinae) and clustering with other members of Dirofilariinae, despite their morphological similarities (Lefoulon et al., 2015).

Excluding Madagascar, the only species described in Africa from the Waltonellinae is *Foleyellides duboisi* (Gedoelst, 1916) found in the Democratic Republic of Congo. This species was described by Gedoelst (1916), based on material collected from unidentified frogs and then recorded again from the marsh frog *Pelophylax ridibundus* (Pallas, 1771) from Palestine (Witenberg and Gerichter, 1944). However, these specimens were reported to contain differences in the number of apical papillae and other morphometric characters. Three further blood parasite biodiversity surveys of different anuran families have also been completed in Africa. The first by Readel and Goldberg (2010) in Uganda, who reported on an unidentified microfilaria parasitising *Leptopelis christyi* (Boulenger, 1912) and *L. kivuensis* Ahl, 1929. The second by Aisien et al. (2015) in Nigeria, who reported on microfilaria parasitising *Aubria subsigillata* (Duméril, 1856), *Hoplobatrachus occipitalis* (Günther, 1858), *Sclerophrys regularis* (Reuss, 1833), *S. maculatus*/*pusilla*, and *S. galamensis* (Duméril & Bibron, 1841). Lastly in South Africa Netherlands et al. (2015) observed microfilaria parasitising *Ptychadena anchietae* (Bocage, 1868) and *Sch. carens.*

Within the Waltonellinae only the life cycles of species of *Foleyellides* have been studied. Causey (Causey, 1939a; Causey, 1939b) was the first to report on the experimental transmission and development of *Foleyellides ranae* (Walton, 1929) from *Rana clamitans* Latreille, 1801 and *Foleyellides dolichoptera* (Wehr & Causey, 1939) from *Rana sphenocephala* Cope, 1886 in the mosquitoes *Aedes aegypti* (Linnaeus in Hasselquist, 1762) and *Culex pipiens* Linnaeus, 1758. Subsequently, Causey (1939c) reported on the development of *Foleyellides brachyoptera* in *Rana catesbeiana* (Shaw, 1802) in *A. aegypti*, *Culex quinquefasciatus* Say, 1823, and *C. pipiens*. Although these mosquito species do not naturally feed on amphibians and high mortality of mosquitoes was observed post feeding, development of infective larvae (third-stage) appeared from 13 days post-infection (dpi) (Causey, 1939a; Causey, 1939b; Causey, 1939c). The life cycle of *F. duboisi* infecting *Pelophylax* kl. *esculentus* from Palestine has also been studied (Witenberg and Gerichter, 1944). These authors describe the adult, microfilaria and developmental stages in

experimentally infected *Culex pipiens molestus*. In that study, twenty per cent of blood fed mosquitoes died within 48 hours, and of those that survived 35% were infected. The sausage-shaped first-stage appeared from the third day, while first infective larvae appeared from approximately 14 dpi. Furthermore, these authors did not consider *C. pipiens molestus* as the natural intermediate host for *F. duboisi*, suggesting that a more abundant mosquito species with a preference for feeding on frog hosts would be a more likely natural vector (Witenberg and Gerichter, 1944). Later, Crans (1969) elucidated the life cycle of a filarial nematode, later designated as *F. flexicauda* (Schacher & Crans, 1973), from the american bullfrog *R. catesbeiana* and the amphibian-feeding mosquito *Culex territans* Walker, 1856 in New Jersey. Crans (1969) reported that in the vertebrate host adult filarial nematodes were found encysted in the intestinal mesentery of the body cavity, with microfilaria released in the peripheral blood. In the invertebrate host infective third-stage larvae were detected within ten dpi. Developmental stages appeared throughout the mosquito's haemocoel, in the abdominal fat bodies, the coxal cavities of the legs, and in the head capsule and proboscis (Crans, 1969).

Based on previous research of herpetofaunal blood parasites in KwaZulu-Natal (KZN) (Netherlands et al., 2014; Cook et al., 2015, 2016; Cook et al., 2018; Netherlands et al., 2018) and the occurrence of microfilaria in anurans (Netherlands et al., 2015), northern KZN is well suited as a model ecosystem for research on blood parasite diversity and their associated life cycles. Members of the Bufonidae are common in this area and have been reported to be parasitised with several blood parasite taxa, including microfilaria (Netherlands et al., 2014; Netherlands et al., 2015). Thus the objectives of this study are first to (1) establish which species from within the Bufonidae in northern KZN (South Africa) contain microfilaria, (2) determine the prevalence, distribution, taxonomic placement, and species identification of any microfilaria species found, and (3) attempt to identify any possible vectors. If the above objectives are achieved, we could (4) ultimately try to elucidate the life history of an anuran filarial nematode.

Material and Methods

Toad collection and study area

In a recent survey on the biodiversity of anuran blood parasites, Netherlands et al. (2015) found the Red toad, *Schismaderma carens* parasitised with microfilaria. Thus, for the current study members of the Bufonidae were selected as the vertebrate hosts to be screened for microfilaria. A total of 128 individuals, representing four bufonid species, were collected at

night via active sampling from several localities throughout northern KZN, South Africa (S1 Dataset). Species comprised 45 Eastern Olive toads (*Sclerophrys garmani*), 73 guttural toads (*Sclerophrys gutturalis*), seven Flat-backed toads (*Sclerophrys pusilla*), and three Red toads (*Sch. carens*), identified using Du Preez and Carruthers ((Du Preez and Carruthers, 2009, 2017). Collected specimens were placed in individual marked containers with sufficient moisture and ventilation, and transported back to a field workstation. Toads were collected at these sites during their breeding season and the southern hemisphere warmer months of April, September, November, and December 2014, September, November and December 2016, February and November 2017, and January and March 2018. All specimens collected prior to November 2017, were released after taking blood samples. Based on the intensity of microfilaria selected individuals, uninfected (n=1) and infected (n=4), collected during and after the November 2017 sampling effort were transported back to the North-West University African Amphibian Conservation Research Group laboratory for further processing and examination. This study received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5) and euthanasia of frogs was performed according to approved SOP using tricaine methanesulfonate (MS222) solution (ethics number: NWU-00492-16-S5).

Processing of blood samples and light microscopy screening

To determine prevalence of infection, blood (>0.2 ml) was first taken from each toad via cardiac or femoral venipuncture and thin blood smears prepared, air-dried, fixed and stained using Giemsa-stain following routine practice (Netherlands et al., 2015). When possible, blood smears were screened for microfilaria in the field at a workstation using a Nikon Eclipse E100 compound microscope. The remaining blood was preserved in 70% ethanol for molecular work (ratio 1:15). Stained blood smears were screened using a Nikon ECLIPSE *Ni* Compound microscope at 1000× and images captured and measured using the imaging software NIS Elements Ver. 4. Parasitaemia was estimated according to the number of microfilaria observed in ten optical fields at 400x magnification. In the current study parasitaemia was regarded as low if one microfilaria per ten fields of view was observed, medium if between one and five microfilaria per field of view was observed, and high if more than five per field of view were observed.

Life history and experimental transmission

Following the screening of blood smears previously collected, the opportunity presented itself to return to one of the collection localities, Sodwana Bay, in November 2017 and again in January and March 2018 (S1 Dataset), to increase the sample size of toads from this

area. The site visited, SB-1 (S27.488591°; E32.664259°), is a permanent and well vegetated wetland with a slow flowing stream. Toads from this area were found positive with micofilaria and during sampling, mosquitoes were observed feeding on calling *S. gutturalis*. To investigate if the mosquitoes observed readily feeding on *S. gutturalis* were the responsible vectors, mosquitoes and toads were collected and processed. Table 2 provides a summary of the process followed to elucidate the life history of the filarial nematode found parasitising toads from Sodwana Bay.

Table 2. Step by step summary of field sampling of infected toads and mosquito collection *in situ* and experimental transmission and life cycle elucidation *ex situ*.

November 2017	Field sampling	• Sampling took place in Sodwana Bay at site SB-1 (S27.488591°; E32.664259°). • Collect *Sclerophrys gutturalis* (n = 8) and blood samples as detailed earlier. • During sampling mosquitoes observed feeding on calling *S. gutturalis*. • Blood fed mosquitoes (n = 5) were collected using an aspirator and temporarily housed in small glass jars with moist cotton wool.
	Laboratory	• Collected mosquitoes identified using Jupp (1996) – fixed whole in 70% ethanol. • Two *S. gutturalis* parasitised with microfilaria – euthanized using tricaine methanesulfonate (MS222) solution and dissected. • Adult filarial nematodes removed from body cavity, washed in saline, and fixed hot and stored in 70% ethanol.
January & March 2018 field sampling	Host collection	• Two subsequent sampling expeditions to Sodwana Bay followed. • Collected toads *S. gutturalis* (n = 35) and *S. garmani* (n = 3) – transported to a field workstation to processed and screened blood as detailed earlier. • Specimens positive were kept in individual containers – the rest released at capture site.

	Vector collection	• Infected toads (n = 3) were transported back to site SB-1 to be used as enticement for collecting mosquitoes *in situ*.
		• Mosquitoes were collected:
		  ○ Via a modified portable Centre for Disease Control (CDC) mosquito trap - fitted with a speaker (instead of a light) to play call of *S. gutturalis* as a lure.
		  ○ Using an aspirator – directly from infected *S. gutturalis* enticed to call using playbacks (playing of the call back through a speaker).
		  ○ With a glass tank fixed with a fine mesh funnel and baited with an infected *S. gutturalis* enticed to call.
		• Calls from several anuran species from the area were played with CDC trap without the same success.

Table 2. Contiuded.

Laboratory phase following January & March 2018 field sampling	• Three individuals, two parasitised with microfilaria and the one not infected – transported to NWU frog lab and housed in vivarium to monitor and complete the life history observations *ex situ.* • Collected mosquitoes (n = 146) were maintained in plastic jars (350 ml) lined at the base with moist cotton wool and transported to NWU. • Mosquitoes collected that did not take a blood meal *in situ*, were released in a glass tank containing a highly infected guttural toad *ex situ*. • Mosquitoes were enticed to feed on the infected toads in the lab at night using playbacks for approximately 4-8 hours. • All engorged mosquitoes were kept separately, according to when their blood meal was taken, and supplied daily with fresh water and a 10% sucrose solution. • At least one mosquito was dissected every day for the first 5 dpi, followed by one or two mosquitoes being successively dissected every three days, spanning a period of 20 days. • This experiment was repeated twice (January and March 2018 field sampling) – fatalities were dissected as soon as possible post-mortem.

Mosquitoes were euthanized with carbon dioxide (CO_2) and dissected under a stereomicroscope using modified entomology pins. Sausage-shape first-stage larvae were prepared for compound microscopy by smearing the dissected contents on a glass slide and removing the larvae from the mosquito's intestines and fat bodies. Subsequently the glass slides were fixed and stained as described above for light blood smear preparation, to screen for microfilaria and any larvae that may have been missed. Late sausage-shape first as well as second and third-stage larvae were removed with one hair of a fine tip paintbrush, washed in saline and fixed in hot 70% alcohol.

The remaining infected *S. gutturalis* (n=2) were euthanized, dissected, and adult filarial nematodes removed as mentioned above. Prior to microscopy examination, adult nematodes were placed in distilled water for about 20 min and subsequently cleared in lactophenol for 30 min. Apical and transverse sections were prepared manually using a thin razor and examined on temporary mounts. Morphology of the nematodes was studied and photomicrographs were taken using the Nikon E800 and Nikon ECLIPSE *Ni* compound microscopes.

All measurements in the text are given in micrometres (µm) unless indicated otherwise. Morphometric data are presented as a range followed by values of holotype or paratype in square brackets and mean values in parentheses. For specimens with a sample size of 30 and above, metrical characters of the coefficient of variation (CV) were calculated as standard deviation (SD) divided by the mean value and presented as a percentage.

Scanning electron microscopy (SEM)

Each of the filarial nematode stages obtained were used for scanning electron microscopy (SEM). Specimens were dehydrated using a gradual ethanol series: nematodes were first added to a small Petri dish with 70% EtOH, continuously and slowly 100% EtOH was added to the Petri dish, and simultaneously the same amount of mixed EtOH removed until concentration reached 100%. After dehydration, nematodes were dried using Hexamethyldisilazane as transition fluid (before clear solution worms were dehydrated in a series of Hexamethyldisilazane mixed 1:1 with 70°, 80°, 90°, 96° and 100° EtOH) or by means of a critical point drier (Bio-Rad, Bio-Rad Microscience Division, United Kingdom) using liquid CO_2 as transition fluid. In order to prevent shrinking of adult nematodes and third-stage larvae, specimens were dissected into two or more pieces before dehydration.
Following the methods of Conradie et al. (2017), fresh blood samples obtained from a highly infected guttural toad (S1 Dataset, AE180124C1) were also prepared for SEM. Thin blood smears were made on glass coverslips and Whatman® qualitative filter paper (Grade 1), and a drop of Todd's fixative (Todd, 1986) placed on the smears prior to drying. After a minute, the smears were submerged in fresh Todd's fixative and allowed to fixate for approximately 2 h. The sample was then gently washed with ultrapure water three times each for 15 min. Post fixation was performed with 2% osmium tetroxide (OsO4) for 90 min. This was followed by rinsing three times in ultrapure water for 10 min, with subsequent dehydration in a gradual ethanol series. The sample was then critical point dried as described above for the filarial nematode stages.

Dried specimens and smears were mounted onto 12 mm aluminium stubs with double-sided carbon tape and sputter-coated for 90 seconds with a gold palladium alloy in argon gas at a pressure of 2 atm (SPI-ModuleTM Sputter Coater, SPI Supplies, West Chester, PA, USA). Specimen stubs were stored in a desiccator for at least 30 min before being examined by SEM at an accelerated voltage of 10 kV (Phenom PRO Desktop SEM, Phenom-World B., Eindhoven, Netherlands).

DNA extraction, PCR amplification and phylogenetic analyses

Ethanol-preserved specimens of the different stages (n=5), sausage-shape first-stage larvae to both adult male and female filarial nematodes, and blood samples from all parasitised toads (n=8) were used for molecular work. Genomic DNA was extracted from the samples following the standard protocol for human or animal tissue and cultured cells as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Duren, Germany). Once extracted, DNA was used for polymerase chain reaction (PCR) amplification. The PCR reactions targeted a fragment of approximately 750 nt of the 18S rRNA gene and 650 nt of

the cytochrome *c* oxidase subunit I (COI) gene. Sequences were amplified using primer sets obtained from a previous study based on filariae of the Onchocercidae (Lefoulon et al., 2015). The 18S rRNA sequence fragment was amplified using F18ScF1 (5′-ACC GCC CTA GTT CTG ACC GTA AA-3′) and F18ScR1 (5′-GGT TCA AGC CAC TGC GAT TAA AGC-3′), and the COI sequence fragment was amplified using COIintF (5′-TGA TTG GTG GTT TTG GTA A-3′) and COIintR (5′-ATA AGT ACG AGT ATC AAT ATC-3′). Conditions for PCR of both primer sets were as follows: 40 cycles, entailing a 95°C denaturation for 30 s, annealing at 58°C (18S) and 52°C (COI) for 30 s with an end extension at 72°C for 90 s; followed by a final extension of 72°C for 10 min. PCR reactions were performed using 12.5 µL OneTaq® 2X Master Mix with Standard, 1.25 µL (10 µM) of each of the primer sets mentioned above, and at least 25 ng DNA. The final reaction volume of 25 µL was made up with PCR-grade nuclease-free water (Thermo Scientific). Reactions were undertaken in an Applied Biosystems SimpliAmp Thermal Cycler PCR machine (Thermo Fisher Scientific, Waltham, MA USA). Resulting amplicons were visualized under ultraviolet light on a 1% agarose gel stained with EZ-Vision® Bluelight DNA dye using an E-BOX CX5 imaging system (Vilber Lourmat Deutschland, Eberhardzell, Germany). PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions. Resultant sequences were assembled, and chromatogram-based contigs were generated and trimmed using Geneious R11 (http://www.geneious.com, (Kearse et al., 2012)). Sequence and species identity was verified against previously published sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Sequences obtained in the current study were deposited in the NCBI GenBank database under the following accession numbers [GenBank: XXX–XXX].

For the partitioned phylogenetic analysis, representative sequences from the different Onchocercidae subfamilies, were downloaded from GenBank and aligned to the sequences generated in the current study (Table 3). *Filaria latala* [GenBank: 18S: KP760135 and COI: KP760186] was chosen as the outgroup, following Lefoulon et al. (2015). A partition homogeneity test (1000 replicates - heuristic search) calculated using PAUP version 4.0a152 (Swofford, 2002) was applied to check whether the 18S rRNA and COI gene trees were sufficiently similar in rates of divergence and branching order and that the datasets could be combine. The partition homogeneity test ($P < 0.97$) supported the combination of the 18S rRNA and COI genes. Concatenated 18S rRNA and COI gene sequences were aligned using the Clustal W 2.1 alignment tool (Larkin et al., 2007) under the default settings and implemented in Geneious R11. The GBlocks server was used to remove any alignment gaps and ambiguities selecting the parameters to allow for smaller final blocks with gap positions (Castresana, 2000; Talavera and Castresana, 2007). The final

alignment consisted of 54 sequences with a 659 nt 18S rDNA and 577 nt COI, with a total of 1280 nt, 82% of the original 1445 positions. A partitioned Bayesian inference (BI) analysis was performed using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001) implemented from within Geneious R11. Prior to the analyses, a model test was performed to determine the most suitable nucleotide substitution model according to the Bayesian information criterion using jModelTest 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012). The model with the best BIC score for the 18S rDNA sequence alignment was the Kimura 2-parameter model (Kimura, 1980) with an estimated proportion of invariable sites and a discrete gamma distribution (K80 + I + Γ). For the COI sequence alignment the General Time Reversible (Tavaré, 1986) with an estimated proportion of invariable sites and a discrete gamma distribution (GTR + I + Γ) was selected as the model with the best BIC score. For the BI analysis, the alignment was partitioned according to the 18S rRNA (1–659 nt) and COI (660–1236 nt) genes, the Markov Chain Monte Carlo (MCMC) algorithm was run for 10 million generations, sampling every 100 generations, and using the default parameters. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. Results were visualized in Tracer (Rambaut et al., 2018) (implemented from within Geneious R11), to assess convergence and the 'burn-in' period.

Table 3. Summary of filarial species, their host, accession numbers and locality, used in phylogenetic analyses in this study.

Subfamilies	Species	Definitive host	Accession numbers (18S; COI)		Locality
Dirofilariinae	*Dofilaria immitis* (Leidy, 1856)	*Canis familiaris*	KP760134	KP760185	Italy
	Foleyella candezei (Fraipont, 1882)	*Agama agama*	KP760136	KP760187	Togo
	Foleyella candezei	*Agama agama*		FR823336	Togo
	Foleyella furcate (Linstow, 1899)	*Furcifer oustaleti*		KM234627	Madagascar
	Foleyella furcata	*Furcifer* sp.		KM234628	Madagascar
	Pelecitus fulicaeatrae (Diesing, 1861)	*Podiceps nigricollis*	KP760161	KP760206	Spain
	Loa loa (Cobbold, 1864)	*Homo sapiens*	KP760143	KP760194	France
Icosiellinae	*Icosiella neglecta* (Diesing, 1851)	*Pelophylax ridibunda*	KP760137	KP760188	Ukraine
	Icosiella neglecta	*Pelophylax* kl*. esculeta*	KP760138	KP760189	France
	Icosiella sp.	*Conraua goliath*		MH182623	Cameroon
Onchocercinea	*Acanthocheilonema odendhali* (Perry, 1967)	*Callorhinus ursinus*	KP760116	KP760168	Alaska
	Acanthocheilonema vitaea (Krepkogorskaya, 1933)	*Meriones unguiculatus*	KP760117	KP760169	FR3 strain
	Breinlia jittapalapongi Veciana, Bain, Morand, Chaisiri, Douanghoupha, Miquel and Ribas, 2015	*Rattus tanezumi*	KP760119	KP760170	Laos
	Brugia malayi (Brug, 1927)	*Meriones unguiculatus*	KP760120	KP760171	FR3 strain
	Brugia pahangi (Buckley and Edeson, 1956)	*Meriones unguiculatus*	KP760121	KP760172	FR3 strain
	Brugia timori Partono, 1977	*Homo sapiens*	KP760122	KP760173	Indonesia
	Cercopithifilaria bainae Almeida and Vicente, 1984	*Canis familiaris*	KP760123	KP760175	experimental
	Cercopithifilaria rugosicauda (Böhm and Supperer, 1953)	*Capreolus capreolus*	KP760124	KC610815	France
	Cruorifilaria tuberocauda Eberhard, Morales and Orihel, 1976	*Hydrochoerus hydrochaeris*	KP760125	KP760176	Venezuela
	Dipetalonema caudispina (Molin, 1858)	*Ateles paniscus*	KP760126	KP760177	Guyana
	Dipetalonema gracile (Rudolphi, 1809)	*Cebus olivaceus*	KP760128	KP760179	Venezuela
	Dipetalonema graciliformis (Freitas, 1964)	*Saimiri scuireus*	KP760131	KP760182	Peru
	Dipetalonema robini Petit, Bain and Roussilhon, 1985	*Lagothrix poeppigii*	KP760132	KP760183	Peru
	Litomosoides brasiliensis Lins de Almeida, 1936	*Carollia perspicillata*	KP760139	KP760190	Peru
	Litomosoides hamletti Sandground, 1934	*Glossophaga soricina*	KP760141	KP760192	Peru
	Litomosoides solaria Guerrero, Martin, Gardner and Bain, 2002	*Trachops cirrhosus*	KP760142	KP760193	Venezuela
	Loxodontofilaria caprini Uni and Bain, 2006	*Naemorhedus crispus*	KP760144	AM749237	Japan
	Mansonella perforate Uni, Bain and Takaoka, 2004	*Cervus nippon*	KP760145	AM749265	Japan
	Mansonella ozzardi (Manson, 1897)	*H. sapiens*	KP760147	KP760195	Haiti
	Madathamugadia heipei Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain, 2000	*Pachycactylus turneri*	KP760146	JQ888270	South Africa

Table 3. Continued.

Subfamilies	Species	Definitive host	Accession numbers (18S; COI)		Locality
	Monanema martini Bain, Bartlett and Petit, 1986	*Arvicanthis niloticus*	KP760148	KP760196	Senegal
	Onchocerca armilatta Railliet and Henry, 1909	*Bos taurus*	KP760153	KP760200	Cameroon
	Onchocerca dewittei japonica Uni, Bain and Takaoka, 2001	*Sus scrofa leucomystax*	KP760154	KP760203	Japan
	Onchocerca eberhardi Uni and Bain, 2007	*Cervus nippon*	KP760155	AM749268	Japan
	Onchocerca gutturosa Neumann, 1910	*Bos taurus*	KP760156	KP760201	Cameroon
	Onchocerca ochengi Bwangamoi, 1969	*Bos taurus*	KP760157	KP760202	Cameroon
	Onchocerca skrjabini Ruklyadev, 1964	*Cervus nippon*	KP760158	AM749269	Japan
	Wuchereria bancrofti (Cobbold, 1877)	*Homo sapiens*	AF227234	JN367461	Mali
	Yatesia hydrochoerus (Yates, 1980)	*Hydrochoerus hydrochaeris*	KP760166	KP760210	Venezuela
Oswaldofilariinae	*Oswaldofilaria petersi* Bain and Sulahian 1974	*Crocodilurus amazonicus*	KP760160	KP760205	Peru
	Oswaldofilaria chabaudi Pereira, Souza and Bain, 2010	*Tropidurus torquatus*	KP760159	KP760204	Brazil
Setariinae	*Setaria tundra* Bain, 1974	*Rangifer tarandus*	KP760165	KP760209	Finland
	Setaria labiatopapillosa (Alessandrini, 1848)	*Bos taurus*	KP760164	KP760208	Cameroon
Splendidofilariinae	*Aproctella alessandroi* Bain, Petit, Kosek and Chabaud, 1981	*Saltator similis*	KP760118	FR823335	Brasil
	Madathamugadia hiepei Hering-Hagenbeck, Boomker, Petit, Killick-Kendrick and Bain, 2000	*Pachycactylus turneri*	KP760146	JQ888270	South Africa
	Rumenfilaria andersoni Lankester and Snider, 1982	*Rangifer tarandus*	KP760163	JQ888273	Finlande
Waltonellinae	*Foleyellides* sp.	*Rana pustulosa*		KC130677	Mexico
	Foleyellides sp.	*Rana pustulosa*		KC130679	Mexico
	Ochoterenella sp. 1	*Rhinella granulosa*	KP760151	KP760198	Venezuela
	Ochoterenella sp. 2	*Rhinella marina*	KP760152	KP760199	Venezuela
	Ochoterenella sp. 3	*Phyllomedusa bicolor*	KP760150	KP760197	French Guyana
	Onchocercid n. gen. n. sp. 1	*Sclerophrys garmani*	XXX	XXX	South Africa
	Onchocercid n. gen. n. sp. 1	*Sclerophrys gutturalis*	XXX	XXX	South Africa
Outgroup	*Filaria latala* Chabaud and Mohammad, 1989	*Panthera leo*	KP760135	KP760186	South Africa

Results

The blood of 128 anuran individuals representing four species, namely *Sclerophrys garmani* (n=45), *Sclerophrys gutturalis* (n=73), *Sclerophrys pusilla* (n=7), and *Schismaderma carens* (n=3) was collected and screened for microfilaria (S1 Table). Eight toads from two species, *S. gutturalis* and *S. garmani*, were found positive with microfilaria, all collected from Sodwana, KZN, South Africa. For *S. gutturalis* 13.5% of the males (7/52) and 6.7% of *S. garmani* females (1/15) were infected with microfilaria, as compared to 0% of the *S. gutturalis* females (0/21) and 0% of the *S. garmani* males (0/30). Based on the morphology of the observed blood stages alone, microfilaria could not be classified to genus or species level. Hence, for further morphological classification (see species description below) both male and female adult specimens were collected from the infected *S. gutturalis* (n=4). The number of adults collected ranged from 10 to 52 specimens across the different individuals dissected.

Description

Phylum: Nematoda Rudolphi, 1808
Class: Chromadorea Inglis, 1983
Order: Rhabditida Chitwood, 1933
Suborder: Spirurida Railliet & Henry, 1915
Superfamily: Filarioidea Weinland, 1858
Family: Onchocercidae Leiper, 1911
Subfamily: Waltonellinae Bain & Prod'Hon, 1974
Genus: Onchocercid n. gen.

Diagnosis

Large elongated nematodes, females generally bigger than males. Oral opening small, oval with two parastomal structures arranged laterally. Buccal capsule small, conspicuous, wider than long. Narrow lateral and caudal alae present in both sexes. Transverse or longitudinal striations absent. Oesophagus visibly divided into muscular and glandular sections. Nerve ring at level of muscular oesophagus posterior quarter. Excretory pore minute, poorly visible, situated at level of oesophageal-intestinal junction. Posterior end of male with narrow caudal alae, four pairs of caudal papillae (one above and three below cloaca). Spicules simply-shaped with sharpened tips, unequal (left longer than right one). Tail tapering with rounded tip. Females viviparous, vulva situated at posterior level of oesophagus. Caudal alae narrow,

tail tapering with rounded tip. Parasites found in body cavity and peripheral blood of anurans.

According to the keys provided by Esslinger (1986a), Purnomo and Bangs (1999), and Bain et al. (2013), onchocercid n. gen. belongs to the family Onchocercidae and subfamily Waltonellinae, based on the presence of distinct parastomal structures, lack of submedian cephalic spines, position of anus not subterminal, possession of vulva posterior to nerve ring in females, possession of several pairs of papillae on somewhat elongated tail in males and parasitising a definitive amphibian host. Within Waltonellinae, the morphological characters of onchocercid n. gen. conform closest with *Foleyellides*, such as the presence of a distinct buccal capsule and parastomal structures, the presence of lateral and caudal alae in both sexes, and the absence of cuticular bends and bosses on the body cuticle. Nonetheless onchocercid n. gen. can be easily distinguished from *Foleyellides* and all other genera from Waltonellinae by the absence of dorsal outer cephalic papillae and the absence of small inner cephalic papillae. Onchocercid n. gen. is characterised by only two enlarged submedian cephalic papillae on the ventral sides of the oral opening, and the absence of other papillae surrounding the oral opening. Since difference in cephalic morphology is commonly used for generic differentiation in many groups of parasitic nematodes and based on the other morphological and molecular differences such as asymmetry, we consider our specimens as belonging to the new genus onchocercid.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:XXX. The LSID for the new onchocercid genus name is urn:lsid:zoobank.org:act:XXX.

Etymology: The generic name is derived from the close morphological resemblance to representatives of *Foleyellides*, with the new genus possessing morphological characters, such as asymmetry and a reduced number of the cephalic papillae generally considered as a progressive character within onchocercids (Anderson and Bain, 1976).

Type species: **onchocercid n. gen. n. sp. 1**

Type-host: *S. gutturalis*

Other host: *S. garmani*

Site in host: body cavity, subcutaneous. Microfilariae were also observed in peripheral blood obtained from the femoral artery or veins, heart and body cavity

Vector: *Uranotaenia* (*Pseudoficalbia*) *mashonaensis, Uranotaenia* (*Pfc*.) *montana*

Site in vector: stomach, intestine, fat body, thoracic and abdominal cavities, head capsule, proboscis

Type-locality: Sodwana, KwaZulu-Natal, South Africa. Coordinates: S27.488591°; E32.664259°

Type-material: Vouche material deposited in the parasite collection of the National Museum, Bloemfontein, South Africa, under accession number [NMB P XXX]. Vouche material, deposited in the collection of the research group Zoology: Biodiversity and Toxicology of Hasselt University (Diepenbeek, Belgium), under accession number [HU XXX].

Representative DNA sequences: The 18S rRNA AND COI gene sequences were submitted to the GenBank database under the accession numbers XXX

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:XXX. The LSID for the new name onchocercid n. gen. n. sp. 1 is urn:lsid:zoobank.org:act:XXX.

Etymology: The species epithet is derived from XXX.

Description

General. Body elongated, cylindrical almost along entire length with rounded anterior and narrowed posterior end. Oral opening small, oval with two parastomal structures arranged laterally (Figs. 1B, 2A, 3C, D). Buccal capsule small, conspicuous, wider than long. In both sexes cuticle forming narrow lateral and caudal alae without any other conspicuous transverse and longitudinal striations. Lateral alae beginning at level of anterior end of muscular oesophagus and terminating close to cloaca (Fig. 2D). Oesophagus visibly divided into short muscular and longer glandular section. Nerve ring encircling glandular oesophagus at level of its posterior quarter. Minute excretory pore (seen only on SEM images) situated al level of oesophageal-intestinal junction.

Males (n=30). Body 15–28, [20] (22±3.1, 14.3) mm long, 97–138 [105] (120±12.0, 10.0), 151–215 [153] (190±22.1, 11.7) and 135–248 [162] (202±29.3, 14.5) wide at nerve ring, oesophageal-intestinal junction, and mid-body level, respectively (Fig.2B). Buccal capsule, 3–6 [3] (4±0.8, 20.1) long, and 6–12 [8] (9±1.5, 18.0) wide. Glandular portion of oesophagus 190–369 [263] (282±41.8, 14.8) long; 27–57 [32] (38±6.1, 16.0), 25–49 [29] (34±5.4, 16.0) and 32–51 [37] (41±5.9, 14.2) wide at anterior, mid-length and posterior level, respectively. Muscular section of oesophagus 658–1212 [704] (918±145.9, 15.9) long; 44–88 [58] (66±11.8, 18.0), 62–157 [71] (106±23.0, 21.7) and 49–158 [49] (96±27.2, 28.4) wide at anterior, mid-length, and posterior level, respectively. Total oesophagus 887–1497 [967] (1204±172.1, 14.3) long spanning 4–7 [5] (6±0.6, 11.5) % of body length. Nerve ring situated at 179–270 [207] (222±25.2, 11.3) from the anterior end of body, spanning 14–24 [21] (19±2.7, 14.2) % of total oesophagus length (Figs. 1C, 2C).

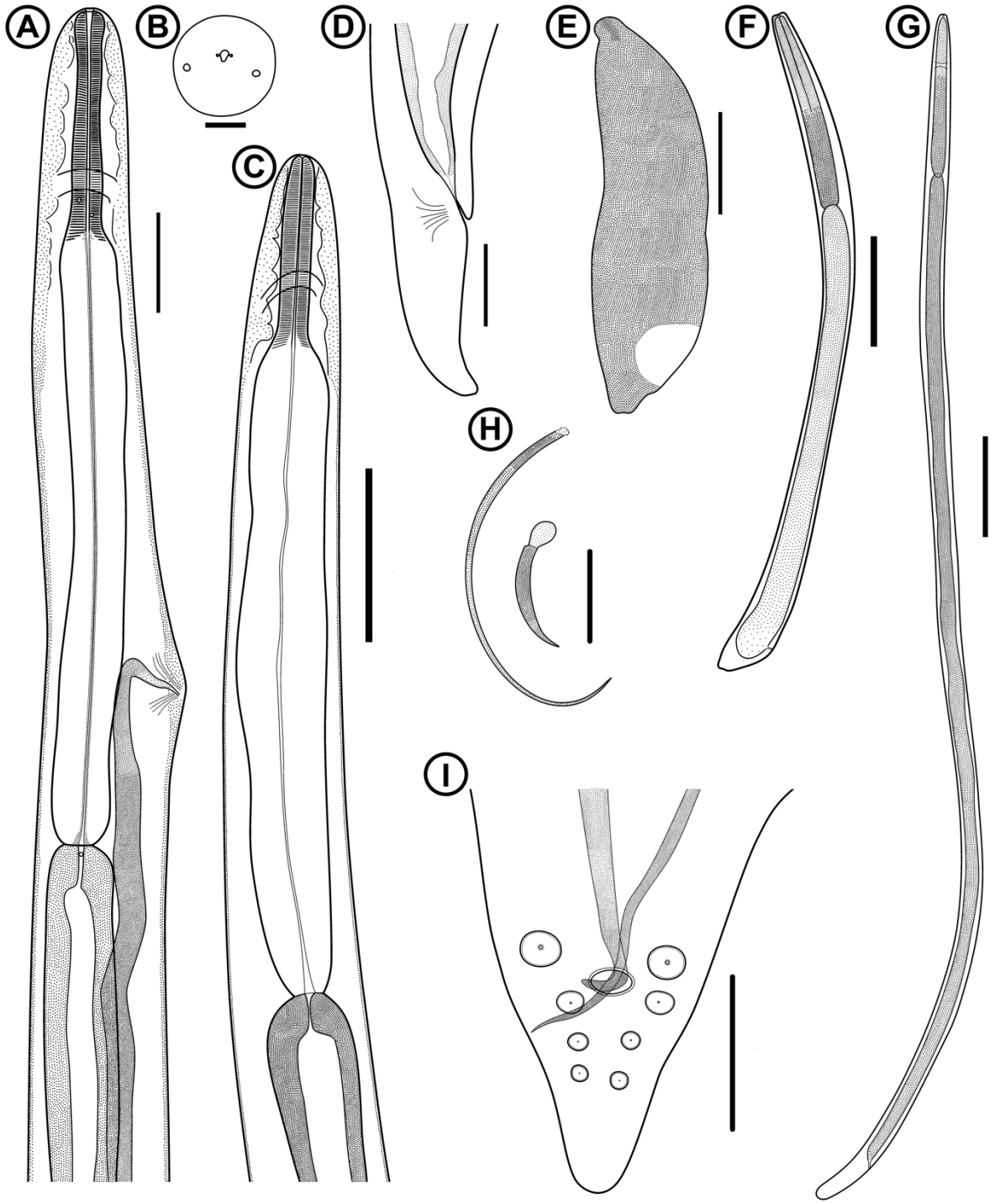

Fig. 1. Illustrations of adult and larval stages onchocercid n. gen. n. sp. 1. A – female, anterior part of body, lateral view; B – female, anterior part of body, apical view; C – male, anterior part of body, lateral view; D – female, posterior part of body, lateral view; E – first-stage sausage-shape larva, lateral view; F – second-stage larva, lateral view; G – third-stage larva, lateral view; H – male, spicules, lateral view; I – male, posterior part of body, ventral view. Scale bars: A, C, D, F-H – 100; B, E – 20; I – 50.

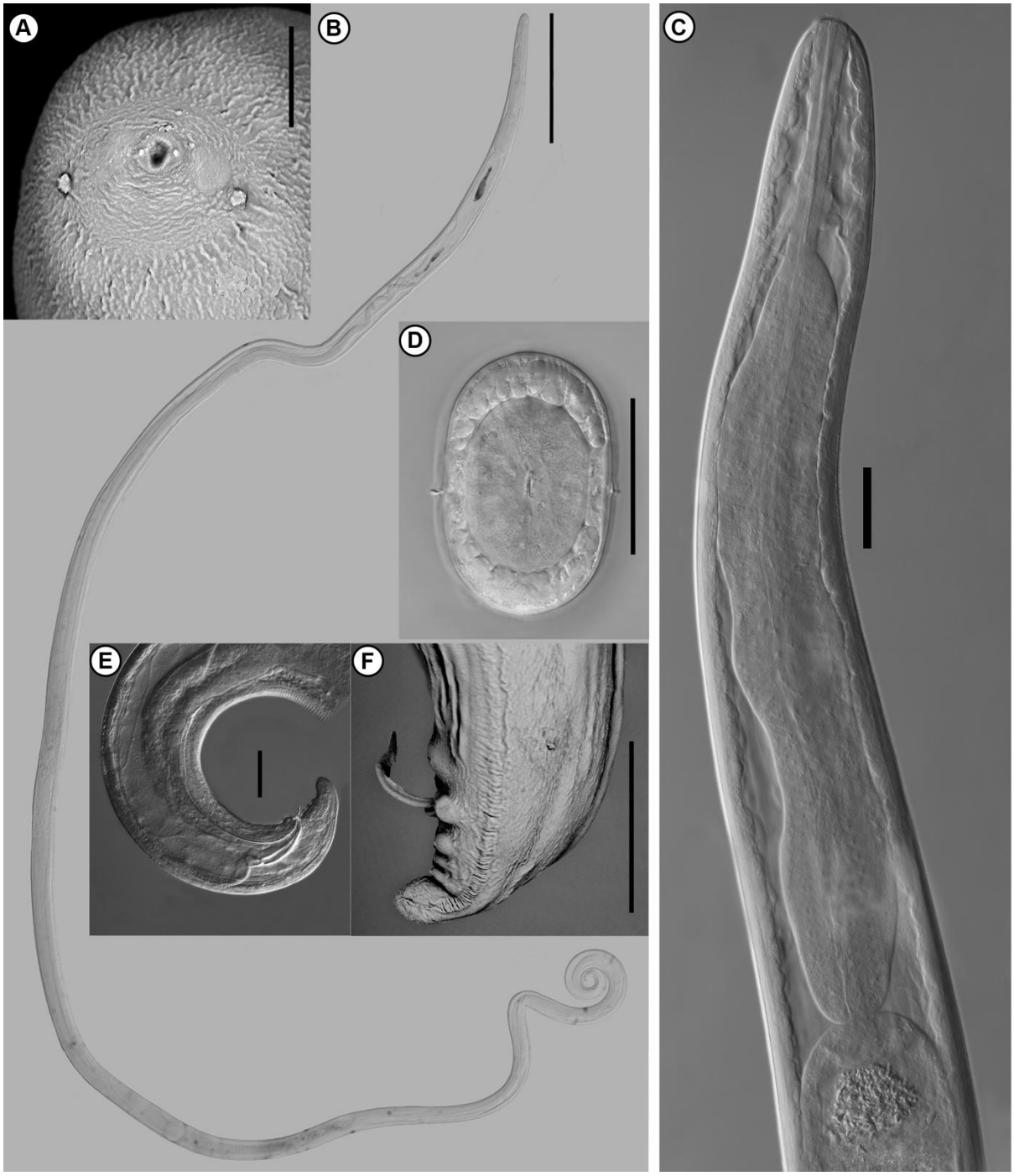

Fig. 2. Photomicrographs of onchocercid n. gen. n. sp. 1 male. A – anterior part of body, apical view (SEM image); B – entire body, lateral view; C – anterior part of body, lateral view; D – cross-section at mid-body level; E – posterior part of body, lateral view; F – posterior part of body, lateral view (SEM image). Scale bars: A – 10; B – 1mm; C – 100; D-F – 50.

Spicules unequal: left elongated, often extending from cloaca, 50–94 [74] (73±9.1, 12.5) long; right shorter and thicker, 91–158 [152] (137±14.7, 10.7) long (Figs. 1H, 2E). Five pairs of sessile papillae located at caudal region: one pair pre-anal, one ad-anal and three post-anal papillae. Size ranges of papillae decrease towards the posterior end. Tail tapering with

rounded tip, 50–94 [74] (73±9.1, 12.5) long. Cuticular ornamentation well developed above cloaca. Narrow caudal alae nearly reaching tail's tip (Figs. 1I, 2E, F).

Females (n=30). Body 16.2–71.5 [6.2] (49.5±15.9, 32.1) mm long, 117–201 [167] (158±23.6, 14.9), 193–390 [368] (291±78.5, 27.0) and 147–441 [402] (330±89.0, 27.0) wide at nerve ring, oesophageal-intestinal junction, and mid-body level, respectively (Fig. 3A). Buccal capsule 3–7 [5] (4±1.1, 23.6) long, and 5–15 [9] (10±2.1, 22.4) wide (Fig. 3C). Glandular section of oesophagus, 215–436 [364] (320±61.1, 19.1) long; 30–56 [30] (40±5.9, 14.7), 25–51 [35] (34±5.2, 15.2) and 25–67 [49] (45±7.7, 17.2) wide at anterior, mid-length and posterior level, respectively. Muscular portion of oesophagus, 490–1798 [1798] (1147±312.9, 27.3) long; 37–105 [70] (74±16.2, 21.9), 55–176 [110] (117±29.6, 25.3) and 56–184 [143] (104±32.7, 31.4) wide at anterior, mid-length and posterior level, respectively. Total oesophagus 722–2164 [2162] (1467±355.0, 24.2) long spanning 2–5 [3] (3±0.6, 20.5) % of body length. Nerve ring at 166–420 [275] (249±56.4, 22.6) from anterior end, spanning 12–31 [13] (17±4.0, 22.8) % of total oesophagus length (Figs. 1A, 3B).

Vulva transversely split, at 687–1882 [1782] (1234±266.1, 21.6) from anterior end, spanning 2–4 [3] (3±0.6, 23.7) % of body length (Figs. 1A, 3B, F). Tail tapering, 123–859 [331] (274±138.5, 50.6) long. Narrow caudal alae almost reaching tail's tip (Figs. 1D, 3E).

Microfilaria. Larvae from anuran blood (n=30) small, elongated, coated in relatively thick sheath (Fig. 4A, 5C). Anterior and posterior ends rounded, maximum width at level of anterior quarter, 66–92 (77±7.04, 9.15) long, and 4–5 (5±0.22, 4.78) wide. Cuticle smooth along entire body. Unsheathed microfilaria from digestive tract of mosquito (n=30) thin, elongated measuring 82–134 (105±15.14, 14.40) long, and 5–8 (6±0.60, 9.49) maximum width (at anterior quarter) (Fig. 4B). Fine transverse striations observed along body cuticle. Anterior end with crown-like structure and small tooth (clearly visible on SEM images) (Figs. 5A, B).

Sausage-shape first-stage larvae (n=37). Body short, almost oval measuring 51–118 (79±18.53, 23.52) long, with maximum width of 12–29 (18±4.03, 22.02) over anterior quarter. Early sausage stage with narrow anterior end, possessing crown-like structure and small tooth (Fig. 4C). Late sausage-shape stage with wider and smoother anterior section, deprived of other structures (Figs. 1E, 4C, 5E).

Second-stage larvae (n=20). Body relatively short, with slightly widened anterior and posterior ends, measuring 315–663 (474) long, and 23–49 (31) wide at mid-body level. Oesophagus narrow at anterior section, uniformly widening towards posterior end, measuring 77–286 (157) long, and spanning 16–47 (31) % of body length. Dissected oesophagus visibly divided on glandular (with nerve ring at posterior quarter) and muscular parts. Tail tapering to rounded tip, measuring 21–51 (39) long; and spanning 4–11 (8) % of body length (Figs. 1F, 5F, G).

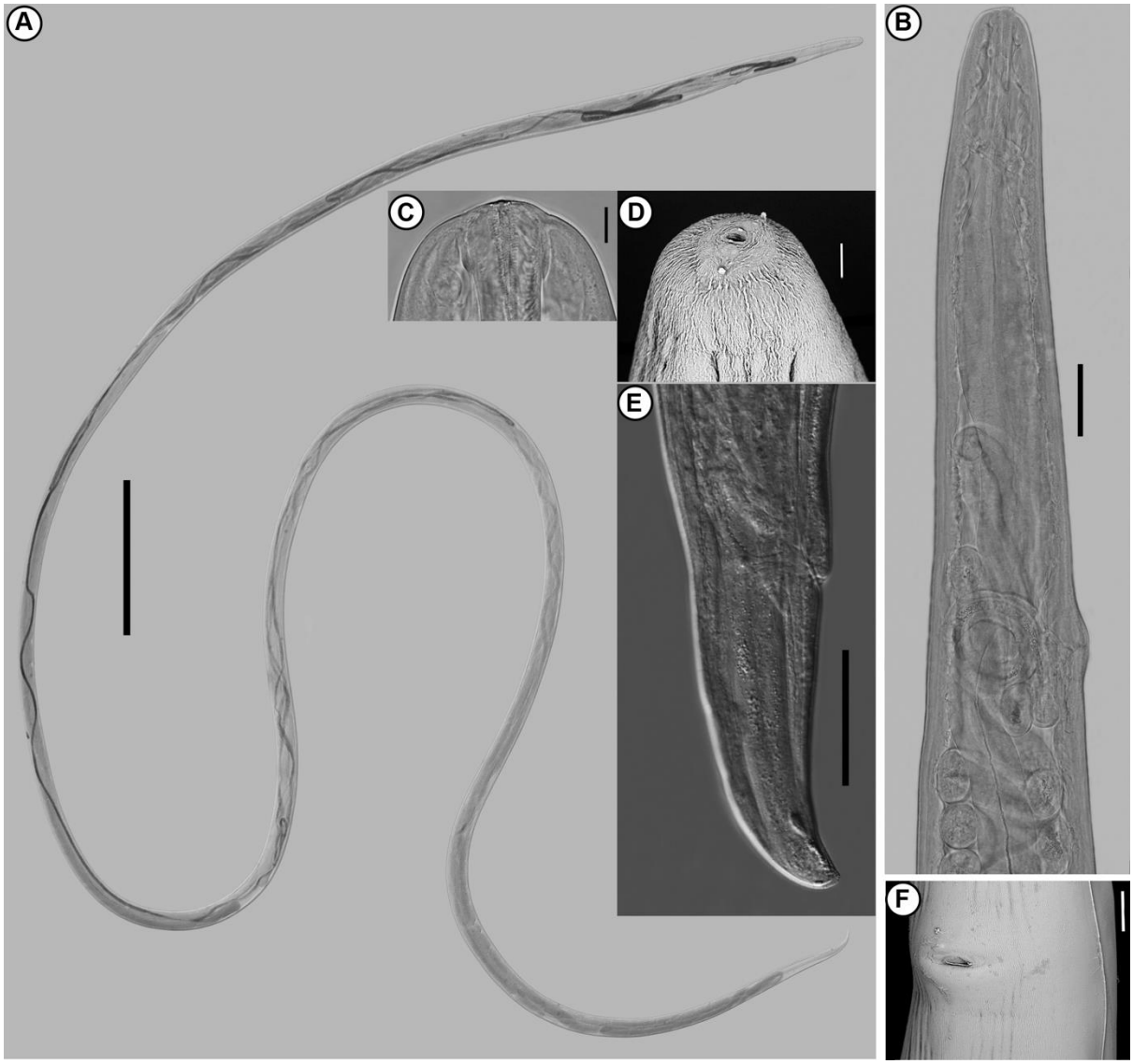

Fig. 3. Photomicrographs of onchocercid n. gen. n. sp. 1 female. A – entire body, lateral view; B – anterior part of body, lateral view; C – anterior part of body, head region, lateral view; D – anterior part of body, apical view (SEM image); E – posterior part of body, lateral view; F – region of vulva, ventral view (SEM image). Scale bars: A – 2mm; B, E – 100; C, D, F – 20.

Third-stage larvae (n=30). Specimens from body cavity thin and elongated, measuring 752–1090 (927±87.1, 9.4) long, with maximum width of 14–27 (19±2.9, 14.9) at level of anterior third of body. Oesophagus short, measuring 103–196 (142±21.9, 15.4) long, spanning 11–21 (15±2.5, 16.4) % of body length. Nerve ring encircling oesophagus at level of its anterior quarter, measuring 48–107 (75±15.1, 20.0) from anterior end of body, spanning 5–11 (8±1.6, 19.5) % and 40–68 (53±5.9, 11.2) % of body and oesophagus length, respectively. Tail short and rounded, measuring 32–55 (45±5.7, 12.7) long, and spanning 3–6 (5±0.6, 11.5) % of body length. Specimens from mosquito head (n=15) relatively small, measuring

798–978 (893) long, with a maximum width of 16–23 (19). Oesophagus 124–208 (146) long, comprising 13–22 (16) % of body length. Position of nerve ring varying within posterior half of muscular oesophagus, measuring 44–49 (47) from anterior end of body, spanning 5–6 (5) % and 29–37 (33) % of body and oesophagus length, respectively. Tail rounded, measuring 32–51 (44) long, and spanning 3–6 (5) % of body length (Figs. 1G, 5H).

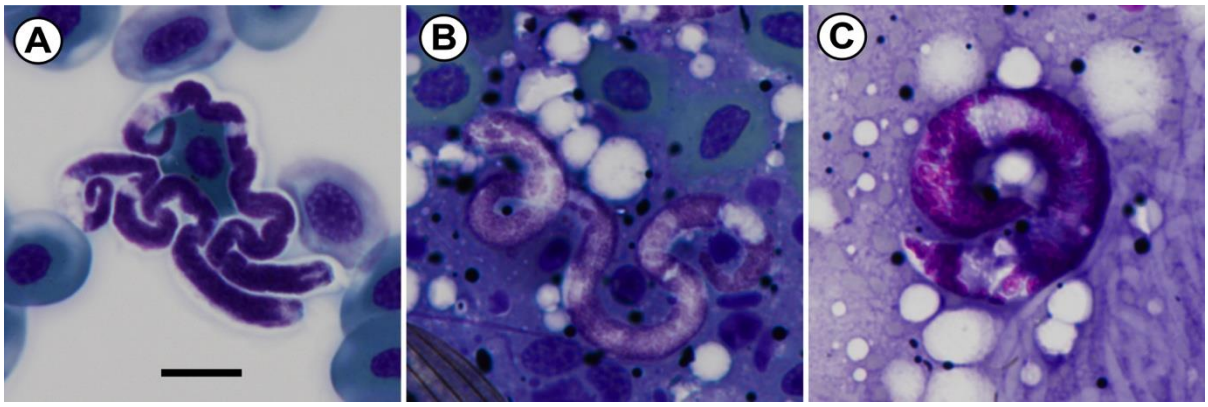

Fig. 4. Photomicrographs of onchocercid n. gen. n. sp. 1 stained larvae. A – microfilaria from amphibian blood; B – unsheathed microfilaria from mosquito blood meal; C – sausage-shape first stage. All images captured from the deposited slides (XXX) Scale bar: 10µm.

Remarks

Several morphological characters are used for differentiation between the various genera and species within Waltonellinae. The most defining characters are based on differences in the apical structures, and the male and female genital system. Onchocercid n. gen. n. sp. 1 can be easily distinguished from all other described species within the Waltonellinae by the absence of dorsal outer cephalic papillae and the absence of small inner cephalic papillae.

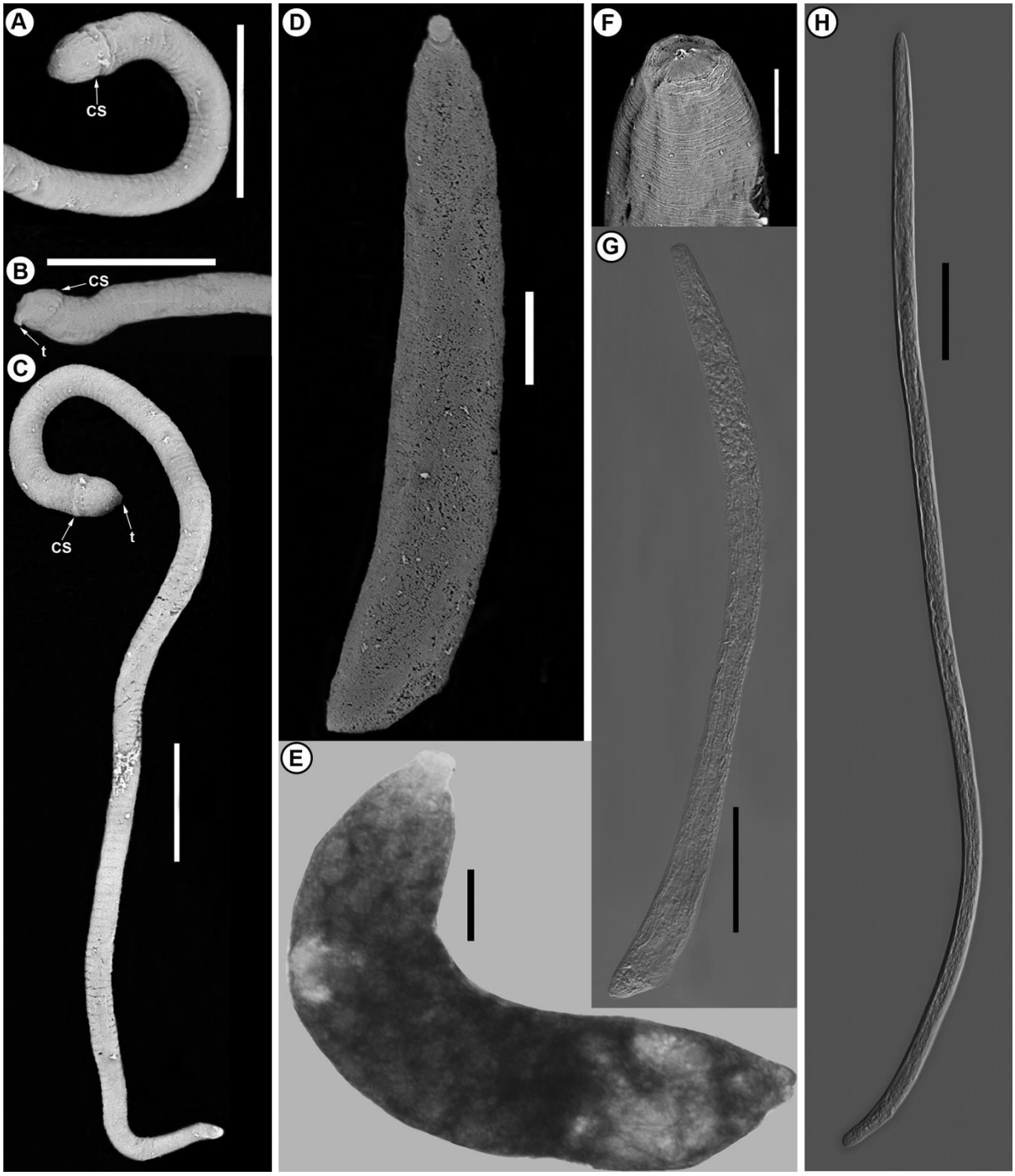

Fig. 5. Photomicrographs of onchocercid n. gen. n. sp. 1 larval stages. A – exsheathed microfilaria from amphibian blood, anterior part of body, subdorsal view (SEM image); B – sheathed microfilaria from amphibian blood, anterior part of body, lateral view (SEM image); C – sheathed microfilaria from amphibian blood, entire body, subdorsal view (SEM image); D – early sausage-shape first-stage, entire body, lateral view (SEM image); E – late sausage-shape first-stage, entire body, lateral view; F – second-stage, anterior part of body, apical view (SEM image); G – second-stage, entire body, lateral view; H – third-stage, entire body, lateral view. t – tooth, cs – crown-like structure. Scale bars: A-D, F – 10; E-H – 100.

Phylogenetic analysis

Amplicons of between 753 and 775 nt (n=15) of the 18S rRNA gene, and approximately 663 nt (n=15) of the COI gene were obtained in the current study. Sequences were derived from the sausage-shape first-stage, second-stage, and third-stage larvae in the mosquito vectors *U.* (*Pfc.*) *mashonaensis* or *U*. (*Pfc*.) *montana*, and from the adult stages in the body cavity of the vertebrate host *S. gutturalis* and from microfilaria in the blood of the hosts *S. garmani* and *S. gutturalis*. All the isolates obtained for onchocercid n. gen. n. sp. 1, from the various stages (from microfilaria to adults) and hosts (toads and mosquitoes) for both the 18S rRNA and COI gene sequence fragments were identical. onchocercid n. gen. n. sp. 1 is represented in this analysis by sequences obtained from microfilaria infected blood samples from *S. gutturalis* and *S. garmani*. For the BI phylogenetic analysis, filariae of the Waltonellinae isolated from anuran hosts are the earliest diverging linages in the ingroup. All other onchocercid taxa used in this analysis, Dirofilariinae, Setariinae, Splendidofilariinae and Onchocercinae, form a monophyly with 0.95 posterior probability support, with taxa from the Dirofilariinae and the Onchocercinae are shown as polyphyletic. onchocercid n. gen. n. sp. 1, is shown as a sister taxon to Icosiellinae, Oswaldofilariinae and all other onchocercids from the Onchocercidae (Fig. 6).

Life history experiment

A total number of 146 mosquitoes were collected *in situ* (see Fig. 7A-B). Of these, 64 were identified as *U.* (*Pfc.*) *mashonaensis* and 42 as *U*. (*Pfc*.) *montana*, the 40 mosquitoes reaming did not take blood meals or died in captivity, and were not included in the experiment. Collected mosquitoes used for experimental transmission consumed blood meals from *S. gutturalis* highly parasitised with microfilaria. Within 24 h post feeding, desheathed microfilaria were observed in the mosquito's intestines, along with undigested erythrocytes. Microfilaria observed in fresh wet blood smears were slightly more active compared to the desheathed microfilaria from mosquitoes. From three days post-infection (dpi) early sausage-shaped first-stage larvae were observed in the intestine, followed by late sausage-shape first-stage larvae appearing approximately three to four dpi in the fat bodies of its host (Fig. 8). Early sausage-shaped larvae seem to be dormant whereas late sausage-shaped larvae were observed having slow movement of the anterior part of the body. Second-stage larvae were found in the fat bodies and body cavity of the abdomen, and in the thorax of the mosquito as from six to 14 dpi (Fig. 8). Second-stage larvae were able to move relatively slowly, although this was faster compared to the first-stage larvae.

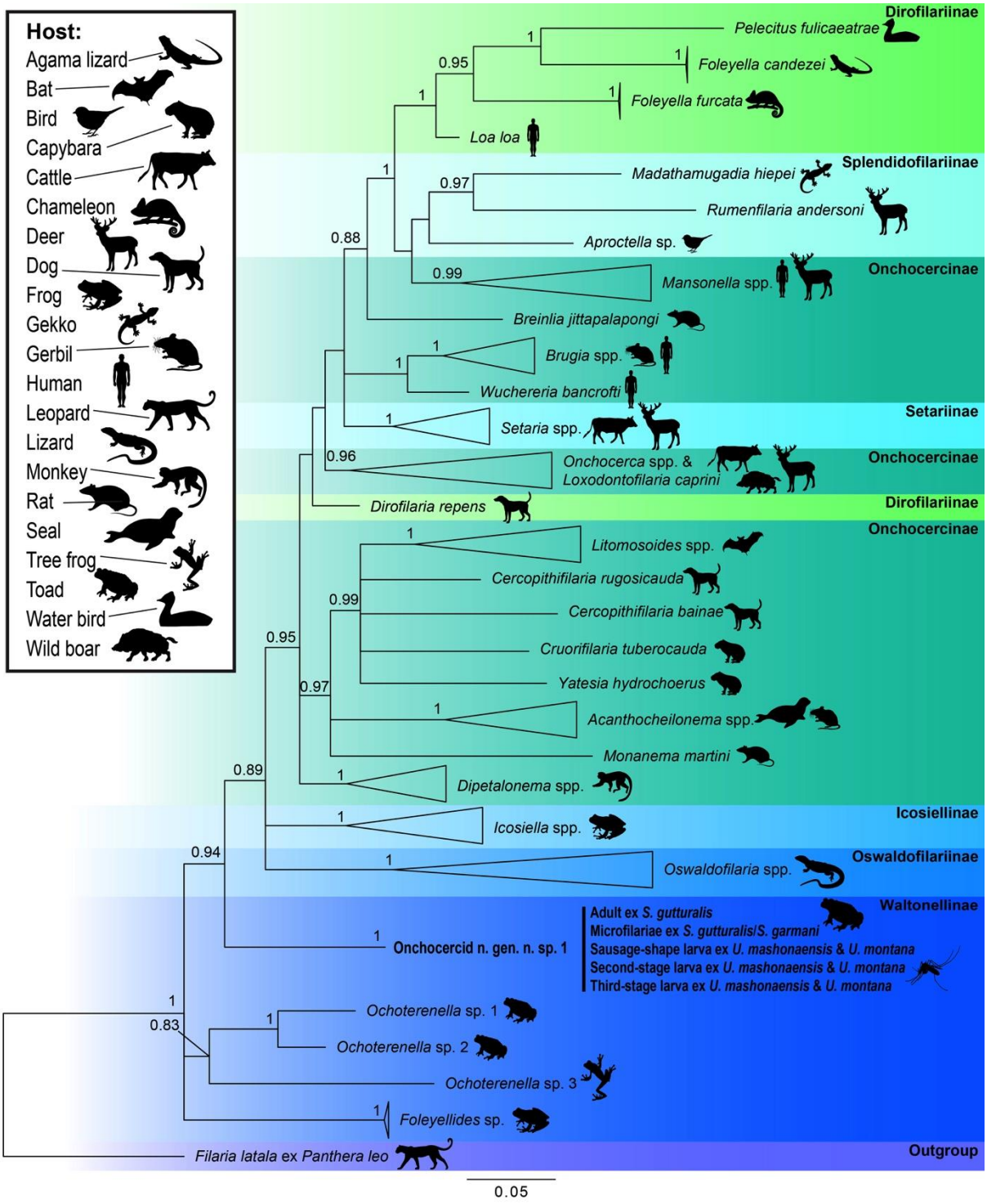

Fig. 6. Phylogeny of selected filarial nematodes from the Onchocercidae, based on partitioned concatenated datasets of 18S rDNA, and COI sequences using Bayesian Inference. The phylogram shows the relationship of onchocercid n. gen. n. sp. 1, compared to species of anuran and other onchocercids. *Filaria latala* [GenBank: 18S: KP760135 and COI: KP760186] was chosen as outgroup. Clades with posterior probability support values lower than 0.80 were removed. The total length of datasets is 1136 nt, and containing 54 taxa. Pictograms are used to illustrate host taxa. The scale bar represents 0.05 nucleotide substitutions per site.

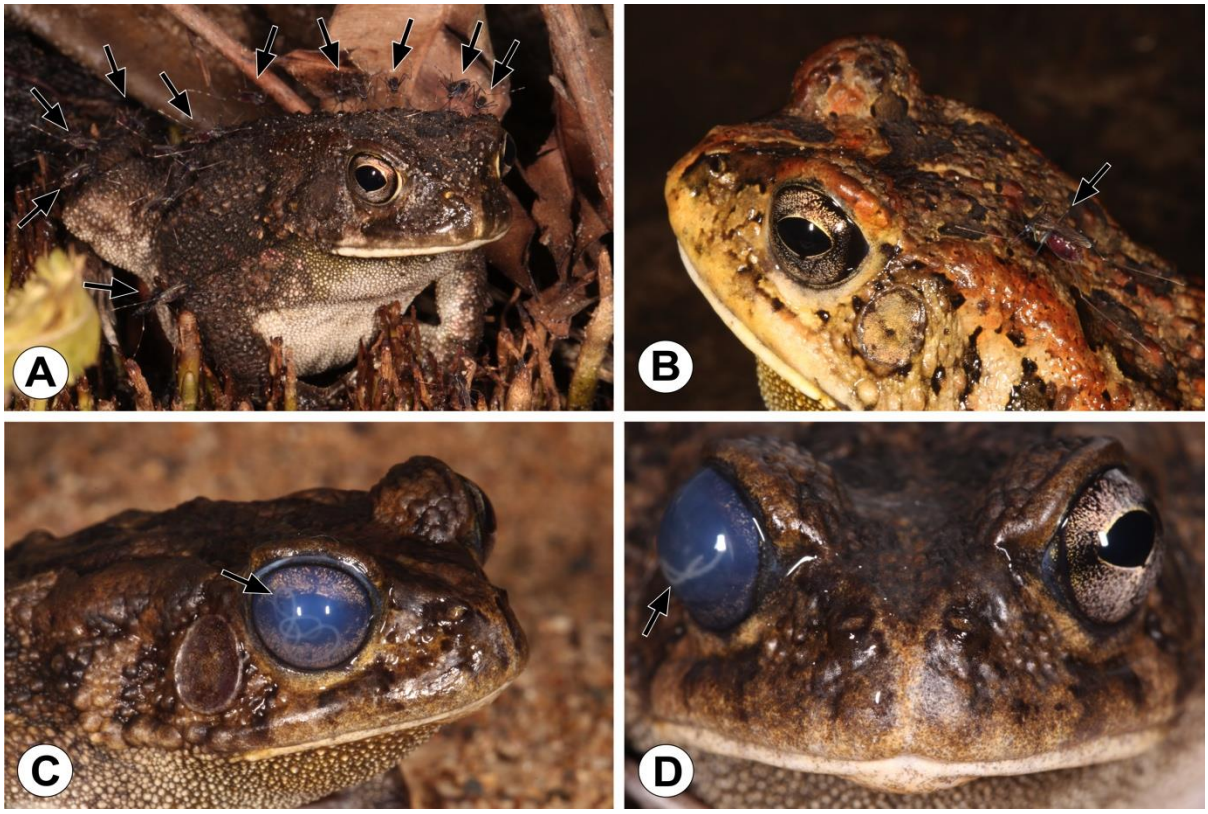

Fig. 7. Mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U.* (*Pfc.*) *montana* feeding on the host *Sclerophrys gutturalis in situ*, as well as the infected eye of one highly parasitised individual. A – Several *U.* (*Pfc.*) *mashonaensis* and *U.* (*Pfc.*) *montana* (arrows) taking a blood meal from a male *S. gutturalis in situ*. B – Close up photograph of engorged mosquito (arrow) taking its blood meal from *S. gutturalis*. C – onchocercid n. gen. n. sp. 1 (arrow) infecting the eye of the definitive guttural toad host, *S. gutturalis.* D – Close up photograph showing *S. gutturalis* infected eye with onchocercid n. gen. n. sp. 1 (arrow) compared to uninfected eye.

Third-stage infective larvae were found primarily in the thorax and head capsule of the mosquito host, roughly from 14 to 18 dpi (Fig. 8). Third-stage larvae moved actively, escaping from the dissected cavity, head or proboscis of the mosquito within several seconds. Development from second- to third-stage larvae was progressively prolonged in highly parasitised individuals, which was in contrast to individuals with a low infection level where all second-stage larvae developed into third-stage larvae simultaneously, and accumulated in the head capsule. In a few dissected mosquitoes, third-stage larvae were found positioned in the proboscis of the mosquito vector, seemingly “ready” to enter into the bloodstream of a new host. In the definitive toad host both adult male and female worms were found in the body cavity or subcutaneously, one individual was parasitised in the eye (see Fig. 7C-D), and microfilaria occurred in the peripheral blood (Fig. 8).

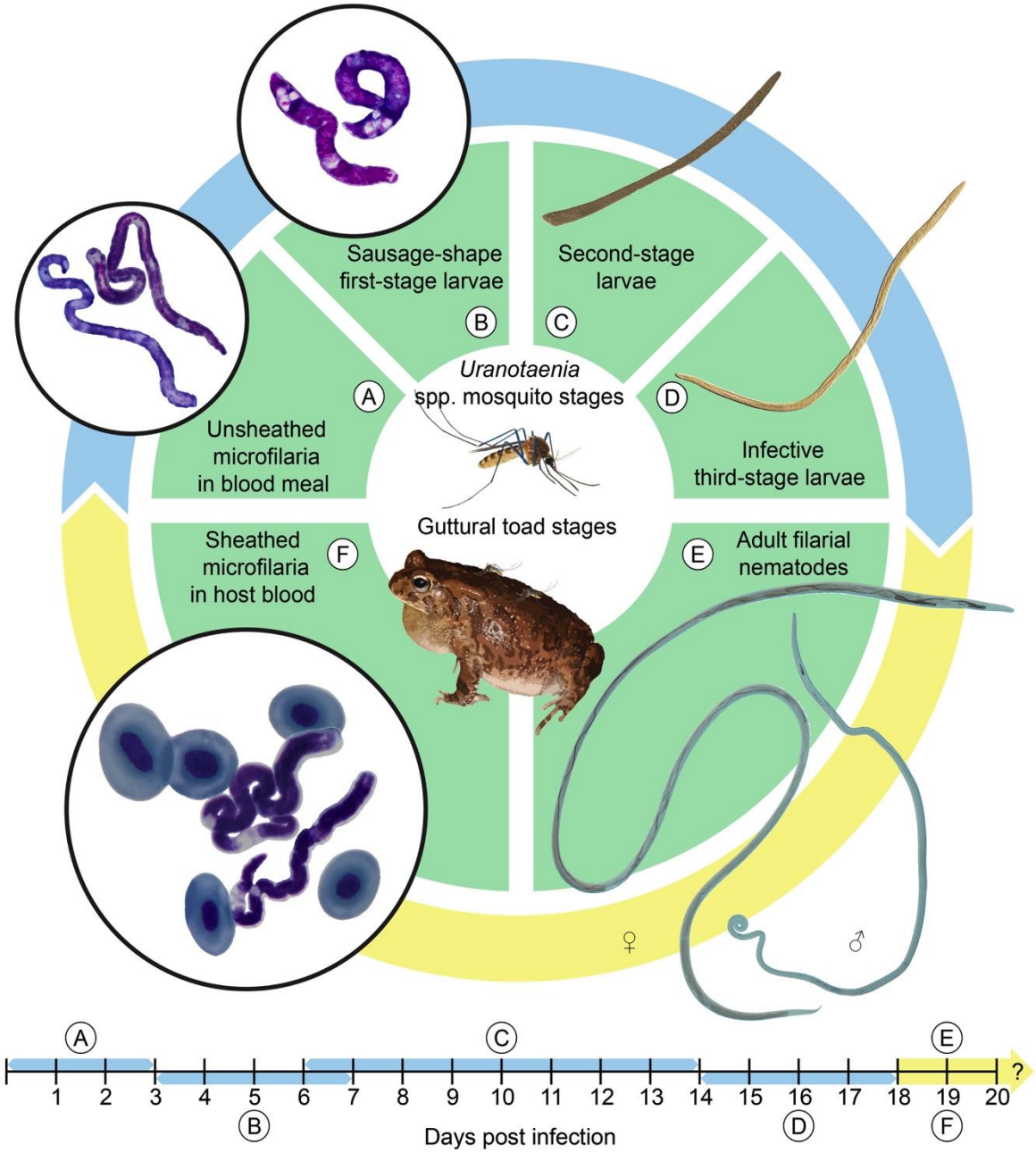

Fig. 8. Graphical representation of the life history of onchocercid n. gen. n. sp. 1 in the invertebrate mosquito vectors *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *U.* (*Pfc.*) *montana*, and in the vertebrate definitive host *Sclerophrys gutturalis*. A-D – onchocercid n. gen. n. sp. 1 development in the mosquito vector. A – Represents unsheathed microfilaria in the blood meal of the mosquito, observed up to three days post infection (dpi). B – Sausage-shaped first-stage larvae, observed from between three and seven dpi. C – Second-stage larvae, observed from between six and 14 dpi. D – Third-stage infective larvae, observed from between 14 and 18 dpi. E-F – onchocercid n. gen. n. sp. 1 development in the definitive guttural toad host, *S. gutturalis*. E – Male and female adult stages. F – Sheathed microfilaria in the peripheral blood of *S. gutturalis*. Images not drawn to scale.

Discussion

In the current study a total of 128 individuals representing four bufonid species were collected in northern KZN, South Africa and screened for the presence of microfilaria. Individuals from two species, *S. garmani* (n = 1) and *Scl. gutturalis* (n = 7) collected in Sodwana, were found positive with microfilaria infections. PCR amplification of blood samples from all specimens positive with microfilaria confirmed that all *S. garmani* and *S. gutturalis* were parasitised with the same filariid species (100% identity for both 18S rRNA and COI gene fragments). To identify and classify this species, adult filarial nematodes were collected from highly infected individuals, and light and scanning electron microscopy was used to study their morphology. Furthermore, *U.* (*Pfc.*) *mashonaensis* and *U.* (*Pfc.*) *montana* mosquitoes were observed feeding on calling guttural toads from Sodwana. These mosquitoes were collected *in situ* for experimental transmission studies. Mosquitoes still requiring blood meals were enticed to feed on infected toads. Blood-fed mosquitoes were progressively dissected, and nematodes extracted and fixed according to the stages of their development. Based on these findings we describe and elucidate the life history of a new amphibian filarial nematode, onchocercid n. gen. n. sp. 1

Morphology

With the exception of the genus *Madachotera*, onchocercid n. gen. and the other genera of the Waltonellinae share characters, such as well-developed cuticular parastomal structures and a buccal capsule. Despite the presence of the buccal capsule in most genera of Waltonellinae, past descriptions failed to provide measurements of this structure. Measurements of the buccal capsule of onchocercid n. gen. n. sp. 1, varied in length and width (in females between 5 and 15) depending on the size of the individual worm (Pearson coefficient of correlation $r = 0.48$ ($p \leq 0.001$) for both sexes). Nonetheless, the sclerotised structure should not be overlooked as it does not alter with fixation and may differ between different species. Cuticular structures e.g. lateral and caudal alae, and bands and bosses over the mid-body region are also used for generic differentiation. As in *Foleyellides*, onchocercid n. gen. posses lateral and caudal alae in both sexes, but lack bands or bosses. Examination of caudal alae and lateral alae on transverse sections, using SEM confirmed their simple shape and small size. However, this was the first study to examine these structures using SEM, thus comparative studies in the future with other species using SEM may reveal additional and new characters for species differentiation. Within the Waltonellinae the male genital system is similar across its different members, possessing an elongated left spicule, a short more cuticularized right spicule, and several large papillae

above and below the cloaca. Number and arrangement of the papillae and the ratio of the spicules are used for species differentiation within *Foleyellides* and *Ochoterenella*. However, onchocercid n. gen. n. sp. 1 contains the lowest number of papillae, one adcloacal and three postcloacal, as in two species of *Foleyellides*, namely *F. confusa* and *F. rhinellae* ((García-Prieto et al., 2014). With regard to the female genital structure, only the position of the vulva has been used to distinguish *Madachotera* (with vulva clearly posterior to the oesophagus) from other genera in the Waltonellinae. In onchocercid n. gen. n. sp. 1, the position of the vulva varies from the posterior end of the oesophagus (90% of oesophagus length) to the anterior end of the intestine (103% of oesophagus length). It was also noted that younger (=smaller) females possess a vulva closer to the anterior end, covering about 70% of the oesophagus length, while older (=larger) specimens usually possess a vulva at the section posterior to the oesophagus, covering 90–100% of its length. Therefore, in our opinion, the position of the vulva should only be used as a reliable species or generic differentiator if a sufficient sample size of nematodes with different body length is used to determine its location.

Scanning electron micrographs illustrated some unique characters of unsheathed microfilaria that are not visible under light microscopy, such as a small tooth, a crown-like structure on the anterior end, and fine transverse striations covering the body. The unique structures possessed by the microfilaria gradually disappeared from the early (younger) to late (older) sausage-shape first-stage larvae. We hypothesize that these characters help the microfilaria to easily penetrate the mosquitoes' stomach and intestinal wall. In comparison, second and third-stage larvae contained relatively simple morphological features. Characteristically for other filarial nematodes, both second and third-stages contained a rounded anterior end without conspicuous apical structures, a prominent rectum, and a rounded tail on the posterior end. The oesophagus and intestine were also clearly visible in both stages, whereas in the second-stage larvae the nerve ring surrounding the oesophagus was only visible once dissected.

No genital primordiums were observed in any of the collected larval stages. All examined stages were coated in a relatively thick sheath, with the exception of desheathed microfilaria observed in the mosquito blood meal. These unsheathed microfilaria seem to lose their sheath shortly after ingestion by their mosquito host. During SEM preparation of microfilaria obtained from the guttural toad's blood, the microfilaria effortlessly desheathed after coming into contact with the filter paper, which is in contrast to the second and third-stage larvae that possess a well-attached sheath. Even dissected second and third-stage larvae still contain a strongly attached sheath. It is possible that the thick sheath covers and conceals certain internal organs and structures, such as the excretory glands, and genital primordium.

In general, the microfilaria and other larval stages of anuran onchocercids are poorly studied. Only for *Paraochoterenella* the absence of a sheath and the shape of the tail in microfilaria stages is used to distinguish it from other genera within the Waltonellinae (Purnomo and Bangs, 1999). In our opinion, meticulous examination of different larval stages can reveal numerous and additional characters for species and generic differentiation.

Analyses of the metric characters of both adult and larval stages showed high variability. The smallest coefficient of variation observed was firstly for the values of the nerve ring for both adult sexes, 22.6 in females and 11.3 in males, and secondly for the male spicules comprising 11.3 and 10.7 for left and right spicules respectively. The high variability observed for all the other characters is possibly due to the large sample size and the large size of the nematodes. The metric characters of the different larval stages also varied greatly, possibly due to their intensive growth rate. Only the body length of the third-stage larvae was rather stable (CV less than 10). Based on the above observations, we suggest that species and generic differentiation of members of the Waltonellinae should be supported by both qualitative morphometric and molecular data.

Potential effects of onchocercid n. gen. n. sp. 1 on its anuran host

The majority of adult specimens of onchocercid n. gen. n. sp. 1 was removed from the body cavity of dissected guttural toads, with a few exceptions in highly infected hosts. Specifically, one and three adult filarial nematodes were found subcutaneously in the two most highly infected toads respectively. In the latter guttural toad (S1 Dataset, AE180124C1), three onchocercid n. gen. n. sp. 1 were attached to the lymphatic tissue, and one immature specimen was observed in its host's eye (see Fig. 7C-D). Although no specific attempts were made to study the specific pathological effects onchocercid n. gen. n. sp. 1 has on its host, some observations may suggest such effects. A heavily (52 specimens) infected *S. gutturalis* contained a visibly enlarged spleen, gall bladder, and liver, the latter also appearing darker than normal. Moreover, the same individual's eye was parasitised with a filarial nematode. Although this nematode died within two weeks after its host was collected, it caused swelling, infection and loss of sight to the infected eye. This individual would potentially have been vulnerable to predators and would likely not have survived long in nature.

Phylogenetic position of onchocercid n. gen. n. sp. 1 within the Onchocercidae

In the current phylogenetic analysis members of the Waltonellinae do not form a monophyletic clade, with *Ochotenerella* and *Foleyellides* represented by a polytomy and

separate from onchocercid n. gen. n. sp. 1 The lack of molecular data of filarial nematodes from cold-blooded vertebrates so far has prevented a detailed phylogenetic comparison of onchocercid n. gen. n. sp. 1 with the other genera and species within the Waltonellinae. Molecular data are only available for three species of *Ochoterenella* (18S rRNA, 28S rRNA, MyoHC, rbp1 and COI, 12S rDNA, hsp70) and one species of *Foleyellides* (COI). In the current study, a BI partitioned phylogenetic analysis was conducted, based on a concatenated dataset of 18S rRNA and COI gene sequences. Although these data revealed generic differences between onchocercid n. gen., *Foleyellides* and *Ochoterenella*, increased sampling of other species and genera from the Waltonellinae is required to obtain a more complete overview of the phylogenetic relationships of this subfamily. It would especially be interesting to compare other genera exclusively found parasitising African anurans, such as *Madochotera* and *Paramadochotera*, to see if these genera from a monophyly with onchocercid n. gen. or if the various genera of the Waltonellinae remain as paraphyletic taxa.

Host-vector and parasite relationships

Recently, a number of general studies on parasites of African anurans and particularly the guttural toad have been performed (Halajian et al., 2013; Kruger and Du Preez, 2015). The current study is the first record of an adult filariid nematode from an anuran in South Africa. Although in the current study filariid nematodes were only found in a single locality (Sodwana), it is highly probable that these parasites have a wider distribution, considering the distribution range of the their vertebrate hosts and invertebrate vectors (Ingram and De Meillon, 1927; Du Preez and Carruthers, 2017). It is also likely that the presence of these parasites is highly dependent on their mosquito vectors, specialised in feeding on these anurans. However, further investigations on the distribution, host specificity and ecology of these mosquito species are required in order to test these hypotheses.

In all previous life cycle studies of *Foleyellides*, commonly cultured species of mosquitoes of the genera *Aedes* and *Culex* were used, but these are doubtfully the natural vectors in the life cycle of these filarial nematodes (Causey, 1939a; Causey, 1939b; Witenberg and Gerichter, 1944). In the present study, two species of *Uranotaenia* Lynch Arribálzaga, 1891 mosquitoes were used for life history and transmission experiments. These mosquito species were selected based on *in situ* observations made of them feeding on *S. gutturalis* at high prevalences (see Fig. 7A-B). Furthermore, we noted that these mosquito species were particularly attracted to the calling male guttural toads. This observation is similar to other reports of species of *Uranotaenia* attracted to calling anurans, namely, *U. unguiculata* Edwards, 1913 from Europe (Camp et al., 2018), *U. lowii* Theobald,

1901 from Costa Rica (Borkent and Belton, 2006), and several species of *Uranotaenia* from Japan (Toma et al., 2014). Our observations were based on two experiments using approximately 64 *U.* (*Pfc*.) *mashonaensis* and 42 *U.* (*Pfc*.) *montana* as potential vectors and two highly parasitised male *S. gutturalis* as the definitive host. Unfortunately, the natural conditions could not be simulated *ex situ* and since attempts to culture these mosquito species failed (data not shown), we had to rely on the mosquitoes collected *in situ* to complete the life history experiments. Due to the limited number of mosquitoes collected, a sufficient sample size of individual mosquitoes could not be examined each dpi. As only an average estimation of the time of development can be given from the experiment in the current study, it is essential to consider that this may not truly reflect the timing of development *in situ*. Nonetheless, the data obtained from our experiment conforms to those from previously published experiments, in that first-stage sausage-shaped larvae were observed from the third dpi, and third-stage larvae from the 15th dpi. Although no larvae were observed in the Malpighian tubes, distant parts of intestine, or coxal cavities of the legs, a few third stage larvae were observed in the abdominal cavity of the mosquito host, with the majority occurring in the thorax or head, and even in the proboscis. It is also important to note for future experimental work that the mosquito species were highly sensitive to decreases in the level of humidity (all specimens kept in plastic containers without moist cotton wool as a source of water died within 4 hours) and temperature, which could also have affected the rate of development of the different larval stages.

In terms of the host-vector and parasite relationships, our observations showed strong evidence of the feeding preference of female *U. (Pfc.) mashonaensis* and *U. (Pfc.) montana* to vocalising male *S. gutturalis*. Playing calls of other anurans (*S. garmani*, *Sclerophrys poweri* (Hewitt, 1935), *Sch. carens*, *Leptopelis natalensis* (Smith, 1849), *Phrynobatrachus nataliensis* (Smith, 1849), and *P. anchietae*) found in this area was not as successful in attracting these mosquito species, with only the call of *S. garmani* working but not as effective as the call of *S. gutturalis*. Once a suitable individual is located, the mosquitoes seem to have the opportunity to take a blood meal with minimal interference from the calling toad. This could prevent large numbers of mosquitoes from being consumed by the toads (we observed toads consuming mosquitoes once calling bouts had ended). However, more data are required before these host-vector relationships can be explained with more certainty. No mosquitoes were observed feeding on female toads, and only male *S. gutturalis* were parasitised with onchocercid n. gen. n. sp. 1, with the exception of one female *S. garmani* (S1 Dataset, AE180124F1). The infection of onchocercid n. gen. n. sp. 1 in this female *S. garmani* seemed unlikely, since female toads do not vocalise, thus having less chance of attracting the mosquito vectors. The infection could have taken place when the female was mistaken for a calling male during breeding activity, or if other vectors not

attracted by the call are also able to transmit onchocercid n. gen. n. sp. 1 A similar situation was observed for *Trypanosoma tungarae* Bernal and Pinto 2016, parasitising túngara frogs and potentially being transmitted by eavesdropping frog-biting midges (Bernal and Pinto, 2016).

Furthermore, almost all the *U. (Pfc.) mashonaensis* and *U. (Pfc.) montana* specimens which fed on infected *S. gutturalis* contained developing larvae of onchocercid n. gen. n. sp. 1, some individuals surviving with intensities of more than 50 developing larvae after a single blood meal. However, the majority (n=8) of mosquitoes that fed on one of the highly parasitised *S. gutturalis* (S1 Dataset, AE180313A1) *in situ*, died one dpi, with only those individuals surviving that did not have sufficient blood meals. This indicates that there may be a threshold in the intensity of infection that an individual mosquito can survive. The high mortality rate of these individual mosquitoes so early post infection could indeed be due to the effect of the microfilaria, which penetrate the gut wall of their host. In the majority of the mosquitoes, larval development was chronological, with only one scenario where sausage-shaped larvae co-occurred with third-stage larvae in the body cavity at the same time. Also, in highly parasitised individuals, development seemed to be gradually prolonged, in that not all the individual larvae developed at the same rate, as compared to the development in mosquitoes with less intense infections. These findings could indicate how onchocercid n. gen. n. sp. 1, have specifically adapted to maximise the period of available third-stage infective larvae to be transmitted to a new host.

Perspectives

Although as mentioned above, third-stage larvae were found in the proboscis of some of the mosquitoes dissected between 14 and 18 dpi, transmission attempts to uninfected *S. gutturalis* hosts were unsuccessful due to mosquitoes not taking a second blood meal. This could be due to a number of factors, such as mosquitoes not being kept at optimal conditions. Likewise, since all mosquitoes were collected *in situ*, there was no knowledge on the number of previous blood meals taken, and specimens could even have been at the end of their life cycle. In addition, as all infected females were highly gravid, conditions may not have been optimal for them to release their egg clutches, reducing the need for an additional blood meal. Although the ultimate test for any potential vector is transmission to a new and uninfected host, in this case, this may only be possible *ex situ* using a large sample of laboratory cultured and reared mosquitoes under naturally-simulated conditions. Several additional questions remain to be answered, such as: are there any triggers that influence the intensity of microfilaria in blood, for example increased testosterone in the breeding season, a chemical reaction caused from the bite of a mosquito,

or even the time of day? Elevated levels of testosterone have been shown to increase parasite transmission potential, especially in male-biased host-parasite occurrences (Grear et al., 2009). The avian malarial parasite *Plasmodium relictum* (Grassi and Feletti, 1891) demonstrates increased parasitaemia in hosts exposed to feeding mosquitoes as compared to hosts not exposed (Cornet et al., 2014). This could be a result of chemical cues given off by the host in reaction to the bite or by the parasites ability to sense the density of mosquitoes feeding (Cornet et al., 2014). Obviously there are several other hormones that could have the same affect, for example species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) have been shown to synchronize their oocyst output with the nocturnally peaking hormone melatonin which coordinates the hosts circadian rhythm (Dolnik et al., 2011; Martinez-Bakker and Helm, 2015). Other heteroxenous parasites have also been shown to maintain or increase certain stages relative to the occurrence or rhythms of their vectors (Martinez-Bakker and Helm, 2015). Microfilaria of *Wuchereria bancrofti* (Cobbold, 1877) are known to alter in intensity based on the activity of the vectors and the time of day (Reece et al., 2017). These scenarios could be the same for anuran fillariids and their host species.

Concluding remarks

The present study contributes to the limited knowledge on the biodiversity, distribution, evolutionary, and ecological data of this group of neglected anuran parasites. Due to the lack of data on these parasites, the application of molecular methods, as well as life cycle elucidation through natural intermediate hosts, are necessary to gain better knowledge of their phylogenetic relationships, the ecology of these parasites *in situ*, and to be able to link different

life stages from various hosts to a particular species. Furthermore, molecular tools are valuable in identifying species, in large scale screening of hosts and possible vectors. Genetic data could shed more light on the history of the interactions between these onchocercids and their amphibian hosts. To obtain our data for this study only a few selected amphibian individuals were sacrificed to yield a maximum number of data. Furthermore, our study provides a template for a full taxonomical account, which includes both morphological and molecular data, as well as an approach to elucidate the life history of anuran filarial nematodes such as onchocercid n. gen. n. sp. 1.

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CHAPTER 5

Positive parasitology: spatial heterogeneity in parasite community composition suggests frog blood parasites as potential indicators of environmental health

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Abstract

Effective conservation efforts require sufficient information on the ecosystem to prevent erroneous or misguided policies. Organisms used as indicators for ecosystem health may provide several advantages over traditional, physicochemical indicators, e.g. dissolved oxygen and nutrient content. Recently, the use of parasites as effect indicator species and their interactions with established bioindicators, e.g. fish and frogs, have become a promising research area. The primary goal of this study was to explore whether frog blood parasites comply with some criteria of bioindicators. Blood parasites infecting grass frogs (*Ptychadena* spp.) from the Phongolo River system in South Africa were used as a case study. Mean intensity of infection, mean abundance of infection, prevalence, and richness of three genera, *Dactylosoma* Labbé 1894*, Trypanosoma* Gruby, 1843 and *Hepatozoon* Miller, 1908, were compared between frogs from anthropogenically-impacted versus less-impacted sites, and dry versus wet season. Significant differences were found in combined parasite prevalence, prevalence of *Dactylosoma* sp., and in richness between seasons; and between locations in combined parasite prevalence, parasite richness, prevalence and mean abundance of an unknown *Dactylosoma* sp., and mean abundance and intensity of multiple *Trypanosoma* spp. In general, frogs from heavier impacted sites harboured more blood parasites than from less-impacted sites. Based on these results, we argue that the blood parasites from our study adhere to several criteria of what is considered a good indicator. We argue that blood parasites hold potential as indicators for healthy ecosystems and intact food webs.

Keywords: Protozoa, blood parasites, haemogregarines, trypanosomes, parasite ecology

Introduction

Bioindicators are taxa that have a response to environmental changes and are used to evaluate the quality of the environment (Holt and Miller, 2010; Russell and Downs, 2012). There are several advantages of using indicator species. First of all, while tradition physicochemical indicators, such as nutrient content, dissolved oxygen, pH, salinity and temperature can give a broad indication of the general ecosystem health (Markert et al., 1999; Maila and Cloete, 2005), they provide no direct information on the trophic and biotic relationships between the organisms in the ecosystem. Hence, bioindicators have the potential to be more representative predictors of the impact of anthropogenic disturbances to ecosystem than traditional physicochemical indicators. The latter, according to Holt and Miller (2010) and Payne (2013) provide information on what is polluting an ecosystem as opposed to how it affects the ecosystem. For example, high phosphorous content can indicate pollution in a riverine system but does not provide comprehensive information on how this affects the biotic communities that reside in this ecosystem. Secondly, bioindicators are integrative over time, meaning that lifespan or residence time of an organism account for temporal variation (Holt and Miller, 2010). Finally, organisms that biomagnify pollutants are highly sensitive bioindicators and can indicate pollution before high concentrations are detected by chemical methods (Markert et al., 1999).

Parasitism is estimated to be the most common life strategy and is thus an essential link in the food web (Cizauskas et al., 2017). Moreover, a diverse community of parasites is actually part of a well-functioning ecosystem (Hudson et al., 2006). Parasites are dependent on their hosts for transmission and survival. For this reason disruption of ecosystem stability will indubitably impact the complex biotic interactions in which parasites are involved. Endoparasites, such as cestodes, are often faced with high concentrations of toxic substances that have accumulated in their host (Morris et al., 2016). Thus, endoparasite taxa that can accumulate toxins have the potential to be sensitive bioindicators that show signs of premature environmental disturbance (Sures et al., 2017). Furthermore, endoparasites do not solely show point disturbances, but are more integrative through time, with a better potential for their use as long-term indicators. For these reasons, endoparasites have been subjects of interest for bioindication, making use of infection intensity, infection abundance, prevalence, or diversity as endpoints of anthropogenic disturbance (Sures, 2001, 2003; Marcogliese, 2005; Koprivnikar et al., 2012; Morris et al., 2016; Brunner et al., 2017; Sures et al., 2017).

Often, blood parasites are heteroxenous (multi-host life-cycle) and can infect hosts in several trophic positions ranging from primary consumers, such as tortoises and cattle

(Cook et al., 2014; Leta et al., 2016; O'Donoghue, 2017), to top predators, such as crocodiles, carnivores, and birds of prey (Fermino et al., 2013; Valkiūnas et al., 2016; O'Donoghue, 2017; Penzhorn et al., 2018). Additionally, the life cycle of most blood parasites includes a vertebrate (e.g. amphibians, reptiles and mammals) and invertebrate host (e.g. leeches and dipterans), as well as both aquatic (e.g. amphibians, fishes, and leeches) and terrestrial hosts (e.g. reptiles and mammals) (Barta, 1991; Smith, 1996; Hayes et al., 2006; Hayes et al., 2014).

Anurans are often key links between aquatic and terrestrial environments serving as intermediate and definitive hosts for parasites. They form an essential element of the food web, and their permeable skin makes them prone to the bioaccumulation of persistent pollutants (Korzh and Zadorozhnyaya, 2013; Wolmarans et al., 2018). However, in a recent case study looking at organochlorine pesticides in aquatic frogs, results indicated that although accumulation did occur it was at sub-lethal levels (Wolmarans et al., 2018). Such findings may require a need to look for other or more indicator taxa that are sensitive to pesticides like dichlorodiphenyltrichloroethane (DDT).

Four species of African grass frogs belonging to *Ptychadena* Boulenger, 1917 (Ptychadenidae) (*P. anchietae* (Bocage, 1868), *P. mossambica* (Peters, 1854), *P. nilotica* (Seetzen, 1855) and *P. oxyrhynchus* (Smith, 1849)) are present in the lower Phongolo River floodplain, KwaZulu-Natal, South Africa. Species of *Ptychadena* are common in savannah bushveld, require a moist habitat near water and have a diet that consists of invertebrates and small amphibians. They are suitable model hosts in environmental parasitology, first of all, because they occur in the proximity of the invertebrate vectors of the blood parasites, namely dipterans and leeches. Secondly, species of *Ptychadena* are successful in various habitats, which could explain the variety of parasites that infect and have co-evolved with these frogs (Du Preez and Kok, 1992; Minter et al., 2004; Du Preez and Carruthers, 2009). Thirdly, it appears that species of *Ptychadena* are susceptible to various blood parasite loads and have the ability to cope with these infections (Du Preez and Kok, 1992; Smith, 1996). Netherlands et al. (2015) found species of *Ptychadena* hosted multiple blood parasite taxa, with *Ptychadena anchietae* exhibiting a high prevalence of *Trypanosoma* spp., a *Dactylosoma* sp. and a *Hepatozoon* sp. Species of *Hepatozoon* and *Dactylosoma* are apicomplexan, intracellular parasites infecting primarily erythrocytes. The life cycles of species of *Dactylosoma* and *Hepatozoon* are comparable, with sexual sporogonic replication occurring in an invertebrate vector, followed by asexual, merogonic replication in the vertebrate host (Barta, 1991; Smith, 1996). *Trypanosoma* spp. are Euglenozoa, extracellular blood parasites with the life cycle of several species infecting anurans already elucidated (Feng and Chung, 1940; Anderson and Ayala, 1968; Martin and Desser, 1991). Their life cycle involves primarily asexual reproduction by multinucleate fission in a dipteran or leech

vector. The subsequent blood meal releases the infective trypomastigote stage into the body fluids and organs of the frog host.

Studying these heteroxenous blood parasites may be advantageous, as they are sensitive to specific ecosystem changes, such as a disrupted food chain, and thus accurately signal a disturbance in the ecosystem (Cadrin et al., 2005). Therefore, the present study assessed the potential of blood parasites in frogs as indicators of ecosystem health by comparing attributes such as diversity, abundance, prevalence, and effect on host between impacted and non-impacted regions.

For this, we test some of the criteria of good bioindicators, using the blood parasites of the grass frogs from the lower Phongolo River floodplain as a case study. These criteria included: being abundantly and consistently present over time (Schmidt et al., 2003; Williams and MacKenzie, 2003; Cadrin et al., 2005; Russell and Downs, 2012), not being detrimental to the host (Bahri et al., 2002; Cadrin et al., 2005), and that the change in their presence or abundance is unequivocally attributed to the environmental change (Russell and Downs, 2012). Additionally, we evaluated the use of multiple species as recommended by Holt and Miller (2010). Our hypotheses for this study were that frog blood parasite infection intensity, infection abundance, diversity, and prevalence is consistent across seasons, and that the parasites do not cause mortality to the host. For direct life cycle parasites, where we expect higher levels of parasitism in impacted areas as a result of impaired immune systems. In complex life cycle parasites, on the other hand, like the blood parasites in the present study, parasite transmission depends on the abundance of vector. Hence, we hypothesised that frog blood parasite infection intensity, infection abundance, diversity, and prevalence will be higher in less-impacted sites as compared to more-impacted sites given the host's, vector's and parasite's dependency on an integral ecosystem, as indicated by Netherlands et al. (2015). Hence, this would make frog blood parasites a suitable indicator for ecosystem health by signalling a robust food web.

Materials & Methods

Study site and sampling

Sampling was conducted twice at several locations along the Phongolo River (Fig. 1), in northern KwaZulu-Natal. The Phongolo River and associated floodplain are of considerable socio-economic importance for the residents of this region because water is one of the necessities for agriculture (Dube et al., 2015). The Phongolo River floodplain comprises the area from the Pongolapoort Dam to the border with Mozambique (Dube et al., 2015). In this

study area, rainfall is considerably higher in what is referred to as the wet season, running from November to April, while May to October is considered the dry season (Dube et al., 2015). Sampling occurred during the southern hemisphere dry winter (September 2016) and wet summer (February 2017) months. Smit et al. (2016) conducted an integrated regional scale risk assessment of physical and chemical stressors on the Lower Phongolo River and floodplain, to evaluate threats to the ecosystem and define risk regions within the area. Sampling locations were selected according to the risk regions defined by Smit et al. (2016), based on the human impacts and population numbers of wards associated with the river and floodplain pans (Fig. 1). The risk regions include all riverine and floodplain pool ecosystems associated with the Phongolo River. According to Smit et al. (2016), Risk Region 1 (RR1), was classified as a higher impacted area compared to Risk Region 2 (RR2), and both RR1 and RR2 are higher impacted areas as compared to Risk Region 3 (RR3). The site 'Dam' is located in RR 1, below the Pongolopoort Dam wall in Jozini, a densely populated town. The site 'PRC' is also located in RR1 and is surrounded by rural villages, industrial and subsistence farmland, with water from the river used to irrigate crops (Table 1; Fig. 1). The sites 'Walkway' and 'Bridge' are located in RR2 (Table 1; Fig. 1), and are surrounded by a small rural town and subsistence farmland. The site 'Bridge' has an artificial barrier of boulders blocking the flow of the river, creating well vegetated stagnant pools. The furthest downstream site "Ndumo Game Reserve" (NGR) is located in RR3 (Table 1; Fig. 1), a protected area in the northern part of the Phongolo River floodplain: the NGR, a 10,117 ha (Netherlands et al., 2015) reserve of high biodiversity with over 10,000 ha of wetland (Dube et al., 2015). Frogs were collected at night by hand and identified to species level using the keys and descriptions in Du Preez and Carruthers (2009). Frogs were held in plastic containers and transported back to a field workstation where the individuals were weighed, measured and sexed. Sterile insulin syringes (1 ml) were used to draw blood followed by staining following Netherlands et al. (2015).

Table 1: Anthropogenic disturbances at the sampling sites.

Site name	Anthropogenic disturbance	Risk region
NGR	None direct (only indirect influence of upstream sources)	RR3
Walkway	Subsistence agriculture (pesticides and fertilisers) and rural town	RR2
Bridge	Rural town	RR2
PRC	Industrial agriculture (pesticides and fertilisers)	RR1
Dam	Urban pollution (i.e. waste disposal and dam constructions)	RR1

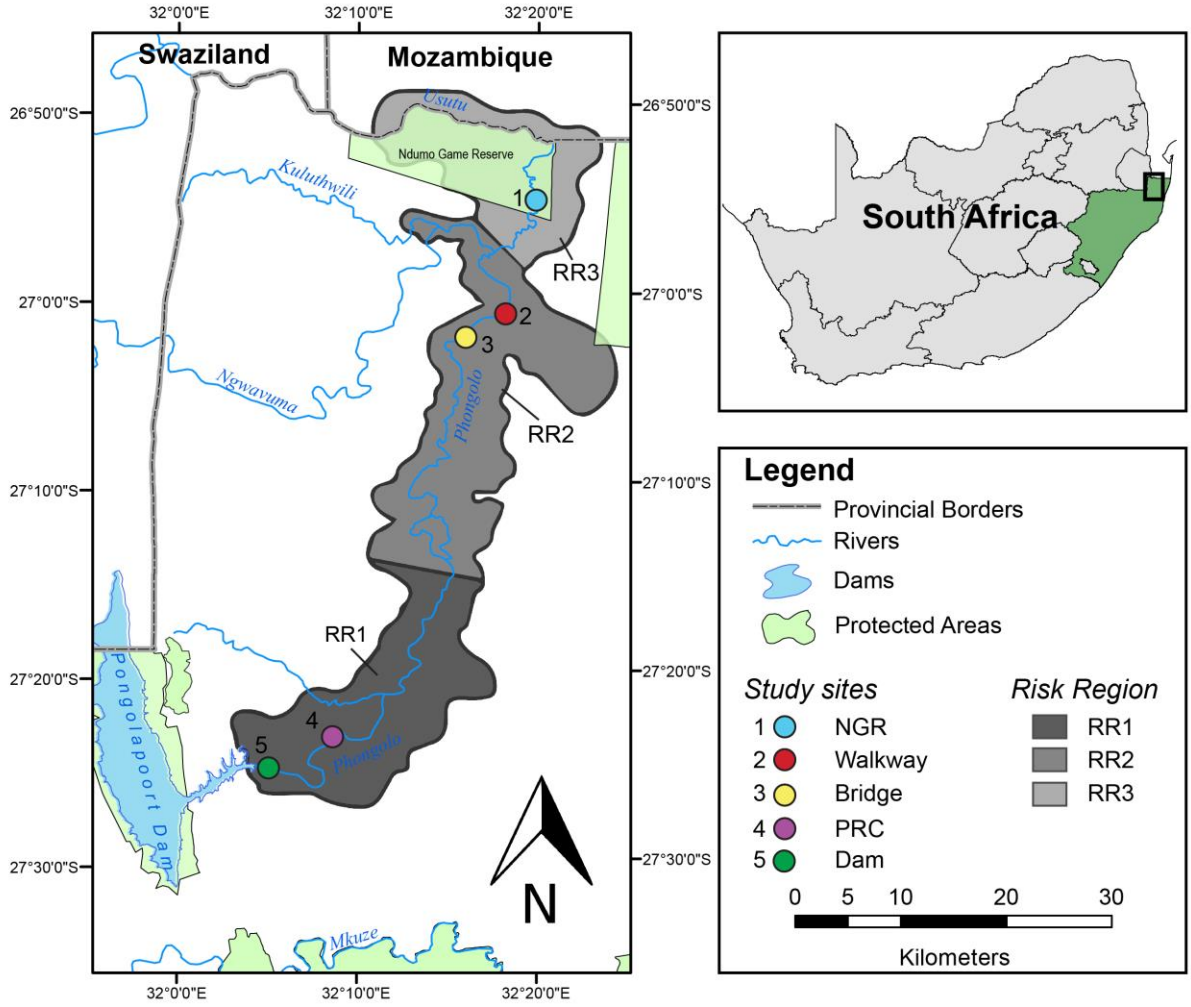

Fig. 1. Map of the study area, including the five sampling sites along the Phongolo River. The map also shows the spatial scope of Risk Regions 1, 2 and 3 adapted from Smit et al., (2016) in a risk assessment of the use of the Phongolo floodplain water resources.

All frogs were released in their original capture location following processing. Three species of *Ptychadena* were sampled: *P. anchietae*, *P. mossambica* and *P. nilotica* (syn. *P. mascareniensis* Duméril & Bibron, 1841). Fulton's index (K) was used as a Body Condition Index (BCI) as a proxy for fat reserves, to assess the health of individuals (Stevenson and Woods Jr, 2006). Fulton's index is calculated as $K = m/(LxW^2)$, with m = mass of the frog, L = length of the frog and W = width of the frog (Stevenson and Woods Jr, 2006; Băncilă et al., 2010).

Microscopical identification and quantification of parasites

Stained blood smears were microscopically screened and blood parasites were identified to genus level based on morphology. Subsequently, the prevalence of the individual parasite

genera was calculated following Cook et al. (2015). Additionally, the presence of any of the parasites was used to determine an “combined prevalence”-parameter, indicating whether at least one type of parasite was present. Species richness was also determined, indicating whether none, one, two or all of the parasite genera were present in an individual. Parasitaemia adapted from Cook et al. (2015) was used to quantify the mean intensity and mean abundance of infection. Parasitaemia was calculated after scanning 40 visual fields of approximately 100 erythrocytes for infections of intracellular parasites (or occurrences of extracellular parasites), resulting in a parasitaemia estimate per ± 4,000 erythrocytes. Mean abundance of infection is calculated as an average of parasitaemia across all hosts, whereas the mean intensity of infection excludes the data from the frogs without infection.

Data analysis

All prevalence data were analysed with a generalised linear model under a binomial error distribution assumption using RStudio (version 1.0.44). Mean abundance, mean intensity and richness were analysed with a generalised linear model under a Poisson error distribution assumption for counts. Host species, sampling site, sampling season, host sex and host BCI were included as fixed factors in the model. The primary model included an interaction effect between season and site, as well as between BCI and sex to incorporate the fact that female frogs are usually larger than males. An exception was made when analysing mean intensity: some sites had too few infected individuals for analysis of mean intensity. Hence, the primary model for mean intensity included sampling season, host sex and host BCI, with an interaction effect between sex and BCI, but not site. Therefore, the factor site was analysed separately via the non-parametric Kruskal-Wallis test using the function kruskal.test() and not admitted in the Poisson model. In the models where the host species was a significant factor, hosts belonging to *P. mossambica* (N=15) and *P. nilotica* (N=5) were omitted from the dataset. This isolation allowed us to assess the influences of the other factors on the parasite prevalence, abundance, intensity and diversity for one host (*P. anchietae*, N=89). The primary model was selected step-wise based on the Akaike Information Criterion (AIC), using the function stepAIC() from the *MASS* (Ripley et al., 2017) package. The model with the lowest AIC score was retained.

A type three between-groups multiway analysis of variance (ANOVA) was performed using the Anova() function from the *car* (Fox et al., 2016) package. For the models with binomial error distributions, overdispersion was assessed using the quasibinomial model: quasibinomial models with an overdispersion parameter ≤ 1 were dismissed, and the binomial model was chosen instead. For the models with Poisson error distributions, overdispersion was assessed using the quasipoisson model: quasipoisson models with an

overdispersion parameter ≤ 1 were dismissed, and the Poisson model was chosen instead. Tukey post-hoc tests compared sites in a pairwise order. In the absence of an interaction effect between season and site, the glht() function from the *multcomp* (Hothorn et al., 2016) package was used. In the presence of an interaction effect, the lsmeans() function from the *lsmeans* (Lenth, 2017) package was used.

A permutational multivariate analysis of variance (PERMANOVA) was performed on presence-absence and parasitaemia data of the parasite communities within the host *Ptychadena anchietae*, using the function adonis() from the *vegan* (Oksanen et al., 2017) package with 10,000 permutations. A PERMANOVA allows determining which factors significantly shape the parasite community. Jaccard and Bray-Curtis distances were used to calculate dissimilarity matrices for presence-absence and parasitaemia data. Factors included in both models consisted of the sampling site, sampling season, host sex, host BCI, and an interaction effect between sex and BCI. Analysis of similarities (ANOSIM) was also performed on the same data using the anosim() function from the *vegan* (Oksanen et al., 2017) package, with a Jaccard dissimilarity matrix for presence-absence data and a Bray-Curtis dissimilarity matrix for parasitaemia data. The ANOSIM allows checking which factors significantly shape similarity patterns in the parasite community.

Results

Frogs hosted *Trypanosoma* spp., a *Dactylosoma* sp. 1, and a *Hepatozoon* sp. 1 (Fig. 2). The combined parasite prevalence was 52% with a prevalence of 34%, 34% and 27% for *Trypanosoma* spp., *Dactylosoma* sp., and *Hepatozoon* sp. 1 respectively (see Table 2). The Body Condition Index (BCI) and sex of the host and the interaction between both had no significant effect on any of the infection parameters considered (Table 3). Host species had a significant effect on combined parasite prevalence, richness and *Dactylosoma* sp. 1 prevalence (Table 4). For these three infection parameters, only data for the most commonly sampled species, *P. anchietae*, was retained to correctly assess influences of the other factors on the parasite prevalence, abundance, intensity and diversity. Tukey post-hoc tests demonstrated that *P. anchietae* had significantly higher prevalence and richness than *P. mossambica* and *P. nilotica*.

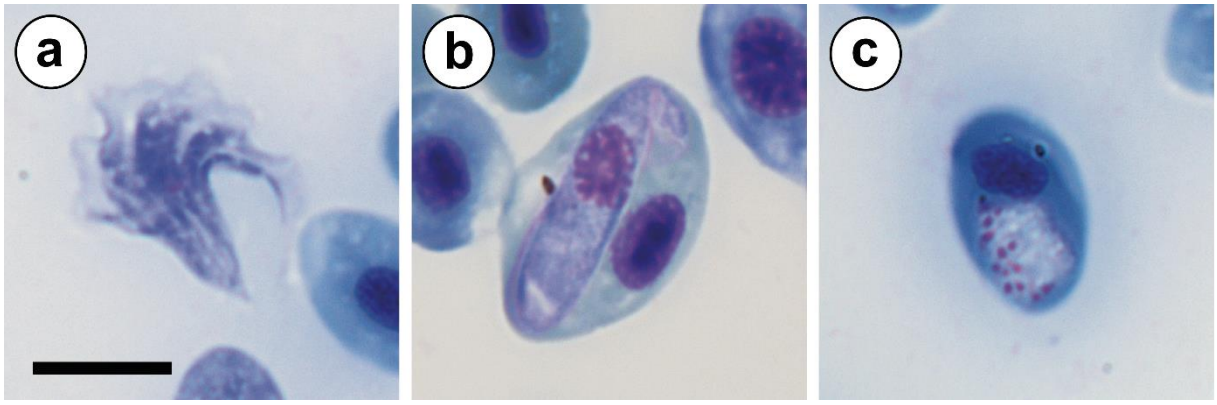

Fig. 2. Light micrographs of the three most common blood parasites found in frogs during the study. (a) The extracellular *Trypanosoma* spp. (b) Intracellular *Hepatozoon* sp. 1 and (c) intracellular *Dactylosoma* sp. 1 Scale bar: 10µm.

Table 2: Table showing the combined, total and seasonal parasite mean prevalence (%) and standard error (SE).

	Combined		*Trypanosoma* spp.		*Dactylosoma* sp. 1		*Hepatozoon* sp. 1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total	52	5	34	5	34	46	27	4
Dry season	64	7	43	7	40	7	34	6
Wet season	40	7	25	6	28	6	19	5

Table 3: Fixed effects of generalised linear models for representatives of *Dactylosoma*, *Hepatozoon* and *Trypanosoma*, and combined blood parasite prevalence and richness. The prevalence models assume a binomial error distribution. The richness, abundance and intensity models assume a Poisson error distribution. P-values in bold are significant at $\alpha \leq 0.05$. Asterisks mark the fact that site was not included in the intensity model, but was done via a Kruskal-Wallis test. Slashes mark the exclusion of the factor from the model: species of the host could not be included when working with a dataset that includes only one host, and a site-season interaction effect could not be included when the factor site was not included in the model. Factors between brackets were excluded from the final model, based on the AIC score. Df = degrees of freedom; X^2 = likelihood-ratio chi-squared test statistic; P = p-value.

***DACTYLOSOMA* SP. 1**

		Prevalence		Abundance		Intensity	
Factor	**Df**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**
Species	2	/	/	1.5329	0.4647	/	/
Site	3/4/3	30.785	**< 0.0001**	22.6175	**0.0002**	3.6005*	0.4628*

Table 3. Continued.

Season	1	5.9503	**0.0147**	0.8994	0.3429	0.0123	0.9117
Site:season	2/3/.	(3.144)	(0.3699)	(6.9641)	(0.1378)	/	/
Sex	1	(0.079)	(0.7789)	0.3605	0.5482	0.3613	0.5478
BCI	1	(0.305)	(0.5808)	(0.0347)	(0.8523)	0.1351	0.7132
Sex:BCI	1	(0.051)	(0.8219)	(0.0443)	(0.8333)	0.0984	0.7538

***HEPATOZOON* SP. 1**

		Prevalence		Abundance		Intensity	
Factor	**Df**	**X²**	**P**	**X²**	**P**	**X²**	**P**
Species	2	5.8916	*0.0526*	1.3839	0.5006	/	/
Site	4/4/3	7.1509	0.1281	6.9587	0.1381	4.5135*	0.3409*
Season	1	0.0000	1.0000	0.0000	0.9999	0.5239	0.4692
Site:season	3/3/.	15.3849	**0.0040**	6.4801	0.1661	/	/
Sex	1	(0.9561)	(0.3281)	2.7544	*0.0970*	0.9702	0.3246
BCI	1	3.5897	*0.0581*	0.0847	0.7710	0.6006	0.4383
Sex:BCI	1	(0.9229)	(0.3367)	2.3872	0.1223	0.8451	0.3579

***TRYPANOSOMA* SPP.**

		Prevalence		Abundance		Intensity	
Factor	**Df**	**X²**	**P**	**X²**	**P**	**X²**	**P**
Species	2	3.0974	0.2125	3.6425	0.1618	/	/
Site	4/4/3	21.848	**0.0002**	29.6817	**< 0.0001**	11.129*	**0.0252***
Season	1	0.0000	0.9999	0.0000	1.0000	1.5449	0.2139
Site:season	3/3/.	16.725	**0.0022**	9.2799	*0.0545*	/	/
Sex	1	(0.6720)	(0.4124)	(0.0654)	(0.7982)	1.7805	0.1821
BCI	1	(1.8314)	(0.1760)	(0.0347)	(0.8523)	0.0023	0.9617
Sex:BCI	1	(0.8272)	(0.3631)	(0.0443)	(0.8333)	1.7824	0.1819

COMBINED

		Prevalence		Richness	
Factor	**Df**	**X²**	**P**	**X²**	**P**
Species	2	/	/	/	/
Site	3	36.291	**< 0.0001**	36.119	**< 0.0001**
Season	1	27.352	**< 0.0001**	19.878	**< 0.0001**
Site:season	3	(0.6444)	(0.8862)	16.530	**0.0009**
Sex	1	(0.2033)	(0.6521)	(0.222)	(0.6372)
BCI	1	(0.0524)	(0.8189)	(0.044)	(0.8336)
Sex:BCI	1	(0.2166)	(0.6416)	(0.270)	(0.6032)

Season had a significant effect on the same three infection parameters as the host species: combined prevalence, richness and *Dactylosoma* sp. 1 prevalence (Table 4). Tukey post-hoc tests demonstrated a higher combined prevalence and richness in the dry season than in the wet season. Site had a significant effect on most of the parameters (7 out of 11), except for *Hepatozoon* sp. 1 prevalence, abundance and intensity, and for *Dactylosoma* sp. 1 mean intensity (Table 4). Furthermore, there was only a single effect significant for *Hepatozoon* sp. 1 infections, namely a site-season interaction effect, significantly influencing *Hepatozoon* sp. 1 prevalence (Table 3). Tukey post-hoc tests revealed that two sites, 'Bridge' (in RR2) and 'NGR' (in RR3), were generally characterised by higher richness, prevalence, mean abundance and mean intensity of infection (Fig. 3 and Supplementary Material 1-5). Season and site had a significant interaction effect on richness, *Hepatozoon* sp. 1 prevalence and *Trypanosoma* spp. prevalence (Table 3). The permutational multivariate analysis of variance (PERMANOVA) showed that for both parasite community measures (presence-absence & parasitaemia), site had a significant effect (Table 5). This was confirmed with an analysis of similarities (ANOSIM) of site: (1) presence: R statistic = 0.1671 with a p-value of 0.001 and (2) parasitaemia: R statistic = 0.2542 with a p-value of 0.001.

Table 4: Significant effects for each of the infection parameters (response variable). For the first three response variables (combined parasite prevalence, richness and *Dactylosoma* sp. 1 prevalence), the data used for this table is based on two models. If the host species was significant, then the significance marks for the other factors (site, season, site:season) were derived from the model where the host species was excluded (only data from *P. anchietae* as the host was included). Factors BCI, sex and the interaction effect BCI:sex were not significant in any of the models. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. Dac = *Dactylosoma*; Hep = *Hepatozoon*; Tryp = *Trypanosoma.*

Response variable	Host species	Site	Season	Site: season
Combined prevalence	****	****	****	
Richness	*	****	****	****
Dac prevalence	*	****	*	
Dac abundance		***		
Dac intensity				
Hep prevalence				**
Hep abundance				
Hep intensity				
Tryp prevalence		***		**
Tryp abundance		****		
Tryp intensity		*		

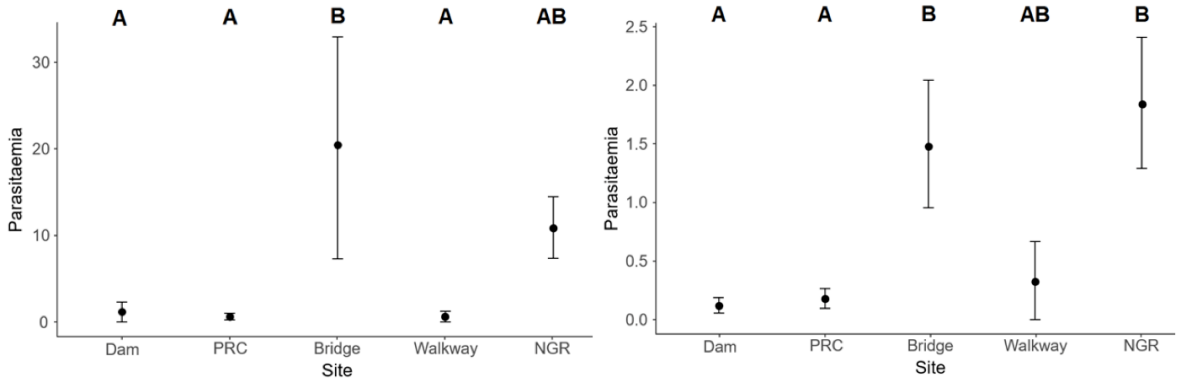

Fig. 3. *Dactylosoma* sp. 1 mean abundance (left) and *Trypanosoma* spp. mean abundance (right), given as parasitaemia per 4,000 erythrocytes, in function of site. Whiskers represent the standard error of the mean. Host sample sizes: Dam n = 25, PRC n = 22, Bridge n = 18, Walkway n = 24, NGR n = 20. Sites with different letters assigned to them are significantly different: the sites marked with an A are significantly different from those marked with a B. Sites marked with an AB are not significantly different from sites marked with either an A or AB.

Table 5: PERMANOVA results on Jaccard or Bray-Curtis distances between parasite communities in *P. anchietae* for presence-absence data or parasitaemia data, respectively. P-values in bold are significant at $\alpha \leq 0.05$. P-values in italics are significant at $\alpha \leq 0.1$. Num Df = numerator degrees of freedom; Den Df = denominator degrees of freedom; SS = sum of squares; MS = mean squares; F = F-statistic; P = p-value.

Community	Factor	Num Df	Den Df	SS	MS	F	R^2	P
Presence	Site	3	53	1.5248	0.5083	2.9584	0.1518	**0.0129**
	Season	1	53	0.0222	0.0222	0.1290	0.0022	0.8536
	Sex	1	53	0.0193	0.0193	0.1225	0.0019	0.8635
	BCI	1	53	0.2404	0.2404	1.3993	0.0239	0.2651
	Sex:BCI	1	53	0.3355	0.3355	1.9530	0.0334	0.1550
Parasitaemia	Site	3	49	2.8390	0.9463	3.4005	0.1771	**0.0002**
	Season	1	49	0.5754	0.5754	2.0677	0.0359	*0.0687*
	Sex	1	49	0.2481	0.2481	0.8915	0.0155	0.4749
	BCI	1	49	0.2825	0.2825	1.0150	0.0177	0.4067
	Sex:BCI	1	49	0.3988	0.3988	1.4332	0.0249	0.1996

Discussion

Our study assessed whether a change in presence or abundance of blood parasites in frogs is related to environmental factors, a precondition to the use of the blood parasites as indicators of ecosystem health. For a parasite to be an optimal bioindicator, it should fulfil specific criteria. The parasite should (1) be abundantly and consistently present over time (Schmidt et al., 2003; Williams and MacKenzie, 2003; Cadrin et al., 2005; Russell and Downs, 2012), (2) be easy and non-lethal to sample (Bahri et al., 2002; Schmidt et al., 2003; Cadrin et al., 2005; Russell and Downs, 2012), (3) not be an opportunistic species, (4) not be detrimental to the host (Bahri et al., 2002; Cadrin et al., 2005), (5) allow for change in its presence or abundance to be unequivocally attributed to the environmental change (Russell and Downs, 2012), (6) be sampled in a well-known study area (Williams and MacKenzie, 2003). Also, (7) the ecology of the organism should be well known (Schmidt et al., 2003; Williams and MacKenzie, 2003), as well as (8) the pollutants and their effects on the host (Schmidt et al., 2003; Williams and MacKenzie, 2003). Furthermore, the use of multiple species is recommended (Holt and Miller, 2010). In the current study, the following criteria for a good parasite bioindicator were tested: 1, 4 and 5, as well as the use of multiple species, and criteria 1, 2, and 4–6, and use of multiple species were considered fulfilled (Bahri et al., 2002; Schmidt et al., 2003; Williams and MacKenzie, 2003; Cadrin et al., 2005; Russell and Downs, 2012).

Interaction between the parasite and host species

Species had a significant effect on richness, combined parasite prevalence and prevalence of *Dactylosoma* sp. 1 (Table 4). While not significant in all cases, the pairwise comparisons showed that *P. anchietae* to harbour parasites more often than the other two frog species. Furthermore, *P. anchietae* carries a variety of parasites (both intracellular and extracellular), lives close to a variety of vectors and is susceptible to various parasite loads without being seemingly impacted (Du Preez and Kok, 1992). Hence we suggest this species is a good model host for ecological parasitology.

Influence of season on parasitism

Richness, combined parasite and *Dactylosoma* sp. 1 prevalence was significantly different between season, with higher parasite prevalence in the dry season than the wet season (Table 4). This is slightly counter-intuitive since rainfall is expected to increase suitable habitat for the parasite vectors (leeches, mosquitoes and putatively other hematophagous invertebrates), namely calm water bodies such as pools or stagnant riverine habitats.

However, out of the ordinary, KZN, and South Africa in general, have been experiencing severe droughts since 2015. The year 2015 marked the worst drought in South Africa in 30 years, with the lowest annual rainfall since recording (Strydom and Savage, 2018). Because of the irregular seasonal rainfall, the results are not representative of typical seasonal dynamics. Hence, we will not further focus on our findings concerning seasonal effects on infection parameters.

Spatial heterogeneity of parasitism

The ANOVA, the PERMANOVA and ANOSIM results show spatial heterogeneity for the richness, prevalence, mean intensity and mean abundance of the assessed parasites (Tables 1 and 4). The PERMANOVA showed that presence and abundance of the parasite in the parasite community differed significantly between sites. Similarly, the ANOSIM indicates that the similarity patterns in the parasite communities differed significantly across sites. Both multivariate analyses demonstrate the potential value of the use of multiple species of parasites as indicators of ecosystem health. According to the ANOVA results, frogs from two sites, ‘NGR' and ‘Bridge', carried consistently more parasites than frogs from the other sites. The ‘NGR' (in RR3) is not subject to direct pollution, although indirect pollution from upstream sites or surrounding sites cannot be excluded, whereas ‘Bridge' (in RR2) was subject to pollution from a rural settlement adjacent to the site (Fig. 1). The site ‘Bridge' also has been modified through the artificial damming of the main flow of the river. This barrier has created stagnant and vegetated habitat suited for the frogs and potential hematophagous invertebrate vectors. Hence, these tests indicate that the spatial heterogeneity could be associated with the degree of anthropogenic disturbance and suggest a potential use of infection parameters of *Dactylosoma* sp., *Trypanosoma* spp., or the blood parasite community as a whole as indicators for anthropogenic stress. Frogs from sites associated with less human disturbance have significantly higher combined parasite prevalence, parasite richness, *Dactylosoma* sp. 1 prevalence and mean abundance of infection, and *Trypanosoma* spp. prevalence, mean abundance and mean intensity of infection. The association between anthropogenic disturbance and parasite diversity and prevalence is consistent with ample other evidence. An inverse relationship between richness or diversity, and the anthropogenic burden has been reported by Sures et al. (2017), showing an increase in helminth diversity in less impacted sites. Netherlands et al. (2015) reported a similar pattern for blood parasites in frogs. A decrease in parasite species richness in the Pumpkinseed, *Lepomis gibbosus* (Linnaeus, 1758; Perciformes) was associated with more disturbed streams (Chapman et al., 2015). Trematode diversity and prevalence were found to inversely correlate with the degree of anthropogenic impact (Shea

et al., 2012; Sures et al., 2017). Moreover, larval trematode diversity in snails belonging to *Physa* Draparnaud, 1801 and *Stagnicola* Jeffreys, 1830 was lower in sites with higher agricultural impact (Shea et al., 2012). Similarly, trematode prevalence in both an intermediate snail host and definitive fish hosts was reduced in polluted sites (Siddall et al., 1993; Valtonen et al., 1997).

The North-Eastern region of KwaZulu-Natal, South Africa is an endemic malaria region where DDT is still used for mosquito vector control (Dube et al., 2015). This is evident in the Ndumo region from recent DDT exposure in domestic poultry (Thompson et al., 2017), terrestrial wildlife (Yohannes et al., 2017) and aquatic organisms such as fish (McHugh et al., 2011; Wepener et al., 2012) and frogs (Smit et al., 2016; Wolmarans et al., 2018). These authors also demonstrated high levels of other persistent organochlorine pesticides (OCPs) in the organisms under investigation. Some OCPs such as DDT are known to impair the immune system in mice (Fagotti et al., 2005). In frogs, although, DDT has been found to affect the endocrinal system (Boyd et al., 1963; Hayes et al., 2010; Viljoen et al., 2016; Trollip, 2017), in a recent study DDT accumulation were found to have sub-lethal responses (Wolmarans et al., 2018). Regardless, studies on the effects of OCPs on the immune system of frogs are lacking. Thus, while it is possible that a potentially compromised immune system may affect intensity of infection, there is no evidence available to support this hypothesis. However, the effects of DDT on potential vectors, such as dipterans (mosquitos and sand flies), despite them not being the intended targets of the application, may explain why frogs from impacted areas were found to carry lower infection intensities compared to non-impacted sites. Individuals with higher parasite prevalence and infection intensities may have been exposed to higher numbers of infected vectors, which continually infect or re-infect the frogs in these non-impacted areas. Indeed, for these complex life cycle parasites, high levels of parasitism imply a stable trophic system with a supposedly health population of vectors and hosts required for the completion of its life cycle. This is reflected in the fact that the infected frogs from Dam (RR1), PRC (RR1) and Walkway (RR2) are parasitised less intensely (Fig. 1). This would be unexpected in direct life cycle parasites, where pollutants result in higher levels of parasitism as a consequence of a suppressed immune system. Studies on the relationship between parasitism and eutrophication – also a consequence of agricultural pollution – show that the response of parasites to eutrophication is generally contrasting to the response of parasites to pesticides (Brunner et al., 2017; Sures et al., 2017). Our results are concurrent with the response of parasites to pesticides, although our study did not include measurements of pesticides nor nutrients in the assessment.

The potential use as indicators of ecosystem health

Our results support some of the criteria for a good indicator of ecosystem health when evaluated for the blood parasites in our study. While traditional indicators provide information on what is polluting an ecosystem, it, however, does not provide information of how it affects the biotic relationships between organisms. If blood parasites could be used as bioindicators, an advantage would be that they could indicate a stable food web, with the presence of vectors, as well as vertebrate hosts, which depend on a healthy aquatic and terrestrial environment. With a combined parasite prevalence of 52% and prevalence of 34%, 34% and 27% of *Dactylosoma* sp.*, Trypanosoma* spp. and *Hepatozoon* sp.*,* respectively, these parasites show sufficiently high prevalence rates to be relevant for bioindication (see Table 2). For comparison, publications on *Hepatozoon canis* (James, 1905) prevalence in dogs report levels between 1.57% and 22.4% (Amoli et al., 2012; Qamar et al., 2017). For African human trypanosomiasis, prevalence rates between 1 and 5% are considered high (Franco et al., 2014). Moreover, all parasites were present in both seasons, with seasonal differences in prevalence being no more than 24%: in the dry season, 64% of frogs were infected by parasites, with 40% infected in the wet season (see Table 2). Hence, the proportion of parasitised frogs remains substantial throughout the year. We also found no evidence that parasites affect the health, measured as BCI, of their host. Most importantly, our study shows an effect of location on blood parasitism, correlated with environmental conditions that may be linked to anthropogenic disturbance. However, more studies are required to be able to unequivocally attribute anthropogenic impact to the spatial differences in blood parasitism, excluding other parameters that could influence the spatial heterogeneity, for instance, environmental variables. Additionally, one assumption of this study is that both vectors and hosts are not too mobile. For instance, we assume that vectors from a less-impacted site is more or less associated with this general area and is not able to disperse or migrate towards a more-impacted site. The PERMANOVA and ANOSIM also showed that the presence of certain parasites in the component community (the parasite community within *P. anchietae*) is highly location-specific. This supports the notion that species composition is a better indicator than individual species as suggested by Holt and Miller (2010). With regard to the criterion that indicator species should not be opportunistic parasite species, the term generalist or specialist is still relatively ambiguous but often linked to host specificity (Poulin et al., 2011; Strona and Fattorini, 2016). Based on the evolution and ecology of host specificity, parasites using only closely related hosts are considered more specialised and host specific as compared to generalist parasites and their hosts that have a broader host range (Strona and Fattorini, 2016). However, host specificity in heteroxenous parasites is especially difficult to define since these parasites have multiple

intermediate or definitive hosts (O'Donoghue, 2017). Hence due to a lack of data, determining whether or not the parasites are considered opportunistic species, based on host specificity cannot clearly be assessed.

Furthermore, the parasites conform to several other criteria. Frog sampling is readily done and is non-lethal since it consists of withdrawing blood from the femoral artery of frogs, a technique causing no permanent harm to the frog, combined with microscopic identification and quantification. The study area is well-known; owing to the high biodiversity of NGR and the socio-economic and ecological importance of the Phongolo River floodplain, a substantial number of biodiversity, ecotoxicological, hydrological and socio-economic studies have been carried out in this region (Heeg and Breen, 1982; Schmidt et al., 2003; Van der Laan et al., 2012; Netherlands et al., 2014; Coetzee et al., 2015; Nell et al., 2015; Netherlands et al., 2015; Smit et al., 2016; Dube et al., 2017; Minter et al., 2017; Phaka et al., 2017; Netherlands et al., 2018).

Nonetheless, two of the criteria for optimal bioindicators are not yet fulfilled. First, the ecology and life cycles of both host and parasite should be thoroughly understood (Schmidt et al., 2003). Thus, more research on this subject is required. Secondly, pollutants and their effects on the host should be well-known. These factors were also not included in our study and require more research. Our study is naive towards the variation in pollutants per location and season. Therefore, we suggest that in future studies on bioindication through assessment of blood parasites, nutrient and pollutant levels should be included, to more accurately reject the possibility of the spatial heterogeneity being the result of a sampling bias. The effect of OCPs on the development and endocrinal system of frogs is known. However, specific immunological research concerning blood parasites and their frog hosts is lacking. It should be mentioned that while BCI can account only for external signs of weakened hosts, parasites, by definition, inflict at least a minimum of detriment to their host. Hence, a BCI can by no means fully assess the health of a host, only external signs of impaired morphometry.

In conclusion, our results show that frog blood parasites from the genera *Dactylosoma*, *Hepatozoon* and *Trypanosoma*, have the potential to be used as indicators of environmental health. The spatial heterogeneity of most infection parameters and the parasite community composition, in relation to environmental conditions potentially linked to anthropogenic disturbance, the lack of visible health effects on the hosts, and the constant and abundant presence of the parasites support our argument for the use of these parasites in bioindication.

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Amphibians are regarded globally as the most threatened vertebrate class, with numbers declining rapidly. This is due to habitat loss, increased pollution, diseases, invasive species and climate change (Beebee and Griffiths, 2005). The focus on these losses has led to an increased interest in research on amphibians and as a result, has increased our knowledge on their biodiversity, including the discovery of new species. Studies of their parasites on the other hand have not followed the same trend, especially those infecting the blood of anurans.

Blood parasites reported from anuran to date include: the adeleorinid haemogregarines, the eimeriorinid haemococcidia, the kinetoplastid trypanosomatids and the microfilarial nematodes. It must be noted that the blood parasites of ectotherms remain poorly studied (Davies and Johnston, 2000). This is probably due to a bias of studies focussed on blood parasites of medically or economically important host groups. In particularly, blood-borne diseases, such as: human malaria, leishmaniasis, African trypanosomiasis, schistosomiasis, filariasis and other neglected tropical diseases are prioritized. Furthermore, since only a few blood parasite surveys on frogs have been carried out in sub-Saharan Africa, information on anuran blood parasite diversity and relevant data remains scanty (Netherlands et al., 2015). Thus, before an assessment of the evolutionary relationships in these parasites may begin, accurate baseline data needs to be provided. From there, the determination of their biodiversity, phylogenetic relationships, possible vectors and life cycles can be documented. Together, this data will provide better insight to answer questions on the bigger picture of blood parasite evolution, such as, the strategies they use for transmission and the effects they have on their host. The present study was designed to address this void.

Overview of the present study

The majority of the fieldwork in this study took place in northern KwaZulu-Natal, South Africa, specifically the area adjacent the Phongolo River and its associated floodplain (Fig

1). However, an opportunity was presented to include samples collected from anurans in the southern regions of the Kruger National Park, South Africa, an important conservation area (see Fig. 1). Additionally, an opportunity was afforded to collect blood samples of frogs in Belgium, which was fortuitous, as Europe is the type locality for many frog blood parasite species and genera. These samples provided essential data for phylogenetic comparisons between the African and European species.

In South Africa, a total of 618 individual frogs from 35 species and 19 genera were collected from various localities (see Fig. 1 for all the species). In Belgium, 25 individuals representing three species, namely *Pelophylax* kl. *esculentus* (Linnaeus, 1758), *Pelophylax ridibindus* (Pallas, 1771) and *Pelophylax lessonae* (Camerano, 1882) were collected from three localities (Haacht, Keerbergen and Rijmenam, Het Ven). The samples were first screened microscopically for the presence of any blood inhabiting organisms. Samples marked positive could then be further analysed according to the aims of the entire study, or more specifically for one of the chapters. Analyses included both morphological and molecular aspects. Molecular analysis was primarily used to assist in the correct classification or to compare the phylogenetic relationships of certain species with one another, or to data from an online database (i.e. GenBank).

In addition to the morphological and molecular aspects, the ecology of these blood parasites formed an important component of this thesis. As highlighted in the introduction, and throughout Chapters 1 – 5, data and research on most, if not all anuran blood parasites, is limited.

What this study revealed

The present study is to date the largest multi-species, generic and family amphibian blood parasite survey to be completed, with a total of 643 anurans of 38 species, 20 genera and 13 families collected and screened for the presence of blood parasites (see Datasheet 1). Most previous amphibian biodiversity blood parasite surveys screened approximately 100 – 250 individuals and between five to 10 species (Mackerras and Mackerras, 1961; Levine and Nye, 1977; Barta and Desser, 1984; Readel and Goldberg, 2010). Although there are studies with a similar or slightly larger dataset, these studies only surveyed a few host species (Mansour and Mohammed, 1962, 1966). The most comparable study, in terms of samples screened, was by Netherlands et al. (2015) from the same region. In that study, a total of 436 individuals from 29 species were screened. From these larger surveys, a glimpse of the ‘true’ diversity becomes apparent and other aspects of these parasites can be appreciated, such as host-parasite interactions, host specificity, and parasite community structure.

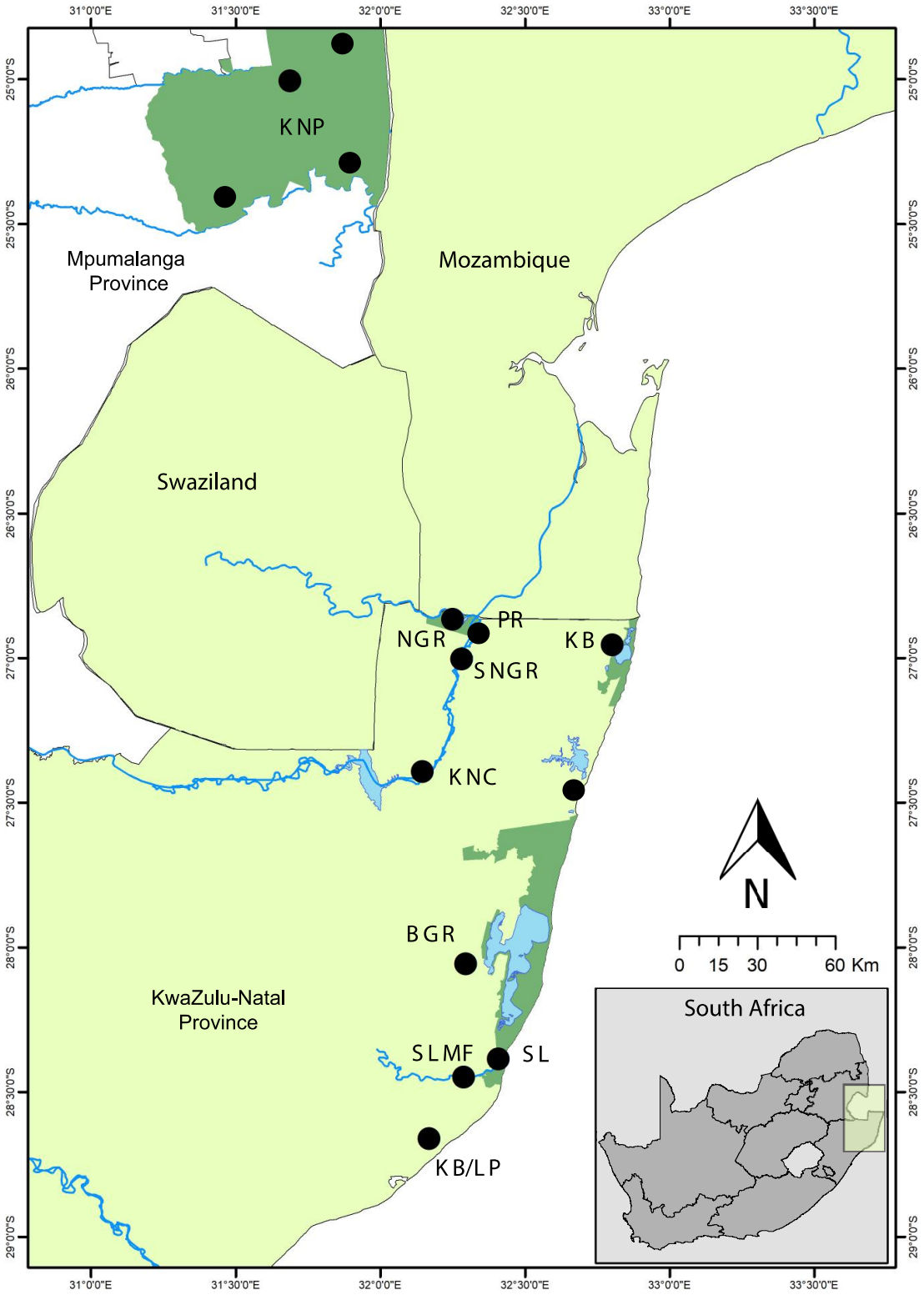

Fig. 1. Map of the main sampling localities in South Africa. Bonamanzi Game Reserve (BGR), Kosi Bay (KB), Kruger National Park (KNP), KwaMbonambi/Langepan (KB/LP), Kwa Nyamazane Conservancy (KNC), Ndumo Game Reserve (NGR), the area directly surrounding the NGR (SNGR), Phongola River (PR), St. Lucia (SL) and St. Lucia Monzi Farm (SLMF).

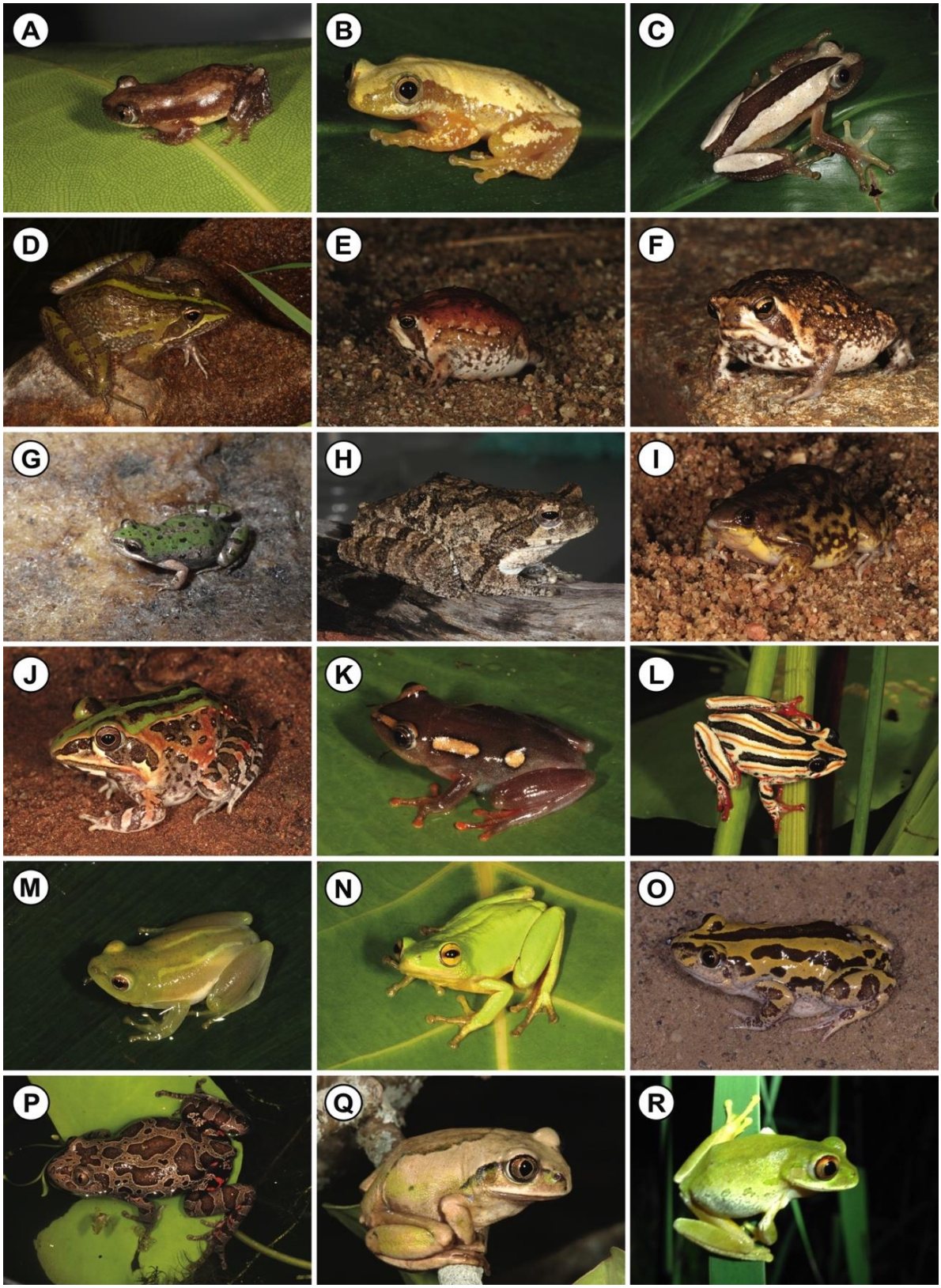

Fig. 2. Photo plate of the frog species collected during the present study in South Africa. A: *Afrixalus aureus.* **B:** *Afrixalus delicatus.* **C:** *Afrixalus fornasini.* **D:** *Amietia delilandii.* **E:** *Breviceps mossambicus.* **F:** *Breviceps passmorei.* **G:** *Cacosternum boetgeri.* **H:** *Chiromantis xerampelina.* **I:** *Hemisus marmoratus.* **J:** *Hildebrandtia ornata.* **K:** *Hyperolius argus.* **L:** *Hyperolius marmoratus.* **M:** *Hyperolius pussilus.* **N:** *Hyperolius tuberilinguis.* **O:** *Kassina senegalensis.* **P:** *Phlyctimantis maculatus* **Q:** *Leptopelis mossambicus.* **R:** *Leptopelis natalensis.*

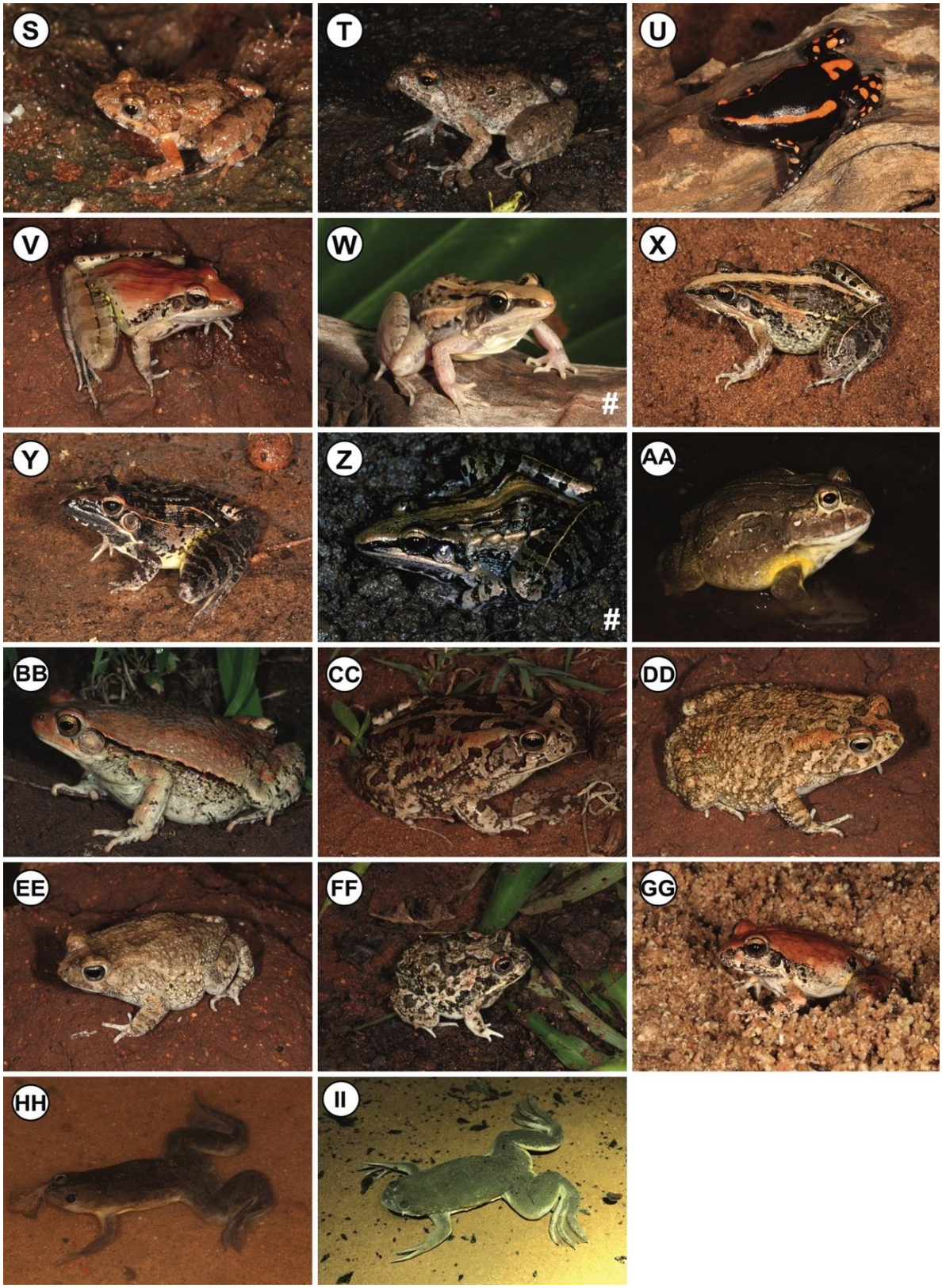

Fig. 2 continued: S: *Phrynobatrachus mababiensis.* **T**: *Phrynobatrachus natalensis.* U: *Phrynomantis bifasciatus.* **V**: *Ptychadena anchietae.* **W**: *Ptychadena mossambica.* **X**: *Ptychadena nilotica.* **Y**: *Ptychadena oxyrynchus.* **Z**: *Ptychadena porosissima.* **AA**: *Pyxicephalus edulis.* **BB:** *Schismaderma carens.* **CC:** *Sclerophrys garmani.* **DD**: *Sclerophrys gutturalis.* **EE:** *Sclerophrys pusilla.* **FF**: *Tomopterna cryptotis.* **GG:** *Tomopterna natalensis.* **HH**: *Xenopus laevis.* **II:** *Xenopus muelleri.* #:Photo L.H. du Preez.

The known diversity

In the present study, different blood parasite taxa were recorded. These taxa included haemogregarine species (Apicomplexa: Adeleiorina) of *Hepatozoon* Miller, 1908 and *Dactylosoma* Labbé, 1894. Also found were haemococcidian species (Apicomplexa: Lankesterellidae) of *Lankesterella* Labbé, 1899 (three species) and a currently undescribed genus. Microfilariae of a filarial nematode were found infecting two species of toads. This species of filarial nematode is considered to belong to a currently undescribed genus and species. Extracellular flagellate parasite species of *Trypanosoma* Gruby, 1843 were observed in ecological studies on grass frogs (*Ptychadena* Boulenger, 1917). Other protozoan blood parasites found, but that did not form the focus of this study, include *Hepatozoon ixoxo* Netherlands, Cook & Smit, 2014, from five different species of anurans; two unidentified species of *Hepatozoon* from tree frogs (*Leptopelis* Günther, 1859); and one unidentified *Hepatozoon* species from *Ptychadena anchietae* (Bocage, 1867) (see Chapter 5). Furthermore, a number of trypanosomes and intraerythrocytic organisms of a viral or bacterial nature, which could not be identified to species level, were also observed in various anurans across the different families.

The first taxonomic contribution presented in this thesis was the description of three new species of *Hepatozoon*, that were described from hyperoliid frogs in northern KZN (Chapter 1) (Netherlands et al., 2018). The species described were: *Hepatozoon involucrum* Netherlands, Cook & Smit, 2018 parasitising *Hyperolius marmoratus* Rapp, 1842; *Hepatozoon tenuis* Netherlands, Cook & Smit, 2018, parasitising *Afrixalus fornasini* (Bianconi, 1849), *Hyperolius argus* Peters, 1854 and *Hyp. marmoratus*; and *Hepatozoon thori* Netherlands, Cook & Smit, 2018 parasitising *Hyp. argus* and *Hyp. marmoratus*. Prior to this study, only two species of *Hepatozoon* from anuran hosts had been described from South Africa, namely: *Hepatozoon theileri* (Laveran, 1905) and *Hepatozoon ixoxo*. *Hepatozoon ixoxo* was described from three species of toads (Bufonidae Gray, 1825), also from northern KZN (Netherlands et al., 2014). Based on morphology, *H. ixoxo* was observed parasitising *Ptychadena mossambica (Peters, 1854)*, *Hemisus marmoratus* Peters, 1854, *Sch. carens*, *Scl. garmani* (also recorded as a host in the original description), and *Scl. pusilla* (type host). Thus, from northern KZN, four recognised blood parasite species occur.

Species of *Dactylosoma* Labbé, 1894 were also screened for and found in the blood of three anuran species, namely: *Pty. anchietae* (Ptychadenidae Dubois, 1987) and *Sclerophrys gutturalis* (Power, 1927) (Bufonidae) from South Africa, and *Pel. lessonae* from Belgium (Chapter 2). *Dactylosoma ranarum* (Kruse, 1890) is the most commonly reported species, recorded to occur in several anuran species globally. This is the only named species reported from Europe and Africa. It was also reported in *Scl. gutturalis* from South

Africa, however, no morphological or molecular data is available from that report for comparison (Fantham et al., 1942). Based on the data obtained in the current study, the dactylosomatid from *Pty. anchietae* and *Scl. gutturalis* belong to the same species, yet are distinct from *D. ranarum* from Europe. All data indicate that this species is an undescribed species, awaiting formal description (Chapter 2). The *Dactylosoma* species found parasitising *Pel. lessonae* from Belgium remains an unidentified species.

Three species of *Lankesterella* were found in the present study. Two were species from Belgium, of which one conforms morphologically to *Lankesterella minima* (Chaussat, 1850) from Europe (type species of the genus). The other, proposed to be a new species (*Lankesterella* sp. 1), was found parasitising *Pel.* kl. *esculentus* and *Pel. lessonae*. From South Africa a single species of *Lankesterella* (*Lankesterella* sp. 2) was found parasitising *Afrixalus delicatus* Pickersgill, 1984 and *Afr. fornasini* from KZN. This is the first report of a lankesterellid from South Africa. In addition to the *Lankesterella* species found, two species of an intraerythrocytic parasite, similar in appearance to the lankesterellids, but enclosed in a greatly expanded parasitophorous vacuole (PV), were observed. These two species were found infecting *Afr. fornasini*, *Phrynobatrachus mababiensis* FitzSimons, 1932*, Pty. anchietae*, and *Pyxicephalus edulis* Peters, 1854. Morphological comparisons of the parasite from the latter three species conformed closely to *Lankesterella ptychadeni* Paperna and Ogara 1996 and these parasites are all considered to belong to the same species. However, based on the unique PV enclosing the mature peripheral blood stages of this parasite, and supported by molecular phylogenetic analyses using nuclear 18S rRNA and mitochondrial COI gene datasets (see Chapter 3), it is proposed that these two species be assigned to a new genus (haemococcida n. gen.).

Microfilariae, the larval stage of filarial nematodes, are easily recognised in the peripheral blood of their hosts. However, in the current study microfilariae were only observed in *Scl. gutturalis* and *Sclerophrys garmani* (Meek, 1897) (Chapter 4). Sampling of these infected toads was restricted to the one area only, namely Sodwana, KZN. Based on morphology and molecular data, this filarial nematode represents an undescribed genus and species. From within the Waltonellinae, and excluding Madagascar, only a single species has been described from Africa, *Foleyellides duboisi* found in the DR Congo (Gedoelst, 1916) and Palestine (Witenberg and Gerichter, 1944).

The unknown diversity

In addition to the data reported in Chapter 1 on *Hepatozoon* species from members of the Hyperoliidae, two species of tree frogs were also infected with an unidentified species of *Hepatozoon*, namely: *Leptopelis natalensis* (Smith, 1849) and *Leptopelis mossambicus*

Poynton, 1985 (Arthroleptidae Mivart, 1869) (unpublished data). In the current study, 34% of the individual grass frogs screened in Chapter 5 were positive with trypanosomes. Although the diversity is not clear, as this group is known to be polymorphic, molecular tools are required to assess the diversity with certainty. Additionally, trypanosomes were observed in several other species and families, namely *Afr. delicatus*, *Afr. fornasini*, *Hyp. argus*, *Hyp. marmoratus*, *Hyperolius tuberilinguis* Smith, 1849 (Hyperoliidae Laurent, 1943); *Hyp. marmoratus* Peters, 1854 (Hemisotidae Cope, 1867); *Leptopelis natalensis* (Smith, 1849) and *Lep. mossambicus*; *Phr. mababiensis* (Phrynobatrachidae Laurent, 1941); *Scl. garmaini,* and *Scl. gutturalis* (Bufonidae) (unpublished data).

Box 1: *Research highlights*

Chapter 1:

- Three new species of *Hepatozoon* described, increasing South Africa's anuran *Hepatozoon* diversity to 38% (5/16) of all species currently described from Africa anurans.
- Increased available 18S rDNA sequence data from five to eight recognised species of *Hepatozoon* from anuran hosts.

Chapter 2:

- First description of anuran species of *Dactylosoma* from Africa.
- First molecular characterisation (18S rDNA) of a species of *Dactylosoma* from Africa.
- Contrary to popular belief – this study provided new data on the possibility of dipteran (*Sergentomyia* spp. and *Uranotaenia* spp.) vectors as opposed to solely being transmitted by leech vectors.

Chapter 3:

- Described two new species of *Lankesterella* of anurans from Belgium and South Africa.
- Redescribed and provided first molecular data of the type species *Lankesterella minima.*
- A new genus and species of haemococcidia of anurans from Africa is described.
- Provide 18S rDNA and COI gene sequence data for different Haemococcidia.

Chapter 4:

- New genus and species of filarial nematode (Ochocercidae) from South African anurans described.
- Elucidated all the different life stages of the filarial nematode.
- Established the mosquitoes *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* and *Uranotaenia* (*Pfc.*) *montana* as the natural (*in situ*) vectors of this filarial nematode.

Chapter 5:

- Provides evidence of anuran blood parasites as sensitive multi-trophic indicators of environmental health and intact food webs.

Infected vs uninfected hosts

In South Africa, 209 individuals (16 species) were infected with at least one blood parasite species, with 409 individuals (19 species) not infected. Table 1 provides a summary of the infected and non-infected host species, along with the infection prevalence given as a percentage. From the 19 uninfected species found in the present study, four species were reported infected in the survey by Netherlands et al. (2015). The negative results from the present study could be a result of small sampling sizes for these species.

In Belgium, from the 25 individuals screened (from three species), eight individuals (from two species) were infected with at least one blood parasite species, with 17 individuals (from 3 species) not infected. From the seven individuals of *Pel.* kl. *esculentus* screened, 28.6% were infected with species of *Lankesterella* and 28.6% with species of *Trypanosoma*. From the 14 individuals of *Pel. lessonae* screened, 21.4% were infected with species of *Lankesterella*, 7.1% with a species of *Dactylosoma* and 7.1% with species of *Trypanosoma*.

Why some anuran species are hosts for blood parasites, and other seemed to be free of infection is an intriguing question, and deserves further discussion (see sections that follow).

A story of blood-and-guts, and the in-between

"The blood is the life" Dracula.

The ecological niche is the position or status an organism occupies in an ecosystem, and the specific link it forms with that ecosystem (Jones et al., 1997). For blood parasites, that niche is their host's blood and associated organs. These parasites have evolved specialised traits to help them not only survive within the hosts blood system, but to also make use of a vector to reach the host initially (Desser et al., 1995). The link between these different organisms is the blood. Without it, the host, the parasite, nor the vector can survive. In the rest of this chapter, the different host-parasite interactions will be discussed in more detail regarding scenarios observed in the current study.

Table 1. Summary of the infected and non-infected host species, along with the infection prevalence

Frog species	Infected	Screened	*Hepatozoon*	*Dactylosoma*	Haemococcidia	Microfilaria	*Trypanosoma*
Afrixalus aureus	✗	6	-	-	-	-	-
Afrixalus delicatus	✓	24	-	-	20.8 %	-	12.5 %
Afrixalus fornasini	✓	20	50.0 %	-	10.0 %	-	30.0 %
Amietia delilandii	✗	2	-	-	-	-	-
Breviceps mossambicus	✗	1	-	-	-	-	-
Breviceps passmorei	✗	3	-	-	-	-	-
Cacosternum boetgeri	✗	1	-	-	-	-	-
Chiromantis xerampelina	✗*	2	-	-	-	-	-
Hemisus marmoratus	✓	5	20.0 %	-	-	-	20.0 %
Hildebrandtia ornata	✗*	2	-	-	-	-	-
Hyperolius argus	✓	4	75.0 %	-	-	-	25.0 %
Hyperolius marmoratus	✓	39	28.2 %	-	-	-	15.4 %
Hyperolius pussilus	✗	6	-	-	-	-	-
Hyperolius tuberilinguis	✓	26	-	-	-	-	23.1 %
Kassina senegalensis	✗*	6	-	-	-	-	-
Leptopelis mossambicus	✓	7	14.3 %	-	-	-	14.3 %
Leptopelis natalensis	✓	22	50.0 %	-	-	-	63.6 %
Phlyctimantis maculatus	✗	2	-	-	-	-	-
Phrynobatrachus mababiensis	✓	22	-	-	4.5 %	-	-
Phrynobatrachus natalensis	✗	5	-	-	-	-	-
Phrynomantis bifasciatus	✗*	6	-	-	-	-	-
Ptychadena anchietae	✓	163	39.9 %	37.4 %	3.7 %	-	36.2 %
Ptychadena mossambica	✓	24	4.2 %	-	-	-	4.2 %
Ptychadena nilotica	✓	5	20.0 %	-	-	-	-
Ptychadena oxyrynchus	✗	12	-	-	-	-	-
Ptychadena porosissima	✗	12	-	-	-	-	-
Pyxicephalus edulis	✓	45	-	-	11.1 %	-	-
Schismaderma carens	✗*	3	-	-	-	-	-

Table 1. Continued.

Sclerophrys garmani	✓	45	6.7 %	-	-	2.2 %	2.2 %
Sclerophrys gutturalis	✓	73	2.7 %	12.3 %	-	9.6 %	6.8 %
Sclerophrys pusilla	✓	7	14.3 %	-	-	-	-
Tomopterna cryptotis	✗	6		-	-	-	-
Tomopterna natalensis	✗	1		-	-	-	-
Xenopus laevis	✗	2		-	-	-	-
Xenopus muelleri	✗	9		-	-	-	-

*** Indicates species infected in the study by Netherlands et al. (2015)**

Host susceptibility and parasite specificity

One of the fundamental properties of any parasitic organism is to exploit its host's resources. Some parasites have evolved or adapted certain strategies to either make use of many hosts i.e. parasites that are highly host-specific will occur in a single host species, whereas generalist parasites will be dispersed among individual hosts from several different host species (Lajeunesse and Forbes, 2002; Little et al., 2006). Basic host specificity can be defined as the number of host species successfully exploited by a particular parasite (Lajeunesse and Forbes, 2002; Poulin et al., 2011; Vanhove and Huyse, 2015). From an evolutionary perspective, host specificity reflects the parasite's historical associations with its host (Page, 2003). This interaction, or lack thereof, can be attributed to a number of factors exerted from the parasite to the host or conversely from the host to the parasite. In most studies on host-parasite interactions, specificity is assumed to have a genetic basis (Little et al., 2006). However, there are alternatives, for example phenotypic plasticity, in which the parasite adapts to overcome the hosts defence mechanisms (Little et al., 2006). This adaptation is also frequently assumed in studies of microorganisms (Rainey, 2004; Little et al., 2006). Furthermore, there are two kinds of evolutionary events in which a parasite can increase its host range. First, if the original parasite's host speciates then the parasite may be capable of infecting both daughter host species. This event would result in the parasite not only occurring on the ancestral host, but also in the two closely related host species (Poulin et al., 2006). The second and probably more frequent event is host-switching. Host-switching is the process of a certain parasite linage successfully colonising a new host species, and initiated by a few founder individuals (parasite) infecting a new host species (Ròzsa et al., 2015; Vanhove and Huyse, 2015). For this process to occur the parasite requires several events to establish a relationship with a new host. The parasite must have the opportunity to host switch, for example the chance of co-existence or contact between the host and parasite (temporally and spatially) (Brooks, 1979; Araujo et al., 2015; Vanhove and Huyse, 2015). The switching parasite lineage and host should be compatible by overcoming barriers imposed by the host, most such switches result in immediate mortality of the parasite or in its new host population, preventing establishment of the parasite (Araujo et al., 2015; Ròzsa et al., 2015). The host organism should provide an adequate and accessible resource for the parasite (Araujo et al., 2015). Lastly the parasite and the host should overcome subsequent conflicts or barriers emerging from the basic dynamics of co-adaptation (Brooks, 1979; Araujo et al., 2015). When the invaders successfully establish a viable population on the new host species a host-switching event has occurred. Furthermore, this event my lead to speciation of the parasite, for example if the parasite becomes reproductively isolated within the boundaries of its new host species, such as in

the case of sexually or socially transmitted parasites, the parasite may develop into a separate species (Ròzsa et al., 2015).

More susceptible hosts are host species that are prone to infection by multiple parasite species or taxa at any given time, season, or locality (see Fig. 3A). Hosts that harbour more genera of parasites may belong to a larger systematic group (Brooks, 1979). For example, a large host population that continually expands its distribution range comes into contact with more and different species, which include parasites. Over time this long co-evolutionary history may lead to rich parasite assemblages for these hosts (Ròzsa et al., 2015). On the other hand isolated host populations may be more susceptible and less able to develop appropriate immune reactions to parasites only “recently” acquired (Ròzsa et al., 2015). This high parasite susceptibility could also be due to the host’s own behaviour, genetics, host co-existence to various parasite, or even the evolutionary relationships that multiple the parasite has perfected over time to have minimal impact on the host. For example, in the present study the most susceptible host by far was the plain grass frog, *Pty. anchietae*. Remarkably, of the 163 individuals collected, only 51 (31%) were found uninfected. Cases, where more individuals are infected as compared to non-infected individuals, were rare, in fact, this was only also observed in the natal tree frog, *L. natalensis* where 16 of 22 frogs (73%) were infected. Also, in the present study, *Pty. anchietae* was infected with all the blood parasite groups, which were investigated, namely intracellular apicomplexans (haemogregarines and haemococcidia) and the extracellular flagellates (trypanosomes), excluding microfilariae (see Table 1). However, Netherlands et al. (2015) reported on microfilariae infection in this host at low prevalence. The haemogregarines (*Hepatozoon* sp. and *Dactylosoma* sp.) and the trypanosomes (*Trypanosoma* spp.) all infected more than 30% of the screened individuals. The guttural toad, *Scl. gutturalis*, seems to be also highly susceptible to blood parasite infections. This is in terms of blood parasite diversity found in this host, but not with regard to the number of infected individuals.

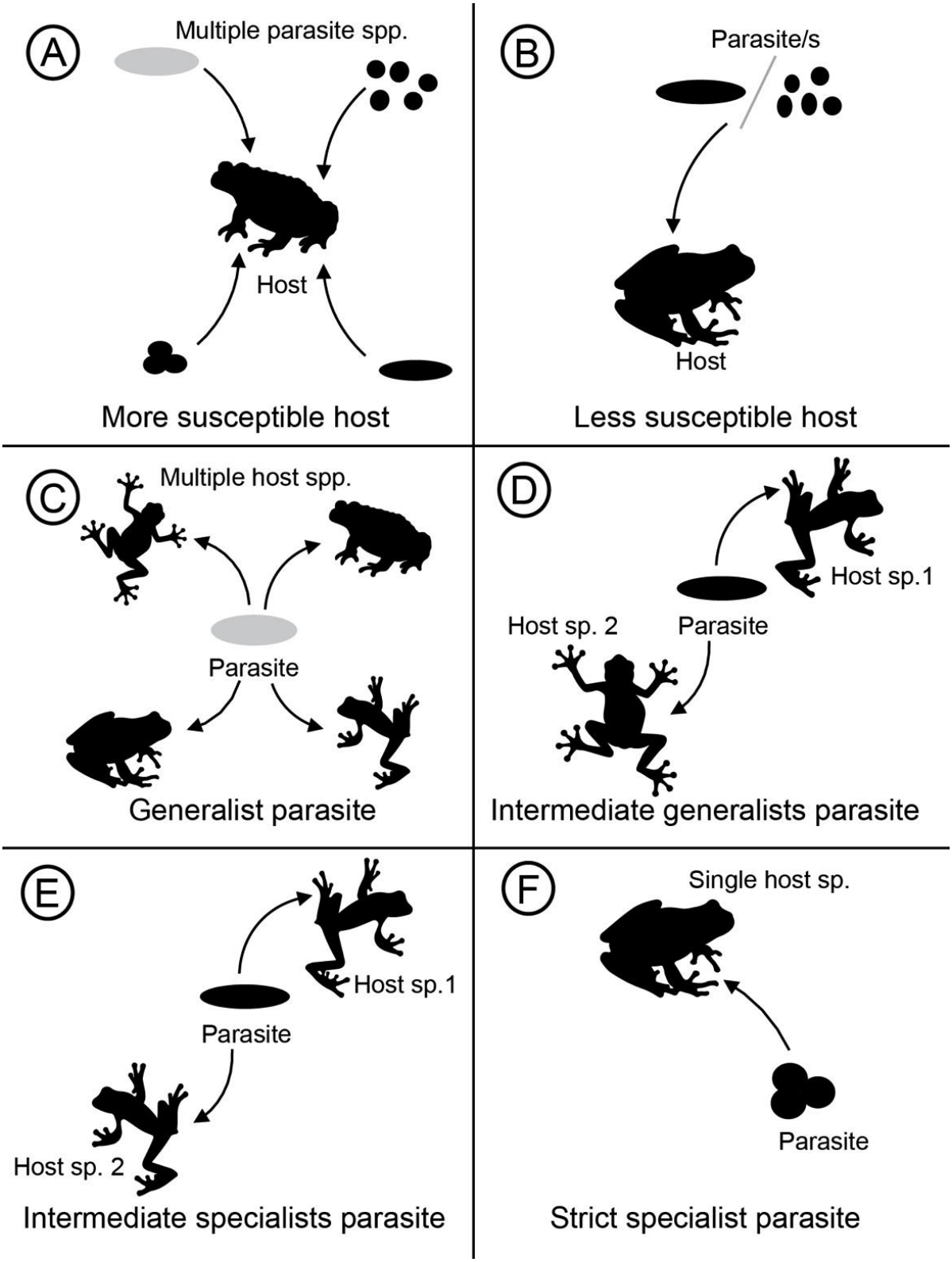

Fig. 3. Illustrations of different examples of blood parasites and host occurrences in terms of susceptibility and specificity. A: More susceptible host infected by multiple parasite species or taxa. **B:** Less susceptible host infected by a limited number parasite species or taxa. **C:** Generalist parasite infects multiple host species from different genera and families. **D:** Intermediate generalist parasite infects two or more host species from one family. **E:** Intermediate specialist parasite infects two host species from one genus. **F:** Strict specialist parasite infects a single host species only.

In contrast to hosts that are susceptible to multiple parasite infections, some host hosts seem to be less susceptible or more resistant and are infected with fewer parasites species or taxa (see Fig. 3B). These hosts have evolved certain defence mechanisms, including avoidance of infection (behavioural and physiological), the controlled population growth rate, and a complex immune system (Decaestecker et al., 2002; Boots et al., 2012). Host resistance can also occur when the host undergoes changes in its ecology, for instance if the host occupies a novel environment, it may obtain a new combination of traits, that in turn may release it form infection by the parasite (Brockhurst et al., 2005; Poisot, 2015). Host resistance, is difficult to quantify and in most cases has not been experimentally measured or the host not sufficiently screened throughout its host range (Dobson et al., 2008). Data collected from studies based parasite presence or absence, don't necessarily provide concrete evidence on host resistance. However, reports on the presence or absence of different parasite taxa remain important and may provide at least some indication of species infections in a particular area. In the present study, six species (see Table 1) were found infected with two different taxa and four host species infected with only one parasite species or taxa (see Table 1). For example, *Leptopelis natalensis* seems to be less susceptible to infection by several different blood parasite taxa and was found infected by only two parasite taxa, but in high prevalence (more than 50%). The African bullfrog, *Pyx. edulis*, was infected with only a single haemococcidium species. Although prevalence was low, intensity of infection ranged from high to low. Furthermore, 19 species were found not infected with any blood parasite species (see Table 1). This could be due to patterns of life history, genetics, or even evolution. However, five of these species were reported to be parasitised by one or more blood parasite species or taxa in the study by Netherlands et al. (2015). In the case of these five species, it is most likely that a small sample size (less than six individuals per species) led to the negative results in the present study. However, this could also be due to other factors such as the locality or season. Nonetheless, there are certain species, such as the rain frogs (*Breviceps* spp.) that don't seem (based on the current data and available literature) infected with blood parasites (Netherlands et al., 2015). Their lack of blood parasites could be due to their behaviour or lifestyle, as these frogs spend the majority of their time underground, only emerging during or after heavy rain (Minter et al., 2004). Furthermore, these frogs have a specialised diet, consisting mostly of ants or termites, which could eliminate transmission from dipteran vectors through ingestion (trophic transmission) e.g. species of *Hepatozoon*. However it is important to note that rain frogs aren't entirely parasite free and have been reported to be infected with parasitic nematodes, which are easily transmitted due to these fogs prolonged contact with the soil (Baker, 1982). Susceptibility to blood parasites must be considered a possibility, however, specialist host behaviour could be an important factor regarding the absence of blood

parasites in those particular hosts. Further genetic or experimental investigations and increased sampling over a larger area are essential (Little et al., 2006; Poulin et al., 2011). This highlights the importance of acquiring data with a sufficient sample size, over a wide area, and across different seasons for any parasite-based surveys, before making any statements on the parasite susceptibly of a host. Due to the limited of data available on frog blood parasites from South Africa and the limited number of samples obtained for certain species. Species that were found not infected were regarded as data deficient.

Generalist parasites are those that are able to infect multiple host species from different genera and families (Šimková and Morand, 2015; Vanhove and Huyse, 2015). These parasites have little restricting their survival and transmission to a new and different host species. A number of scenarios are possible with regards to the success of generalist parasite species. Generalist parasites have been shown to have higher phenotypic and genetic variability, most likely to accommodate the heterogeneity of their different hosts (Desdevises et al., 2002; Poisot, 2015). According to Kaci-Chaouch et al. (2008) generalist species of *Lamellodiscus* Johnston and Tiegs, 1922 monogeneans have more variability than specialist species both in terms of morphology and genetics. Generalist species of *Plasmodium* Marchiafava and Celli, *1885* and *Haemoproteus* Kruse, 1890 have been shown to be better colonisers of island avifauna compared with specialist parasites, occasionally causing mortalities in native bird species (Atkinson and LaPointe, 2009; Ewen et al., 2012; Moens and Pérez-Tris, 2016). For example, generalist species of *Haemoproteus* parasites have caused mortality in various species of captive parrots throughout Germany, and *Plasmodium relictum* (Grassi and Feletti, 1891) and its vector *Culex quinquefasciatus* Say, 1823 caused major declines of native species of honeycreepers the Hawaii archipelago (Atkinson and LaPointe, 2009; Moens and Pérez-Tris, 2016). The mammalian species *Trypanosoma cruzi* Chagas, 1909, also responsible for Chagas disease in humans, is widely accepted as generalist parasite reported from a large number of mammal species (Lima et al., 2013; Pinto et al., 2015; Jansen et al., 2018). This parasite species is also transmitted via several species of triatomine bugs (insects of the order Hemiptera), in which the parasite establishes permanent infections (Jansen et al., 2018). Another example of a generalist trypanosome is the interesting scenario of *Trypanosoma dionisii* Bettencourt and França, 1905 that has been reported from reptiles, humans, bats, and marsupials (Jansen et al., 2018). A number of frog blood parasite species and taxa are generalist parasites that are not restricted to a particular species, genus or family. A good example is *H. ixoxo*, which, although was first only described infecting three species of toads from Bufonidae (Netherlands et al., 2014), has been shown in subsequent reports as well as in the present study to have infected several other species not belonging to Bufonidae. The morphology of *H. ixoxo* may provide some indication of why this parasite that is able to parasitise a wide

range of host species. Conradie et al. (2017), hypothesised that the polar cap contained by *H. ixoxo* is a storage organ for waste, preventing the leakage of pigment into the host cell or blood plasma. The polar cap of *H. ixoxo* may, cause minimal haemoglobin depletion and preventing the premature death of its host cell, ultimately increasing its chance of vector transmission. Other blood parasites that can be considered as generalists are anuran trypanosomes. Although, as mentioned earlier, these parasites are polymorphic and often difficult to identify, several similar looking morphotypes were observed in different hosts.

An intermediate generalist parasite (Fig. 3D) infects two or more host species from one family and an intermediate specialist parasite (Fig. 3E) infects two closely related host species from the same genus (Šimková and Morand, 2015; Vanhove and Huyse, 2015). According to Šimková et al. (2013), two closely related cyprinids, and their hybrids were susceptible to infection by intermediate generalist parasites. However, prevalence and intensity of infection were higher in one species. This finding indicates that intermediate generalist parasites may prefer certain species within its host range, as suggested by ŠImková et al. (2006). In the current study, good examples of these types of parasites are species of *Hepatozoon* that infect members of the Hyperoliidae. *Hepatozoon tenuis* is an example of intermediate generalist parasite parasitising the leaf folding frog *Afr. fornasini*, and the reed frogs *Hyp. argus* and *Hyp. marmoratus.* The haemogregarine *Hepatozoon thori* is an intermediate specialist parasite parasitising the reed frogs *Hyp. argus* and *Hyp. marmoratus* (Netherlands et al., 2018). Thus far, these hemogragarines have only been found in hyperoliid frogs, showing intermediate levels of host specificity in terms of the hosts they infect (restricted to the same family), and that these parasites only specifically infect certain members of this family.

Specialist parasites are found to infect a single host species only (Fig. 3F). Although, common among other parasite groups, for example, monogenean polystomes (Héritier et al., 2015), blood parasites appear to be more generalist parasites. Specialist blood parasites, especially in frogs, seem rare. This is probably why in the past so many blood parasite species were described exclusively on the basis of host range and now need to be revised (Hoare, 1932; Telford, 2009). In the present study, although rare, certain blood parasite species do seem to be restricted to a single host. For example, *Hepatozoon involucrum* is considered a specialist parasite, found only parasitising *Hyp. marmoratus* throughout the study area.

Another specialist parasite is the species of *Hepatozoon* found only parasitising *Pty. anchietae*. This is the most common specialist parasite encountered in this study, with 39.9% of the individuals screened positive for infection. It is an interesting observation, that such a highly susceptible host was found only to be parasitised by a single species of *Hepatozoon*, and that this haemogregarine is found only in this host. This parasite seems to

rather follow the same aggressive life strategy as *H. theileri*. This is in terms of the notable modifications and effects on its host cells. Instead of preserving the host cell as in *H. ixoxo*, the resources from the infected erythrocyte, such as the haemoglobin are used up by the parasite (Conradie et al., 2017). It is likely that these modifications have some pathological effect on the host. However, as in some other apicomplexans such as *P. falciparum* (Welch, 1897), there is probably some alteration of protein gene expression, allowing the parasite to avoid detection or attack by the hosts immune system (Kilejian et al., 1977).

The reason for such specificity could be due to the aggressive nature of this parasite. However, field observations indicate that it does not alter the host's behaviour and is well tolerated. The absence of one parasite does not necessarily equal presence of another. However, it does seem that when the host is infected with one of the three protozoans, namely: *Hepatozoon*, *Dactylosoma* or *Trypanosoma* species, then there is a good chance it will also be infected with either one or both of the remaining parasites. This could indicate a slight imbalance in an infected host's immune system. It would be interesting to determine if one of these parasite taxa has a direct effect on its host's immune system, specifically making it possible for the other parasites to more easily infect that particular host. Dual infection is common among protozoans in ectothermic vertebrate hosts (Hayes et al., 2006; Van As et al., 2013; Cook et al., 2014). However, the role of the vector should not be forgotten in such cases of multiple infections. Hayes et al. (2006) observed developmental stages of a trypanosome and haemogregarine in their marine leech vector taken from infected fishes, suggesting that some vectors are able to transmit multiple parasites simultaneously.

The question is raised whether the different blood parasites are mutualistic or not? In the current study, a number of parasites occur sympatrically, for instance in the host *Afr. fornasini,* the haemococcidium parasite appears to favour blood cells already occupied by the haemogregarine *H. theuis* (see Fig. 4). In some cases, blood cells are parasitised with up to four haemococcidium parasites sharing the same host cell (Fig. 4C). This parasite-parasite interaction seems to be a clear strategy used by one of these taxa, almost as if the one needs the other to weaken the cell for the other to enter. It is unfortunate that only a single host was infected and available to examine. It would be interesting to see if individuals of these two species of parasite also infect the same blood cells in less intense infections. Species of *Hepatozoon* are also often found in the same host cell (Bashtar et al., 1991; Van As et al., 2013; Cook et al., 2014; Han et al., 2015; Van As et al., 2015), probably so they are ready to fuse and start development as soon as a vector takes them up. It may also simply be that it is more energy conserving for different individual parasites (from the same species or not) to share a host cell. The already occupied blood cells defensive system is compromised, thus taking less energy for the second parasite to enter the already occupied

blood cell. However, parasite-parasite interactions, especially if they are mutualistic are probably far more complicated.

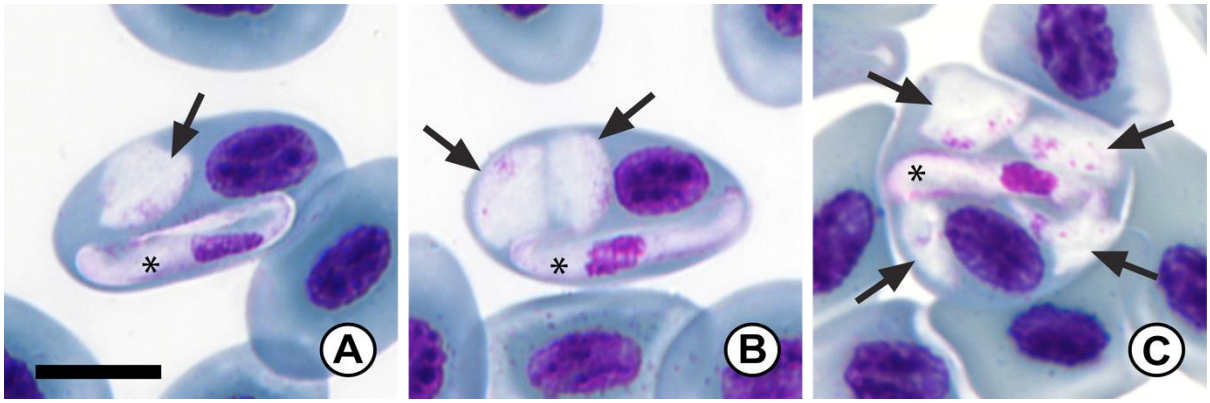

Fig. 4. Light micrographs of a haemococcidian (arrows) and a haemogregarine, *Hepatozoon tenuis* (asterisk) parasitising the same host cell of *Afrixalus fornasini*. Scale bar 10 μm.

The vector

The vector of any blood parasite plays a crucial role in its survival and successful transmission to a new host (Smith, 1996). Some vectors are the definitive host, where sexual reproduction takes place i.e. for the species *Plasmodium* and *Hepatozoon*. Others are merely paratenic or mechanical transport hosts i.e. for haemococcidia (Desser, 1993; Valkiūnas, 2004).

Mosquitoes, among other haematophagous invertebrates, are well known vectors of filarial nematodes (Ferguson and Smith, 2012). In the current study, microfilariae (onchocercid n. gen.) were found infecting the toads *Scl. gutturalis* and *Scl. garmani*. This parasite was restricted to a single locality within the study area. Although these two species are relatively similar, occurring sympatrically, only a single *Scl. garmani* was found to parasitised. This could be due to an insufficient sample size of *Scl. garmani* from that area. Alternatively, the mosquito vectors could play a role in the prevalence and distribution of these parasites, limiting their distribution to the range and type of habitat that these mosquitoes live in.

In situ observations on the feeding habits of the mosquitoes *Uranotaenia* (*Pseudoficalbia*) *mashonaensis* Theobald, 1901 and *Uranotaenia* (*Pfc.*) *montana* Ingram & Meillon, 1927, revealed interesting findings on the preference and methods these mosquitoes use to locate their host. In the initial field observations of these mosquitoes, it was noted that they were only found on male *Scl. gutturalis* that were actively calling.

Recently several species of *Uranotaenia* mosquitoes have been reported to be attracted to frog calls (Camp et al., 2018). To test this concept, calling toads were located at night, checked for the presence of mosquitoes and then watched using an infrared light, not to disturb the toads and mosquitoes. If toads were reluctant to call, playback-calls were broadcast and a response call was waited for. Remarkably, once a toad started to call, mosquitoes would almost instantly hone in on the calling individual. Frog feeding mosquitoes are known to possess different sensory apparatuses as compared to their counterparts with preference for feeding on mammals (Ferguson and Smith, 2012). This method is efficient, with high numbers of mosquitoes feeding on calling hosts. Prior to this study, only three studies provided evidence of species of *Uranotaenia* mosquitoes and their attraction to the sound of their host species (Borkent and Belton, 2006; Toma et al., 2014; Camp et al., 2018). However, none of these studies provided field-based observational data of this host-vector interaction. In these previous studies, traps baited with a speaker playing frog calls were used to collect mosquitoes. Thus, to date almost no data is available on the behaviour of these mosquitoes attracted to calling anurans.

Additionally, these mosquitos don't seem to be only the vector for a single parasite species (filarial nematode), but also the possible of vector of the species of *Dactylosoma* found parasitising a population of the guttural toads, *Scl*. *gutturalis*. However, dactylosomatids are thought to be transmitted solely by a leech vector (Barta, 1991), which is strange as no leeches were found feeding on any anurans in the current study despite the number of parasites taxa thought to be transmitted by leech vectors i.e. dactylosomatids and certain trypanosomes.

At the end of the day who sits on the iron throne?

Host-parasite and vector interactions are complex processes that have adapted over time and withstood pressures from changing environments to the host's defence system (Takken and Koenraadt, 2013). One of the questions that come to mind when studying host-parasite interactions is who is in control? The basic process of vector-transmitted parasites is the same, in that a parasite is taken up by the vector and then transmitted to a new host. However, certain blood parasites use a vertebrate and others an invertebrate as definitive host (Desser et al., 1990). Some are transmitted through ingestion of the invertebrate vector (trophic transmission). Others are transmitted via the bite from an infected vector (salivary or injected transmission) (Barta and Desser, 1989; Desser, 1993; Desser et al., 1995). In each of these scenarios the host and vector play a different role, and serve a specific function. The only real constant is the blood parasite. The question thus arises whether the parasite is purely involved by chance, or does it play an active role in enhancing its chance of

successful transmission? Indeed the latter option would be more advantageous to the parasite, this would mean that these parasites have not just evolved to survive in this unique niche, but that they are able to alter both the host and vector's behaviour to suit their needs, increasing their chance of long-term survival.

Manipulation of host behaviour or chemical signals from the host is a widespread strategy employed by parasites to increase their chance of transmission (Moore, 1993; Thomas et al., 2005). The haemogregarines, *Hepatozoon sipedon* Smith, Desser & Martin, 1994 and *Hepatozoon clamatae* (Stebbins, 1905) have been shown to affect host-seeking behaviour, increasing the feeding potential of their invertebrate vectors on infected hosts (Ferguson et al., 2013). Host manipulation may include changes in composition of epidermal microbial flora populations, consequently modifying host odours and kairomone profiles, or olfactory cues such as increased levels of CO_2 or specific odour profiles released by the host that attract target mosquito species (Ansell et al., 2002; O'Shea et al., 2002; Cooperband et al., 2008; Prugnolle et al., 2009; Ferguson et al., 2013). These types of host manipulation by the parasite would mean that they play an active role to increase their change of transmission to new hosts.

However, many haematophagous invertebrates are often highly adapted to locate a suitable and specific host or host group (Borkent and Belton, 2006; Ferguson and Smith, 2012; Kvifte and Wagner, 2017; Reeves et al., 2018). In the present study the elucidation of the filarial nematodes life history provide interesting observations of host, vector and parasite interactions. The mosquito vectors showed high levels of attraction to vocalising guttural toads. Attraction to the call of its host is a particularly effective host-seeking approach, especially if the host’s call is used to ensure its own chance of finding a mate (Ferguson and Smith, 2012). However, anurans are known to consume large numbers of invertebrates that include mosquitoes (Hirai and Matsui, 1999). In fact guttural toads have been used as a bio-control for mosquitoes in Mauritius and Reunion Island (Kraus, 2008). If the parasite is transmitted via injection, and mosquitoes that are consumed in large numbers, then chances of successful transmission decrease. However, certain mosquito species infected with malaria have been shown to decreased levels of attraction to their host with early stage infections and increased attraction with late-stage infections (Cator et al., 2013). Thus, reducing risky host-seeking behaviour during parasite development would increase the chance of parasite transmission as a result of vector survival (Gleave et al., 2016).

In the current study, parasite manipulation of the vector, to reduce risk taking could not be determined. However the mosquito’s behaviour and feeding habits do seem to reduce the chance of consumption by the toad host. For example, as mentioned above mosquitoes were attracted to calling guttural toads, and would almost instantly hone in on a calling

individual. However, as soon as the toad stopped calling the mosquito would find a suitable place to land and wait. As soon as the toad resumed calling the mosquitoes, depending on its distance to the toad, would hop or fly onto the calling individual and start feeding. This start-stop strategy reduces the risk of consumption by the host; firstly by approaching a preoccupied calling toad that is less likely to feed; and secondly, immediate landing ensures the mosquito does not attract the toad's attention in between calling bouts. This strategy may ensure the parasites survival in the mosquitos and increases chance of transmission to a new host. Vocal signals that male anurans use to call for females are crucial for reproductive success in frogs. However calling is an energy taxing activity, favouring larger and stronger frogs that are able to indicate their fitness through continuously calling efforts (Phelps et al., 2005; Wells and Schwartz, 2007). These traits are not only attractive to female anurans, but can also increase preference of micropredators, such as eavesdropping flies (Bernal et al., 2006; de Silva et al., 2015; Bernal and Pinto, 2016). According to Bernal et al. (2006) eavesdroppers use host signals to assess quality and fitness, preferentially feeding on louder or larger individuals. Host fitness, if selected for by the vector, can also be advantageous for the parasite. For example in the present study "louder" calls (using play backs) were observed to attract higher numbers of mosquitoes. Thus if the vector prefers feeding on hosts with the loudest call, then the parasite has an increased chance of being transferred to a stronger, larger or fitter individual. A healthy host is important for the survival of the parasite and continuance of transmission to new vectors and ultimately new hosts (Tseng and Myers, 2014). Other possibilities for increased transmission success of the parasite could be if the adult nematodes are able to detect elevated levels of testosterone in the host during breeding. This has been shown to increase parasite transmission potential, in male-biased host-parasite occurrences (Grear et al., 2009). Several parasitic groups and taxa, including blood parasites, possess adaptations that synchronize the intensity of their infective stages to the feeding habits of their vectors (Dolnik et al., 2011; Cornet et al., 2014; Martinez-Bakker and Helm, 2015; Reece et al., 2017). Although most of these scenarios are based on chemical cues, an alternative prospect could be a physical signal that synchronizes parasite infective stages (e.g. microfilariae) with vector presence. For example if the vibrations caused by the call of the toad both attract mosquitoes to feed and stimulated the adult filarial worms to release their microfilariae. This would be interesting to test experimentally. Based on the scenarios discussed above, it is clear that although some blood parasites have the ability to alter host and vector behaviour, both the parasite and the vector are reliant on the host for survival. Thus, in general not the host, nor the parasite or the vector can be considered the dominant group.

How many blood parasites of anurans are there?

Parasite systematics provides the foundation for our understanding of parasitism in ecosystems (Combes, 1996; Marcogliese and Cone, 1997; Adlard et al., 2015). Parasites are also often claimed to represent a large portion of the Earth's total biodiversity (Dobson et al., 2008; Poulin, 2014). However, the inability to estimate the true parasite diversity of almost any parasitic group highlights the large gap in our understanding of the biological world. Although it seems like an impossible task to gather enough information to be able to estimate total parasite biodiversity, it is important to realise that each contribution is valuable, given that it is done accurately and correctly. Determining the true parasite diversity will require numerous resources as well as data on parasite taxa through extensive field-based research efforts focused on parasite diversity, and geographical ranges (Adlard et al., 2015). Poulin (2014), further highlights that it is not worth attempting predictions of any parasite diversity, in groups or from areas with limited data available.

Anuran blood parasites are no exception, with still many species continually being discovered. In the present study, where a small area was extensively surveyed, lead to the discovery and description of several species. However, this was in a biodiversity hotspot, thus an area with high host species richness, is according to Poulin (2014) one of the main drivers of parasite species richness. Future multi-approach studies on anuran blood parasites, from different areas and host species will undoubtedly contribute to our understanding of the diversity of these parasites. However, the availability of host species could control the parasite richness if the host diversity is low, parasite transmission among hosts can decrease (Moens and Pérez-Tris, 2016). Thus although it may not be impossible to predict total anuran blood parasite diversity, one may be able to predict the diversity of an area base on the host species richness.

Societal relevance

According to the South African National Environmental Management Biodiversity Act 10 of 2004 (NEMBA) and the Global Taxonomy Initiative, South Africa's biodiversity is a national priority that is important and should be managed correctly (Klopper et al., 2002; NEMBA, 2004). To achieve this, management and decision-making of biodiversity conservation and its sustainable use in the relevant economic sectors should be addressed. Policy-makers rely on information and data provided by scientists in the relevant fields of conservation biology, biodiversity, and ecology, to help make informed decision on the way forward. South Africa's 2nd National Biodiversity Strategy and Action Plan 2015-2025 (NBSAP) declares that biodiversity provides South Africa's people with a rich heritage of nature based cultural traditions, reiterating the significance of wildlife to the country's cultures (NBSAP, 2015). The

NBSAP acknowledges that biodiversity is not as well understood as it should be, thus there is a need to communicate better to the general public.

Box 2: *National Acts on research and biodiversity*

NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT 10 OF 2004

To provide for the management and conservation of South Africa's biodiversity within the framework of the National Environmental Management Act, 1998; the protection of species and ecosystems that warrant national protection; the sustainable use of indigenous biological resources; the fair and equitable sharing of benefits arising from bioprospecting involving indigenous biological resources; the establishment and functions of a South African National Biodiversity Institute; and for matters connected therewith (NEMBA, 2004).

NATIONAL RESEARCH FOUNDATION ACT 23 OF 1998

To provide for the promotion of research, both basic and applied, and the extension and transfer of knowledge in the various fields of science and technology and indigenous technology; and for this purpose to provide for the establishment of a National Research Foundation; and to provide for incidental matters. NRFA

Through sharing the outcomes of this research, the relationship with the authorities of Ezemvelo KZN Wildlife, specifically those of the Ndumo Game Reserve and Tembe Elephant Park has been strengthened. In doing so these parks have made themselves accessible and have helped us engage with the local community, providing the perfect opportunity to broaden the public's knowledge about the complexity of biodiversity and how its conservation is inevitably linked to their well-being.

Throughout this PhD we have worked continually with the local community within the study area, and have held several workshops to inform and train members form the community and Ezemvelo KZN wildlife staff from the Ndumo Game Reserve and Tembe Elephant Park. This was to educate them on 1) the rich amphibian biodiversity present in the area, 2) why it is important to protect this biodiversity, and 3) how the diversity of these frogs can be used as a sustainable eco-tourism initiative (see Fig. 5A-C). As a by-product of the research data obtained on the frog hosts during this PhD study, a Masters project on amphibian diversity and community-based ecotourism in Ndumo Game Reserve, South Africa was co-supervised and completed (Phaka, 2018). The aim of this study was to "bridge the gap" between the "researcher" and local members of the community, providing them with knowledge on the importance of conserving biodiversity and how frogs can serve as a tool in eco-tourism. Furthermore, this masters study led to the publication of the first bilingual field guide on the frogs of Zululand in both the English and isiZulu languages (see Fig. 5D)

(Phaka et al., 2017), aiming to educate and raise awareness in the broader community which is necessary for amphibian and habitat conservation efforts. This guide was freely distributed throughout northern KZN, primarily at schools. Although, this transfer of knowledge is more focused on the host and not the parasites themselves, it is firstly important transfer knowledge to the tangible biodiversity (the frog hosts), and the role these frogs play in the ecosystem, for example, the controlling of diseases by the frogs consuming large amounts of vectors, before focusing on the diversity of the parasites found in the frogs.

Fig. 5. Illustrates some of the societal impacts this study had directly on the broader community within the study area. A: Advertising sign for frog eco-tourism within the Ndumo Game Reserve, run by local guides. **B:** Group photo taken after an educational workshop held for community members from the Tembe tribe in northern KZN. **C:** Training and educational workshop held for the local guides and game rangers. **D:** The front cover of the bilingual field guide to the frogs of Zululand.

These educational engagements align with the National Research Foundation's mandate to “Support and promote research through funding, human capacity development and the provision of the necessary research facilities, in order to facilitate the creation of knowledge, innovation and development in all fields of science and technology” as outlined in the National Research Foundation Act 23 of 1998.

This multi-approach study of frog blood parasites in northern KZN provides a lager dataset contributing to our knowledge on the diversity, taxonomy and in some cases their host-parasite relationships. In the long run, this research may help piece together the complex evolution of these microscopic yet economically and ecologically important organisms. Furthermore, parasites are recently been more widely accepted as major influencers in functioning ecosystems, and studying the relationships between the host, parasite, and vector provides data on the ecological integration between trophic levels in an ecosystem (Combes, 1996; Poulin, 1999). In the current study results indicate that frog blood parasites are sensitive to their surrounding ecosystems and could potentially indicate healthy ecosystems and intact food webs.

Conclusion

This study explored the efficacy of a large multi-species, multi-approach survey to explore the diversity of the anuran blood parasites from northern KwaZulu-Natal, South Africa. Knowledge of species diversity is one of the primary biological building blocks to understanding any group of organisms. From there one can gather knowledge of ecology and evolutionary biology of that organism or group of organisms. Globally, data and diversity of anuran blood parasites is underestimated. The current global diversity of anuran blood parasite species comprises 48 species of *Hepatozoon*, three species of *Dactylosoma*, 10 species of haemococcidia, approximately 67 species of *Trypanosoma*, and 32 species of filarial nematodes (Waltonellinae) (Bardsley and Harmsen, 1973; Ray and Choudhury, 1983; Barta, 1991; Paperna et al., 2009; Bain et al., 2013; Netherlands, 2014; Attias et al., 2016; Bernal and Pinto, 2016; Netherlands et al., 2018). Compared to the present study, that found six species of *Hepatozoon*, a single species of *Dactylosoma*, three species of haemococcidia, several trypanosome morphotypes, and a single species of filarial nematode (Waltonellinae). These results established a foundation of the blood parasite biodiversity in northern KZN, indicating that this area is not only rich in anuran diversity, but also their blood parasites. Furthermore, this study provides a baseline for future taxonomic and ecological work on these parasite groups, not only in South Africa but also globally.

Future work and perspectives

This thesis provides a baseline for future studies of diversity, ecological, and phylogenetic relationships. Taxonomy remains an important component for any biodiversity related studies (Poulin, 2014). Thus it is important to continue describing new species encountered and to document the host and geographical distribution of species already described. From the data collected in the present study several species across the different blood parasite groups remain undescribed and these description will be completed in the future. Although a seemingly high number of species of *Trypanosoma* were also observed infecting several different anurans from the study area, these species still require morphological and molecular characterisation to determine their diversity and identify (new or valid species). Other future prospects include expanding the dataset to include different habitat types and different host species. South Africa has a rich anuran fauna with an increase of species diversity northwards, stretching along the coast from the southwestern Cape to KZN, with an increase of endemic species southwards towards the southwestern Cape (Du Preez and Carruthers, 2017). Comparing the blood parasite species diversity found in northern KZN (current study), to other areas may provide us with better insights to the biogeographical distributions of these parasites. Also, comparing parasite distribution to host distribution may provide insight on the parasite sensitivity to varying ecosystem. For example certain anurans may have distribution ranges covering several different biomes or habitats, which could influence parasite occurrences. Although northern KZN is known for its rich anuran diversity, there are less endemic species as compared to the southwestern Cape. Could that mean that blood parasites found in the endemic hosts are more likely to be specialist parasite species? Or would these areas favour more robust generalist parasite species? The anurans from northern KZN are assumed to contain a high diversity of blood parasites, however, no comparative studies have been carried out in other areas of South Africa. Only once comparative data is collected, can a conclusion on the diversity of blood parasites found in KZN be debated.

This thesis has provided a suitable framework for future ecological studies on anuran blood parasites. Several species of haematophagous dipterans were observed feeding on anurans within the study area. These dipterans are the potential vectors of at lease some, if not several blood parasite species and genera. In the current study mosquitoes were transported to the lab to complete the life history of a filarial nematode. If these mosquitoes could be cultured in a laboratory, then more robust experimental studies on the life cycle and transmission can be conducted. This may help determine the time it takes for the infective larvae transmitted by the mosquitoes to reach sexual maturity in the vertebrate host, or even how many new hosts one parasitised mosquito can infect. Cultured mosquitoes can also be

used to experimentally elucidate the life history of other parasite taxa. For example, although developmental stages of a species of *Dactylosoma* were observed in mosquitoes collected in the current study, the life history still needs to be elucidated, before these mosquitoes can be considered the vectors of these parasites. Furthermore, although observations on vector manipulation by blood parasites were not observed in the present study, it is possible that certain species, do manipulate their host in increase chance of transmission. If this is the case then it would be interesting to determine if the generalist or specialist parasites are more prone to host manipulation, as this would provide insight of the strategies these parasites may use to increase chance of transmission.

The use of molecular tools was shown to be a major advantage in the determining species identity and classification, especially for species with minimal morphological variations, however this was mainly based on a single locus (18S rDNA). Furthermore, mixed infections of blood parasites are common in anurans, and conservative markers have trouble in distinguishing between closely related genera. Recently the complete mitochondrial genome of *Hepatozoon catesbianae* (Stebbins, 1904) was obtained (Leveille et al., 2014), which may provide a bases from which to work in the development of faster evolving markers, for more insightful phylogenetic comparisons between apicomplexan blood parasites. Whole-genome sequencing using Next-Generation Sequencing (NGS), may enhanced our understanding of the adaptations of blood parasites to their respective host environments (Kelly et al., 2014). Host-parasite coevolution is also an attractive and relevant field of study, co-phylogenetic studies are based on the assessment of the similarity between a host tree and a parasite tree and assessing the relationship contribution of co-speciation, duplication, host-switching and sorting (Vanhove and Huyse, 2015). This is particularly of interest for blood parasites, since some of these parasites seem to have a wide range of host species.

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APPENDIX CHAPTER 2

Dataset S1. Sampling data of anurans collected from several sampling localities in South Africa and Belgium.

Dataset S1 is available in Mendeley Data (http://dx.doi.org/10.17632/b88z6k5hjz.1)

Table S1 (1-18). Model corrected estimates of divergence using between partial 18S rDNA sequences from the haemogregarines species used in the current study. Distance matrix showing ranges for the model-corrected genetic distances between the sequences downloaded from GenBank and compared to *Dactylosoma* sp. 1 isolated from *Ptychadena anchietae* and *Sclerophrys gutturalis* from South Africa and *Dactylosoma* sp. (*splendens*) isolated from *Pelophylax lessonae* from Belgium (represented in bold). Alignment length 1637 nt. Genetic distances shown as percentage (%).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. MG041595 *H. tenuis* ex *Afrixalus fornasini*																		
2. MG041591 *H. involucrum* ex *Hyperolius marmoratu*	1																	
3. MG041605 *H. theileri* ex *Ametia delalandii*	1,7	2																
4. MG041604 *H. ixoxo* ex *Sclerophrys pusilla*	1,3	1,6	1,4															
5. MG041600 *H. thori* ex *Hyperolius marmoratus*	1,7	1,8	1,9	1,4														
6. HQ224963 *H.* cf. *clamatae* ex *Lithobates clamitans*	2,4	2,5	2,5	1,7	2,3													
7. HQ224954 H. cf. catesbianae ex Lithobates catesbeianus	2,3	2,5	2,4	1,6	2,2	0,3												
8. HQ224960 *H. magna* ex *Pelophylax* kl. *esculentus*	1,8	2,2	2,1	1,8	1,8	1,9	2											
9. MG519501 *H. angeladaviesae* ex *Philothamnus semivariegatus*	7,8	8,7	8,3	7,9	8,2	8,2	8,2	7,7										
10. MG519504 *H. cecilhoarei* ex *Philothamnus natalensis natalensis*	7,6	8,5	8,2	7,8	8,1	8,3	8,2	7,6	0,9									
11. JN181157 *H. sipedon* ex *Nerodia sipedon sipedon*	9,1	9,3	9,3	9	9,5	9,8	9,7	8,9	8,9	9,2								
12. AY600625 *H.* cf. *erhardovae* ex *Clethrionomys glareolus*	5,9	6,4	5,8	5,9	6,1	5,7	6	5,5	7	7,3	7,7							
13. EF222259 *H.* sp. ex *Sciurus vulgaris*	8	8,5	7,8	8,2	8,3	8	8,1	7,8	9,2	9,5	9,7	3						
14. FJ719815 *H.* sp. ex *Abrothrix olivaceus*	5,9	6,3	5,6	5,9	6,1	5,7	5,9	5,1	7,2	7,7	7,9	1,4	3,2					
15. EF157822 *H. ayorgbor* ex *Python regius*	5,6	6,1	5,4	5,7	6	5,5	5,8	5,4	6,7	7	7	1	2,7	1,1				
16. KF939620 *H. chinensis* ex *Elaphe carinata*	4	4,2	3,8	4	4,1	4,2	4	3,5	5,9	5,8	6,8	0,8	2,5	0,6	0,3			
17. FJ719813 *H.* sp. ex *Dromiciops gliroides*	4,5	4,9	4,1	4,4	4,6	4,5	4,5	4,4	6,3	6,2	6,3	3	4,8	3,1	2,3	1,5		
18. KF022102 *H. peircei* ex *Oceanodroma melania*	8,4	8,6	8,2	8,3	8,7	8,4	8,2	8,3	9,9	9,7	9,6	6,5	9	6,4	6,2	4,5	4,6	
19. KF992698 *Hemolivia mauritanica* ex *Testudo marginata*	6,6	6,9	6,6	6,8	6,8	7	7	6,1	7,3	7,4	8,7	4,2	6,3	4,3	3,8	2,5	2,5	5,2
20. KR069083 *Hemolivia parvula* ex *Kinixys zombensis*	5	5	4,8	4,9	4,7	5,1	4,9	4,1	5,6	5,8	7,9	3,1	4,5	2,7	2,5	2,1	1,4	4
21. KF992713 *Hemolivia* sp. ex *Rhinoclemmys pulcherrima manni*	5,8	6,1	5,7	6	6	6,2	6,2	5,3	6,6	6,7	7,6	3,3	5,3	3,4	2,9	2	1,6	4,3
22. KF992711 *Hemolivia mariae* ex *Testudo graeca*	6,8	6,9	6,7	6,6	6,8	7,2	7,1	6,2	7,1	7,4	8,4	4,3	6,4	4,2	3,7	2,5	2,6	5,6
23. KP881349 *Hemolivia stellata* ex ex *Rhinella marina*	5,4	5,7	5,2	5,6	5,5	5,5	5,8	4,9	6,6	6,7	7,4	3,6	5,4	3,5	3	1,9	2	5,2
24. JX531941 *H.* sp. ex *Algyroides marchi*	4,1	4,4	4,3	4,5	4,5	4,9	4,7	4	6,9	6,9	7,7	3,3	5,2	2,7	2,7	2,6	1,8	5,1
25. JX531928 *H.* sp. ex *Podarcis bocagei*	4,3	4,5	4,5	4,8	4,8	5	4,8	4,3	7,1	7,1	7,6	3,4	5,3	2,8	2,8	2,6	2	5,2
26. KJ461939 *Karyolysus latus* ex *Podarcis muralis*	8	8,3	7	8,3	8,9	8,6	8	7,2	13,2	14	14,4	6,5	6,3	5	5	4,5	3,7	9,4
27. KJ461941 *Karyolysus* sp. ex *Lacerta viridis*	7	7,7	6,5	8,3	8,3	8,5	8	7,1	13,1	14	14,2	6	6,2	5	4,5	4,1	3,2	8,3
28. KJ461940 *Karyolysus lacazei* ex *Lacerta agilis*	7,2	8	6,7	8,6	8,6	8,8	8,3	7,4	13,5	14,3	14,5	6,3	6,5	5,3	4,8	4,3	3,4	8,6

Table S1. Continued (1-18).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
29. JX244269 *H.* sp. ex *Hemorrhois hippocrepis*	7	8	6,8	7,7	8	7,9	7,3	6,7	11	11,9	13,4	6	5,9	4,5	4,3	4	3	8,8
30. KX011039 *Karyolysus paradoxa* ex *Varanus albigularis*	6,2	7,3	5,8	7,3	7,3	7,5	7	6,3	9,8	10,4	11,9	5,2	5,4	4,3	3,9	3,5	2,2	7,6
31. AY471615 *H. canis* ex *Pseudalopex gymnocercus*	7,3	7,6	7,1	7,5	7,1	7,2	7,6	6,4	8,7	8,6	9,7	6	7,9	5,5	5,3	3,5	3,9	7,6
32. EF222257 *H.* sp. ex *Martes martes*	6,2	6,3	5,9	6,2	6,5	6,2	6,4	5,9	7,4	7,4	8,2	5,2	6,9	5	4,4	3,3	2,7	6,2
33. AY620232 *H. felis* ex *Felis catus*	6,2	6,4	6	6,5	6,1	6,3	6,5	5,7	8,4	8,2	8,6	4,8	6,7	4,3	4,1	2,5	2,8	6
34. HQ224959 *Haemogregarina balli* ex *Chelydra serpentina*	10,2	10,6	9,6	10,4	10,3	9,1	10,5	9,4	10	11,6	11,9	7,3	10	7,3	7,1	4,6	6,1	9,5
35. KF992697 *Haemogregarina stepanowi* ex *Mauremys caspica*	9	9,5	8,6	9,2	9,2	9	9,1	8,3	9,5	9,7	9,8	5,8	8,5	6	5,6	4,3	4,7	7,9
36. KM887507 *Haemogregarina sacaliae* ex *Sacalia quadriocellata*	8,7	9	8,3	8,7	8,9	8,5	8,7	7,7	9,5	9,7	9,6	5,7	8,2	5,7	5,7	4,6	4,8	7,7
37. KM887508 *Haemogregarina pellegrini* ex *Malayemys subtrijuga*	8,6	9,1	8,4	9	8,2	8,6	8,8	7,8	9,6	9,3	10,4	5,9	8,5	6,4	5,9	4,5	4,8	8
38. HQ224957 *Dactylosoma ranarum* ex *Pelophylax* kl. *esculentus*	8,1	8,3	8,1	8,4	8,6	8,7	8,7	8,5	9,7	9,7	9,7	6,4	8,1	6,4	5,8	4,6	4,6	7,8
39. *Dactylosoma* sp. (*splendes*) ex *Pelophylax lessonae*	7,7	7,9	7,6	8	8,2	8,2	8,3	8	9,3	9,2	9,3	6	7,7	6,1	5,5	4,3	4,2	7,5
40. *Dactylosoma* sp. 1 ex *Pty. anchietae* / *Scl. gutturalis*	7,6	7,7	7,5	8,1	8,1	8,1	8,1	7,9	9,2	9,1	9,2	5,9	7,6	5,9	5,3	4,3	4,1	7,4
41. DQ096835 *Adelina dimidiata* ex *Scolopendra cingulata*	15,6	15,4	15,5	15,7	15,4	16,1	16,7	15,6	17	17,1	19	13,5	16,2	13,7	13,2	10,8	11,8	15,7
42. DQ096836 *Adelina grylli* ex *Gryllus bimaculatus*	14,1	14,1	14	14,3	14,5	14,3	14,9	14	15,9	15,9	17,4	11,4	13,9	12	11,2	8,7	10,1	13,5
43. HQ224955 *Klossia helicina* ex *Cepaea nemoralis*	16,8	15,9	16,3	16,4	16,5	16,4	17,3	16,1	18,4	18,1	18,7	14,4	16,4	14,6	13,9	11,2	12,5	16,3

Table S1. Continued (19-43). Model corrected estimates of divergence using between partial 18S rDNA sequences from the haemogregarines species used in the current study.

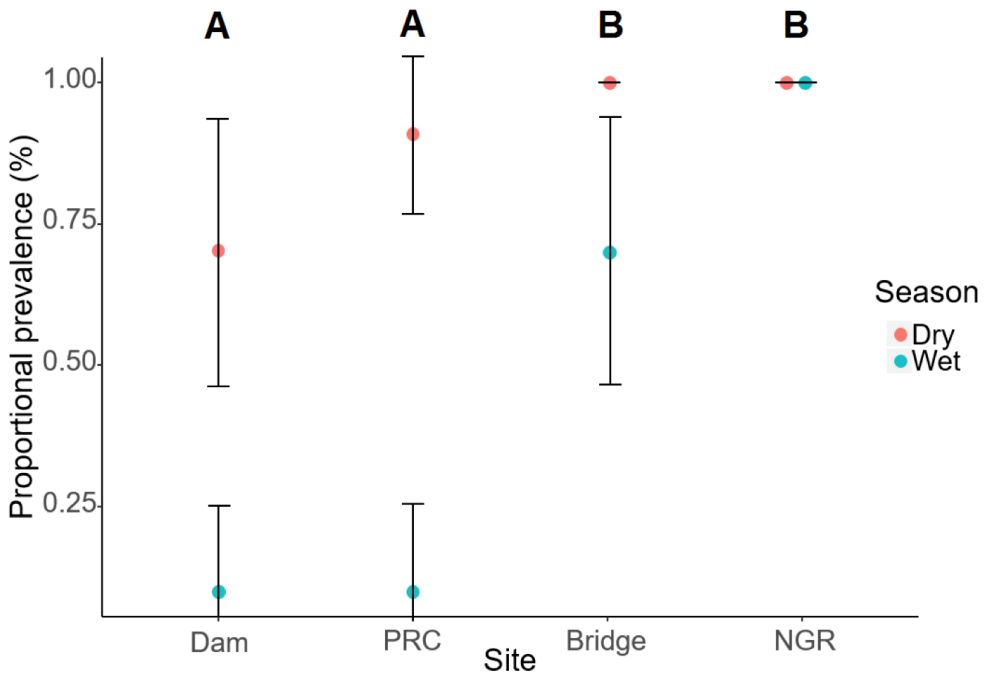
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1. MG041595 *H. tenuis*																									
2. MG041591 *H. involucrum*																									
3. MG041605 *H. theileri*																									
4. MG041604 *H. ixoxo*																									
5. MG041600 *H. thori*																									
6. HQ224963 *H.* cf. *clamatae*																									
7. HQ224954 *H.* cf. catesbianae																									
8. HQ224960 *H. magna*																									
9. MG519501 *H. angeladaviesae*																									
10. MG519504 *H. cecilhoarei*																									
11. JN181157 *H. sipedon*																									
12. AY600625 *H.* cf. *erhardovae*																									
13. EF222259 *H.* sp.																									
14. FJ719815 *H.* sp.																									
15. EF157822 *H. ayorgbor*																									
16. KF939620 *H. chinensis*																									
17. FJ719813 *H.* sp.																									
18. KF022102 *H. peircei*																									
19. KF992698 *Hem. mauritanica*																									

Table S1. Continued (19-43).

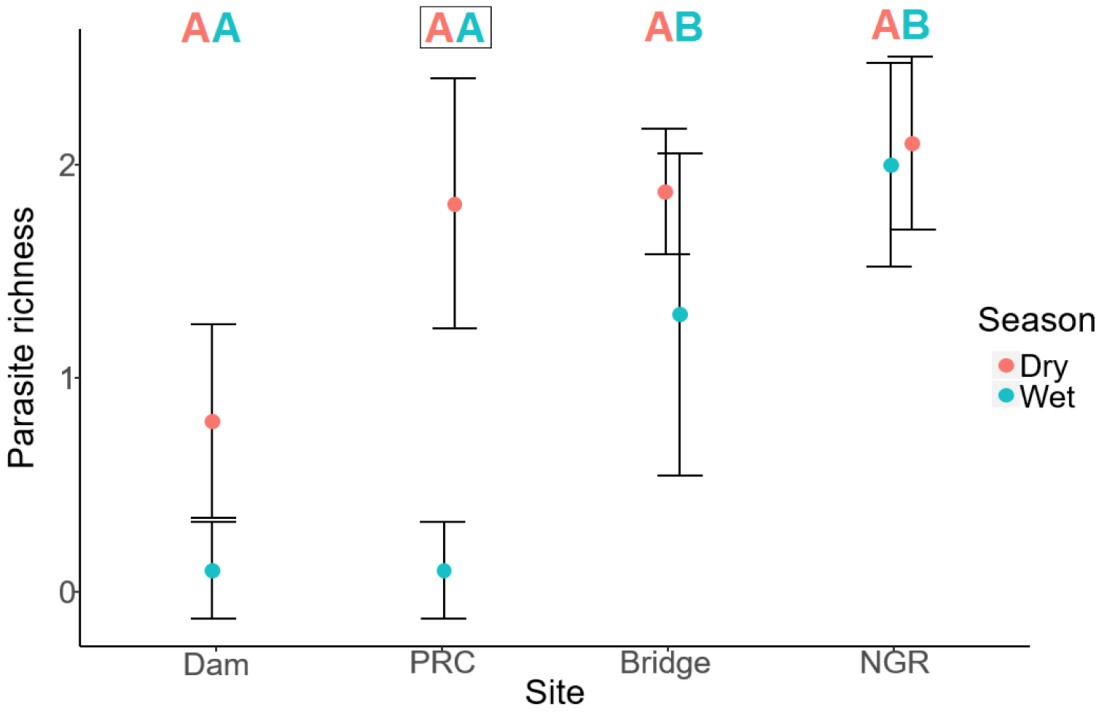
	19	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**
20. KR069083 *Hem. parvula*	0,7																								
21. KF992713 *Hemolivia* sp.	1	0,7																							
22. KF992711 *Hem. mariae*	2	0,8	1,3																						
23. KP881349 *Hem. stellata*	1,5	0,8	0,7	1,3																					
24. JX531941 *Hepatozoon.* sp.	3	2,8	2,5	2,7	2,2																				
25. JX531928 *Hepatozoon.* sp.	3,2	3,1	2,7	2,9	2,5	0,4																			
26. KJ461939 *K. latus*	5,1	4,4	4,4	4,2	4	0,9	1,3																		
27. KJ461941 *Karyolysus* sp.	4,6	4,3	3,9	4	3,5	0,9	1,3	1,1																	
28. KJ461940 *K. lacazei*	4,8	4,6	4,1	4,2	3,7	1,1	1,5	1,3	0,2																
29. JX244269 *Hepatozoon* sp.	4	3,3	3,5	3,1	3,3	2	2,5	2	1,8	1,8															
30. KX011039 *K. paradoxa*	3,2	2,8	2,8	2,8	2,8	2,2	2,6	2,7	1,9	2,1	1,5														
31. AY471615 *H. canis*	4,6	3,2	3,8	4,5	4,2	2,9	3,2	6,3	6,5	6,7	7	5,1													
32. EF222257 *Hepatozoon* sp.	4,5	2,9	3,6	4,1	3,6	2,6	2,9	4,2	3,7	3,9	3,8	3,4	3,7												
33. AY620232 *H. felis*	4,1	2,5	3,2	3,9	3,3	1,8	2	3,5	3,4	3,7	3	3,2	3,3	2,7											
34. HQ224959 *Haem. balli*	5,6	4,6	5	5,7	6,1	4,5	4,8	7,4	7,3	7,4	6,6	5,9	7,9	7,4	6,8										
35. KF992697 *Haem. stepanowi*	5,4	4,6	4,5	5,3	4,5	4,2	4,4	7,4	7,3	7,4	6,9	5,9	6,5	5,8	5,3	0,6									
36. KM887507 *Haem. sacaliae*	5,3	4,7	4,6	5,3	4,6	4,3	4,5	7,6	8,1	8,1	6,9	6,5	6,1	5,8	5,3	1,2	0,7								
37. KM887508 *Haem. pellegrini*	5,1	4,4	4,4	5,4	4,4	4,3	4,5	7,5	7,5	7,6	6,8	6,1	6	5,9	5,2	1,4	0,8	0,9							
38. HQ224957 *D. ranarum*	5,4	4,5	4,7	5,6	4,9	4,8	5,1	9	8,4	8,7	9,3	6,7	6	5,4	5,5	6,6	5,2	5,3	5						
39. *Dactylosoma* sp. (*splendes*)	5,1	4,2	4,4	5,3	4,6	4,5	4,8	8,6	8,1	8,3	9,3	6,5	5,6	5,1	5,2	6,2	4,9	5	4,7	0,2					
40. *Dactylosoma* sp. 1	5	4,1	4,2	5,2	4,4	4,4	4,7	8,5	7,9	8,2	9,1	6,3	5,5	4,9	5,1	6,1	4,8	4,9	4,6	0,5	0,2				
41. DQ096835 *Ade. dimidiata*	11,7	9,9	11	12,3	11,2	10,4	10,8	17,6	17,5	17,9	17,6	13,4	13,2	13,7	13,1	13,9	11,9	12,4	11,7	12,1	11,6	11,7			
42. DQ096836 *Ade. grylli*	10,7	8,7	9,9	11,2	9,5	9,1	9,4	16	16,7	17	17,4	13,8	11,7	11,7	10,9	12,3	11,1	11,2	10,8	10,1	9,7	9,5	3,9		
43. HQ224955 *Klo. helicina*	12,8	11,1	12,1	12,8	12,2	10,9	11,3	17,1	17,5	17,9	17	15,9	14	13,6	13,3	15,5	13,8	13,8	13,8	12,9	12,4	12,5	6,9	6,4	

APPENDIX CHAPTER 3

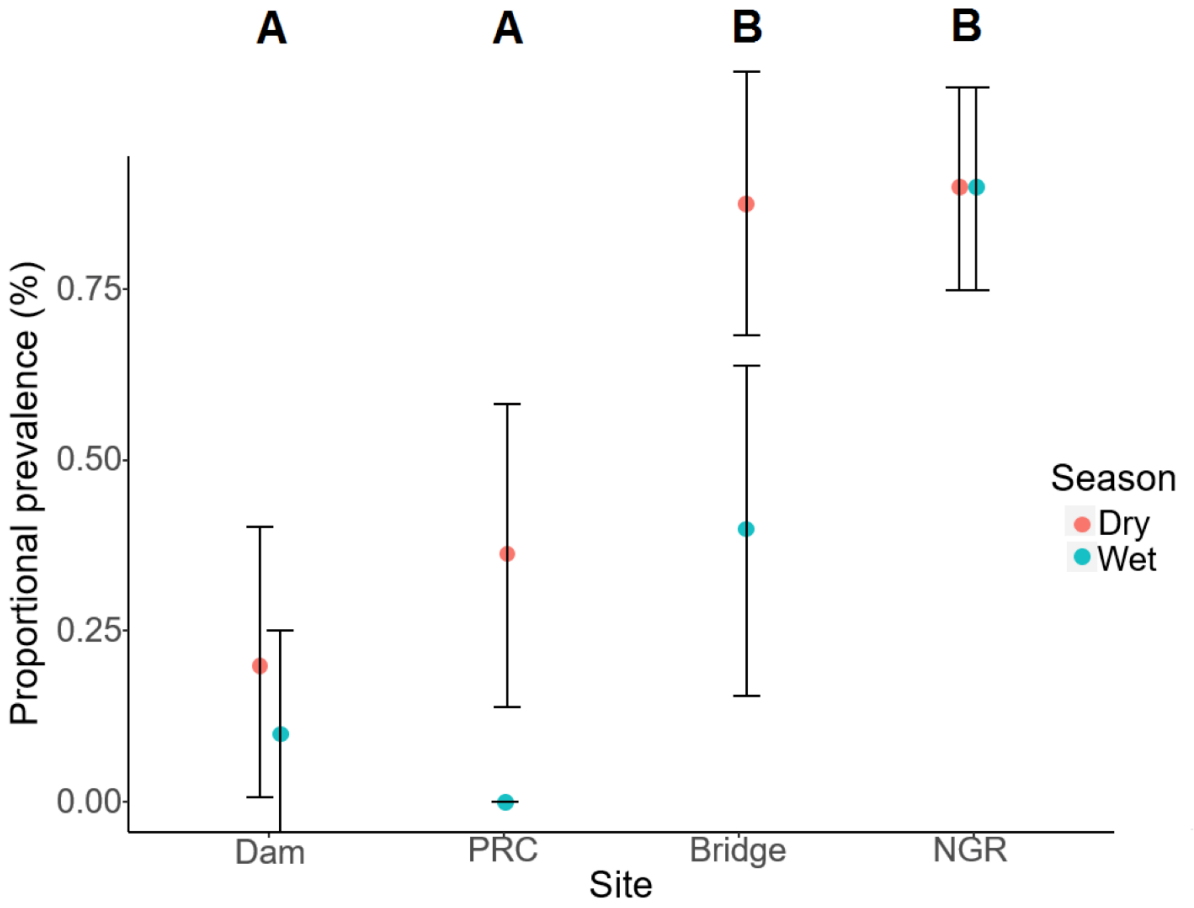
Dataset S1. Sampling data of anurans collected from several sampling localities in South Africa and Belgium. **Dataset S1** is available in Mendeley Data (http://dx.doi.org/10.17632/b88z6k5hjz.1)
Dataset S2. Estimates of divergence between partial 18S rDNA sequences used in the concatenated phylogenetic analysis in the current study. Asterisks (*) indicate the sequences obtained in the current study. Distance matrix showing ranges for the uncorrected p-distances between the sequences. Alignment length 1518 nt. Genetic distances shown as percentage (%). Sequences 1 – 61 corrospond to the COI Dataset S3. **Dataset S2** is available in Mendeley Data (http://dx.doi.org/10.17632/b88z6k5hjz.1)
Dataset S3. Estimates of divergence between partial COI gene sequences used in the concatenated phylogenetic analysis in the current study. Asterisks (*) indicate the sequences obtained in the current study. Distance matrix showing ranges for the uncorrected P-distances between the sequences. Alignment length 758 nt. Genetic distances shown as percentage (%). Sequences 1 – 61 correspond to the 18S Dataset S2. **Dataset S3** is available in Mendeley Data (http://dx.doi.org/10.17632/b88z6k5hjz.1)



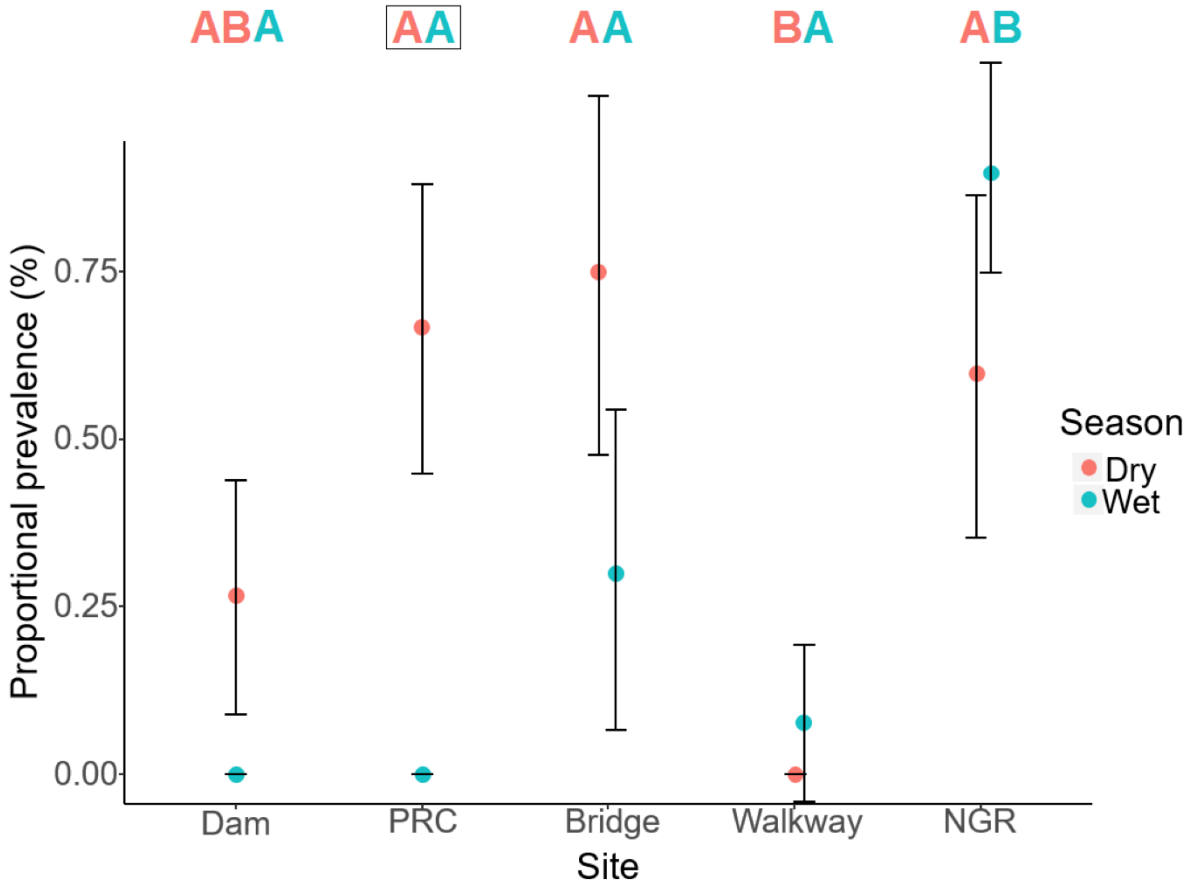
SM 1. Combined parasite prevalence, given as the proportion of individuals infected, in function of sites and season. Whiskers represent the standard error of the mean. Host sample sizes: Dam in the dry season n = 10, Dam in the wet season n = 10, PRC in the dry season n = 11, PRC in the wet season n = 10, Bridge in the dry season n = 8, Bridge in the wet season n = 10, NGR in the dry season n = 10, NGR in the wet season n = 10. Sites with different letters assigned to them are significantly different. For example: the sites marked with an A are significantly different from those marked with a B; Dam and PRC are significantly different from both Bridge and NGR. Data is separated by season because of a significant effect of season. Walkway is not included as site because too few *P. anchietae* hosts were sampled for an accurate analysis.



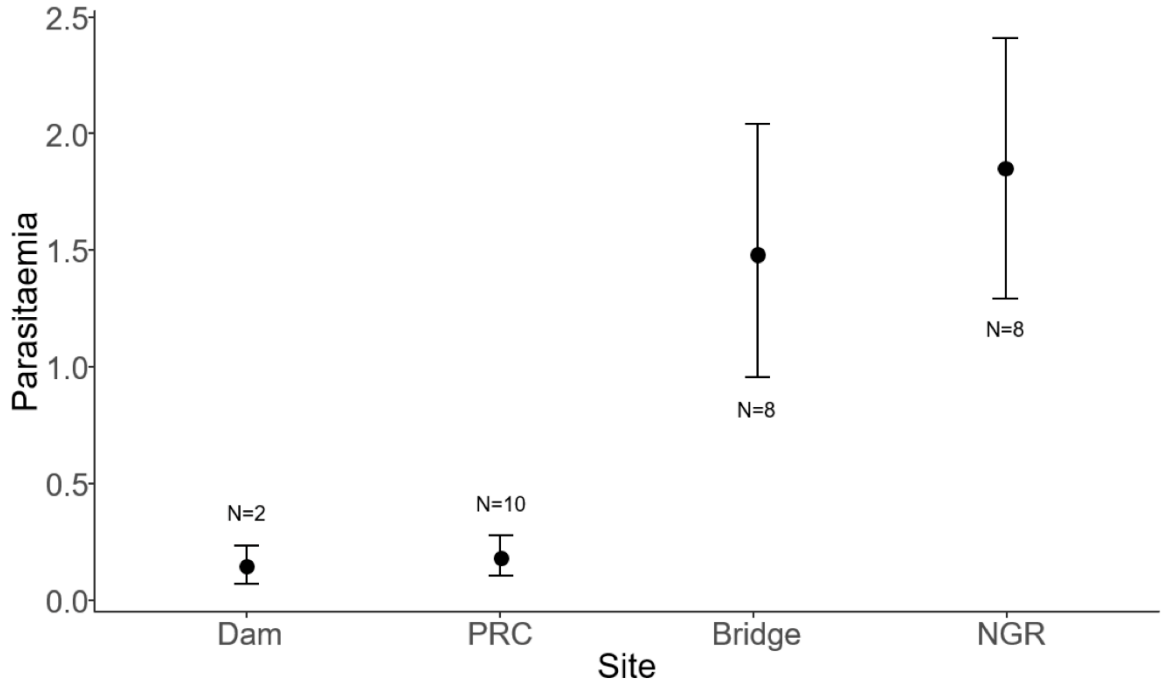
SM 2. Parasite richness in function of sites and season. Whiskers represent the standard error of the mean. Host sample sizes: Dam in the dry season n = 10; Dam in the wet season n = 10; PRC in the dry season n = 11; PRC in the wet season n = 10; Bridge in the dry season n = 8; Bridge in the wet season n = 10; NGR in the dry season n = 10; NGR in the wet season n = 10. Sites with different letters assigned to them are significantly different, colour of the letter corresponds with the season and a rectangle around the letter means that the site is significantly different between seasons. For example: in the wet season, Dam and PRC are significantly different from Bridge and NGR. Seasons had to be assessed separately due to a significant interaction effect (Table 3 and 4), hence the different colours of the letters.



SM 3. *Dactylosoma* sp. 1 prevalence, given as the proportion of individuals infected, in function of sites and season. Whiskers represent the standard error of the mean. Host sample sizes: Dam in the dry season n = 10; Dam in the wet season n = 10; PRC in the dry season n = 11; PRC in the wet season n = 10; Bridge in the dry season n = 8; Bridge in the wet season n = 10; NGR in the dry season n = 10; NGR in the wet season n = 10. Sites with different letters assigned to them are significantly different. For example: the sites marked with an A are significantly different from those marked with a B; Dam and PRC are significantly different from both Bridge and NGR. Data is separated by season because of a significant effect of season. Walkway is not included as site because too few *P. anchietae* hosts were sampled for an accurate analysis.



SM 4. *Trypanosoma* spp. prevalence, given as the proportion of individuals infected, in function of sites and season. Whiskers represent the standard error of the mean. Host sample sizes: Dam in the dry season n = 15, Dam in the wet season n = 10, PRC in the dry season n = 12, PRC in the wet season n = 10, Bridge in the dry season n = 8, Bridge in the wet season n = 10, Walkway in the dry season n = 11, Walkway in the wet season n = 13, NGR in the dry season n = 10, NGR in the wet season n = 10. Sites with different letters assigned to them are significantly different, the colour of the letter corresponds with the season and a rectangle around the letter means that the site is significantly different between seasons. For example: in the wet season, NGR is significantly different from the other sites. Seasons had to be assessed separately due to the interaction effect (Table 3 and 4), hence the different colours of the letters.



SM 5. *Trypanosoma* spp. mean intensity, given as parasitaemia per 4,000 erythrocytes, in function of site. Whiskers represent the standard error of the mean. Walkway is not included as site because there were too few infected individuals for an analysis of mean intensity. A non-parametric Kruskal-Wallis test showed a significant effect of site, but an adequate pairwise post-hoc test could not be performed.

LIST OF PUBLICATIONS

1. Phaka, FM, **Netherlands EC**, Kruger DJ, du Preez, LH. (2019) Folk taxonomy and indigenous names for frogs in Zululand, South Africa. *Journal of Ethnobiology and Ethnomedicine*, 15, 17. https://doi.org/10.1186/s13002-019-0294-3
2. Penzhorn BL, **Netherlands EC**, Cook CA, Smit NJ, Vorster I, Harrison-White RF, Oosthuizen MC. (2018) Occurrence of *Hepatozoon canis* (Adeleorina: Hepatozoidae) and *Anaplasma* spp. (Rickettsiales: Anaplasmataceae) in black-backed jackals (*Canis mesomelas*) in South Africa. *Parasites & Vectors*, 11 (1), 158. https://doi.org/10.1186/s13071-018-2714-y
3. Cook CA, **Netherlands EC**, Van As J, Smit NJ. (2018) Two new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) parasitising species of *Philothamnus* (Ophidia: Colubridae) from South Africa. *Folia Parasitologica*, 65(1), 04. https://doi.org/10.14411/fp.2018.004
4. **Netherlands EC,** Cook CA, du Preez LH, Vanhove MPM, Brendonck L, Smit NJ (2018). Monophyly of the species of *Hepatozoon* (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa. *Parasitology*, 145 (8), 1039–1050. https://doi.org/10.1017/S003118201700213X
5. Minter LR, **Netherlands EC**, du Preez LH (2017): Uncovering a hidden diversity: two new species of Breviceps (Anura: Brevicipitidae) from northern KwaZulu-Natal, South Africa. *Zootaxa,* 4300 (2), 195–216. https://doi.org/10.11646/zootaxa.4300.2.3
6. Welicky R, De Swart J, Gerber R, **Netherlands EC**, Smit NJ (2017): Drought-associated absence of alien invasive anchorworm, Lernaea cyprinacea (Copepoda: Lernaeidae), is related to changes in fish health. *International Journal for Parasitology: Parasites and Wildlife,* 6 (2017), 430–438. https://doi.org/10.1016/j.ijppaw.2017.01.004
7. Conradie R, Cook CA, du Preez LH, Jordaan A, **Netherlands EC** (2017): Ultrastructural comparison of *Hepatozoon ixoxo* and *Hepatozoon theileri* (Adeleorina: Hepatozoidae), parasitising South African anurans. *Journal of Eukaryotic Microbiology*, 64, 193–203. https://doi.org/10.1111/jeu.12351
8. Van As J, Cook CA, **Netherlands EC**, Smit NJ (2016): A new lizard malaria parasite *Plasmodium intabazwe* n. sp. (Apicomplexa: Haemospororida: Plasmodiidae) in the Afromontane *Pseudocordylus melanotus* (Sauria: Cordylidae) with a review of African

saurian malaria parasites. *Parasites & Vectors* 9(1), 437. https://doi.org/10.1186/s13071-016-1702-3

9. Cook CA, **Netherlands EC**, Smit NJ (2016): Redescription, molecular characterization and taxonomic re-evaluation of a unique African monitor lizard haemogregarine *Karyolysus paradoxa* (Dias, 1954) n. comb. (Karyolysidae). *Parasites & Vectors*, 9, 347. https://doi.org/10.1186/s13071-016-1600-8
10. Cook CA, **Netherlands EC**, Smit NJ (2015): First *Hemolivia* from southern Africa: reassigning chelonian *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) to *Hemolivia* (Adeleorina: Karyolysidae). *African Zoology,* 50(2), 165–173. http://dx.doi.org/10.1080/15627020.2015.1044467
11. **Netherlands EC**, Cook, CA, Kruger DJ, du Preez, LH, Smit NJ (2015): Biodiversity of frog haemoparasites from sub-tropical northern KwaZulu-Natal, South Africa. *International Journal for Parasitology: Parasites and Wildlife,* 4(1), 135–141. http://dx.doi.org/10.1016/j.ijppaw.2015.01.003
12. **Netherlands EC**, Cook CA, Smit NJ (2014): *Hepatozoon* species (Adeleorina: Hepatozoidae) of African bufonids, with morphological description and molecular diagnosis of *Hepatozoon ixoxo* sp. nov. parasitising three Amietophrynus species (Anura: Bufonidae). *Parasites & Vectors*, *7*(1), 552. https://doi.org/10.1186/s13071-014-0552-0
13. **Netherlands EC**, Cook CA, Smit NJ, du Preez LH (2014): Redescription and molecular diagnosis of *Hepatozoon theileri* (Laveran, 1905) (Apicomplexa: Adeleorina: Hepatozoidae), infecting *Amietia quecketti* (Anura: Pyxicephalidae). *Folia Parasitologica*, 61(4), 293–300. https://doi.org/10.14411/fp.2014.046

Books:

1. Phaka, FM, **Netherlands EC**, Kruger DJ, du Preez, LH (2017): A Bilingual Field Guide to the Frogs of Zululand. *South African National Biodiversity Institute,* Pretoria. 978-1-928224-19-8