Parasite introduction to the endangered western leopard toad: Spill over or spill back?

N Kruger

22808019

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Supervisor: Prof L du Preez

Co-supervisor: Dr J Measey

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This Dissertation is dedicated to my Father, Mother, Brother, Sister, and Grandparents.

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"The six most important words: 'I admit I made a mistake'. The five most important words: 'You did a good job'. The four most important words: 'What is YOUR opinion?' The three most important words: 'If you please'. The two most important words: 'Thank You'. The one most important word: 'We'. The least important word: 'I'."

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ABSTRACT

South Africa has a diverse anuran fauna consisting of 161 described species. Amphibians are suffering large-scale regressions due to various threats: (1) pollution, (2) habitat engineering and (3) invasive species. The endangered Western Leopard toad (*Sclerophrys pantherina)* endemic to the Western Cape is currently experiencing major external pressures from these above-mentioned threats. The latter being a local invader, namely the Guttural toad (*Sclerophrys gutturalis*), which (through human-assisted translocation) was introduced from KwaZulu-Natal into the native range of the Western Leopard Toad. The direct effects such as predation and competition have received extensive attention, seeing that invasive species can have devastating effects on native fauna.

However, a more neglected field of research is the indirect threats invasive species pose to the native fauna such as interactions with infectious agents, which include spill back and spill over of parasites. The present study aimed to understand this interaction by focusing on the relationship between parasites, native species, and invasive species. The research examined mechanisms such as spill back and spill over for a better understanding of the indirect drives and consequences invasive species may hold. Morphological markers (light microscopy and scanning electron microscopy) as well as molecular markers (COI and 28S) were applied to survey parasites found in collected toads from five populations: (1) native Guttural Toad in KwaZulu-Natal; (2) invasive Guttural Toad isolated in Western Cape; (3) invasive Guttural Toad and native Western Leopard Toad in Western Cape; (4) native Western Leopard Toad isolated in Western Cape; and (5) native Guttural Toad from Potchefstroom. Parasites that were observed was a nasal mite *Lawrencarus eweri* (Lawrence, 1952); a lung nematode *Rhabdias* cf. *africana* Kuzmin, 2001; intestinal nematode *Cosmocerca* sp. Diesing, 1861; intestinal trematode *Mesocoelium* cf. *monodi* Dolfus, 1929; two blood parasites *Hepatozoon ixoxo* Netherlands, Cook, & Smit 2014, as well as *Trypanosoma* sp. Gruby, 1843.

It was found that the introduced species may have vacated their parasites throughout the invasion, possibly due to the ‘enemy release hypothesis’ (Marr *et al.* 2008). This can enhance the invaders competitive ability as defence costs against parasites can be decreased and reproductive rates can increase (Hatcher & Dunn, 2011).

The native Western Leopard Toad population co-existing with invasive Guttural Toad was found to contain less parasites than the isolated native Western Leopard Toad population. In this case, it is possible that the invader decreased the parasite loads of the native population by acting as a ‘sink’ for native parasites (Kelly *et al.,* 2009). If so, native parasites are taken

up by the invader but fail to complete their life cycle due to disorientation and lack of co-evolutionary history (Hempel *et al.,* 2003).

Encysted nematodes (presumably third-stage larvae), collected from native Western Leopard Toads and invasive Guttural Toads from the Western Cape, appear to have a host-size and niche specificity rather than a specificity to the host itself. Few life cycles have been described for nematodes in South African fauna to which a toad acts as an intermediate host for the third-stage larvae. Thus, without further identification and molecular studies it is uncertain whether these cysts in native Western Leopard Toads and invasive Guttural Toads are a result of spill back or spill over.

However, each case of parasite-host relationship is unique since the relationship can be dynamic. This makes it difficult to predict the consequences. Furthermore, as is the case with restoration ecology, irreversible changes may have to be accepted in certain ecosystems that are subject to invading parasites and their introduced hosts (Dunn & Hatcher, 2014).

Keywords: Amphibian, macro-parasite, light-microscopy, scanning electron-microscopy, PCR, spill back, spill over.

OPSOMMING

Suid-Afrika huisves ’n diverse anuran fauna wat tans uit 161 beskryfde spesies bestaan. Amfibieë ly grootskaalse regressie weens verskeie bedreigings: (1) besoedeling, (2) habitatverandering, en (3) indringerspesies. Die bedreigde Westelike Luiperd-skurwepadda (*Sclerophrys pantherina*) wat endemies aan die Wes-Kaap is, is tans onder ingrypende eksterne druk weens hierdie bogenoemde bedreigings. ’n Voorbeeld van laasgenoemde is 'n plaaslike indringer, die Gorrel-skurwepadda (*Sclerophrys gutturalis*) wat (as gevolg van antropogeniese translokasie) van KwaZulu-Natal af ingevoer is in die inheemse terein van die Westelike Luiperd-skurwepadda. Die regstreekse gevolge hiervan, soos predasie en kompetisie, het wyd aandag getrek van navorsers omdat indringerspesies ’n verwoestende uitwerking op die inheemse fauna kan hê.

Tog is daar 'n navorsingsterrein wat meer verwaarloos is. Dit behels die onregstreekse bedreigings wat indringerspesies inhou vir die inheemse fauna, soos interaksie met parasiete wat oorvloei (spill over) en terugvloei (spill back) van parasiete insluit. Die doel van die huidige studie was om hierdie vorm van interaksie te verstaan deur te fokus op die verhouding tussen parasiete, inheemse spesies, en indringerspesies. Meganismes soos oorvloei en terugvloei is ondersoek om 'n duideliker begrip te vorm van die onregstreekse aandrywing en gevolge wat indringerspesies moontlik vir inheemse spesies kan inhou. Morfologiese merkers (ligmikroskopie en skandeer-elektronmikroskopie) asook molekulêre merkers (COI en 28S) is gebruik om parasiete te monitor wat gevind is in versamelde skurwepaddas uit vyf bevolkings: (1) inheemse Gorrel-skurwepadda in KwaZulu-Natal; (2) indringer Gorrel-skurwepadda geïsoleer in Wes-Kaap; (3) indringer Gorrel-skurwepadda en inheemse Westelike Luiperd-skurwepadda in Wes-Kaap; (4) inheemse Westelike Luiperd-skurwepadda geïsoleer in Wes-Kaap; en (5) inheemse Gorrel-skurwepadda van Potchefstroom. Die volgende parasiete is waargeneem: ’n nasale myt *Lawrencarus eweri* (Lawrence, 1952); 'n long-nematode *Rhabdias* cf. *africana* Kuzmin, 2001; derm-nematode *Cosmocerca* sp. Diesing, 1861; derm-trematood *Mesocoelium* cf. *monodi* Dolfus, 1929; en twee bloedparasiete *Hepatozoon ixoxo* Netherlands, Cook, & Smit 2014, en *Trypanosoma* sp. Gruby, 1843.

Daar is gevind dat die indringerspesies moontlik hulle parasiete kan ontruim deurlopend deur die inval in die nuwe omgewing, moontlik as gevolg van die sogenaamde ‘vyand-vrylatingshipotese’ (Marr *et al.,* 2008). Dit kan die kompeterende vermöe van die indringer spesie verbeter as gevolg van verdediging kostes teen parasiete wat verminder en reproduktiewe kostes toeneem (Hatcher & Dunn, 2011).

Die inheemse Westelike Luiperd-skurwepadda bevolking wat met die indringer Gorrel skurwepadda bevolking in die Wes-Kaap saambestaan, bevat minder parasiete as die geïsoleerde inheemse Westelike Luiperd-skurwepadda bevolking. In hierdie geval is dit moontlik dat die indringer die parasietlading van inheemse spesies verminder het deur as ‘resevoir’ vir die plaaslike parasiete te dien (Kelly *et al.* 2009). Indien wel, word die inheemse parasiete deur die indringerspesie opgeneem, maar slaag nie daarin om hulle lewensiklus te voltooi nie, weens disoriëntasie en 'n gebrek aan mede-evolusionêre geskiedenis (Hempel *et al*., 2003).

Geënsisteerde nematodes (vermoedelik derde-stadium larva), versamel van inheemse Westelike Luiperd-skurwepadda en indringer Gorrel-skurwepadda van die Wes-Kaap, wil voorkom om ‘n gasheer-grootte en nis spesifisiteit te hê eerder as ‘n spesifisiteit vir die gasheer self. Min lewensiklusse is beskryf vir nematood spesies in Suid-Afrikaanse fauna vir wie die skurwe padda as intermediêre gasheer dien. Dit is onseker of die geënsisteerde nematodes ‘n gevolg is van oorvloei of terugvloei sonder verdere identifikasie en molekulêre studies te doen.

Tog moet elke geval as uniek beskou word, aangesien die verhouding tussen parasiete en gashere dinamies kan wees. Gevolglik is dit moeilik om die gevolge te voorspel. Voorts, soos sake met restourasie-ekologie verloop, moet onomkeerbare veranderings verwag word in bepaalde ekosisteme wat oorgeneem is deur indringerparasiete en hulle ingevoerde gashere (Dunn & Hatcher, 2014).

Sleutelwoorde: Amfibieë, makro-parasiete, lig mikroskopie, skandeer elektron mikroskoop, PCR, terugvloei, oorvloei

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AMPHIBIAN INVASIONS AND PARASITES

CHAPTER LAYOUT

This chapter is divided into two main themes. (1) Amphibian invasions and invaders, which will explore amphibian invasions globally and more specifically to the Western Cape, SA. This part will also review the focal species in the current study: *Sclerophrys gutturalis* (Guttural Toad) and *Sclerophrys pantherina* (Western Leopard Toad). (2) Introduced parasites and effects, which will review historical data of parasites found in Africa. This part will also review the role that parasites play during the invasion and after the invasion.

1.1. AMPHIBIANS IN DECLINE

South Africa has a diverse anuran fauna with 161 known species of 33 genera assigned to 13 families. Scientists have identified the leading global threats to amphibian biodiversity as (1) the alteration of the urban and agricultural habitats, (2) collecting and acquiring of natural resources, (3) introduction of species, and (4) general climate change (Rahbek & Colwell, 2011). Furthermore, researchers estimate that the present extinction rate of species globally is 100-1 000 times faster than pre–human rates (Pimm *et al.*, 1995). Anthropogenic activities are affecting all vertebrate classes, but amphibians appear to be impacted more severely affected than other vertebrates (Beebee & Griffiths, 2005).

Conferring to historical statistics; amphibians are experiencing large-scale regressions in species variety since at least the 1970's (Stuart *et al.,* 2004). During the First World Congress of Herpetology (1989), the concerns over the decline of amphibian populations were highlighted, and the need for an accurate assessment of the level of these populations globally became obvious. It was established that a form of population decline was apparent in 43.2% (2 468) of amphibian species, with 32.5% (1 856) listed on the IUCN Red Data List as globally threatened. However, alarmingly 22.5% (1 294) of species was found data deficient, which may imply that the level of threat is grossly underestimated, and it is greatly feasible that a noteworthy portion of these species are threatened worldwide (Stuart *et al.*, 2004).

1.2. Introduction of invasive species

The swelling ecological, conservational, and economic problems in ecosystems in the world are caused by invasive species and the threats they pose (Dunn *et al.*, 2014; Pimentel, 2002; Wilcove *et al.*, 1998; Kraus, 2009).

1.2.1. Concept of invasive species

The Invasive Species Advisory Committee (ISAC, 2006) explains invasive species as "a species that is non-native to the ecosystem under consideration and whose introduction does or is likely to cause economic or environmental harm or harm to human health" (Dunn *et al.*, 2014).

Invasions is one of the main originators of ecological novelty (Dunn *et al.*, 2014) and introduced species can disturb ecological organization by imposing novel species interactions and altering current ones (Sax *et al.*, 2005). Anthropogenic impact on the biological and physical system of the Earth caused dramatic changes to the distribution of species. One of the major routes to introduce most invasive species is through anthropogenic transport. Global transport of animals increased exponentially and as a result opportunities to translocate species with the prospective potential to spread diseases and parasites are growing, coupled with the growth in economic expansion, global trade, and transportation (Dobson *et al.*, 2008).

Invaders can alter (often reduces) the native species abundance (Thieltges *et al.*, 2009). This occurs owing to a range of direct interactions such as habitat engineering, predation, and competition, which heightens the concern for the native species. However, a more understated and unrecognised indirect consequence of invaders is their potential interference with interactions of native parasite hosts (Thieltges *et al.*, 2009; Taraschewski, 2006). Invaders may affect these even though they seldom develop infections with native parasites since they are often host specific (Thieltges *et al.*, 2009; Torchin *et al.*, 2003).

Even if introduced species are not sympatric in their distribution with native species, certain environmental characters such as river systems and wetlands can function as connectivity corridors to distribute parasites. This complicates the outcomes among native and invasive species by the intimate associations established concerning parasites in wildlife (Adlard *et al.,* 2014). The aquatic ecosystem can function as an important corridor to translocate and transmit aquatic parasites as well as parasites with free-living stages. It also facilitates transmission of infective stages to intermediate or final hosts. It is speculated that parasitism arose in aquatic ecosystems, and thus parasites are extraordinarily diverse (Adlard *et al.*,

2014). The high parasite diversity and richness that can be observed currently in aquatic ecosystems reflects the extended evolutionary history of host and parasite life as it is found in freshwater and marine environments (Rohde, 2005). Among the utmost diverse parasites recognized from aquatic organizations are cestodes, monogeneans and trematodes, with numerous species recognized from each taxon (WoRMS, 2014).

1.2.2. Amphibian invasions

Human assisted distribution of vertebrate fauna (whether unintentional or deliberate) covers an immense list of species, however, only moderately few amphibians have succeeded to establish invasive populations in their novel habitats. The sustainability of the novel habitat, the prevailing climate, and the ability of the introduced species to withstand these changes can all influence the probability of successful invasions (Pitt *et al.*, 2005; Dunn & Hatcher, 2014).

Amphibians are often characterised as generalist, which exhibit several characteristics such as a high reproductive rate allowing for rapid population growth, and the ability to withstand random occurrences. These species are usually inconspicuous, which allows them to remain undetected until the population has been established. A further characteristic is the species' generalised diet that helps them exploit the resources available in the novel habitat (Pitt *et al.*, 2005). These customarily are the traits of the most successful invaders, even though the probability of establishing a successful invasion often relies on both suitable climate and habitat (Simberloff & Von Halle, 1999).

Globally, a limited number of amphibians have become problematic both ecologically and economically due to translocation (Kraus, 2009). However several of these introductions have a devastating effect on the native biota. Threats such as habitat loss, pollution, and climate change are presently being rivalled by the disruptions caused by introduced species (Kraus, 2009). This can be a result of the irreversible nature of several alien invasions, these invasion are less susceptible to rectification, unlike numerous other ecological problems.

Scientists discovered that numerous amphibians have been introduced across the globe as indicated by a compendium of anuran introductions compiled by Kraus (2009). An estimate of approximately 81 amphibian species has proven to be successful invaders, and several to be particularly damaging. These invaders include the following:

- the American bullfrog, *Lithobates catesbeianus* (Shaw, 1802);
- the Cane Toad, *Rhinella marina* (Linnaeus, 1758);
- the Guttural Toad, *Sclerophrys gutturalis* (Power, 1927);
- the African clawed frog, *Xenopus leavis* (Daudin, 1802);

- the common Coqui, *Eleutherodactylus coqui* Thomas, 1966;
- the Cuban Tree frog, *Osteopilus septentrionalis* (Dumeril & Bibron, 1841); and
- The Asian Toad, *Duttaphrynus melanostictus* (Schneider, 1799).

Of the above-mentioned invaders, *L. catesbeianus*, *R. marina* and *E. coqui* have been registered by the 'Global Invasive Species Database as part of the top 100 of the world's worst invasive species' (Lowe *et al.*, 2000). A recent study by Measey *et al.* (2016) outlined the importance of assessing the impact of alien species on an environment. The author found that some amphibians can have devastating effects to the environment of their novel (introduced) ranges.

The Cane toad, *Rhinella marina,* is one of the invasive amphibians that are most widely researched. The distribution of this species occupies a wide range: from southern Texas, USA, and extending south through Central America, until northern South–America (Telford, 2015; Slade & Moritz, 2013). This species has also been introduced into, and established at various regions around the world such as Hawaii, Australia, and Bermuda, to mention a few (Kraus, 2009). The species as such was introduced as measure for bio–control in 1935 in northern Queensland, Australia. The original aim was to regulate the sugar cane pests: grey-backed beetle (*Dermolepida albohirtum*), and the Frenchi beetle (*Lepidiota frenchi*) (Slade & Moritz, 2013).

These toads, however, failed to control the pests and ultimately flourished as an invasive species by exploiting ecosystem functions and expanding rapidly throughout immense ranges of Australia (Lampo & De Leo, 1998). *Rhinella marina* has proven to be a potential ecological hazard. The ecological effects within Australia indicate that the species can alter communities severely and impact dynamics of ecosystems significantly.

To illustrate the point above: a recent study by Lettoof *et al.* (2013) assessed the effect of *R. marina* on the parasite burdens of the native Australian frogs. These scholars found that contrary to the belief that toad invasion is connected with reduced parasite burdens in native frogs, it rather proved that these toads did not seem to transmit any new parasites to native frog populations. Instead, this species may have reduced frog-parasite quantities by assimilating native parasites that are then destroyed by the toad's immune defences (Lettoof *et al.,* 2013). The impact of this introduction on Australia's native biota are severe and particularly concerning as the region is home to numerous endemic, rare, and endangered species. The mentioned study, among several others, highlights the potential effect of a toad with generalist life-history characters when introduced to novel ranges. Although not always negative, these forms of impact should be examined to assess the changes in community structure and diversity.

1.2.3. Amphibian invasions in the Western Cape.

There are 3 globally recognised biodiversity hotspots in South Africa and the Western Cape (Western Cape) plays host to one. This province contains a variety of endemic amphibian diversity. This is owing to the compatibility of the diverse topography and hydrological stability of the Cape Fold Mountains (Measey & Davies, 2011; Poynton, 1964). Two local amphibian invaders have managed to establish populations within the Western Cape region (Measey & Davies, 2011). These invaders are the Guttural Toad, *Sclerophrys gutturalis*, and the Painted Reed Frog, *Hyperolius marmoratus* (Rapp, 1842).These species have successfully invaded and established themselves in this region. As a result, problematic populations in the Western Cape are emerging and they pose a variety of threats to the native biota (Measey *et al.,* in press)

Hyperolius marmoratus was first noticed in the Cape as recent as 2006 and today it is widely distributed throughout the province. At present this species is encountered in garden ponds and farm dams across most of the parts of the province with the wettest climate (Davies *et al.,* 2015). It was found that the invasive populations were established as a result of multiple human-mediated introductions. These entail jump dispersals from their ancestral ranges in the northern and central KwaZulu-Natal, the Eastern Cape and the Southern Cape, as indicated by Tolley *et al.* (2008).

These findings are comparable to the 'translocation hypothesis for *S. gutturalis'.* Therefore, it is assumed that this frog species was introduced accidentally through vectors such as landscaping, hitchhiking on cars, trains, boats, caravans, or the moving of building material (Measey & Davies, 2011; Telford, 2015). A recent study by Telford (2015) proved that the invasive population in Cape Town originated from an 'eastern clade population', which in turn has a wide-ranging dispersal from southern KwaZulu-Natal northwards into Limpopo and Mpumalanga provinces.

1.3. *Sclerophrys gutturalis*: A case study

1.3.1. *Sclerophrys gutturalis* (Power, 1927)

Sclerophrys gutturalis, also known as the Guttural Toad, is a large toad with a snout–vent length of up to 140 mm widely distributed species (Channing, 2001). This species ranges from Kenya, and Somalia to South Africa and Lesotho (Channing, 2001; Du Preez *et al.*, 2004). However, it is absent from more arid regions in southern Africa such as southern Namibia and the southern part of South Africa (as seen in Fig. 1.1 below) (Channing, 2001). Guttural Toads are habitable in a wide variety of environments such as savannahs, grassland, and thickets that range from sea level to ~1 900 m. Hence, this species is highly adaptable to changing environments and can adjust to urban areas where it often occupies garden ponds (Channing, 2001; Du Preez *et al.,* 2004).

The toads are prolific breeders and one breeding couple can lay 15 000 to 25 000 eggs in a single clutch (Wager, 1986; Channing, 2001; Du Preez *et al.*, 2004). One clutch contains two singular gelatinous strings of eggs. These are laid in shallow water at the edge of water dams, and are often coiled in and around aquatic vegetation (Channing, 2001). Females are capable of breeding in tropical and subtropical regions and will often produce two clutches annually. However, they can also reproduce once seasonally in the more arid southern regions, during the winter rainfall seasons (Channing, 2001).

Figure 1.1: Natural distribution area of *Sclerophrys gutturalis* (The IUCN Red List, 2004).

The diet of the Guttural Toad consists mostly of insects, gastropods, and other invertebrates (Wager, 1986; Channing, 2001; Du Preez *et al.*, 2004; Measey *et al.,* 2015). They also act as prey for a variety of predators such as snakes, birds and small mammals (Channing, 2001).

The southern borders of their natural distribution are being breached by the rapid growth of their population range. However, to date there have been limited studies on the specific Guttural Toad invasion in South Africa. The current establishment of Guttural Toad in Cape Town, South Africa, is assumed to be an unintentional introduction through landscaping development (De Villiers, 2006). It is still unknown when the introduction might have occurred. However, in January 2000, toads' calls were registered for the first time in the Cape Town district of Constantia (De Villiers, 2006).

Enlargements of this invasive population were witnessed in 2007 and during the breeding season of 2008 and 2009. The City of Cape Town charted the first degree of their initial range as $5km^2$ in Constantia (Richardson, 2014). Unfortunately, this expanding Guttural Toad population was found in the natural breeding grounds (see Fig. 1.2 below) of the endangered *Sclerophrys pantherina* (Western Leopard Toad) as listed by The IUCN Red List (2004) (SA–FRoG, 2010).

In response to this invasion, an eradication programme was launched to curb the spread of the population. This was undertaken by the Nature Conservation Corporation (NCC) contracted by the City of Cape Town. Results and the impact of eradication efforts are still unknown and there is no sign of decline in population (Telford, 2015). An examination of the locality data from the eradication programme indicates that the invasive Guttural Toad range seemingly has expanded significantly over the past few breeding seasons (Telford, 2015). However, due to differently applied methods and different employees, the range data taken during the mentioned breeding seasons should be considered questionable (Richardson, 2014).

At this point, researchers have become increasingly concerned with the direct and indirect impact of this species on the native endangered toad populations. Therefore, it was realised that further studies are necessary to determine the full impact. It is also vital to evaluate the potential influence of the specie's invasion on nearby ranges, which contain the habitats of other critically endangered species (e.g. the Table Mountain Ghost Frog, *Heleophryne rosei* Hewitt, 1925). The rapid migration of these toads and their widespread distribution means that their control or eradication do not seem to be feasible as yet.

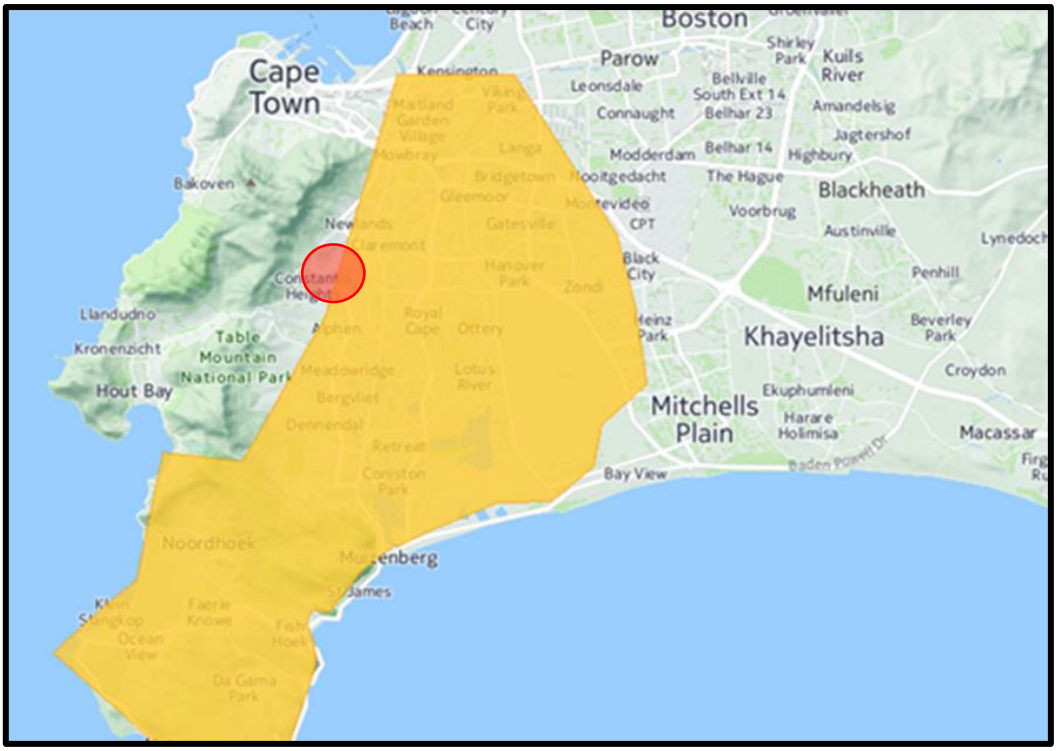

Figure 1.2*:* Distribution area of the endangered Western Leopard Toad. Yellow indicates the natural distribution area; red dot marks the overlapping area with *Sclerophrys gutturalis* (IUCN Red List).

1.3.2. *Sclerophrys pantherina* (Smith, 1828).

Sclerophrys pantherina, commonly known as the Western Leopard Toad, is a bulky toad and may reach a snout–vent length of 140 mm in females. These species are endemic to the south-western tip of South Africa and is classified as endangered by the IUCN Red List (IUCN *et al.,* 2011). The Cape Fynbos biome provides a habitat for these species, with this region also known as the winter-rainfall region. Extirpations have occurred over the past 20 years in populations that distributed naturally in the Kleinmond and Pringle Bay areas (Measey & Tolley, 2011).

This species can be found adjacent water forms such as marshes, swamps, dams, and pools (De Villiers, 2004), and often take up habitation in residential gardens, or on farm land. Accessibility of toads to specific breeding grounds determines the current distribution, however, this is not limited to immaculate natural habitats. (Du Preez & Carruthers, 2009). This natural distribution occurs in terms of two distinct populations in the Cape Fynbos biome (as seen in Fig. 1.3 below).

Figure 1.3: Natural distribution area of *Sclerophrys pantherina* indicating two distinct populations

The breeding behaviour of these mentioned populations corresponds with the commencement of the winter rainfall season during July through August.They are 'explosive breeders' that meet at breeding spots including dams and small meres for a period of approximately one week. During this period, males attract mates, emitting a sound that can be described as a "slow snore" (De Villiers, 2004). To elude predators such as fish and birds, the threads of approximately 25 000 eggs are laid within shallow marshy areas. Once they hatch the tadpoles fodder on large algae and after approximately 10–12 weeks, metamorphosis takes place (Du Preez & Carruthers, 2009).

As is evident from Fig. 1.3 above, their present distribution encompasses two separate populations. These populations are separated by 100 km. with one population located in the greater Cape Town Area and the other in the Overstrand region. These populations are estimated to have been separated for approximately 5 000 years (Measey & Tolley, 2011). Anthropogenic activity is highly present in both habitats which these populations occupy.

1.4. Invasive species and parasites

When an animal is introduced, it may expose the system to new stresses. In this regard, novel occupant host species are exposed to the particular parasites of the system or undergoes the transference of novel parasites (Weir, 1977; Holmes, 1979).

1.4.1. Invasive parasites

Parasites comprise more than half of all living organisms and perform a significant role in the functioning of ecosystems (Clayton *et al.*, 2003). Somewhat 10% of the metazoans are living at the cost of free-living parasites. As Hatcher *et al.* (2012a), explains, "Parasitic disease of wildlife, managed or human populations is cited as a driver behind the impact of nearly a quarter of species on the IUCN list of the World's Worst Alien Species."

Research suggests that parasitism most likely arose in aquatic ecosystems, and thus parasites are extraordinarily diverse (Adlard *et al.,* 2014). The extended evolutionary history of host and parasite life found in freshwater and marine environments can be observed currently in aquatic ecosystems in terms of the high diversity and richness of parasites (Rohde, 2005).

Due to co-evolution between host and parasite, the latter had the opportunity to thrive in a healthy and stable natural environment. This co-evolution causes certain pathogenic effects in uninfected host animals, as it is known that the main goal of parasites is to not kill off its host (Smit, 1996). However, when introduced to the system, the parasites may be transferred to a novel host. This may cause apparent pathogenic effects, which can lead to the destruction and destabilisation of the host population (Smit, 1996).

Parasites can have detrimental effects when introduced (Alexander & Holt, 1998) such as disturbances through trophic levels (Hatcher *et al.*, 2012b), host behaviour and survival (Werner & Peascor, 2003), as well as density and affecting the characteristics of invasive and native populations (Hatcher *et al.*, 2012b).

Disturbances through trophic levels include the novel traits that hosts can inherit from infection with parasites. Such characteristics can change the interactions of the host within the biological communities (Dunn & Hatcher, 2014). Biodiversity can be enhanced by parasites that curb competitively dominant host species (Janzen, 1970). However, those parasites with greater damaging effects on fragile competitors are projected to lessen species' coexistence. The characteristics of food webs can be altered radically (Lafferty *et al.*, 2006), and parasites can affect the forms of interactions that are most frequently observed in ecological systems such as apparent competition (Dunne *et al.*, 2013). This can impact the community's stability as a whole.

Disturbances can occur with host behaviour and regarding survival by decreasing the competitive ability of the native host and increasing the invasion. These parasites can suppress the native species and reduce their growth rate, thereby diminishing the native population's ability to exclude invaders competitively (Wells *et al.*, 2014). Strong host-

specificity is cultivated between parasites and its hosts due to the close link that develops between them (Poulin, 2007).

Due to disturbances in density and affected traits of the invasive and native populations, the interaction can vary between these two species. Parasites can alter the competitive dynamics between native and invasive species (Alexander & Holt, 1998). The disturbed relationship between native and invasive hosts can have equally significant consequences for the invader (Lafferty *et al.*, 2005; Phillips *et al.*, 2010), and the native wildlife. This is the case, particularly if parasites passed by the invaders are proficient of infecting the native species (Gozlan *et al.*, 2005). Invasive species can drive variations in the diversity and richness of the host species, often in aggregation with other forms of environmental modification (Dunn & Hatcher, 2014). Thus, parasites can be key partners in the operation of ecosystems (Hudson *et al.*, 2006). In this sense, parasites are important components of biological diversity (Dobson *et al.*, 2008).

An overlapping susceptibility of parasites is caused by phylogenetic affinity and ecological similarity of hosts, which may lead to switching of hosts by parasites (Holmes, 1979). This compatibility between closely related hosts, therefore, can result in successful host switching (Poulin, 2007). If the hosts are analogous in morphology and behaviour, then switches also can occur even though the two are not related closely (Clayton *et al.*, 2003).

New combinations of parasites and hosts may arise from the spreading of invasive species (Dunn & Hatcher, 2014). Even if the host species do not intermingle directly, outcomes of coexistence can also be influenced by shared parasites (Hudson & Greenman, 1998). This is possible since certain environmental characters can act as connectivity corridors between hosts to help distribute parasites. Such a condition further complicates the outcomes, seeing that the intimate links between diseases in wildlife can be transformed (Adlard *et al.*, 2014). A crucial corridor to translocate and transmit aquatic parasites as well as parasites with free-living stages is the aquatic ecosystem (Adlard *et al.*, 2014).

When hosts interact directly, numerous amounts of them can be infected by several parasites. Therefore, in this case, prevalence of parasites depends on the community's arrangement, which impacts the interaction and spread rates between viable hosts (Dunn & Hatcher, 2014). In such a situation, several processes can occur: (1) Invaders may profit by parasite loss. (2) Novel parasites can be introduced into inhabitant populations. (3) Introduced hosts can obtain new parasites themselves from the native populations (Dunn, 2009).

Ultimately, host-switching by parasites hinges largely on host-specificity. The evolution of such specificity is moulded by degrees of encounter and compatibility with the host's

morphology, physiology and immunology. In certain conditions, ambient fauna can influence the switching from hosts as certain parasites follow a free-living stage – usually aquatic. These parasites may fall prey to various new predators in the novel environment (Thieltges *et al.*, 2008). This can cause a dilution of parasite fauna, which in turn impairs the transmission of parasites with incompetent hosts. As a result, it distracts infectious phases from true hosts, decreasing their infection stages (Keesing *et al.*, 2006).

1.4.2. Amphibian parasites

Amphibians, in particular frogs, performs as hosts to all key assemblages of animal parasites: Protozoa, Trematoda, Cestoda, Acanthocephala, and Nematoda, which provides a rich parasite fauna to study (Smyth & Smyth, 1980). The following section is a review of the parasites found in toads in Africa and their biology.

1.4.2.1. Acari

The ticks and mites (Acari) are a remarkably assorted group of Arachnida (Cheliserata), in both form and life strategies (Fayaz & Khanjani, 2013). A limited portion of the real taxonomic diversity is represented by a mammoth 50 000 named species, which is occasionally projected at more than one million species (Alberti, 2005). A vast number of mites are commensals and parasites that attach themselves to a correspondingly great diversity of plant and animal hosts.

Acariformes, a superorder of Acari, is reflected to consist of four main groups of diverse taxonomic ranks: Astigmata, Endeostigmata, Oribatida (=Cryptostigmata), and Trombidiformes (=Prostigmata) (Lindquist & Evans, 1965). The family Ereynetidae Oudemans 1931, of the Trombidiformes, is divided into Speleognathinae Womersley 1936, Ereynetinae Oudemans, 1931, and Lawrencarinae Fain, 1957 (Fain, 1962). Lawrencarinae have been observed in the nasal cavities of amphibians while Speleognathinae are nasal parasites that frequent birds and mammals (Fain, 1962). Only one genus has been reported to parasitize on toads in Africa, namely *Lawrencarus eweri* (Lawrence 1952).

1.4.2.2. Helminths

Helminths are known as parasitic worms such as flukes, tapeworms, or roundworms. They are large multicellular organisms, which when matured, generally can be observed with the naked eye. They typically feed on a living host to gain sustenance and security, while instigating deprived absorption of nutrients, as well as weakness and disease in the host

(Mandal, 2014). Helminths have been reported from 25 (21%) of the 117 South African anurans (Halajian *et al.,* 2013). Relevant helminths of African toads are discussed subsequently.

1.4.2.2.1. Nematoda

Nematodes are known as roundworms, which indicate that they have a cylindrical body shape. These organisms also have lips, teeth and dentary plates, and can be either male or female (Mandal, 2014). Currently, there are approximately 2,271 described genera in 256 families. It is also estimated that approximately 33% of all the defined nematode genera function as parasites of vertebrates (Anderson, 1992). These nematodes presumably are derived from soil nematodes (Chitwood & Chitwood, 1950; Chabaud, 1954). The nematode parasites probably did not appear and evolve until the introduction of terrestrial vertebrates (Anderson, 1984). Several taxa of nematodes contain amphibian parasitic round worms. African toad species contain several nematode species that are distributed widely across Africa, as indicated by Table 1.1 below.

The family Rhabdiasidae Rialliet, 1915, is a small assemblage of nematodes that presently include eight genera of which the adult members function as parasites in lungs, oesophagus and mouth of amphibians and reptiles globally. The immense majorities of the rhabdiasids belongs to the genus *Rhabdias* Stiles & Hassal, 1905, and contain approximately 70 nominal species (Kuzmin, 2001). These nematodes function as parasites solely in the lungs of their hosts. *Rhabdias bufonis* (Shrank, 1788), is a parasite specific to toads. Research found that the eggs of these parasites passed in the faeces of the toad host, hatched, and its larvae produced a free-living generation of adult worms (Whicker & Lanter, 1968; Baker, 1987). It is common to find mature nematodes in the lungs of amphibians caught in the wild, as well as numerous sub-adults in the body cavity (Baker, 1987). Another big genus, *Entomelas* Travassos 1930, contains nine types of rhabdiasid species, however only a single one, *Entomelas sylvestris* Baker 1982, is parasitic to amphibians (Kuzmin, 2001; Tkach *et al.*, 2014a).

The family Cosmocercidae Travassos, 1925, is a widely distributed group of nematodes consisting of as many as 95 species and are parasites present in the gut of amphibians and reptiles. The subfamily, Cosmocercinae Railliet 1916, is found mainly in amphibians. Before establishing themselves in the intestines, certain species are known to undergo a period of development in the lungs (Anderson, 1992). The cosmopolitan species, *Aplectana macintoshii* (Stewart, 1914), is found in the rectum of amphibians (Baker, 1987). Furthermore, *Cosmocerca commutata* (Diesing, 1851) is a parasite of toads and frogs, and

Cosmocercoides variabilis (Harwood, 1930) is a common parasite present in the rectum of toads (Vanderburgh & Anderson, 1987).

Five species of Cosmocercoidea Travassos, 1925 have been described in Southern Africa. These are:

- *Aplectana capensis* Baker, 1981;
- *Aplectana degraafi* Baker, 1981*;*
- *Aplectana macintoshii,* (Stewart, 1914);
- *Aplectana chamaeleonis* (Baylis, 1929); and
- C*osmocerca ornata* (Dujardin, 1845).

Three of these species can be found in the Western Cape: *A. chamaeleonis, A. capensis,* and *C. ornata.* Only two of these species are native to KwaZulu–Natal: *A. chamaeleonis* and *C. ornata.*

Aplectana chamaeleonis was found previously in the toad hosts *Sclerophrys capensis* Tschudi 1838 in KwaZulu–Natal, and *Vandijkophrynus angusticeps* Smith 1848, in the Cape Province. *Cosmocerca ornata* was found in *Capensibufo rosei* Hewitt 1926, in the Cape Province, and *Schlerophrys rangeri* Hewitt 1935, in KwaZulu–Natal. *Aplectana capensis* emerged in *C. rosei* in the Cape Province (see Fig. 1.4 below).

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Figure 1.4: Localities in southern Africa where cosmocercoids were collected from frogs.
1. *Aplectana chamaeleonis*. **2.** *Cosmocerca ornata.* **3.** *Aplectana capensis* (adapted from Baker, 1981)

The superfamily Ascaridoidea, Baird, 1853, consists mainly of medium-sized to large nematodes that typically dwell in the stomach and intestine of the final host, and thus ingest food consumed by the host. The subfamily Angusticaecinae Skryabin & Karokhin, 1945, is confined to terrestrial reptiles. A few of these species, which probably are captures from reptiles, have been reported in amphibians. Species of the genera *Amplicaecum* Baylis, 1920 is also reported in amphibians (Anderson, 1992).

The superfamily Camallanoidea Travassos, 1920 is parasites of the stomach and intestines of lower predacious vertebrates (Chabaud *et al.*, 1961). Of eight clearly described genera consisting of 150 species, approximately 40 species are found in amphibians and reptiles (Baker, 1987).

Table 1.1: Nematode species reported for Bufonid members from Africa (adapted from Canaris & Gardner, [2002])

Family	Species	Host	Location	Source
Ascarididae Baird, 1853	*Amplicaecum africanum* Taylor, 1924	*Sclerophrys gutturalis*	West Africa	Taylor (1924)
	Amplicaecum gedoelsti Yorke & Maplestone, 1926	Bufonidae	Central Africa	Yorke & Maplestone (1926)
	Amplicaecum involutum Gedoelst, 1916	*Sclerophrys gutturalis*	Central Africa	Gedoelst(1916)
	Orneoascaris chrysanthemoides (Skrjabin, 1916)	Bufonidae	East Africa	Yorke & Maplestone (1926)
Cosmocercidae Travassos, 1925	*Aplectana capensis* Baker, 1981	*Capensibufo rosei*	South Africa	Travassos (1931)
	Aplectana chamaeleonis (Baylis, 1929)	*Sclerophrys capensis*	South Africa	Travassos (1931)
	*Aplectana dogieli***(Skrjabin, 1916)	Bufonidae	Africa	Travassos (1931)
	Aplectana macintoshii (Stewart, 1914)	*Sclerophrys garmani (*Meek, 1897)	South Africa	Travassos (1931)
	Cosmocerca ornata (Dujardin, 1845)	*Sclerophrys garmani*	South Africa	Diesing(1861)
Camallanidae Railliet & Henry, 1915	*Camallanus mazabukae* Kung, 1948	Bufonidae	South Africa	Kung (1948)
	Procamallanus brevis Kung, 1948	Bufonidae	South Africa	Kung (1948)

Table 1.1. *continued.*

Onchocercidae Yamaguti, 1961	*Foleyella bouillezi* Witenberg & Gerichter, 1944*	*Sclerophrys regularis* (Reuss, 1833)	Central Africa	Witenberg & Gerichter (1944)
	Foleyella leiperi (Railliet, 1916)*	*Sclerophrys regularis* (Reuss, 1833)	East Africa	Witenberg & Gerichter (1944)
Rhabdiasidae Rialliet, 1915	*Rhabdias africanus* Kuzmin, 2001	*Sclerophrys garmani*	South Africa	Kuzmin (2001)
	Rhabdias picardiae Junker, Lhermitte–Vallarino & Bain, 2010	*Sclerophrys gutturalis*	South Africa	Junker *et al*. (2010)
	Rhabdias sylvestris (Baker, 1982)	*Sclerophrys maculata* (Hallowel, 1854).	Africa	Halajian *et al.* (2013)
Strongyloididae Chitwood & McIntosh, 1934	*Strongyloides prokopici* Moravec, Barus & Rysavy, 1987	*Sclerophrys xeros* (Tandy, Tandy, Keith, and Duff–MacKay, 1976)	North Africa	Moravec, Barus & Rysavy (1987)

*According to Esslinger (1986) these identifications are doubtful.

** According to Baker (1980) these can be considered a species dubia.

1.4.2.2.2. Platyhelminthes

Platyhelminthes includes Trematoda which are flatworms that are leaf–shaped and unsegmented. They are hermaphroditic, and can establish themselves in a wide variety of vertebrates (Mandal, 2014). Amphibians harbour a significant number of trematode parasites in their internal organs by taking in its larval forms from the intermediate host. Digenetic trematoda are endoparasites found as adults in amphibian hosts' digestive, respiratory system, gall bladder, bile duct, liver, pancreatic duct, et cetera. A number are known to inhibit the connective muscle tissues of their hosts. The most commonly preferred habitat is the digestive tract. Trematoda of amphibians are residing within their host without causing them any harm and showing a striking example of symbiotic effect.

Host-specificity in trematoda can range from wide to narrow. They can be associated with phylogenetically close or even distant groups of animal hosts, which are, however,

connected by an ecological similarity. Certain trematode families are distributed widely between vertebrate groups, i.e. the families Plagiorchiidae and Lecithodendriidae whose representatives frequent amphibians, reptiles, birds, and mammals (Ginetsinskaya, 1968).

Members of the family Brachycoelidae Looss, 1899, are digeneans that parasitise amphibians and reptiles, and occasionally mammals (Pojma'nska, 2008). This family consists of two subfamilies: Brachycoeliinae Loos, 1899; and Mesocoeliinae Dolfus, 1929. The genus *Mesocoelium* Odhner, 1911, consists of 41 species, which can be found in the intestines of amphibians, reptiles, and fish. However, members of this genus tend to have extremely similar morphologies, which increase the difficulty of resolving species by using the key characteristics which typically distinguish these members (Goldberg *et al.,* 2002). Some trematode species are described in Africa for toads (Table 1.2 below).

Table 1.2: Platyhelminthes species reported for Bufonid members from Africa (adapted from Canaris & Gardner [2002])

Family	Species	Host	Location	Source
Polystomatidae Gamble, 1896	*Eupolystoma alluaudi* (de Beauchamp, 1913)	*Sclerophrys regularis*	East Africa	De Beauchamp (1913)
	Polystoma africanum Szidat, 1932	*Sclerophrys regularis*	Africa	Szidat(1932)
	Polystoma mashoni Beverley–Burton, 1962	*Sclerophrys regularis*	Central Africa	Beverley–Burton(1962)
	Eupolystoma anterorchis (Tinsley, 1978)	*Sclerophrys pantherina*	South Africa	Tinsley (1978)
	Eupolystoma namibiensis Du Preez, 2015	*Poyntonophrynus hoeschi* (Ahl, 1934)	North Africa	Du Preez (2015)
	Eupolystoma vanasi (Du Preez, Tinsley & de Sa, 2003)	*Schismaderma carens* (Smith, 1848)	North Africa	Du Preez, Tinsley & de Sa (2003)
Mesocoeliidae Odhner, 1901	*Mesocoelium monodi* Dollfus, 1929	*Sclerophrys regularis*	West Africa	Dollfus (1929)
	Mesocoelium schwetzi Dollfus, 1950	*Sclerophrys regularis*	Central Africa	Dollfus (1950)

1.4.2.3. Apicomplexa and Euglenozoa

Amphibians host a number of these blood parasites, which can range from protozoans, apicomplexans, to microfilaria (Netherlands *et al.*, 2015).

One of the least studied groups is Apicomplexans, parasites that are unicellular and recorded as taken from an extensive array of tetrapod vertebrates (Smith, 1996). This group presently consists of three families: Heamogregarinidae Leger, 1911, Hepatozoidea Wenyon, 1926, and Karyolysidae Wenyon, 1926. Six genera of blood parasites can be found in these families (Netherlands *et al.*, 2014). Only two heamogregarine genera are known to parasitise amphibian hosts. These genera are *Hemoliva* Petit, Landau, Baccam and Lainson, 1990, and *Hepatozoon* Miller, 1908.

Hepatozoon species can be considered the most common blood parasites, with more than 300 species currently assigned to it (Smith, 1996) and the majority of these parasites can be found parasitising the amphibian family Bufonidae (Netherlands *et al.*, 2014). Another parasite species to consider is the trypanosomes, which are flagellated blood parasites capable of infecting virtually all classes of vertebrates (Hoare, 1972; Botero *et al.*, 2013). This species' information is provided in Table 1.3 below.

Table 1.3: Apicomplexan and Euglenozoan species reported for Bufonid members from Africa (adapted from Netherlands [2014])

Family	Species	Host	Location	Source
Hepatozoidae Wenyon, 1926	*Hepatozoon aegyptia* (Mohammed & Mansour, 1963)	*Sclerophrys regularis*	North Africa	Younis & Saoud (1969)
	Hepatozoon assiuticus (Abdel–Rahman, El–Naffar, Sakla & Khalifa, 1978)	*Sclerophrys regularis*	North Africa	França (1910)
	Hepatozoon boueti (França, 1910) [syn., *Hepatozoon boneti* França, 1925 orTuzet & Grjebine (1957)]	*Sclerophrys regularis*	North Africa	Mohammed & Mansour (1966)
	Hepatozoon faiyumensis (Mansour & Mohammed, 1966	*Sclerophrys regularis*	North Africa	Mohammed & Mansour(1966)
	Hepatozoon francai (Abdel–Rahman, El–Naffar, Sakla & Khalifa, 1978)	*Sclerophrys regularis*	North Africa	Abdel–Rahman *et al*. (1978)
	Hepatozoon froilanoi (França, 1925)	*Sclerophrys regularis*	North Africa	França (1925)
	Hepatozoon ixoxo Netherlands, Cook & Smit, 2014	*Sclerophrys garmani, Sclerophrys gutturalis, Sclerophrys maculatus*	South Africa	Netherlands, Cook & Smit (2014)
	Hepatozoon lavieri (Tuzet & Grjebine, 1957)	*Sclerophrys regularis*	North Africa	Tuzet & Grjebine(1957)
	Hepatozoon magni (Hassan, 1992)	*Sclerophrys regularis*	North Africa	Hassan, (1992)

Table 1.3. *continued.*

	Hepatozoon moloensis (Hoare,1920)	*Sclerophrys* sp.	North Africa	Hoare (1920)
	Hepatozoon pestanae (França, 1910)	*Sclerophrys regularis*	North Africa	Mohammed & Mansour(1910)
	Hepatozoon tunisiensis (Nicolle, 1904)	*Sclerophrys mauritanicana* (Schlegel, 1841)	North Africa	Nicolle (1904)
Trypanosomatidae	*Trypanosoma bocagei* França, 1910	*Sclerophrys regularis*	North Africa	França (1910)
	Trypanosoma elegans França & Athias, 1904	*Sclerophrys regularis*	Central Africa	França & Athias(1904)
	Trypanosoma loricatum (Mayer, 1843)	*Sclerophrys regularis*	North Africa	Netherlands *et al.* (2014)
	Trypanosoma rotatorium (Mayer, 1843)	*Sclerophrys regularis*	North Africa	Netherlands *et al.* (2014)
	Trypanosoma somalense Brumpt, 1906	*Sclerophrys xeros* (Tandy, Tandy, Keith, & Duff–MacKay, 1976)	North Africa	Brumpt(1906)
	Trypanosoma sp.	*Sclerophrys garmani*	South Africa	Netherlands, Cook, Kruger & Du Preez (2015)

1.5. THE CONCEPTS OF SPILL BACK AND SPILL OVER

Extensive research has been conducted on invasions; however, evidently two highly significant concepts of invasion are receiving considerably less attention than others, namely *spill back* and *spill over* (Kelly *et al.*, 2009). Numerous researchers have concentrated on the parasites' direct effect on biological invasion, and on parasites that themselves are invasive (Hatcher *et al.*, 2012b). However, the part that parasites play in invasions may encompass

well past such revealed direct distresses. Parasites are interacting at all trophic levels (Kuris *et al.*, 2008), including those with discrete hosts (Lello *et al.*, 2004). Therefore, indirect effects also are expected on other species than their hosts (Wells *et al.*, 2014).

Through the process of spill over, an invader can assist the dispersion of novel parasites to native species (Kelly *et al.,* 2009). The definition parasite spill over has been utilized to designate the spread of diseases from wild faunas to other animals or humans (Wolfe *et al.*, 2007). Generally, parasite epidemics in wildlife occur mostly overlooked unless humans are affected, thus there are extremely few noted examples of spill over (Otterstatter & Thomson, 2008; Wells *et al.*, 2014).

However, an even more neglected concept in the mentioned field of study is parasite spill back (Kelly *et al.*, 2009; Helen & Handley, 2012). When an alien species acts as proficient host for a native parasite or pathogen, then parasite spill back can occur. In this case, spill back means an introduced host acquires native parasites, and this is a more possible incidence than that of the host presenting new parasites (Holmes, 1979). However, when an alien species does acquire native parasites and pathogens, this will not certainly lead to spill back into native wildlife; the practice rests on the alien species distributing the parasite or pathogen and performing as a reservoir. In this case it will likely lead to an increase of the parasite burden to the native host as the reservoir host would increase the number of infective stages of a given parasite in the environment. It is conceivable that alien species might be sinks for the pathogen or parasite and thereby make the infection less prevalent in the native fauna (Heimpel *et al.*, 2003; Helen & Handley, 2012). This condition depends on the occurrence and richness of resident hosts (Holmes, 1979). Spill back can cause an increase in the parasite range if the host is suitable (Holmes, 1979). Thus, the novel host could act as a population amplifier for parasites (Holmes, 1979).

If spill back occurs, there may be two possible outcomes. Firstly, parasites could undermine the fitness of the new host individuals (Holmes, 1982;; Holmes & Price, 1986), which can cause a drop in the species' richness (Holmes, 1979). Secondly, the attained parasite could co adapt with its novel host species but with no noticeable damaging effect on its wellbeing (Holmes, 1979).

1.6. Parasites and phylogenetics

Molecular phylogenetics has become a common, highly valuable tool that is particularly useful in cases when morphology is insufficient for identification and phylogenetic reconstruction. Molecular techniques can be applied as powerful instruments to assess species' boundaries (Fontaneto *et al.*, 2009). Parasites can be extremely difficult to identify

based on morphology alone. The reasons are that interactions rarely are observed in the wild, and parasites are often enigmatic and covert.

Two of the most frequently used groups in phylogenetics are the nuclear ribosomal (28S) and the mitochondrial genes (COI). Cells contain a large number of mitochondrial genes that are easy to utilise and are usually sufficient for identification. Nevertheless, such genes can be variable and sometimes unreliable due to the wide spectrum they cover (Dabert *et al.*, 2010). Ribosomal genes are more problematic to align but are more probable to contain more revealing sequences (Dabert *et al.*, 2010).

The success of molecular work hinge on quality of information, and a lack of it can impact the study negatively. Only limited information is available on GenBank for African toad parasites, as illustrated in Table 1.4 below.

Table 1.4: GenBank accession no. for African toad parasites with corresponding gene.

Type	Species	Accession number on GenBank	Gene
Nematoda	*Rhabdias africanus*	KF999598	28S
	Rhabdias bufonis	KF999593	28S
	Rhabdias sylvestris	KJ018777	28S
Apicomplexa	*Hepatozoon ixoxo*	KX512803	18S

Only two genera namely *Rhabdias* and *Hepatozoon* are represented from parasites in Africa. Despite the limited information, without enquiry a sequence of DNA molecules delivers the most contingent set of data for phylogenetic studies (Dabert *et al.*, 2010).

1.7. Aims and objectives of the study

The understanding of parasite-host interactions between Guttural Toad and Western Leopard Toad, are fundamentally important. It helps improve eradication programmes and provide insight into of the processes of biological invasion and parasite transfer. The present study focused on the relationship between parasites, native species and invasive species, and examined the mechanisms of spill back and spill over, as well as the consequences that these interactions hold for species, communities, and ecosystems.

The present study had the following aims:

Aim 1: Identify parasites and parasite composition from each range:

a. Native Guttural Toad population in KwaZulu-Natal range;

b. Invasive Guttural Toad population in the core of the invasive range which has not been exposed to the native range of Western leopard Toad population in the Western Cape;
c. Invasive Guttural Toad population at the periphery of the invasive range exposed to the native range of Western Leopard Toad population in Western Cape where population ranges overlaps;
d. Native Western Leopard Toad population in the core of the native distribution which has not been exposed to the invasive Guttural Toads in the Western Cape; and
e. Native Guttural Toad population in Potchefstroom.

Aim 2: Determine the increase/decrease of parasite diversity from the native Guttural Toad population in KwaZulu-Natal to the invasive Guttural Toad population in Western Cape.

Aim 3: Ascertain whether spill over of parasites occurred from invasive Guttural Toad to native Western Leopard Toad.

Aim 4: Ascertain whether spill back of parasites occurred from native Western Leopard Toad to invasive Guttural Toad and whether this eventually leads to increased infection rates in the Western Leopard Toad.

In order to achieve the above-mentioned aims, the following objectives were formulated:

Objective 1: Undertake a comprehensive survey to collect individual toads of each population, examine and extract parasites. In addition, identify parasites through historic data sampling, morphology, and molecular techniques. Native Guttural Toads from Potchefstroom is used as reference population. Add new data to historic data, for a more comprehensive view of past and present parasite diversity, and to eliminate the chance of capturing 'snap-shot data'. Thus, collect new data on parasites by active sampling of toads, examination and identification of parasites and adding to historic data.

Objective 2: Compare identified parasites (historic and new data) of native and invasive Guttural Toads.

Objective 3: Examine parasite's diversity and richness in all three populations, and apply molecular techniques if needed to identify parasite spill over.

Objective 4: Examine parasites' diversity and richness in the two Western Cape populations, and apply molecular techniques if needed to identify parasite spill back.

CHAPTER 2:

MATERIALS AND METHODS

CHAPTER LAYOUT

This chapter will discuss the various sites sampled in the current study as well as methods concerning the study. Methods discussed include (1) collection of toads and parasites (2) examination through morphological and molecular techniques (3) determination of parasite diversity.

2.1. STUDY SITES

For the purpose of this study three main study areas were identified for sampling. They included the Cape Peninsula and in particular the Constantia area, KwaZulu–Natal where we focussed on the Durban and Kosi Bay areas and lastly Potchefstroom in the North–West Province.

STUDY AREA 1: KWAZULU–NATAL.

This study area was divided into two main study sites: northern KwaZulu-Natal and Durban (as seen in Fig. 2.1. below).

Figure 2.1. Map of KwaZulu–Natal indicating study area 1. Site 1a indicating northern KwaZulu-Natal sampling area, and site 1b indicating Durban sampling area (Google Maps).

Site 1a: Northern KwaZulu-Natal is considered to be an amphibian diversity hotspot containing a high variety of microhabitats. Sampling was conducted in the summer rainfall season (January-February 2016) in and around the Kosi Bay area (-26.94121, 32.81501).

Site 1b: Durban (-29.782294, 31.030196) was selected as relevant study site as it is characterised by peri-urban landscape which is very similar to Constantia and the trade between the two cities is very frequent in terms of ornamental plant trade (which could facilitate the accidental translocation of the species). Sampling was done in an area of approximately 5 km^2 in the summer rainfall season (February-March 2015).

Study area 2: Western Cape.

This study area was divided into three main study sites: Silverhurst, Noordhoek, and Bishopscourt (as seen in Fig. 2.2 below).

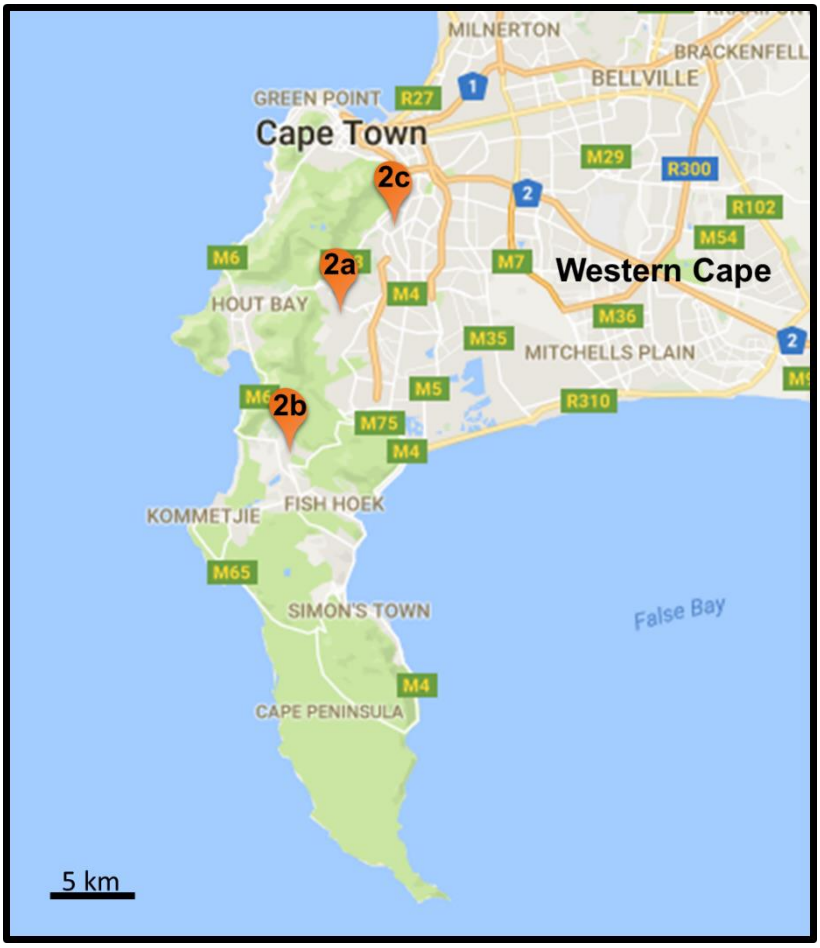

Figure 2.2. Map of Cape Peninsula in the Western Cape indicating study area 2. Site 2a indicating Silverhurst sampling area; site 2b indicating Noordhoek sampling area and site 2c indicates the Bishopscourt sampling area (Google Maps).

Site 2a: Silverhurst (-34.017122, 18.441168) is a residential estate surrounded by wine estates and other urban areas. Sampling was done in an area of approximately 2 km^2 and was conducted in the summer (February 2015) and winter rainfall season (August 2015).

Site 2b: Noordhoek (-34.62583, 18.234113) is a residential area containing a variety of isolated natural areas such as horse farms and winelands. Sampling was done in an area of approxamitely 5 km^2 and was conducted in the winter rainfall season (August 2015).

Site 2c: Bishopscourt (-33.993099, 18.449200) is a peri-urban area containing large residential properties with lots of small ponds and waterbodies. Sampling was done in an area of approximately 1 km^2 and conducted in the summer (February 2015).

Study area 3: Potchefstroom

Potchefstroom (-26.4338.97, 27.55849) was chosen as reference site (see Fig. 2.3. below). This area is mainly residential; however, it contains many water bodies such as dams, marshes, and ponds. Sampling was conducted in the summer (October 2015-March 2016). This area was chosen as reference site due to its importance as a core area in the natural distribution range of Guttural toads and immediate availability.

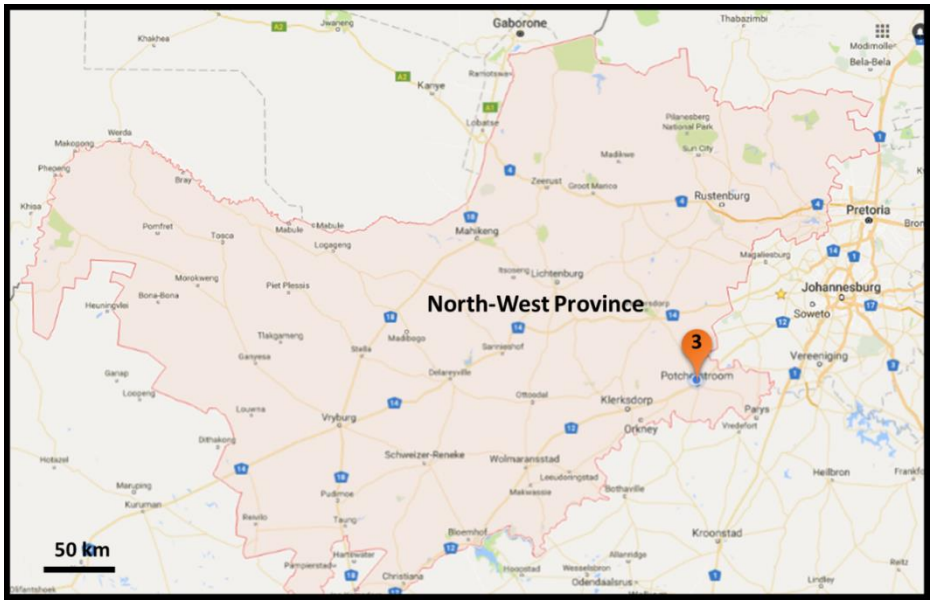

Figure 2.3. Map of the North-West province indicating study area 3 in Potchefstroom (Google Maps).

2.2. Collection of toads

Guttural Toads were actively collected at sites 1a, 1b, 2a, 2c, and 3. (Permit no. OP 4374/2015 and 0056-AAA008-00049). According to law Western Leopard Toad may not be collected and euthanized, and road killed toads were collected by The City of Cape Town

ToadNuts volunteer community at sites 2a, and 2b, and frozen (Permit no. 0056–AAA008–00049). These toads were collected and defrosted and examined.

2.3 Collection of parasites

Toads were euthanized using 3% ethyl–4–aminobenzoate (MS222) solution.

3.3.1. Preliminary examination:

Using a stereo microscope the external surface and buccal cavity of toads were examined for the presence of mites and ticks. The nasal cavities of the toads were inspected by opening the mouth and inspecting the internal passages. Once mites were observed, small incisions were made to open the entire nasal cavity and mites were extracted, and transferred to saline.

The toad was then pinned to the dissection dish. The body cavity was opened and using a 1 mm Insulin syringe blood was drawn from heart. For live toads, blood was drawn from the femoral artery using 1 ml Insulin Syringe (Permit no. 0056–AAA008–00049). One small drop of blood was placed in the centre of two separate labelled (name, ID and location) microscope slides. Using a clean slide the drop was dragged across the surface of the labelled slide creating a blood smear. The blood smear was left to air dry for approximately 10 mins and then covered with Methanol to fix blood cells and any parasites onto the slide. After blood smear has air–dried they are stained with giemsa stain (FLUKA, Sigma–Aldrich, Steinheim, Germany). The remaining blood was fixed in 70% EtOH for molecular analysis.

2.3.2. Examination of the viscera

The body cavity and surface of organs was examined for cysts and coelomic parasites. Cysts can occur between the dermis and the epidermis and are usually encapsulated structures sometimes white or yellow (Smyth & Smyth, 1980). The position of cysts was noted and they were carefully removed and placed in a watch glass of saline. The eyes, nostrils and body surface were inspected for any signs of free–living or encapsulated parasites.

2.3.3. Detailed examination of organs

All potentially infected organs were removed and placed in saline filled Petri dishes. These include lungs, heart, liver, alimentary canal and kidneys. The bladder was carefully examined before removing it by carefully cutting it out and placing it in a petri dish. An incision was made above the oesophagus and underneath the rectum to remove the entire

gut. It was placed in a petri dish and slit longitudinally, so that the confined parasites may be released. Parasites from the different regions of the alimentary canal were processed separately. Protozoans were removed from rectum and placed in 1.5. ml cryo vials containing 70% molecular EtOH. Trematodes and nematodes were removed from duodenum and rectum and placed in watch glass filled with saline. Nematodes retrieved from lungs were placed in saline filled watch glasses. Kidneys were teased out in a watch glass of saline and examined.

Information for each parasite was carefully noted such as prevalence, and location.

2.4. Examination of parasites

2.4.1. Preparation and fixing for light microscopy

Parasite specimens were fixed according to type and stained (see Table 2.1. below) to examine and measure diagnostic characters. Composite focused images for identification and measurements of characteristics were obtained using a Nikon AZ100 microscope (Nikon, Amsterdam, Netherlands) fitted with a motorized Z–drive and a Nikon DIS–Fi2–U2 digital camera. Images were captured using Nikon NIS–Elements D imaging software. To study the morphological characters of each parasite different techniques were applied to fix the specimens so they could be highlighted for examination:

Table 2.1: Fixing and staining methods for different parasite groups used in this study:

Parasite group	Fixing method	Staining method
Acari	70% EtOH	70% EtOH
Platyhelminthes	70% EtOH	Acetocarmine
Nematodes	Heated 70% EtOH	Lactophenol
Apicomplexa and Euglenozoa	Methanol	Giemsa Stain

2.4.2. Preparation and fixing for Scanning electron Microscopy

Specimens prepared for observing surface morphology were dehydrated, critically point dried in a Polaron CO_2 critical point dryer and mounted on carbon tape on a 12 mm aluminium SEM stub. Subsequently, specimens were coated using an SPI Module sputter coater (SPI–Module$^{TM.}$Sputter Coater, SPI Supplies, West Chester, PA, USA) fitted with a gold palladium source, and specimens were then studied using a Phenom pro–desktop scanning electron microscope (Phenom PRO Desktop SEM, Phenom–World B., Eindhoven, Netherlands) at a power 5 kV.

2.4.3. Preparation and fixing for Phylogenetic studies

For molecular work, whole specimens fixed in 70% molecular EtOH were used. Using a rapid DNA extraction method as detailed in the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) genomic DNA of the specimens was extracted.

2.5. Molecular phylogeny

2.5.1. Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were performed in a total volume of 25 µL, with 12.5 µL Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl2), 1.25 µl of each primer (10mM concentration), and 1 µl DNA. The final reaction volume was made up with Milli–q water. Primers for specific specimens are detailed in Table 2.2.

Table 2.2. Specific primer name and primer details for each parasite group

Parasite Group	Primer name	Primer sequence 5'–'3
Nematoda	LCO1490	GGTCAACAAATCATAAAGATATTGG
	HC02198	TAAACTTCAGGGTGACCAAAAAATCA
	ritf	GCGGCTTAATTTGACTCAACACGG
	1500R	GCTATCCTGAGGGAAACTTCG
	ITS5	GGAAGTAAAAGTCGTAACAAGG
	ITS 4	TCCTCCGCTTATTGATATGC
	300R	CAACTTTCCCTCACGGTACTT
	ECD2	CTTGGTCCGTGTTTCAAGACGGG
Platyhelminthes	LSU5	TAGGTCGACCCGCTGAAYTTAAGCA
	1500R	GCTATCCTGAGGGAAACTTCG
	300F	CAAGTACCGTGAGGGAAAGTT G
	ECD2	CTTGGTCCGTGTTTCAAGACGGG

PCR conditions for each primer set are detailed in Table 2.3. The PCR reactions were carried out using a ProFlex™ PCR thermal cycler (applied biosystems by life technologies).

Table 2.3. Detailed PCR conditions for specific primer sets.

Primer set	PCR conditions
LCO1490, HCO2198	Initial denaturation at 95 °C for 5 min, followed by 35 cycles, entailing 95 °C denaturation for 30 s, annealing at 45 – 50 °C for 30 s with an end extension at 72 °C for 1 min, and following the cycles a final extension of 72 °C for 10 min
Rift, 1500R, and internal primers: ITS4, ITS5, 300R, and ECD2	Initial denaturation at 94 °C for 2 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 53 °C, 2 min at 72 °C, and a final extension step of 7 min at 72 °C
LSU5, 1500R, and internal primers 300F, and ECD2.	Initial denaturation at 95°C for 5 min, followed by 40 cycles (30 s denaturation at 95°C, 30 s primer annealing at 55°C, and 2 min at 72°C for primer extension), and a final extension step of 7 min at 72°C.

2.5.2. Sequencing

PCR products were directed 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 Ver. 9.1 (created by Biomatters. Available from http://www.geneious.com). Sequences were identified using the NCBI GenBank database.

2.5.3. Analysis

Uncorrected pair-wise distances (p–distance) and base pair differences for specific parasites were determined with the MEGA7 bioinformatics software program (http://www.megasoftware.net).

Comparative sequences for specimens were downloaded from GenBank and aligned to the sequences generated within this study. Suitable outgroups were identified for each specimen:

2.5.3.1. Lung nematodes: The focus was on a closely-related species of *Rhabdias africanus*. Comparative sequences from the genus *Rhabdias* were downloaded from GenBank (see Table 2.4) and outlined in the phylogenetic tree. *Serpentirhabdias fuscovenosa* (Rialliet, 1899) (GenBank: KF999588) was chosen as suitable outgroup according to Tkach *et al*., 2014b.

Table 2.4: Comparative sequences of the organisms used in the phylogenetic study for lung Nematoda as dowloaded from GenBank with accession no. for 28S.

Family	Species	Accession no. from GenBank	Source
Rhabdiasidae	*Rhabdias eustreptos*	JX826441	(Langford & Janovy, 2013)
	Rhabdias nicaraguensis	KF999605	(Tkach *et al.,* 2014b)
	Rhabdias bakeri	JX826433	(Langford & Janovy, 2013)
	Rhabdias cf. *joaquinensis*	KF999608	(Tkach *et al.,* 2014b)
	Rhabdias ranae	JX826444	(Langford & Janovy, 2013)
	Rhabdias sylvestris	KJ018777	(Tkach *et al.,* 2014a)
	Rhabdias elegans	KF999604	(Tkach *et al.,* 2014b)
	Rhabdias ambystomae	KF999590	(Tkach *et al.,* 2014b)
	Rhabdias americanus	KF999589	(Tkach *et al.,* 2014b)
	Rhabdias cf. *africanus*	KF999598	(Tkach *et al.,* 2014b)
	Rhabdias kongmongthaensis	KF999599	(Tkach *et al.,* 2014b)
	Rhabdias bulbicauda	KF999600	(Tkach *et al.,* 2014b)
	Rhabdias bermani	KF999610	(Tkach *et al.,* 2014b)
	Rhabdias cf. *bufonis*	KF999609	(Tkach *et al.,* 2014b)
	Rhabdias bufonis	KF999593	(Tkach *et al.,* 2014b)

2.5.3.2. INTESTINAL NEMATODES: For the closely-related species of *Cosmocerca* sp. Comparative, sequences of the family Cosmocercidae were downloaded from GenBank (see Table 2.5 below) and outlined in the phylogenetic tree. *Parapharyngodon* sp. (GenBank: KC130695) adhering to relevant phylogenetic outgroup as provided by Prosser *et al.* (2013).

Table 2.5: Comparative sequences of the organisms used in the phylogenetic study for intestinal Nematoda as downloaded from GenBank with accession no. for CO1.

Family	Species	Accession no. from GenBank	Source
Cosmocercidae	*Aplectana* sp. 1	KC130733	(Prosser *et al.,* 2013)
	Aplectana sp. 2	KC130720	(Prosser *et al.,* 2013)
	Aplectana sp. 3	KC130672	(Prosser *et al.,* 2013)
	Aplectana sp. 4	KC130668	(Prosser *et al.,* 2013)
	Cosmocercoides pulcher	LC052771	(Sato *et al.,* 2015)
	Cosmocerca japonica	LC052756	(Sato *et al.,* 2015)

2.5.3.3. PLATYHELMINTHES: It focuses on closely-related species of *Mesocoelium monodi*. Comparative sequences from the genus *Mesocoelium* were downloaded from GenBank (see Table 2.6 below) and utilised in the phylogenetic tree. Grounded on the topologies in the phylogenetic trees of the Digenea published by Olson *et al.* (2003), *Brachycoelium salamandra* (Frölich, 1789) (AF151935) was used as the outgroup. The alignment was then trimmed to the length of the shortest sequence.

Table 2.6: Comparative sequences of the organisms used in the phylogenetic study for Platyhelminthes as downloaded from GenBank with accession no. for 28S.

Superfamily	Species	Accession no. from GenBank	Source
Plagiorchioidea	*Mesocoelium* sp.	AF433677	(Tkach, 2002)
	Mesocoelium sp.	AY222277	(Olson *et al.,* 2003)

Sequences were aligned using the MUSCLE alignment tool (Edgar, 2004) instigated from within Geneious Ver. 9.1. To infer the phylogenetic relationship of specimens, a Bayesian Inference (BI) and Maximum Likelihood (ML) method was used.

JModelTest 21.7 (Darriba *et al.,* 2001) was used to perform an all-inclusive model test to define the most appropriate nucleotide substitution model, conferring to the Akaike information criteria (AIC). The general time reversible model, with estimates of gamma distributed among-site rate variation (GTR + G) was selected. The BI analysis was implemented within Geneious 9.1 using the MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001) plugin. The Markov Chain Monte Carlo (MCMC) algorithm was run for 3–10 million generations. The Markov chain was sampled every 100 cycles. The log-likelihood values of the sample point were plotted against the generation time and the first 25% of the trees were

discarded as ‘burn-in’ with no ‘burn-in’ samples being retained. Results were visualized in Tracer (Geneious ver 9.1) to assess convergence and the ‘burn–in’ period.

2.6. Statistical analysis

2.6.1. Parasite Prevalence and Mean Intensity

Data used in analysis from current study had to meet several requirements to be included in the analysis: the parasites had to be fully censured, some quantitative estimate of infection (prevalence % and mean intensity) was provided. The amphibian species had to be represented by at least nine individuals per survey.

From each survey, the total number of parasite species found (n), average number of species per host individual, and the mean abundance of parasites per host were determined. These measurements represent measures of community richness and abundance.

2.6.2. Parasite diversity

Statistical analyses were performed on a data set comprised of diversity of parasites for each individual in all the populations using Graphpad Prism 5. Parasite diversity was calculated for each individual in all populations (native Western Leopard Toad populations were combined due to small sample size). The specific analyses performed were chosen based on composition normality of the data. Normality was tested using Shapiro-Wilk and Kolmogorov-Smirnov tests to determine whether data sets conform to a Gaussian distribution, which in turn determined the type of analysis performed. The one-way ANOVA along with Tukey’s multiple Comparison Post Hoc test was performed on the diversity of parasites for each population.

CHAPTER 3:

RESULTS

CHAPTER LAYOUT

This chapter will provide the results accumulated for the current study. This chapter comprises of: a general set of results; descriptions of each parasite collected; and parasite community composition of each population sampled. All species descriptions include measurements indicated by a mean value followed by the range value and is measured in the units provided in the character description tables.

3.1. GENERAL RESULTS

All sites were intensely sampled. A total of 34 individual native Guttural Toads were collected at study area 1. In study area 2 invasive Guttural Toad and native Western Leopard Toad were found to overlap, a total of 59 individual invasive Guttural Toads was collected; data and blood of 37 individual native Western Leopard Toads were collected, however, only 15 individual road kills were collected and examined for internal parasites: three at site 2a and 12 at site 2b. Site 2b represents a reference site for Western Leopard Toad not exposed to the distribution range of Guttural Toads. Site 2c represents a reference site for Guttural Toad. In study area 3 a total of nine individual native Guttural Toads were collected. All toads were measured and weighed at each site (see Table 3.1 below):

Table 3.1: Mean and Max Snout–to–Urostyle length; and Mean and Max weight for all *Sclerophrys gutturalis* (GT) and *Sclerophrys pantherina* (WLT) collected at all three study areas.

Site	Species	Native/ Invasive	Sex	n	Mean SUL (mm)	Max SUL (mm)	Mean Weight (g)	Max weight (g)
1a	GT	Native	F	6	58	65	24	31
1a	GT	Native	M	10	59	67	26	35
1b	GT	Native	F	7	63	74	33	56
1b	GT	Native	M	11	62	69	29	43
2a	GT	Invasive	F	21	77	88	53	76
2a	GT	Invasive	M	16	61	72	28	43
2a	WLT	Native	F	3	101	104	117	140
2a	WLT	Native	M	7	83	91	77	90
2b	WLT	Native	F	12	85	100	95	140
2b	WLT	Native	M	15	83	93	58	90
2c	GT	Invasive	F	8	73	80	53	64
2c	GT	Invasive	M	14	68	72	39	49
3	GT	Native	F	1	77	77	59	59
3	GT	Native	M	8	73	83	40	59

Specimens were examined and parasites were extracted and studied according to taxa:

3.2. Acari

Of the 16 native Guttural Toad specimens collected at site 1a, five males were found to be infected with respectively one, one, two, two, and two mites in the nasal cavity and one female infected with three mites (n=11) (prevalence 37.5%, mean intensity 2). Based on morphological traits collected mites were identified as belonging to the genus *Lawrencarus*. Morphological markers (light microscopy and SEM) were applied to identify developmental stage of each specimen (n=7). Specimens used for morphological analysis were first sorted from adults to larva (this includes four stages: adult, deutonymph, protonymh and larva) and each individual was labelled specimen one to seven for individual examination according to measurements (see Table 3.2 below) and chaetotaxy.

Table 3.2: Morphological characters selected to be measured on collected mites based on Fain (1957)

Abbreviation	Character measured
MLi (µm)	Length of the idiosoma (excluding the gnathosoma)
MWi (µm)	Maximum width of the idiosoma
MLg (µm)	Maximum length of the gnathosoma from the base to edge prior the hypostoma
MWg (µm)	Maximum width of the gnathosoma
Lg (µm)	Length of the genital slit
Wg (µm)	Maximum width of the genital slit (male) or transverse portion of the slit (female)

3.2.1. Morphological analysis:

Based on the presence of three pairs of legs specimen 7 was identified as being a larva (see Fig. 3.1a below). Specimens one to five were according to size and body shape and chaetotaxy identified as adults. While specimen six was identified as nymph (see Fig. 3.1b below)

Scanning electron micrographs of specimen one to six were measured (Table 3.3 and Table 3.4) according to standard methods (see Fig. 3.1b-d below). No clear conclusion could be made upon measurements as they were not corresponding to any measurements provided by the previous study (Fain, 1957).

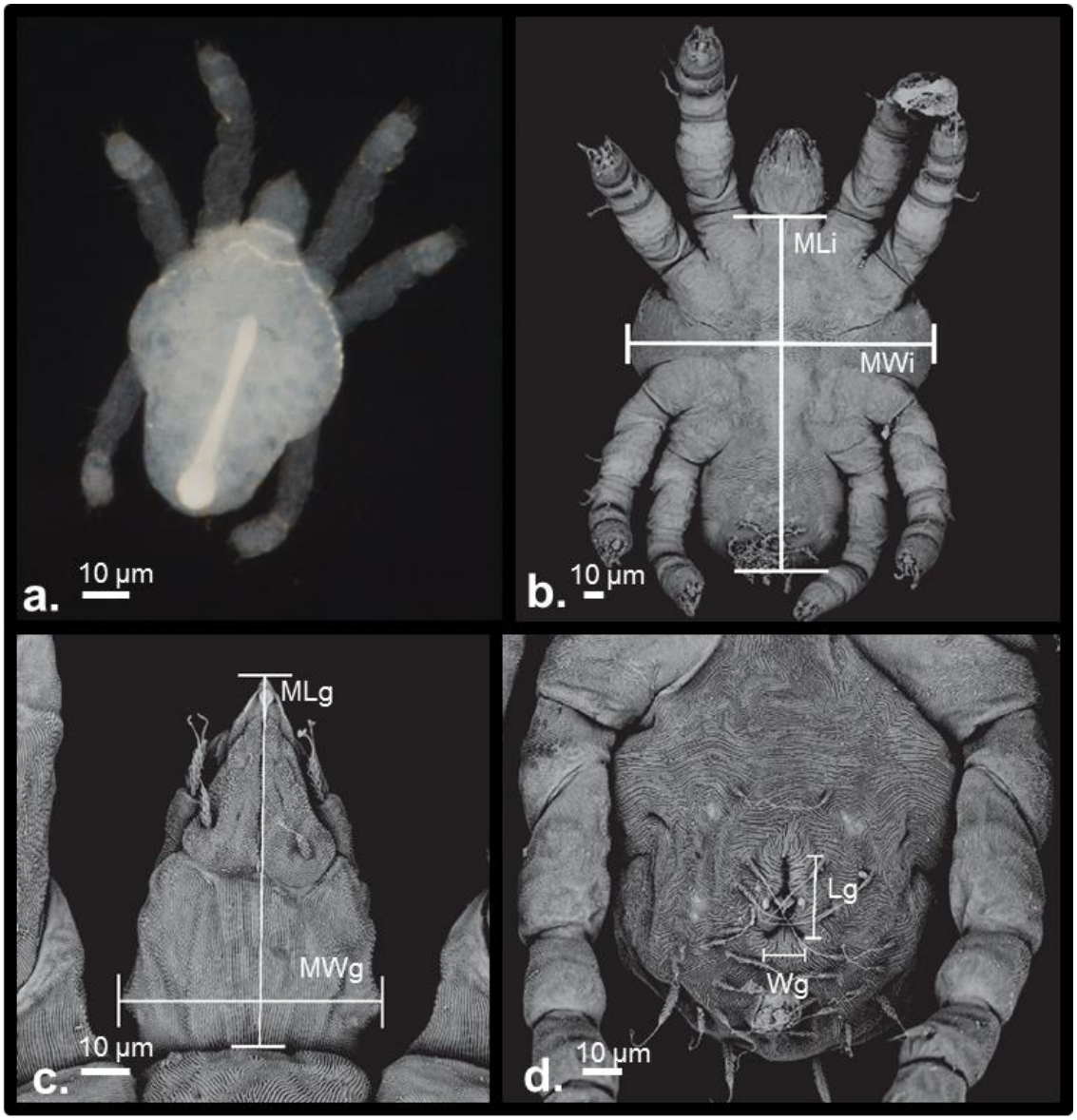

Figure 3.1: *Lawrencarus eweri* (Lawrence, 1952) **a)** Light micrograph of dorsal view of specimen 7 illustrating that it is still a larva. **b)** Scanning electron micrograph of the ventral view of a protonymph. **c)** Scanning electron micrograph of the ventral view of an adult gnathosoma. **d)** Scanning electron micrograph of the ventral view of a female adult genital slit. **Abbreviations:** MLi- Maximum length of the idiosoma, MWi- Maximum width of the idiosoma, MLg- Maximum length of the gnathosoma, MWg- Maximum width of the gnathosoma, Lg- Maximum length of the genital slit, and Wg- Maximum width of the transverse portion of the genital slit.

Table 3.3: Comparing observed and measured characteristics (this study) with published records for described adult *Lawrencarus eweri* collected from *Sclerophrys gutturalis* (Fain, 1957).

Characters	Specimen 1	Specimen 2	Specimen 3	Specimen 4	Specimen 5	Adult female (Fain, 1957)	Adult male (Fain, 1957)
MLi (µm)	304	323	334	352	390	410	380–460
MWi (µm)	250	191	241	250	283	320–420	290–325
MLg (µm)	72	83	81	73	72	85–93	70–86
MWg (µm)	56	47	56	60	41	67–72	62–64
Lg (µm)	35	23	26	27	–	33–60	36–45
Wg (µm)	20	14	15	15	–	23–33	18–22

Table 3.4: Comparing observed and measured characteristics (this study) with published records for described deutonymph and protonymph *Lawrencarus eweri* collected from *Sclerophrys gutturalis* (Fain, 1957).

Characters	Specimen 6	Specimen 7	Deutonymph (Fain, 1957)	Protonymph (Fain, 1957)	Larva (Fain, 1957)
MLi (µm)	218	183.2	352	285	180
MWi (µm)	172	145.9	262	270	165
MLg (µm)	52.4	41.51	–	–	–
MWg (µm)	41.2	36.19	–	–	–
LC i–ii (µm)	8.8–9.2	–	–	13–14	11
LC iii–iv (µm)	–	–	17	12	11

The chaetotaxy of specimens one to five was formulated (see Table 3.5 below). The coxa, genu, tibias, and tarsus formula as well as amount of genital hair of specimen one is in accordance with the provided chaetotaxy of the male adult of *L. eweri.* Specimen's two to four has some inconclusive chaetotaxy as the hair on the genu, tibias, tarsus, and genital was not observable on the scanning electron micrograph. The coxa, and tarsus formulas as well as amount of genital and anal hair for specimen four are in accordance with provided formulas for an adult female. The genu and tibias formulas for specimen five was in accordance with provided formulas for an adult.

The chaetotaxy of specimen six was formulated (see Table 3.6 below). The coxa, genu, and tarsus formulas of specimen six are in accordance with provided formulas of that of a protonymph.

Genital and anal regions of each specimen were inspected (see Fig. 3.2 below). Specimen one has two internal posterior hair located on the inside of the genital opening (see Fig. 3.2a below). Specimen's two to four has a genital slot that contains a horizontal portion and a longitudinal portion that affects the shape of an inverted T (see Fig. 3.2b below). Specimen six contains 4 pairs of perigenital discs and no clear genital opening can be observed (see Fig. 3.2c below).

Table 3.5: Comparing chaetotaxy (this study) with published records for described adult *Lawrencarus eweri* collected from *Sclerophrys gutturalis* (Fain, 1957).

Characteristics			Specimen 1	Specimen 2	Specimen 3	Specimen 4	Specimen 5	Adult (Fain, 1957)
Legs	**Coxa 3–4**		1–0	1–0	1–0	1–0		1–0
	Genu1–2–3–4		4–4–3–1	4–4–3–1	3–2–3–0	4–4–3–1	4–4–3–1	4–4–3–1
	Tibias 1–2–3–4		4–3–2–2	4–3–2–2	2–3–2–2	4–3–2–2	4–3–2–2	4–3–2–2
	Tarsus 1–2–3–4		12–8–7–7	12–8–7–7	12–8–7–7	11–8–7–7	–	12–8–7–7
Genital hair	**Female**	**Lateral**	0	7	8	7	–	7–8–
	Male	**Lateral**	8	0	0	0	–	7–8–
		Internal hair	2	0	0	0	–	2
	Anal hair		18	18	18	18	–	18–31

Table 3.6: Comparing chaetotaxy (this study) with published records for described deutonymph and protonymph *Lawrencarus eweri* collected from *Sclerophrys gutturalis* (Fain, 1957).

Characteristics		Specimen 6	Deutonymph (Fain, 1957)	Protonymph (Fain, 1957)
Legs	**Coxa 3–4**	1–0	1–0	1–0
	Genu1–2–3–4	4–4–3–0	4–4–3–1	4–4–3–0
	Tibias 1–2–3–4	–	4–2–2–1	4–2–2–1
	Tarsus 1–2–3–4	10–6–5–5	10–8–7–7	10–6–5–5
Genital hair		0	2–2–	0
Anal hair		–	23–17	17

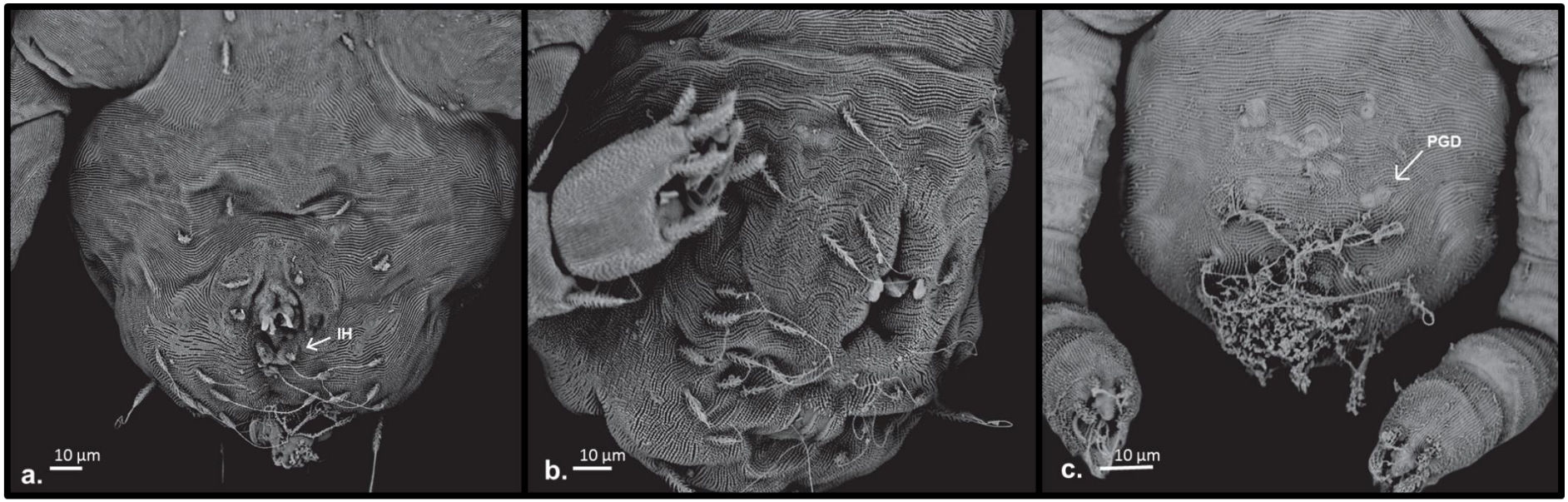

Figure 3.2*:* Scanning electron micrographs of *Lawrencarus eweri* (Lawrence, 1952) **a)** Ventral view of genital and anal region of specimen 1 illustrating two internal hairs located in the genital opening as illustrated by the arrow, **b)** Ventral View of genital and anal region of specimen 2 illustrating inverted T formed by the horizontal and longitudinal portion of the vulvar slit, **c)** Ventral view of anal region of specimen 6 illustrating the perigenital discs surrounding the genital area. **Abbreviations:** IH- Internal hairs, and PGD- Perigenital discs.

3.2.2. Supplementary description *Lawrencarus eweri* (Lawrence 1952) Fain 1957

Taxonomic summary:

Phylum: Arthropoda.

Family: Ereynetidae Oudemans, 1931.

Genus: *Lawrencarus* Fain, 1957.

Syn: *Ricardoella eweri* Lawrence, 1952.

Locality: Northern KwaZulu-Natal, Manguzi municipality grounds, (–26.94121, 32.81501)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Nasal cavities

Specimens on which description is based: Females (n=3), Male (n=1), Protonymph (n=1), and Larva (n=1)

Morphology:

Body shape can be described as inverted pear shape. Gnathosoma is triangular in shape and sometimes reflect as bullet–shaped. Four pairs of legs appear to separate into two pairs of legs on the ventral anterior region of the idiosoma and ventral posterior region of the idiosoma. Each leg is divided into six segments: trochanter, femur, coxa, genu, tibias and tarsus, however, the coxa region gives the impression to be the largest. Hair found only on leg segments coxa, genu, tibias, and tarsus appear to be feathered. Ereynetal organ appears to present on leg one close to the sensory hair. Adults contain a large amount (18) of hair around the anus.

Female (n=3): Female idiosoma can be described as pear-shaped 336 (323–352) x 227 (191–250). The gnathosoma of the female is 79 (73–83) x 54 (47–60). The genital slit is in the form of an inverted T with the median limb pointing towards the anus. The genital slit is 25 (23–27) x 14 (13–15). Four pairs of genital discs are present around the genital opening, two of which are laterally opposite to the anterior end and two other which are opposite to the posterior end of the aperture. The first pair of legs is slightly longer than the rest, whereas the second and third pair is larger than the fourth. Legs 1, 2, and 4 contain no hair on the coxa, however, leg 3 contain a single hair on the coxa. Legs 1 and 2 contain four hairs on the genu whereas leg 3

contains three and leg 4 a single hair. Leg 1 contains four hairs on the tibias whereas leg 2 contains three and legs 3 to 4 contain two. The tarsus of leg 1 contains 12 (11–12) hairs; leg 2 contains eight, and leg 3 to 4 contains seven hairs. In the peri-genital area seven genital hairs were observed and 18 anal hairs were also observed around the anus.

Male (n=1): Male idiosoma can be described as more egg shaped and is 304 x 191. The gnathosoma is more compact and is 72 x 56. The genital opening of the male is nearly oval with well chitinised lateral edges and is 36 x 20. In the central part of the opening there appears to be a strongly chitinised, cylindrical structure. Four pairs of genital discs like those described in the female are present in males. The male contains two internal hairs at the edge of the genital opening. Legs 1, 2, and 4 contain no hair on the coxa, however, leg 3 contain a single hair. Legs 1 and 2 contain four hairs on the genu whereas leg 3 contains three and leg 4 a single. Leg 1 contains four hair on the tibias whereas leg 2 contains three and legs 3 to 4 contain two. The tarsus of leg 1 contains 12 hairs; leg 2 contains eight, and leg 3 to four contains seven hairs. In the peri-genital area eight hairs can be found lateral from the genital opening and 18 anal hairs was observed around the anus.

Protonymph (n=1): Protonymph idiosoma is more compact and smaller than adults and is 218 x 172. The gnathosoma is rounded and is 52 x 41. No clear genital opening can be observed for the protonymph, however, four clear genital discs can be observed in the posterior genital field. Legs 1, 2, and 4 contain no hair on the coxa, however, leg 3 contain a single hair on the coxa. Legs 1 and 2 contain four hairs on the genu, whereas leg 3 contains three, and leg 4 none. Hair could not be observed for the tibias in this specimen. The tarsus of leg 1 contains 10 hairs, leg 2 contains eight hairs, and leg 3 and 4 contain five hairs. No hair could be observed in the anal area (mostly due to position of mite on SEM stub).

Larva (n=2): Larva idiosoma can be described as more compact and smaller than protonymph. The gnathosoma is round of shape. Larva contains only three pairs of legs: two pairs of legs on the ventral anterior region of the idiosoma and one pair the ventral posterior region.

3.3 Nematodes

Three types of nematodes were collected in toads from the current study: (1) Lung nematodes (2) intestinal nematodes and (3) encysted nematodes

3.3.1. Lung Nematodes: *Rhabdias* cf. *africanus*

Of the 18 native Guttural Toad specimens collected at site 1b, five males were found to be infected respectively with one, one, one, 14, and 70 lung nematodes and two females were found to be infected respectively with two and four lung nematodes (n=93) (prevalence 39%, mean intensity 13). Morphological marker (light microscopy) was applied to identify specimens (n=3), and molecular marker (28S) was applied to confirm identification (n=3). All specimens were inspected and confirmed as *Rhabdias* cf. *africanus* by R. Svitin and Y. Kuzmin (personal communication Nov 2017).

Of the nine native Guttural Toad specimens collected at site 3, three males were found to be infected respectively with one, one, and three lung nematodes respectively (n=5) (prevalence 33%, mean intensity 2). Morphological marker (light microscopy) was applied to identify specimens (n=1), and molecular marker (28S) was applied to confirm identification (n=3). All specimens were inspected and confirmed as *Rhabdias* cf. *africanus* by R. Svitin and Y. Kuzmin (personal communication Nov 2017).

3.3.1.1. Morphological analysis:

Characters have been identified according to relativity (see Table 3.7 below). These characters were measured and noted (see Table 3.8 below).

Table 3.7: Characters selected to be measured based on Junker *et al.,* (1957)

Abbreviation	Characters measured
ML (mm)	Length
MW (µm)	Width at mid–body (with vesicle)
BCL (µm)	Buccal capsule length
BCE (µm)	Buccal capsule max. external diameter
BCR (µm)	Buccal capsule ratio
OL (µm)	Oesophagus length
OL%	Oesophagus length as % of body length
OW (µm)	Oesophagus width at mid–length
BD (µm)	Bulb diameter
BW (µm)	Body width at bulb (with vesicle)
HV (µm)	Head to vulva
HV%	Head to vulva as % of body length
TL (µm)	Tail length
TL%	Tail length as % of body length
AW (µm)	Width at anus (with vesicle)
ANR (µm)	Apex to nerve ring

Certain characters such as body shape and position of oesophagus shoulder et cetera was noted and observed (see Fig. 3.3 below). These are discussed in species descriptions.

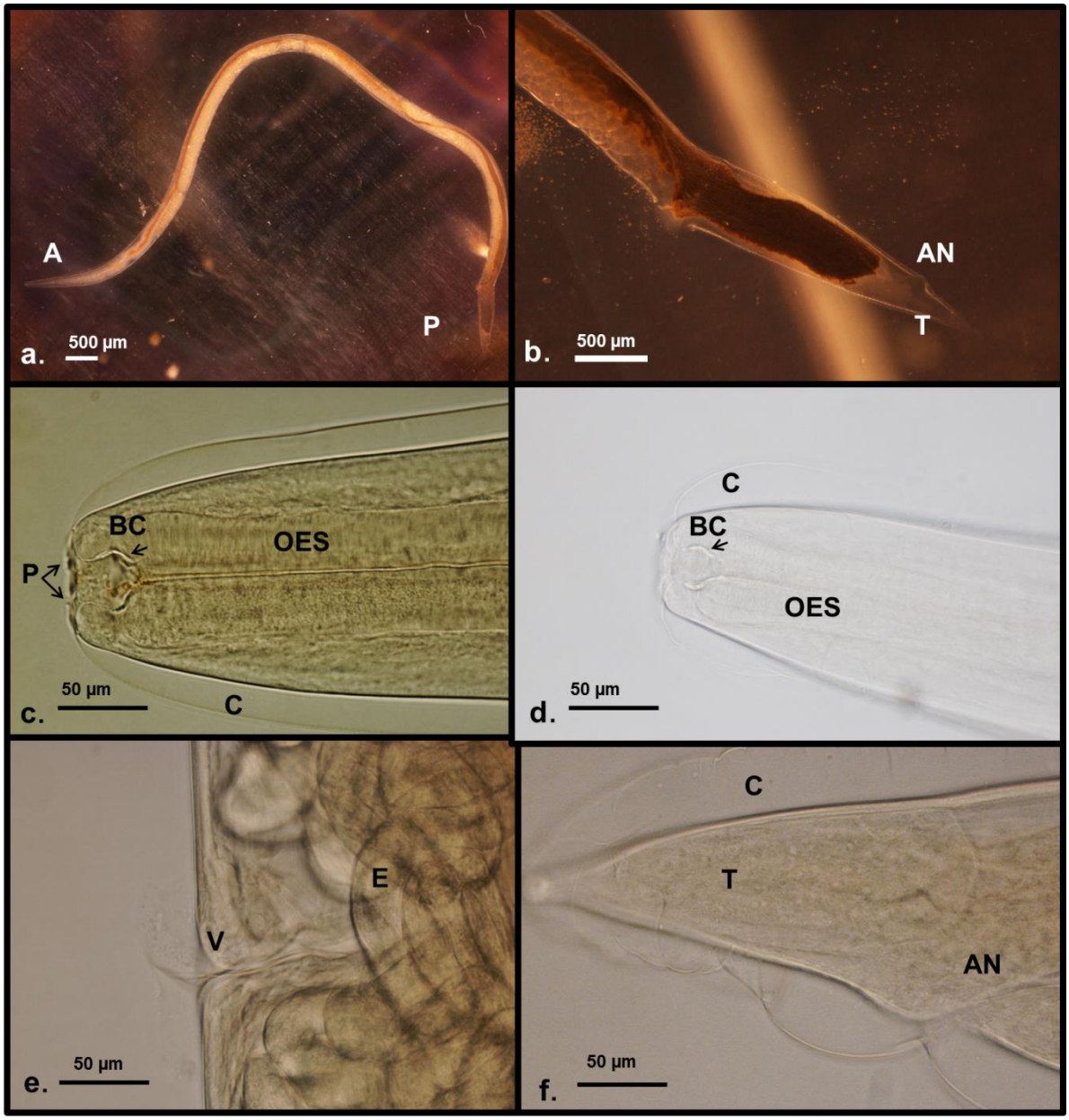

Figure 3.3: *Rhabdias* cf. *africanus* Kuzmin, 2001. **a)** Whole mount. **b)** Tail. **c)** Ventral view of the head. **d)** Ventral view of the head. **e)** Genital opening. **f)** Tail. **Abbreviations:** A– Anterior side, P– Posterior side, AN– Anus, T– Tail, P– Pappilae, BC– Buccal Cavity, OES– Oesophagus, C– Cuticle, V– Vulva, and E– Eggs.

Table 3.8: Comparing character metrics of this studywith published records for described adult *Rhabdias* species collected from *Sclerophrys* toads in Africa adapted from Junker *et al.* (2010).

Species	***Rhabdias* cf. *africanus***		***Rhabdias* cf. *africanus***	***Rhabdias africanus* Kuzmin, 2001**		***Rhabdias bufonis* (Schrank, 1788)**		***Rhabdias picardiae* Junker, Lhermitte–Vallarina & Bain 2010**	
Host	*Sclerophrys gutturalis*		*Sclerophrys gutturalis*	*Sclerophrys garmani &*		*Sclerophrys regularis*		*Sclerophrys gutturalis*	
Country	South–Africa (site 1b, Durban)		South–Africa (site 3, Potchefstroom)	South–Africa		North–Africa		South–Africa	
Source	Current study		Current study	Kuzmin (2001)		Junker *et al.*(2010)		Junker *et al.* (2010)	
	(n=3)		(n=1)	(n=9)		(n=10)		(n=7)	
	Range	**Mean**	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
ML	7–9	8	6	13–20	16	3–13	8	8.0–8.4	8.2
MW	215–244	230	333	300–450	378	163–476	320	460–600	530
BCL	16–19	18	13	15–20	18	15	15	7–10	8.5
BCE	22–25	24	23	20–23	22	21	21	23–25	24
BCR	1:0.7–1:0.8	1:0.8	1:0.6	1:0.7	1:0.7	1:0.7	1:0.7	1:0.3–1:0.4	1:0.4
OL	592–655	624	926	570–710	632	288–510	399	690–790	740
OL%	6%–8%	7%	15%	3%–5%	4%	–	–	9%–10%	9.5%
OW	45–50	48%	86	–	–	–	–	60–70	65
BD	58–76	67	95	65–80	73	57–72	65–	95–130	113

Table 3.8. *continued.*

			280						
HV	**3850–4212**	**4031**	**3904**	**–**	**–**	**–**	**–**	**4535–5050**	**4765**
HV%	48%–53%	50%	62%	47%–50%	48%	–	–	55%–62%	59%
TL	264–429	347	310	250–400	325	144–420	282	270–350	310
TL%	3.3%–3.9%	3.6%	4.9%	–		–	–	3.5%–3.8%	3.6%
AW	107–130	119	156	–	–	–	–	120–440	280
ANR	229–285	257	519	–	–	168–240	204	235–250	243

3.3.1.2. Molecular analysis

Amplicons between 1100 –1200 nt were derived from eight of the *Rhabdias* cf. *africanus* specimens. For the phylogenetic analysis, two sequences were used, one from the *Rhabdias* cf. *africanus* (1) from site 1b, and one from the *Rhabdias* cf. *africanus* (2) from site 3. The sequences was analysed together with 15 28S sequences from species of the genus *Rhabdias*. The BI tree (see Fig. 3.4 below) was divided into four well-supported monophyletic clades. Sequences from the current study forms a monophyletic clade with strong nodal support with the sequence of *Rhabdias* cf. *africanus* (GenBank: KF999598), suggesting that these two sequences belongs to this species.

Uncorrected pair-wise distances (p–distance) and base pair differences were determined for these three sequences. The p-distance differences based on the base differences per site between these sequences confirmed that the sequences from the current study in fact belong to the species *Rhabdias africanus*. The alignment of 1100–1200 nt sites had a difference of six sites and a p–distance value of 0.3%.

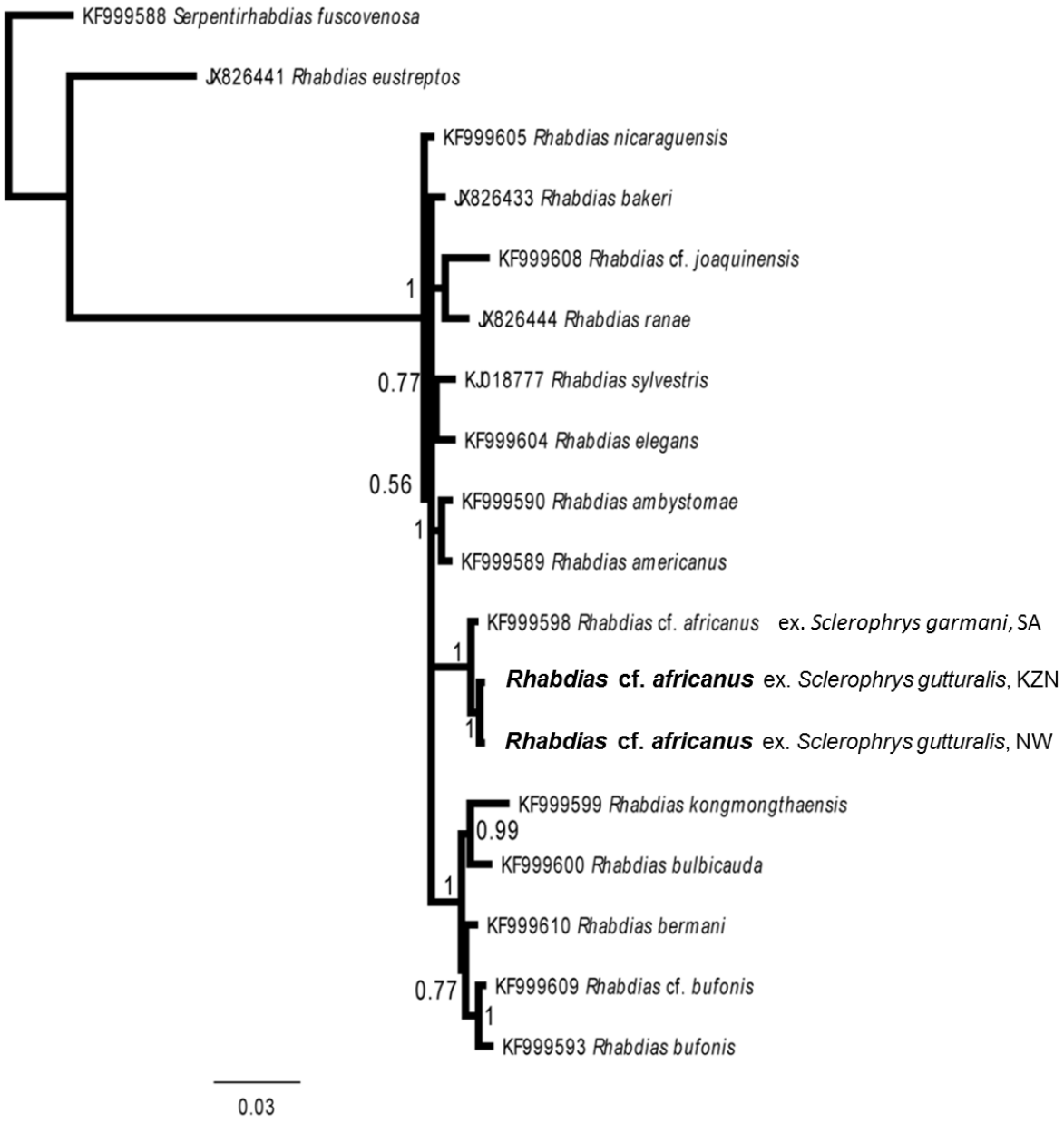

Figure 3.4: Phylogenetic analysis of 28S gene sequences generated by this study for *Rhabdias* cf. *africanus* and closely related lung nematodes belonging to genus *Rhabdias* based on 28S gene sequences collected from GenBank. Bayesian Inference (BI) analysis illustrates the phylogenetic relationship for 16 lung nematode species. Nodal support is provided by posterior probability values.

3.3.1.3. Supplementary description *Rhabdias* cf, *africanus* Kuzmin, 2001

Taxonomic summary:

Phylum: Nematoda

Family: Rhabdiasidae Railliet, 1916.

Genus: *Rhabdias* Stiles & Hassall, 1905.

Locality: Durban (-29.782294, 31.030196), Potchefstroom (-26.4338.97, 27.55849)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Lungs

Specimens on which description is based: Adults (n=4).

Morphology:

Elongated body is 8 mm (6 – 9 mm) x 274 (215–333). Cuticle present, however, thin or indistinct along the body but widens near the tail. Two small papillae is visible adjacent oral opening. Buccal capsule 16 (13–19) x 24 (22–25). Buccal capsule ratio 1:0.8 (1:0.7–1:0.8). Oesophagus with shoulders terminating near or next to the apex of the buccal capsule 761 (592–929) reaching 10.3% (6.0%–14.6%) of the total body length. Oesophagus width at mid-length 66 (45–86), gradually widening to 77 (58–95) at the bulb. Body width at the bulb level 218 (156–280). Oesophagus width decreases near the nerve ring. The nerve ring appears 374 (229–518) from the head. Vulva appears to be slit-like, inconspicuous, situated in the mid-region of the body at 4031 (3850–4212) from the head or 54.7% (47.5%–62.0%) of the body length. Tail is conical in shape measured at 347 (264–429) or 4.1% (3.3%–4.9%) of the body length. Body width at anus 131.3 (107.0–155.7).

Molecular (n=4):

The phylogenetic analysis confirmed the placement of *Rhabdias* cf. *africanus* as a monophyletic clade formed between the *Rhabdias* sp. provided by the current study. A p–distance of 0.3 % confirms this placement.

3.3.2. Intestinal nematodes: *Amplicaecum* sp.

Two different genera of intestinal nematodes have been identified: *Amplicaecum* sp. and *Cosmocerca* sp.

Of the 18 native Guttural Toad specimens collected at site 1b, four males were found to be infected respectively with one, two, five, and seven *Amplicaecum* sp. and two females were found to be infected with one, and three *Amplicaecum* sp. (n=19) (prevalence 33%, mean intensity 3). These intestinal nematodes were identified as *Amplicaecum* sp. by R. Svitin (personal communication Nov 2016). Morphological markers (light microscopy) were applied to confirm identification (Fig 3.5)

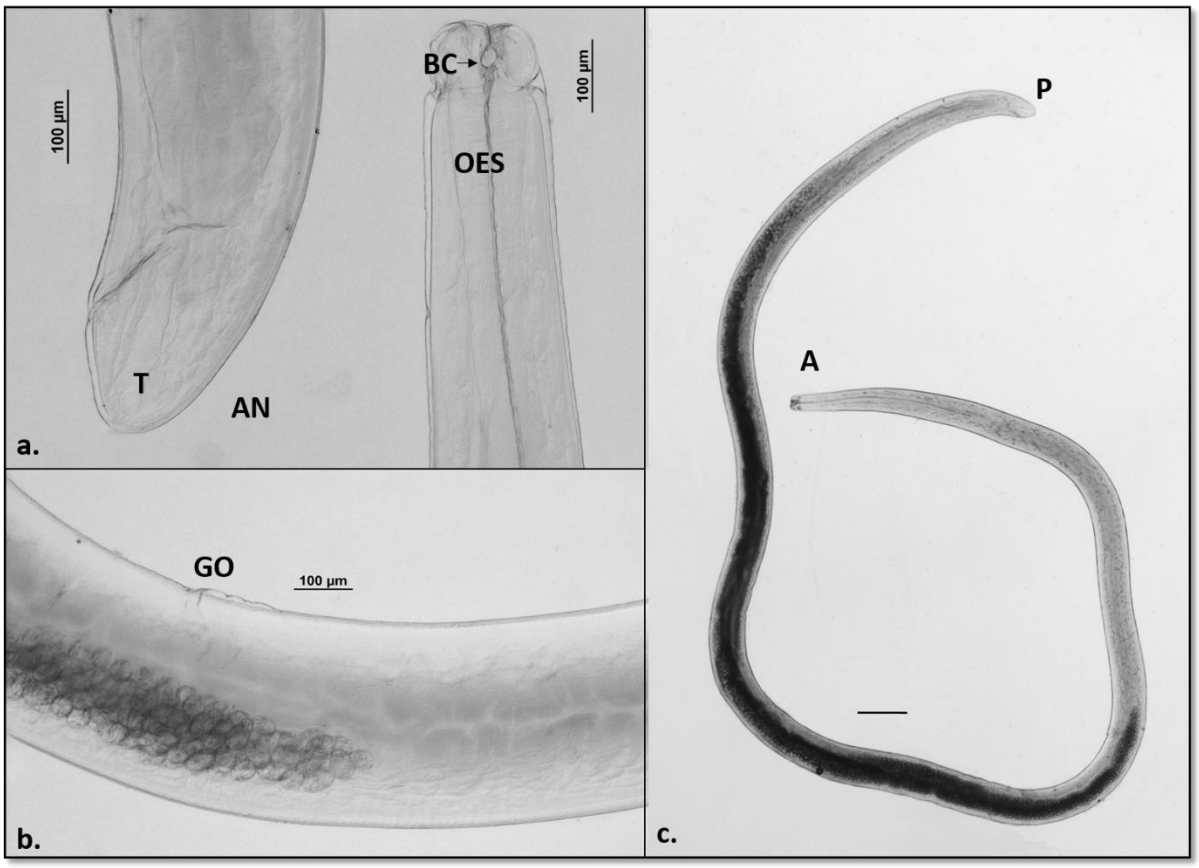

Figure 3.5: *Amplicaecum* sp. Baylis, 1920. a) Posterior and anterior parts. **b)** Genital opening. **c)** Whole mount. **Abbreviations:** AN- Anus, T- Tail, BC- Buccal cavity, OES- Oesophagus, GO- Genital opening, A- Anterior end, and P- Posterior end.

3.3.2.1. Supplementary description *Amplicaecum* sp. Baylis, 1920.

Taxonomic summary:

Phylum: Nematoda.

Family: Ascaridae Baird, 1853.

Genus: *Amplicaecum* Baylis, 1920.

Locality: Durban (-29.782294, 31.030196)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Intestine

3.3.3. Intestinal nematodes: *Cosmocerca* sp.

Of the 16 native Guttural Toad specimens collected at site 1a, two males were found to be infected respectively with five, and 30 *Cosmocerca* sp. and two females were found to be infected with seven, and 15 *Cosmocerca* sp. (n=57) (prevalence 25%, mean intensity 14).

Of the 18 native Guttural Toad specimens collected at site 1b, fourmales were found to be infected respectively with two, five, eight, and 25 *Cosmocerca* sp. and four females were found to be infected with two, 12, 13, and 15 *Cosmocerca* sp. (n=82) (prevalence 44%, mean intensity 10). Morphological marker (light microscopy) was applied to identify specimens (n=4), and molecular marker (COI) was applied to confirm identification (n=3).

Of the 12 native Western Leopard Toad specimens collected at site 2b, one female was found to be infected with five *Cosmocerca* sp. (n=5) (prevalence 8.3%, mean intensity 5). Morphological marker (light microscopy) was applied to identify specimens (n=2), and molecular marker (COI) was applied to confirm identification (n=3, of these n=2 was also used to identify according to morphological work before molecular work).

Of the nine native Guttural Toad specimens collected at study area 3, six males were found to be infected with one, two, four, 13, 20, and 30 *Cosmocerca* sp. respectively (n=70) (prevalence 67%, mean intensity 12).

Only specimens of *Cosmocerca sp.* collected at site 1b and site 2b were analysed utilizing morphological and molecular markers due to relevance to study.

3.3.3.1 Morphological analysis:

Characters have been identified according to relativity (see Table 3.9 below). These characters were measured and noted (see Table 3.10 below).

Table 3.9: Characters selected to be measured based on Ryzhikov *et al.* (1980).

Abbreviation	Characters measured
ML (mm)	Length
MW (µm)	Width at mid–body (with vesicle)
OL (µm)	Oesophagus length
OW (µm)	Oesophagus width
OL%	Oesophagus length as % of body length
OBL (µm)	Oesophagus bulb length
OBW (µm)	Oesophagus bulb width
TL (µm)	Tail length
TL%	Tail length as % of body length
AP (µm)	Tail appendage length
ANR (µm)	Apex to nerve ring
EL (µm)	Egg length
EW (µm)	Egg width
PAP (µm)	Papillae

Certain characters such as body shape and position of oesophagus shoulder et cetera was noted and observed (Fig. 3.6) below. These are discussed in species descriptions.

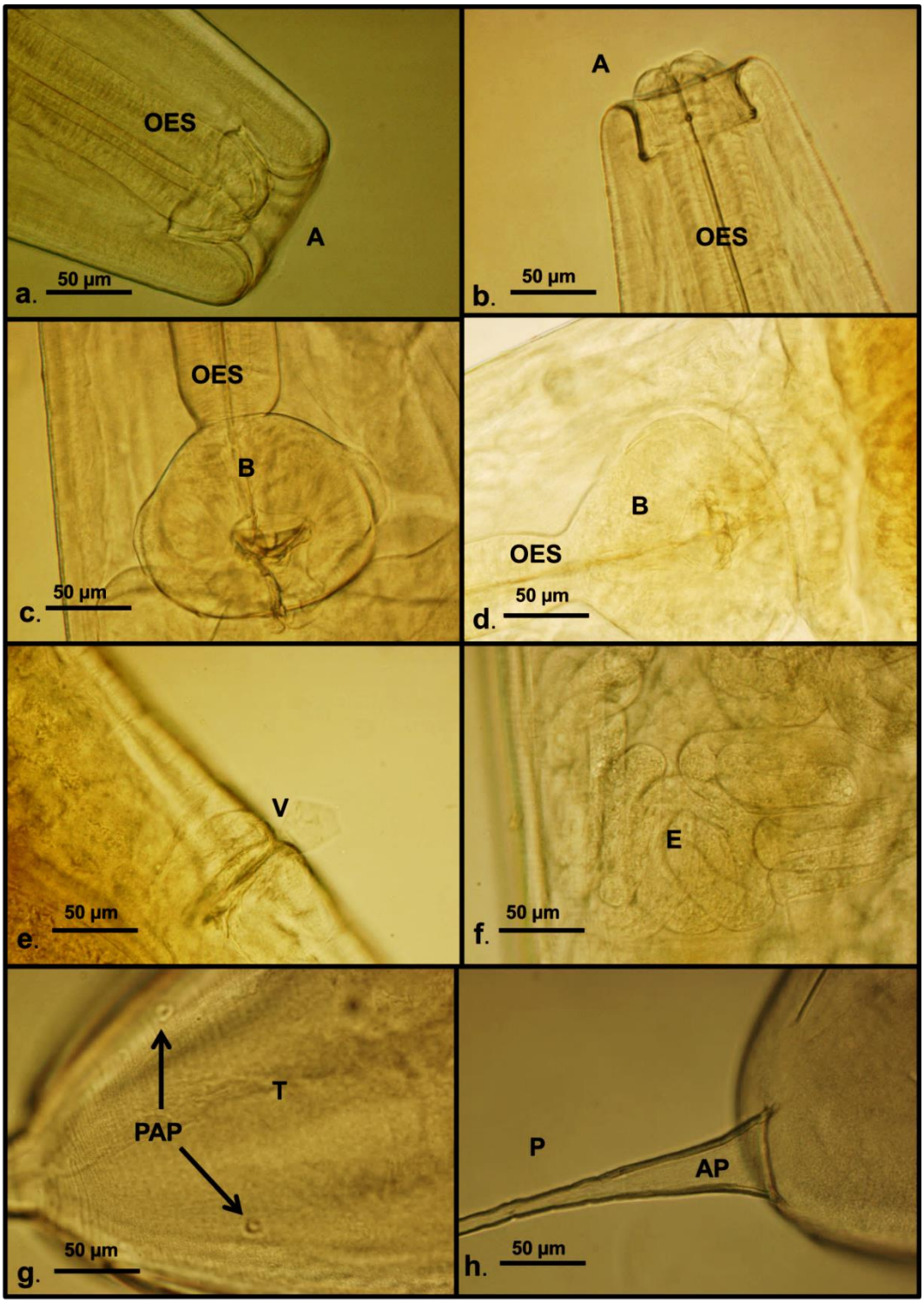

Figure 3.6: *Cosmocerca* sp. **a)** Ventral view of head. **b)** Anterior part. **c)** Bulb at the end of oesohagus. **d)** Bulb at the end of oesohagus. **e)** Genital opening. **f)** Eggs. **g)** Posterior end. **h)** Posterior end displaying appendage. **Abbreviations**: A– Anterior end, P– Posterior end, OES– Oesophagus, B– Bulb, V– Vulva, E–Eggs, T– Tail, AP– Appendage, and PAP– Papillae.

Table 3.10: Comparing character metrics of this study with published records for described adult *Cosmocerca* species collected from *Sclerophrys* toads in Africa adapted from Ryzhikov *et al.* (1980).

Species	***Cosmocerca* sp. 1**		***Cosmocerca* sp. 2**		***Cosmocerca ornata* (Dujardin, 1845) Redescription done by Ryzhikov *et al.* (1980)**		***Cosmocerca ornata* (Dujardin, 1845) Redescription done by Bala (2016)**	
Sex	Female		Female		Female		Female	
Host	*Sclerophrys gutturalis*		*Sclerophrys pantherina*		Grass frog		*Duttaphrynus melanostictus* (Schneider, 1799)	
Country	South–Africa (site 1b, Durban)		South–Africa (site 2b, Noordhoek)		Ukraine		India	
Source	Current study		Current study		Ryzhikov *et al.* (1980)		Bala (2016)	
	(n=4)		(n=2)		(n=unknown)		(n=unknown)	
	Range	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
ML	2.8–4.2	3.5	2.7–3.2	2.9	4.4–7.8	6.1	5.8–8.9	7.3
MW	244–294	269	316–545	431	250–550	400	390–620	505
OL	484–617	550	417–483	450	360–480	420	450–590	520
OW	55–69	62	57	57	–	–	–	–
OL%	15%–17%	16	15%	15%	6%–8%	7%	–	7%
OBL	130–161	145	155–172	165	90–120	105	160–180	170
OBW	149–169	159	173–175	174	130	130	130–160	145
TL	188–218	203	303–591	447	400–490	445	495–568	532

Table 3.10. *continued.*

TL%	5%–7%	6%	11%–18%	15%	6%–9%	7%	–	7%
AP	232–385	309	230–415	323	–	–	198–239	219
ANR	272–298	285	207–264	236	–	–	–	–
EL	76–142	109	102	102	110–148	129	94–112	103
EW	42–70	56	82	82	70–82	76	58–77	67.5
PAP	5x5	5x5	2x2	2x2	5x5	5x5	–	–

3.3.3.2 Molecular analysis

Amplicons of 377 nt were derived from three *Cosmocerca* sp. from site 1b and four *Cosmocerca* sp. from site 2b. For phylogenetic analysis, two sequences were used, one from the *Cosmocerca* sp. (1) from site 1b, and one from the Cosmocerca sp. (2) from site 2b. The sequences was analysed together with six COI sequences from species of the family Cosmocercidae. The BI tree (see Fig. 3.7 below) was divided into two large monophyletic clades. The first monophyletic clade comprises only representative sequences from the genus *Aplectana*. This clade forms a sister clade to the monophyletic clade comprising of the sequences generated in the current study. Based on this molecular evidence the sequences were identified as *Cosmocerca* sp.

Uncorrected pair-wise distances (p–distance) and base pair differences were determined. The p–distance differences based on the base differences per site between these sequences confirmed that the two *Cosmocerca* sp. are indeed two different species as the 604 site alignment between the *Cosmocerca* sp. from Durban differ with 46 nucleotides with the *Cosmocerca* sp. from the Western–Cape. These two specimens have a 7% p–distance value.

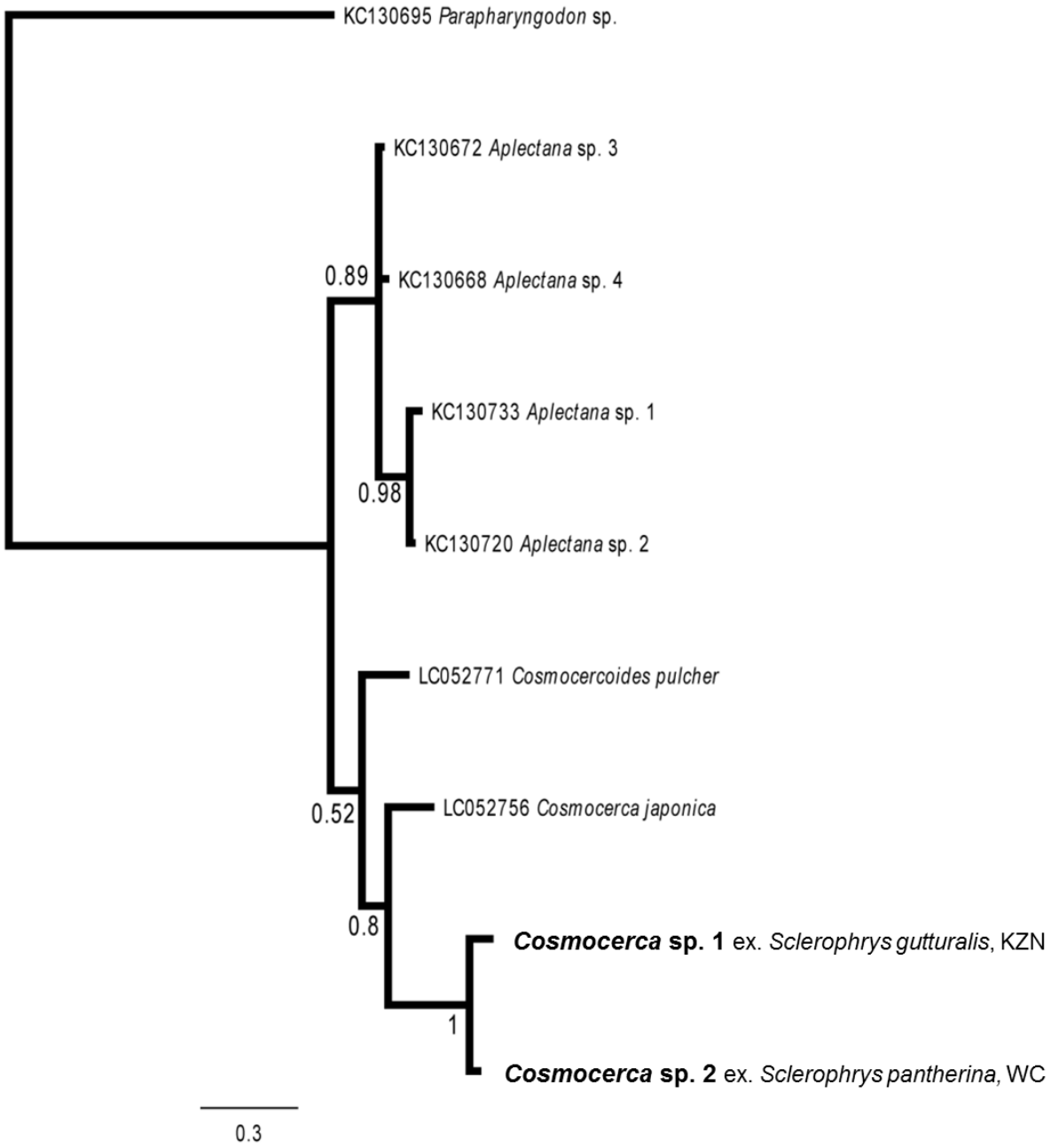

Figure 3.7: Phylogenetic analysis of COI gene sequences generated by this study for *Cosmocerca* and closely related intestinal nematodes belonging to the family Cosmocercidae based on 28S gene sequences collected from GenBank. Bayesian Inference (BI) analysis illustrates the phylogenetic relationship for 7 intestinal nematode species. Nodal support is provided by posterior probability values.

3.3.3.3. Supplementary description *Cosmocerca* sp. 1

Taxonomic summary:

Phylum: Nematoda.

Family: Cosmocercidae (Railliet, 1916).

Genus: *Cosmocerca* Diesing, 1861.

Locality: Durban (-29.782294, 31.030196)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Intestine

Specimens on which description is based: Adult females (n=4).

Morphology:

Small, whitish in colour, stubby/stout shaped.

Female (n=4): Body short 3.5 (2.8–4.2) mm x 269 (244–294). The oesophagus is 550 (484–617) x 62 (55–69) with oesophageal bulb 145 (130–160) x 159 (149–169). The oesophagus uptakes 16% (15%–17%) of the total body length. Oesophagus width decreases near the nerve ring 285 (272–298) from the apex. Tail conical in shape 203 (118–218) and uptakes 5.8% (5.0%–6.7%) of the total body length. Eggs 109 (76–142) x 56 (42–70). Appendages on tail extend 309 (232–385) from tip of tail. Five pairs of papillae were noted in alternate rows lateral from each other.

Molecular (n=4):

The *Cosmocerca* sp.1 forms a monophyletic clade with other species from the genus *Cosmocerca*. This confirmed the placement *Cosmocerca* sp.1 in the genus.

3.3.3.4. Supplementary description *Cosmocerca* sp. 2

Taxonomic summary:

Phylum: Nematoda.

Family: Cosmocercidae (Railliet, 1916).

Genus: *Cosmocerca* Diesing, 1861.

Locality: Noordhoek (-34.62583, 18.234113)

Host in current study: *Sclerophrys pantherina*

Location of parasite: Intestine

Specimens on which description is based: Adult Female (n=2).

Morphology:

Small, whitish in colour, stubby/stout shaped.

Female (n=2): Body short 2.9 (2.7–3.2) mm x 450 (417–483). The oesophagus 450 (417–483) x 57 with oesophageal bulb 165 (155–172) x 174 (173–175). The oesophagus uptakes 15% of the total body length. Oesophagus width decreases near the nerve ring 236 (207–264) from the apex. Tail conical in shape 447 (303–591) and uptakes 15% (11%–18%) of the total body length. Eggs 102 x 82. Appendages on tail extend 323 (230–415) from tip of tail. Two pairs of papillae were noted in alternate rows lateral from each other.

Molecular (n=4):

The *Cosmocerca* sp.2 forms a monophyletic clade with other species from the genus *Cosmocerca*. This confirmed the placement *Cosmocerca* sp.2 in the genus.

3.3.4. Encysted nematodes

Encysted nematodes was subjected to morphological placement analysis as well as molecular placement analysis but unfortunately yielded no positive identification.

Of the 16 native Guttural Toad specimens collected at site 1a, three males were found to be infected respectively with one, two, and six encysted nematodes and one female infected with one encysted nematode on the surface of the organs (n=10) (prevalence 25%, mean intensity 3).

Of the 18 native Guttural Toad specimens collected at site 1b, four males were found to be infected respectively with one, two, two, and three encysted nematodes and three females infected with one, two, and three encysted nematodes (n=14) (prevalence 39%, mean intensity 2).

Of the 37 invasive Guttural Toad specimens collected at site 2a, two males were found to be infected respectively with one, and four encysted nematodes and two females infected with three, and five encysted nematodes (n=13) (prevalence 10.8%, mean intensity 3).

Of the 12 native Western Leopard Toad specimens collected at site 2b, one male was found to be infected with two encysted nematodes and one female was found to be infected with two encysted nematodes (n=4) (prevalence 16.6%, mean intensity 2).

Of the 22 invasive Guttural Toad specimens collected at site 2c, one male was found to be infected with one encysted nematode and one female was found to be infected with two encysted nematodes (n=3) (prevalence 9%, mean intensity 3).

Of the nine native Guttural Toad specimens collected at study area 3, one male was found to be infected with 18 encysted nematodes (n=18) (prevalence 11%, mean intensity 18).

3.4. Platyhelminthes: *Mesocoelium* cf. *monodi*

Of the 18 individual native Guttural Toad specimens collected at site 1b, one male was found to be infected with six *Mesocoelium* cf. *monodi* in the intestine (n=6) (prevalence 5%, mean intensity 6). All six trematodes were retrieved and molecular marker (28S) was applied to identify species (n=4) and morphological markers (Light microscopy) were applied to confirm identification (n=2).

Specimens collected have been assigned to *Mesocoelium* cf. *monodi* due to similar diagnostic characters but specimens did contain some discrepancies to original description, which will be explained in the discussion.

3.4.1. Molecular analysis

Amplicons between 500–700 nt were derived from four of the trematode specimens. For the phylogenetic analysis, only a single nucleotide sequence was used, as the sequences generated and obtained were identical. This sequence was analysed together with two 28S sequences from the genus *Mesocoelium*. The BI tree (see Fig. 3.8 below) was grouped into one monophyletic clade. Sequence generated in this study is represented as *Mesocoelium* cf*. monodi* on the BI tree. Available sequences for the genus *Mesocoelium* cluster together.

3.4.2. Morphological analysis

After molecular identification morphological marker (Light microscopy) was applied to confirm this. Characters were chosen based on relativity and importance for identification (see Table 3.11 below). Each individual was examined and measured (see Table 3.12 below).

Table 3.11*:* Characters selected to be measured based on Dronen *et al.* (2012).

Abbreviation	Character measured
ML	Maximum length of body
MW	Maximum width of body
OSL	Oral sucker length
OSW	Oral sucker width
VSL	Ventral sucker length
VSW	Ventral sucker width
OSW to VSW ratio	Oral sucker width to Ventral sucker ratio
PL	Pharynx length
PW	Pharynx width
OW	Oesophagus width
CSL	Cirrus–sac length
CSW	Cirrus–sac width
RTL	Right testes length
RTW	Right testes width
LTL	Left testes length
LTW	Left testes width
OVL	Ovary length
OVW	Ovary width
EL	Egg length
EW	Egg width
FBL	Forebody length
FBL (%)	Length of forebody as a proportion of body length
POFL	Post–ovarian field length
POFL (%)	Length of post–ovarian field as a proportion of body length
VL	Viteline field length
CL	Ceca length
CLPOF	Ceca length surpassing the post–ovarian field
%CL	Percentage ceca surpass ovary into postovarian space

Certain characters such as body shape and length of ceca et cetera was noted and observed (Fig. 3.9) below. These are discussed in species descriptions.

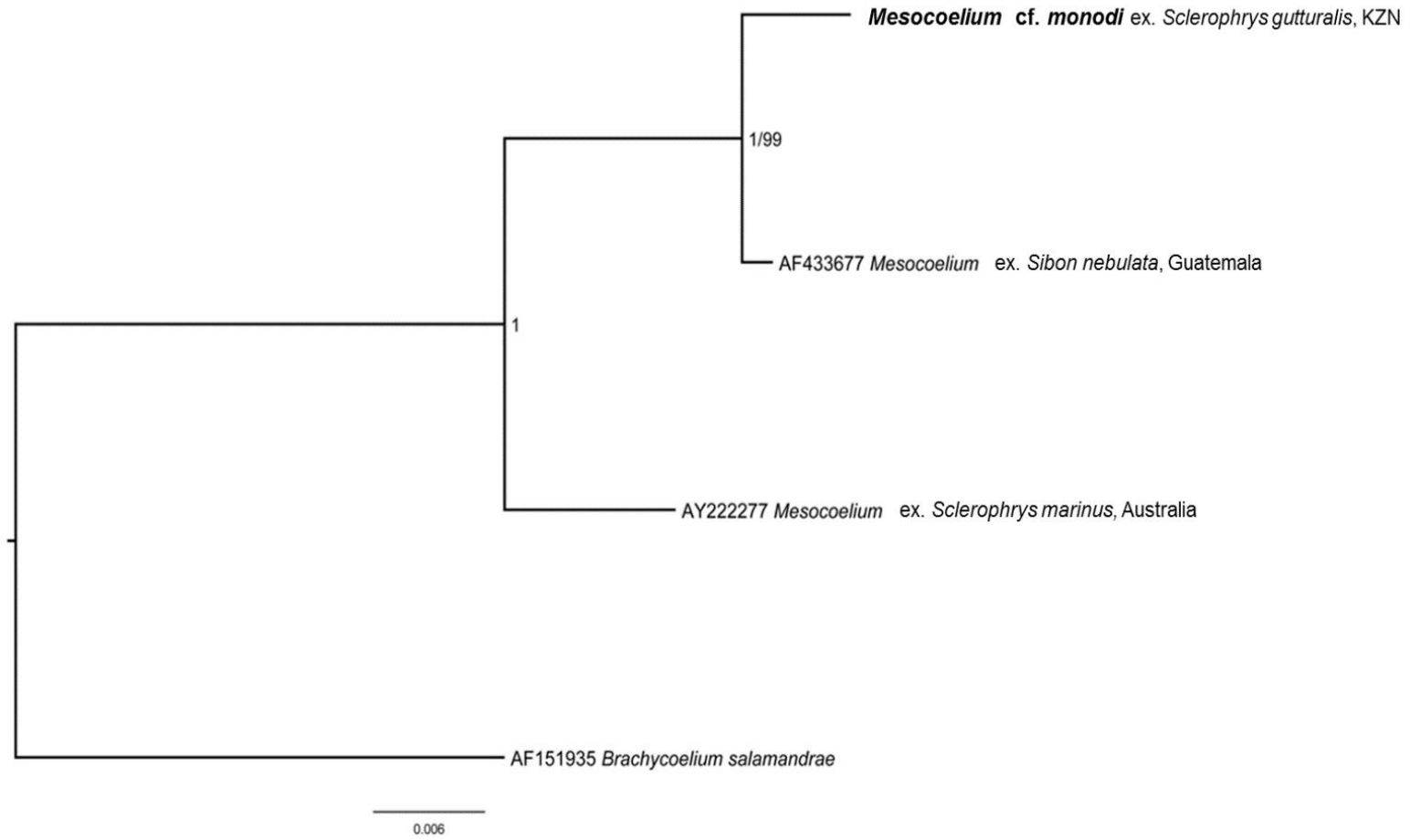

Figure 3.8*:* Phylogenetic analysis of 28S gene sequences generated by this study for *Mesocoelium* cf. *monodi* and closely related trematodes belonging to the family Brachycoeliidae collected from GenBank. Bayesian Inference (BI) analysis illustrates the phylogenetic relationship for 4 trematode species. Nodal support is provided by posterior probability values and Maximum Likelihood scores are illustrated to the right.

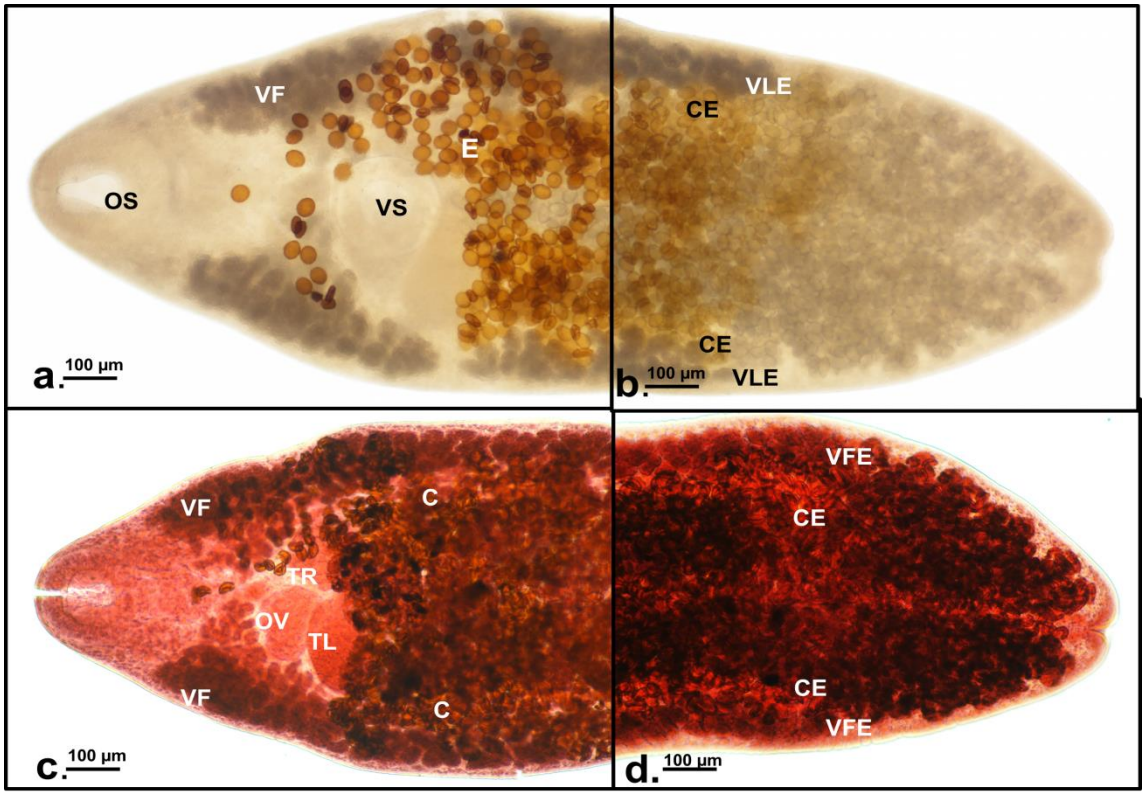

Figure 3.9: *Mesocoelium* cf. *monodi* Dolfus, 1929. **a)** Anterior part of unstained adult. **b)** Posterior part of unstained adult. **c)** Anterior part of stained adult. **d)** Posterior part of stained adult. **Abbreviations**: OS– Oral sucker, VS– Ventral sucker, VF– Vitelline field, E– Eggs, C– Ceca, CE– Ceca ends, VLE/VFE– Viteline line/field ends, OV– Ovary, TR– Right testis, and TL–Left testis

Table 3.12: Comparing character metrics of this study with published records for described adult *Mesocoelium* species collected from *Sclerophrys* toads in Africa (adapted from Dronen *et al.,* 2012).

Species	***Mesocoelium* cf. *monodi***		***Mesocoelium monodi* Dolfus, 1929**		***M. cameroonensis* Saoud, 1964**		***M. schwetzi* Dollfus, 1950**	
Host	*Sclerophrys gutturalis*		*Sclerophrys regularis*		*Sclerophrys regularis*		*Sclerophrys regularis*	
Country	South–Africa (site 1b-Durban)		Central Africa		West–central Africa		Central Africa	
Source	Present study		Dronen *et al.* (2012)		Dronen *et al.* (2012)		Dronen *et al.* (2012)	
	(n=2)		(n=40)		(n=4)		(n=4)	
	Range	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
ML	1614–1806	1710	1450–3513	2317	1750–1975	1833	1260–2410	1835
MW	615–714	664	525–1263	816	900–1206	1054	–	–
OSL	202–205	203	183–330	254	295–375	355	–	–
OSW	180–209	194	178–300	267	290–380	373	169–175	172
VSL	137–144	141	135–260	205	271–320	288	–	–
VSW	158–175	166	143–260	189	290–370	327	–	102
OSW to VSW ratio	1:1.14–1:1.2	1:1.16	1:1.1–1:1.6	1:1.3	1:1.1–1:1.3	1:1.2	–	–
PL	84–80	82	75–145	108	80–93	85	–	–
PW	72–73	73	68–170	129	125	125	–	60
OW	48–81	65	28–105	58	–	–	–	–
CSL	172	172	88–325	220	175–225	195	–	115
CSW	63	63	38–105	67	69–78	74	–	–

Table 3.12. *continued.*

RTL	123–128	126	108–300	203	205–380	300	–	–
RTW	168–170	169	95–228	197	298–331	310	–	55
LTL	139–160	150	93–283	204	215–330	300	–	–
LTW	129–165	147	175–225	190	280–317	300	–	–
OVL	134–146	140	118–345	207	109–178	153	–	–
OVW	156–211	184	103–300	193	145–205	175	–	70
EL	38–41	39	32–44	38	35–42	39	34–37	36
EW	26–28	27	18–25	40	20–28	23	22–24	23
FBL	405–473	439	320–850	643	470–515	478	–	307
FBL (%)	25%–26%	26%	21%–30%	25%	26%	26%	–	31%
POFL	969–1102	1036	850–2150	1542	1150–1525	1338	–	578
POFL (%)	57%–64%	60%	47%–66%	57%	58%–87%	73%	–	59%
VL	987–996–	992	20–108	65	25–68	46	–	–
CL	769–798	783	–	–	30–38	34	–	–
CLPOF	602–615	609	–	–	–	–	–	–
%CL	35%–36%	36%	35%–36%	36%	24–28%	26%	–	43%

3.4.3. Supplementary description *Mesocoelium* cf. *monodi* Dolfus, 1929

Taxonomic summary:

Phylum: Platyhelminthes.

Family: Mesocoeliidae Odhner, 1901.

Genus: *Mesocoelium* Odhner, 1901.

Locality: Durban (-29.782294, 31.030196)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Intestine

Specimens on which description is based: Adults (n=2)

Morphology:

Mesocoelium body type is small, oval, and spinose shape measured 1710 (1614–1806) x 664 (615–714), the fore body 439 (405–473) long uptakes 26% (25–26%) of the body length. The specimen has moderately long ceca, and a genital pore that is prebifurcal and submedian. Oral sucker is shaped spherical and placed slightly sub terminal and is measured 203 (202–205) x 194 (180–209). Pharynx is shaped sub–spherical and is measured 82 (80–84) x 73 (72–73), oesophagus is 65 (48–81) wide. There appears to be caecal bifurcation near the midlevel of the fore body, ceca extends some distance posterior to the ovary, occupying 36% (35%–36%) of the post ovarian space. Ventral sucker can be located near the anterior to midlevel region of the body; smaller than the oral sucker measuring 141 (137–144) x 166 (158–175). Oral sucker width to ventral sucker width ratio 1:1.16 (1:1.14–1:1.2). Testes are smooth, diagonal, situated at the level of ventral sucker. Right testis 126 (123–128) x 169 (168–170), left testis 150 (139–160) x 147 (129–165). Cirrus sac enclosing seminal vesicle is situated between the pharynx and the ventral sucker and measured at 172 x 63. Ovary is smooth, and is measured 140 (134–146) x 184 (156–211). Postovarian space measured 1036 (969–1102) and uptakes 60% (57%–64%) of the total body length. Vitelline fields; 992 (987–996), distributed along the ceca, 783 (769–798) from level of oral sucker posteriorly to near midlevel of body or more posterior, terminating near to, or surpassing the caecal ends. Eggs (n=12) opperculate measured at 39 (38–41) x 27 (26–28).

Molecular (n=4):

The phylogenetic analysis confirmed the placement of the specimen into the genus *Mesocoelium.*

3.5. Apicomplexa and Euglenozoa

Of the 16 individual native Guttural Toad specimens collected at site 1a, three females were infected with blood parasites. These blood parasites were identified as *Hepatozoon ixoxo* (prevalence 19%) and *Trypanosoma* sp. (prevalence 6%) by E.C. Netherlands (personal communication, 7 Nov 2016). Morphological markers (light microscopy) were applied to confirm identification (Fig. 3.10).

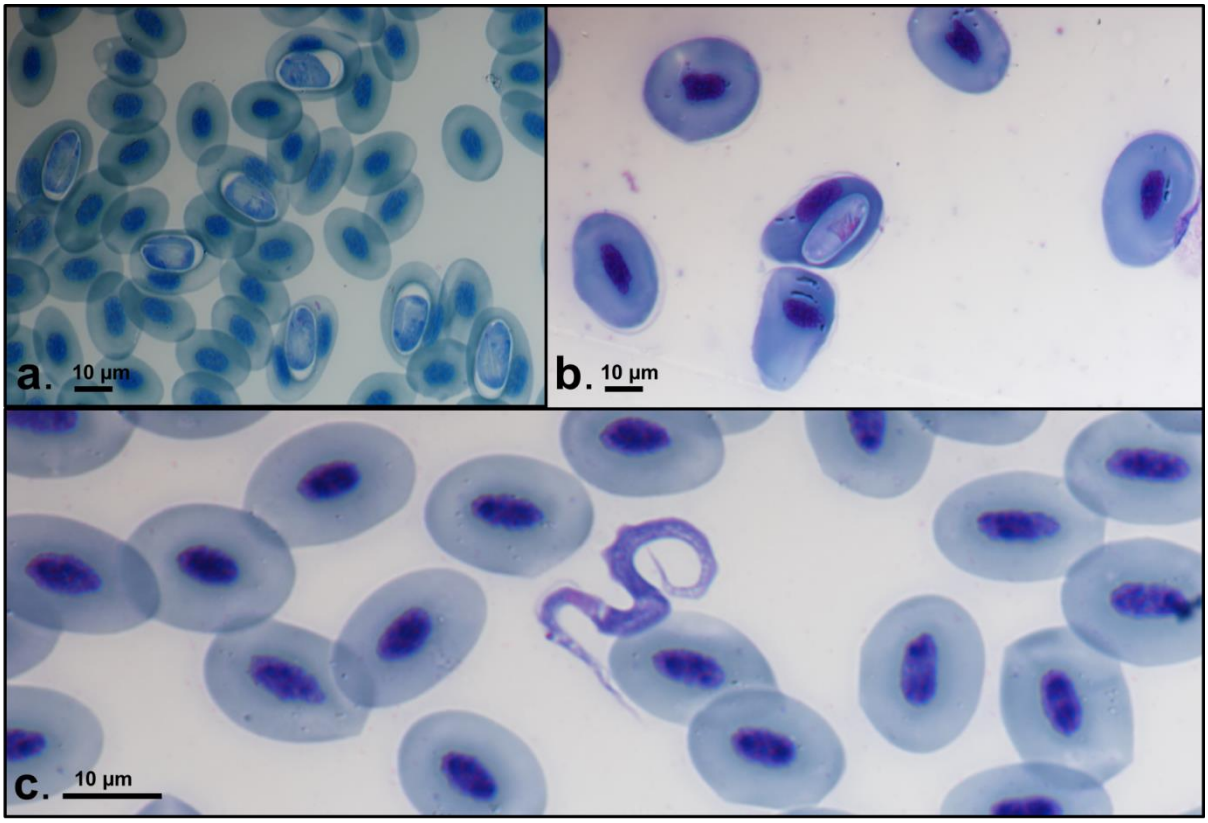

Figure 3.10: *Hepatozoon ixoxo* Netherlands, Cook & Smit, 2014 and *Trypanosoma* sp. Gruby, 1843. **a)** Intracellular *Hepatozoon ixoxo*. **b)** Intracellular *Hepatozoon ixoxo.* **c)** Extracellular *Trypanosoma* sp.

3.5.1. Supplementary description *Hepatozoon ixoxo* Netherlands, Cook, and Smit, 2014

Taxonomic summary:

Phylum: Apicomplexa Levine, 1970.

Family: Hepatozoidae Wenyon, 1926.

Genus: *Hepatozoon* Miller, 1908.

Locality: Northern KwaZulu-Natal (-26.94121, 32.81501)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Blood (Intracellular)

Remarks: The *Hepatozoon ixoxo* was found intracellular and are ovoid shaped. Pink to purple stained Nucleus can be seen.

3.5.2. Supplementary description *Trypanosoma* sp.

Taxonomic summary:

Phylum: Euglenozoa.

Family: Trypanosomatidae Doflein, 1901.

Genus: *Trypanosoma* Gruby,1843.

Locality: Northern KwaZulu-Natal (–26.94121, 32.81501)

Host in current study: *Sclerophrys gutturalis*

Location of parasite: Blood (extracellular)

Remarks: *Trypanosoma* was found extracellular and is elongated in shape. Nucleus can be found centrally inside the cell. Flagellum can be seen which extends from the anterior pole, and undulating membrane is visible.

3.6. Statistical analysis

3.6.1. Parasite Prevalence and Mean Intensity.

Composition of parasite fauna varied markedly among the populations of amphibians examined in the current study (Table 3.13).

Five different parasite species was observed in the native Guttural Toads collected from site 1a. Of these *Rhabdias* cf. *africanus* had the highest prevalence and *Trypanosoma* sp. the least. Four different parasite species was observed in the native Guttural Toads collected from site 1b. Of these *Cosmocerca* sp.1 had the highest prevalence and *Mesocoelium* cf. *monodi* the least.

One parasite was observed in the invasive Guttural Toads collected from site 2a: Encysted nematodes. No parasites were observed in the native Western Leopard Toads collected from site 2a. Two parasites were observed in the native Western Leopard Toads collected from site 2b. Of these encysted nematodes had the highest prevalence and *Cosmocerca* sp. 2 had the least. Encysted nematodes was also observed in invasive Guttural Toads collected from site 2c.

Three different parasite species was observed in the Guttural Toads collected from site 3. *Cosmocerca* sp. had the highest prevalence.

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Table 3.13: Parasites collected from *Sclerophrys gutturalis* (GT) and *Sclerophrys pantherina* (WLT) from different study sites, Prevalence (P%) and mean intensity (MI) provided for parasites collected in certain hosts.

Site	Species	*Lawrencarus eweri*			*Rhabdias* cf. *africanus*			*Amplicaecum* sp.			*Cosmocerca* sp.1			*Cosmocerca* sp.2			Encysted nematodes			*Mesocoelium* cf. *monodi*			*Hepatozoon ixoxo*	*Trypanosoma* sp.
		n	P%	MI	n	P%	MI	n	P%	MI	n	P%	MI	n	P%	MI	n	P%	MI	n	P%	MI	P%	P%
1a	GT	11	37.5	2	93	39	13	–	–	–	57	25	14	–	–	–	–	–	–	–	–	–	19	6
1b	GT	–	–	–		–	–	19	33	3	82	44	10	–	–	–	14	39	2	6	5	6	–	–
2a	GT	–	–	–		–	–	–	–	–	–	–	–	–	–	–	13	10.8	3		–	–	–	–
2a	WLT	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–
2b	WLT	–	–	–		–	–	–	–	–	–	–	–	5	8.3	5	4	16.6	2	–	–	–	–	–
2c	GT	–	–	–		–	–	–	–	–	–	–	–	–	–	–	3	9	3	–	–	–	–	–
3	GT	–	–	–	5	33	2	–	–	–	70	67	12	–	–	–	18	11	18	–	–	–	–	–

3.6.2. Parasite Diversity

No significant difference was found in parasite diversity of individuals compared to each site separately (Fig. 3.11), the P-value of the medians are 0.15 (P>0.05). From this graph it is indicated that parasite diversity in individuals have no significant difference, however a difference can be seen between study area 1 and study area 2. Study area 1 containing the highest diversity of parasites and study area 2 the least.

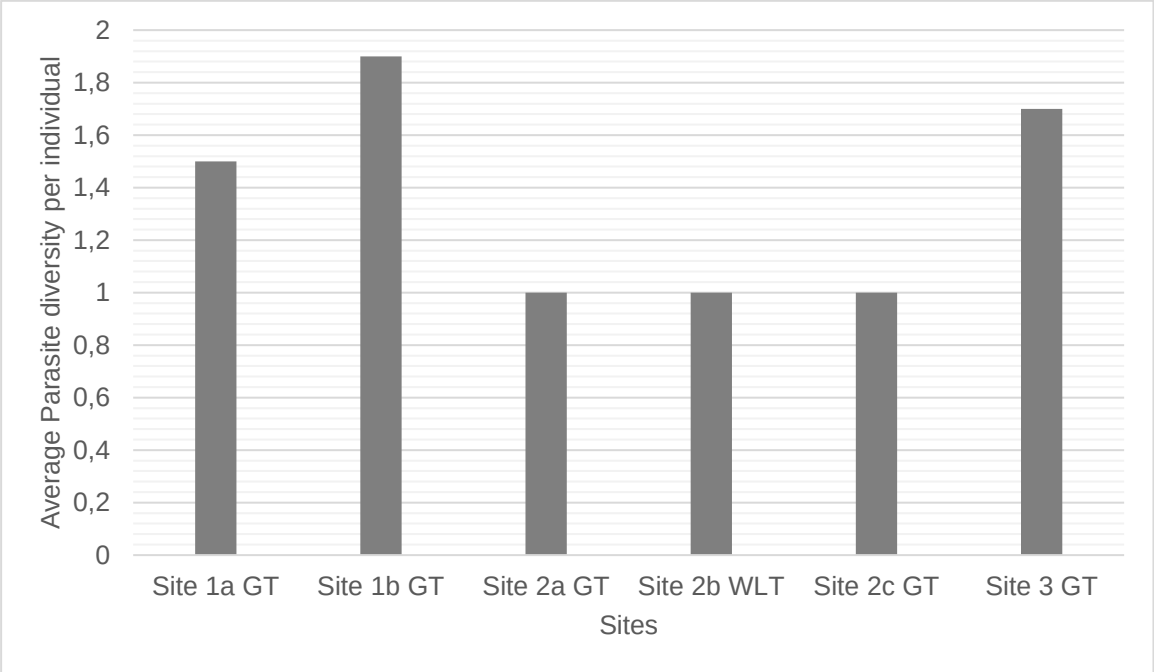

Figure 3.11. The average parasite diversity for individuals of each site. **Abbreviations:** GT- Guttural Toad, and WLT- Western Leopard Toad.

CHAPTER 4:

DISCUSSION

CHAPTER LAYOUT

This chapter will discuss results generated by the current study by the hand of previous findings and research. Parasites collected and observed in toads will be discussed as well as concepts such as ‘enemy release hypotheses’, ‘reservoirs’ and ‘sinks’. Recommendations on future research are provided for each main focus.

4.1. GENERAL

Anuran fauna in southern Africa comprises a wide variety of habitats and environments, which expose them to certain threats and offer them particular opportunities (Du Preez & Carruthers, 2009). Trade in especially plants and vegetables between provinces provides an opportunity for individuals to translocate, which may then pose new threats to native fauna The KwaZulu-Natal province contains several of these habitats such as tropical regions and even grasslands. This province supports the richest diversity of anurans throughout southern Africa (Alexander *et al.,* 2004). It also contains one of the country’s most popular maritime harbours, thus creating numerous opportunities for translocation. The harbour route can be one of many methods of translocating to new environments.

Invasive Guttural Toads may spill over parasites to the native Western Leopard Toads, however a spill back of parasites can also occur from native Western Leopard Toads to invasive Guttural Toads, which then acts as a reservoir for parasites and can in turn transfer parasites to uninfected native Western Leopard Toads. Parasites identified in the current study are discussed and incorporated to establish whether this is the case.

4.2. Acari

The morphological description for this Acari parasite along with molecular characterisation represents the first combined description for *Lawrencarus eweri*, additionally confirming their placement within the super-family Tydeoidea.

The study by Fain (1975) provides most of the baseline morphological data from which to work, however the method of fixation or preparation of the mites is not provided. Potential morphological plasticity emerges with different processes of slide production used in light microscopy. It should be noted that the preparation of specimens for the scanning electron microscope can also cause variation in size during critical-point drying as observed while assessing the effects such drying has on the size of smooth cultured muscle cells. The present study found that reduced sizes are almost unavoidable (see also, Lee *et al.,* 1979). This may explain why measurements in this study differ from those indicated by Fain (1975).

From the findings in the present study, it is clear that the use of measurements for identification or classification may be unreliable until a standard method of preparation and measuring is established. Thus, it is suggested that other diagnostic characteristics should be applied such as the chaetotaxy, the amount of anal and perigenital hair, as well as the shape and form of the body structure. These characteristics should be used to identify the genus or species and to classify the different developmental stages and classification of specimens.

Morphological analysis: Lutfy (1960) describes the *Lawrencarus eweri's* body as pear-shaped or heart-shaped, and even 'egg'-shaped. The author describes this shape as formed by the narrowest part being the opistosoma and the broadest part of the shape the metapodosoma. This comprises four pairs of legs described as two pairs facing forward (possibly on the anterior part of the idiosoma) and two pairs facing backward (possibly on the posterior part of the idiosoma) (Lutfy, 1960).

Fain (1957) typifies the amount of anal hair present in species from the genus *Lawrencarus* as diagnostic, which can be used to identify individuals. For *Lawrencarus eweri* particularly 18 to 30 pairs of hairs can be observed around the anal area whereas others from the same genus consist of only one or two pairs of anal hair (Fain, 1957).

The individuals of *L. eweri* can be distinguished in terms of developmental stage and sex through morphology, more specifically the chaetotaxy and diagnostic characters. The female *L. eweri* contains a vulvar slot with a horizontal and a longitudinal portion, which together resembles an inverted T (Fain, 1957; Andre & Fain, 1999). Females also contain seven to

ten lateral genital hair (Fain, 1957). The male *L. eweri* can present more hair outside the genital slit, however, there may be one or two internal hairs located in paramedian immediately behind the male port itself (Fain, 1957; Andre & Fain, 1999). These are termed perigenital hair and are absent in females. Adults and nymphs of *L. eweri* duplicate the perigenital discs found around the genital area. Nymphs also undergo an enlargement of the cis-acetabular area (Andre & Fain, 1999).

Incorporating the information from previous studies, it was confirmed that the specimens collected from Guttural Toad in northern KwaZulu-Natal, South Africa, was indeed *L. eweri* and contained one male, three females, one unidentified adult, one protonymph, and one larva.

4.3. Lung nematodes

Of the genus *Rhabias* little is known about the individuals infecting amphibians in Africa. Kuzmin (2001) identifies a species found in the lungs of two Bufonid species: *Sclerophrys maculata* (Hallowell, 1845) and *Sclerophrys garmani* (Meek, 1897) in South Africa (Kruger National Park): *Rhabdias africanus* Kuzmin 2001.

Morphological analysis: Kuzmin (2001) describes the body of this species as elongated, with a seemingly swollen cuticle covering the body. In certain areas the cuticle folds upon itself. Papillae are present on the mouth of these species. Furthermore, a buccal capsule is apparent, from a lateral view described as barrel-shaped. The walls of the buccal capsule can be described as sclerotised and the oesophagus as club-shaped. The width of the oesophagus decreases near the surrounding nerve ring. Its tail and rectum are short, and covered with a thick cuticle. Vulva lips are reduced and appear slit-like.

Molecular analysis: The phylogenetic analysis of the 28S gene supports the morphological placement of *Rhabdias* cf. *africanus* in the monophyletic clade consisting of specimens of the genus *Rhabdias*.

Future research: This can focus on clarifying the morphology for *Rhabdias africanus* in the Potchefstroom area. In addition, molecular work can be considered with mitochondrial genes as well as nuclear genes, to provide further molecular information for *Rhabdias africanus.*

4.4 Intestinal nematodes: *Cosmocerca.*

Only a single species of the genus *Cosmocerca* has been described in South Africa: *Cosmocerca ornata* (Dujardin, 1845). This species was described from the intestinal tract of *Capensibufo rosei* (Hewitt, 1926) in the Western Cape (Cape Town); in *Sclerophrys capensis* Tschudi, 1838 in KwaZulu-Natal (Coleford Nature Reserve); in *Sclerophrys gutturalis* (Power, 1927) in Mpumalanga (Kruger National Park) and Gauteng (Pretoria); in *Sclerophrys maculata* (Hallowell, 1854) in Mpumalanga (Kruger National Park); and in *Sclerophrys garmani* (Meek, 1897) in Mpumalanga (Kruger National Park) (Baker, 1981).

Morphological analysis: Dujardin (1845) describes the body of *Cosmocerca ornata* as fusiform shaped and white in colour. The head of the organisms is described as small and broad with a retractile triangular mouth. An inconspicuous oesophageal bulb is visible just below the cylindrical oesophagus. The vulva of females is positioned in the middle of the body and the uteri contain oblong elliptic eggs (Dujardin, 1845; Ryzhikov *et al.*, 1980). Ryzhikov *et al.* (1980) describes the proximal ends of both ovaries as located in front of the oesophagus's base. Its tail is conical and acute with a protruding posterior appendage (Dujardin, 1845). Bala (2016) describes numerous amounts of papillae: eight pairs ventral, three pairs subventral, four pairs lateral, and one pair subdorsal. However, the specimens identified in the present study contain two to five pairs of papillae and cannot be confirmed as *Cosmocerca ornata.*

Molecular analysis: The phylogenetic analysis of the COI gene supports the morphological placement of *Cosmocerca* sp.1 and *Cosmocerca* sp.2 in the monophyletic clade consisting of specimens of the genus *Cosmocerca*.

Future research: Researchers should focus on elucidating the type of *Cosmocerca* sp., collected in the present study, through further morphological studies.

4.5. Encysted nematodes

Encysted nematodes can be found on the surface of organs such as the stomach, liver, and bladder. Kelehear & Jones (2010) states that larvae of nematodes can be enclosed in cysts. Cysts are present in the gastric muscle of the stomach and intestinal tract, however, they have also been known to frequent the submucosal layer of the liver. Encysted nematodes are surrounded by dense layers of fibrous tissue. These cysts have been found to have an extremely minimal pathologic effect on cellular and tissue function. However, in heavy infections, cysts may cause discomfort. The minimum effect on hosts may suggest an extensive association between parasites and hosts (Kelehear & Jones, 2010).

4.6. Platyhelminthes

The morphological description along with molecular characterisation represents the first combined description for *Mesocoelium* cf. *monodi.*

Morphological analysis: According to Dronen *et al.* (2012), members of the family Mesocoelidae, particularly the genus *Mesocoelium,* are relatively small, elongated, spinose shaped flatworms. The oral suckers of the specimens among these genera are subterminal and its mouth opening presents as a ventrally, short prepharynx above the muscular pharynx, oesophagus, with its intestinal bifurcation immediately preacetabular. In certain species of the genera, the ceca do not extend the ovary, and in others such as *Mesocoelium monodi,* the ceca extend the post-ovarium space by 36% and can be considered as a diagnostic character (Dronen *et al.*, 2012). The ventral sucker occurs above the midlevel of the body. Two testes are present next to each other, usually at the level of the ventral sucker. Ovary occurs post-testiculary. The uterus fills most of the post-ovarian space. The Vitellaria follicular occurs in bands along the ceca; the fields reach the shoulders of the oesophagus anteriorly, and posteriorly in certain species of the genus, terminate just below the ventral sucker and, in *Mesocoelium monodi,* it terminates near or close to the ceca. Dronen *et al.* (2012) explain that *Sclerophrys gutturalis* is a definitive host to the species *Mesocoelium monodi* and were previously found in areas of Africa such as the Democratic Republic of the Congo.

Molecular analysis: The phylogenetic analysis of the 28S gene supports the morphological placement of *Mesocoelium* cf. *monodi* based on morphology in the monophyletic clade, which comprises specimens of the genus *Mesocoelium.*

4.7. Apicomplexa and Euglenozoa

Morphological analysis: Netherlands (2015) describes the *Hepatozoon ixoxo* as elliptical shaped with a well-developed cap/cavity at one pole. *Trypanosoma* sp. is described as elongated and containing a kinetoplast closer to the anterior end, with a slight undulating membrane. The flagellum is present, which extends from the anterior pole (Netherlands, 2015).

4.8. Invasion and parasites

The invasion of Guttural Toad to the Western Cape can have several direct effects on the native fauna. The present study, however, focused its research on the parasitological effects of these populations and assessed whether spill back or spill over occurred.

4.8.1. Parasite Community Composition and Diversity

Comparing previous findings with the findings of the current study will attempt to elucidate uncertainties in the dataset.

The nasal mite *Lawrencarus eweri* was rediscovered after being redescribed by Fain (1962). The presence of the parasite in northern KwaZulu-Natal is not surprising as previous descriptions report these mites from several different host species: *Sclerophrys gutturalis, Schismaderma carens* (Smith, 1848)*, Sclerophrys kisoloensis* (Loveridge, 1932), *Bufotes viridis* (Laurenti, 1768)*, Duttaphrynus melanostictus* (Schneider, 1799) and more from areas such as Congo, Rwanda, Morocco, Italy and South-Africa (Fain, 1957). However, the absence of this species in Durban (site 1b), Constantia (study area 2), and Potchefstroom (study area 3) is also surprising, however the exact range of the species is unknown.

The current study was able to identify nematodes from three genera: *Rhabdias, Amplicaecum, and Cosmocerca.* The lung nematode *Rhabdias africanus* was identified in *S. gutturalis* in northern KwaZulu-Natal (site 1a) and Potchefstroom (study area 3). This species was previously described from *Sclerophrys garmani* (Meek, 1897) and *Sclerophrys maculata* (Hallowell, 1845) in the Kruger National Park which is in the same climatic regime as KwaZulu-Natal (study area 1), thus it was not surprising collecting these parasites in *S. gutturalis* as they share similar niche selections. However, the discovery of *R. africanus* in Potchefstroom (study area 3) was surprising and indicates the wide range of distribution of the species. The failure to detect *R. africanus* from Durban (site 1b) can be due to absence of vector, small sample size, or habitat fragmentation. .

The four species described from the family Ascarididae Baird, 1853; *Amplicaecum africanum* Taylor, 1924; *Amplicaecum gedoelsti* Yorke & Maplestone, 1926; *Amplicaecum involutum* Gedoelst, 1916; and *Orneoascaris chrysanthemoides* (Skrjabin, 1916) have been synonymised by Sprent (1985). These species have been described from Bufonid species across Africa especially *S. gutturalis.* Unfortunately, the specimens collected in this study could only be placed into the genera *Amplicaecum.* Further techniques and studies should be applied to elucidate gaps in the taxonomy and distribution range of this species.

The intestinal nematode *Cosmocerca ornata* (Dujardin, 1845) have been reported from five bufonid species including *S. gutturalis* in Mpumalanga, and Gauteng provinces in South-Africa. The *Cosmocerca* sp. 1 collected in KwaZulu-Natal (study area 1) correlates with previous findings, however, the collection of a genetically different *Cosmocerca* sp. 2 in *Sclerophrys pantherina* in the Constantia area (study area 2) indicates that the original description of *C. ornata* should be reviewed, as it was also reported from *Capensibufo rosei*

(Hewitt, 1926) in the same area, and results in current study clearly indicate the presence of two genetically distinct species. Further studies should be conducted to determine the life cycle and range of these species.

The Platyhelminthes species *Eupolystoma anterorchis* (Tinsley, 1978) was reported to be present in *S. pantherina* from Noordhoek, which is included in the current study as study site 2b, by Tinsley (1978). The current study did not detect any *E. anterorchis* however, this may be due to small available sample size. However, it is highly improbable that this is the case as a recent study conducted by Delport (2007) inspected 113 individual Western Leopard toads in Noordhoek and Clovelly (-341215, 18.4306) and no host was found to be infected with *E. antherorchis*. Delport (2007) explains that this parasites may be experiencing environmental pressures and threats due to the current declining of Western Leopard populations due to habitat loss and fragmentation.

Encysted nematodes have been subjected to morphological and molecular characterisation but yielded no positive identification. Further studies should be conducted to create particular methods of identification for these specimens.

Mesocoelium monodi have not been reported in *S. gutturalis* or *S. pantherina* in previous literature. However, *M. monodi and M. schwetzi* have been reported to be found in *Sclerophrys regularis* in central and west Africa by Dollfus (1929 & 1950). The specimens collected in the current study were assigned to *M. monodi* due to morphological traits diagnostically belonging to the species: ceca extending the post-ovarium space by 36% (Dronen *et al*., 2012). However, due to discrepancies between measurements of collected samples and previously reported samples the specimens were assigned to *Mesocoelium* cf. *monodi.* These specimens was only collected from one male *S. gutturalis* in site 1b and due to lack of literature reporting this species in *S. gutturalis* from South Africa it is unclear why no other individuals involved in this study did not harbour these parasites.

Hepatozoon ixoxo and *Trypanosoma* sp. have been described for *Sclerophrys garmani* and *Sclerophrys gutturalis* in the northern KwaZulu-Natal area (Netherlands, 2015; Netherlands *et al.,* 2014).

Contributors to the absence of parasites in the same species or different species in the same areas can include climatic differences, habitat fragmentation, or absence of vector. Other contributing factors include absence/or change in native intermediate or final hosts. Theoretically, the host and the parasite are in constant dynamic interaction and the parasite population can decrease with the decreasing host population (Todar, 2002).The first aim of the present study was to identify the parasites, and the second aim was to establish the

parasite composition in the populations in KwaZulu-Natal and the Western Cape. The third aim was to determine whether an increase or decrease in parasite diversity in Guttural Toad has taken place during the invasion.

The native Guttural Toad in study area 1 contained the highest diversity of parasites and the invasive Guttural Toad in study area 2 had the least. However, no significant difference was found between the parasite diversities for these two populations. This is contrary to what was expected. According to the invasive Guttural Toads relative recent (10 years) introduction on an evolutionary timescale, the invasive Guttural Toad can be considered a new species to the Western Cape. Thus it would be predicted that the invasive Guttural Toads parasite community composition would consist primarily of native range parasites and generalist parasites that are quick to adapt to a new host.

It was found that introduced species frequently vacate most of their parasites throughout the invasion (Tompkins & Poulin, 2006). This is known as 'the enemy- release hypothesis' (Marr *et al.*, 2008). This can result in enhanced competitive capability of the invasive species in the new range (Torchin *et al.,* 2003). This may be primarily imperative for competitively dominant species that are restricted by parasites in their original range (Reinhart & Callaway, 2006). Unrestricted by parasites in the new range, invaders may select to reduce costly defences in favour of increased reserve acquirement and reproductive rates, thus further improving their competitive supremacy (Hatcher & Dunn, 2011). This is mostly due to the following factors: 'founder effects', small original population amounts (Chitwood & Chitwood, 1950), and truncated concentrations of invaders at the expanding front (Phillips *et al.*, 2010), which drives the transfer of pathogens from the invader to the native fauna.

In other cases, the invaders can reduce parasite loads of native fauna by acting as a 'sink' for native parasites (Kelly *et al.,* 2009). In this case, native parasites are occupied by the invader but fail to complete their life cycle owing to the lack of co-evolutionary history (Heimpel *et al.*, 2003). In study site 2a where native Western Leopard Toad and invasive Guttural Toad co-exist, the least number of parasites were found. This is in line with the hypotheses stating that invaders may uptake native parasites. It may happen that these parasites become disorientated, is attacked by the immune system, eventually are unable to complete its life cycle, and thus die off.

4.8.2. Spill over and spill back

The fourth and fifth aim of the present research were to ascertain whether spill back or spill over occurred. The parasites found in the native Western Leopard Toad population that

coincided with those found in the Guttural Toads of all sites was an intestinal nematode belonging to the genus *Cosmocerca* which was also found in the intestines of native Guttural Toad in study area 1. However, after p–distance was calculated (7%) it was confirmed that these parasites where in fact two different species.

Other coinciding parasites are unidentified encysted nematodes, which could not be placed morphologically or molecularly. Cysts of unidentified nematodes where found in the internal tissues of the Guttural Toad, both in the invasive range in Western Cape and its native range KwaZulu-Natal. Their identity and basic biology remains obscure. A recent comparative study by Kelehear & Jones (2010) investigated a similar scenario concerning the invasive Cane Toad (*Rhinella marina*) and the unidentified encysted nematode parasites found in collected Cane Toads in its invasive range (Australia) and native ranges and compared the results to the native frog fauna of Australia. The author identified eight different species of nematode larvae which was encysted in the gastric tissues of the sampled anurans. However, it was a difficult task to distinguish between the larvae of different species (Kelehear & Jones, 2010). They found that cysts from the order Spirurida were more prevalent in larger anurans. Infected Cane Toads had a mean SUL of 109.3 mm (94.1 mm-135 mm). In the current study only native Western Leopard Toads of site 2a and 2b meets the requirements. Small sample sizes of native Western Leopard Toad in site 2a (n=3) prevents the making of valid conclusions. However, if the larger study area (study area 2) is brought under consideration the native Western Leopard Toads (n=15) (mean SUL 88 mm) contain a higher prevalence of 13% of encysted nematodes than the invasive Guttural Toad (n=59) (mean SUL 70 mm) with a prevalence of 10%. Indicating that infection of encysted nematodes can be due to parasite specificity to host size.

Kelehear & Jones (2010) also found that the invasive Cane Toad, had the highest prevalence and species richness of cysts than in the native frog fauna in Australia. However, in the current study both invasive and native species are toads and are ecologically more similar, than the Cane Toad and its native frog competitors. Thus, the trend of cyst infection can be owned to host's body size and morphology, which in this study is very similar for both native and invasive hosts.

The lifestyle of both Guttural Toads and Western Leopard Toads are ground-dwelling, and like all anurans, require regular access to moisture and thus an overlap in habitat use can occur between these two hosts. Kelehear & Jones (2010) suggest that higher prevalence can also be due to niche selection. However, in the current study invasive Guttural Toads and native Western Leopard Toads have similar niche selections in the Western Cape. Both Guttural Toads and Western Leopard Toads can consume invertebrates (Wager, 1986;

Channing, 2001; Du Preez *et al.*, 2004). However, in the Western Cape they are offered similar prey and thus exposes them to a variety of potential infected intermediate hosts. Cysts of the order Spirurida requires an invertebrate, intermediate host, and the encysting (paratenesis) of the third-stage larvae is common (Anderson, 2000).

There was no evidence that the cysts recovered in the current study were able to mature in these hosts and no evidence was recovered that the cysts are able to transfer to a definitive host if the intermediate hosts has fallen prey. Few life cycles have been described for nematodes in South African fauna to which a toad acts as an intermediate host for the third-stage larvae. Thus, without further identification and molecular studies it is uncertain whether these cysts in native Western Leopard Toads and invasive Guttural Toads are a result of spill back or spill over.

Furthermore, previous data of parasites found in Western Leopard Toad include *Eupolystoma anterorchis,* which was not found in any of the populations of Western Leopard Toad or Guttural Toad. No parasites, except for encysted nematodes, of Western Leopard Toad was found in other populations and vice versa indicating that for some parasites no spill back or spill over has occurred between invasive Guttural Toad and native Western Leopard Toad in the Western Cape.

However, each case is unique since the relationship between parasites and hosts can be dynamic. Several factors may influence the outcome such as parasite loss during breeding season, parasite loss for road killed individuals, and parasite loss for captive individuals. This makes it difficult to predict the consequences. Furthermore, as is the instance with restoration ecology, irremediable alterations may have to be accepted in certain systems that are subject to invading parasites and their introduced hosts (Dunn & Hatcher, 2014).

Future research: can include monitoring parasites under controlled environments for all individual toads. Non-invasive methods can be used to identify whether host is infected with parasites such as egg counting in urine. Individual parasite specificities can be researched such as parasite prevalence in road-killed toads, wild caught toads, and captive breeding toads.

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