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Abstract

Trypanosomes are ubiquitous blood parasites of fishes and at least 16 species were originally described infecting African freshwater fishes. This number was later reduced to six and in the late 1990s it was proposed that most records of freshwater fish trypanosomes across Africa are *Trypanosoma mukasai* Hoare, 1932. Recently, results from a molecular analysis of fish trypanosomes from the Okavango Delta, Botswana, reported the presence of at least two genotypic groups and concluded that the identification of *T. mukasai* remains problematic. The aims of the present study were thus to elucidate the life cycle of a freshwater fish trypanosome from southern Africa and to do a morphological and molecular characterization of this parasite from both the fish host and leech vector. To locate trypanosome stages, leeches were removed from fishes captured in the Phongolo River, South Africa, and fish blood films and leech squashes were Giemsa-stained and screened. To determine whether trypanosome stages in fishes and leeches were of the same genotype, DNA was extracted and fragments of the 18S rDNA gene were amplified and sequenced. Trypanosomes were detected in the fish families Cichlidae, Clariidae, Mochokidae and Schilbeidae. Sequence data showed that the trypanosome from one of the leeches, identified as *Batracobdelloides tricarinata* (Blanchard, 1897), was highly similar to those obtained from the plain squeaker, *Synodontis zambezensis*, with 0.7% difference recorded between them. From morphological and molecular data presented here it is clear that the trypanosomes from Phongolo are closely related to those of the Okavango and should be considered as a single diverse species with genetic differentiation between 0.4–2.9%, under the 3–5% differences expected to be seen between true distinct species within the rRNA. Development stages of the trypanosome found in the leech *B. tricarinata* supports its status as the vector and the molecular evidence show the relationship between the trypanosome in the fish and leech, but also illustrates the exceptional genetic and morphological diversity of a single species of trypanosome between host species. The work presented here provides us with clear information

to take further steps in resolving the taxonomy and systematics of African freshwater fish trypanosomes.

Keywords

Fish parasites, Leeches, Trypanosomes, 18S rRNA sequences

1. Introduction

The *Trypanosoma* are kinetoplastid flagellates that have been described to parasitise all classes of vertebrates with more than 500 species currently known (Spodareva et al., 2018). The focus of Kinetoplastid research has been on species of medical importance, especially *Trypanosoma cruzi* Chagas, 1909 causing Chagas disease and *Trypanosoma brucei* Plimmer and Bradford, 1899 causing African sleeping sickness, as well as those of veterinary importance that predominantly infect domestic ungulates, such as *Trypanosoma congolense* Broden, 1904 and *Trypanosoma vivax* Ziemann, 1905 (Hamilton et al., 2007). In comparison, in-depth research into the ecology and pathogenicity of most of the other known *Trypanosoma* species that infect terrestrial and aquatic wildlife have been considerably neglected. This is particularly true for trypanosomes of fishes, with some 200 known species described from both marine and freshwater environments (Eiras et al., 2012; Ferreira and Avenant-Oldewage, 2013).

In Africa, information on fish trypanosomes is scarce, with previous studies recording trypanosomes parasitising fishes in only nine of the 54 countries: Botswana, Egypt, French West Africa, the Congo, the Sudan, Uganda, Mozambique, Namibia and South Africa (Baker, 1960; 1961; Smit et al., 2004; Hussein et al., 2010; Ferreira and Avenant-Oldewage, 2013; McHugh et al., 2016, Scholtz et al., 2018). Most of these studies were descriptions of trypanosomes named as distinct species based on the hosts from which they were identified. Although at least 16 species were originally described infecting African freshwater fishes, currently only six species are considered to be valid, namely *Trypanosoma toddi* Bouet, 1909, *Trypanosoma mukasai* Hoare, 1932,

Trypanosoma tobeyi Dias, 1952, *Trypanosoma alhussaini* Mohamed, 1978, *Trypanosoma cyanophilum* Mohamed, 1978 and *Trypanosoma mansouri* Mohamed, 1978 (see Scholtz et al., 2018). Of these species, *T. mukasai* is considered to have a pan African distribution, having been reported, sometimes tentatively, from a variety of fishes throughout Africa since its description almost a century ago (Smit et al., 2000; Smit et al., 2004; Davies et al., 2005; Ferreira and Avenant-Oldewage, 2013; Scholtz et al., 2018).

The most comprehensive studies on trypanosomes of freshwater fishes in southern Africa were those of Smit et al. (2000; 2004) and Davies et al. (2005), all highlighting the difficulty in trypanosome identification and differentiation on a morphometric basis, particularly that of *T. mukasai*. In an initial attempt to resolve this issue Davies et al. (2005) molecularly characterized fish trypanosomes from the Okavango Delta, Botswana, illustrating that there were two distinct trypanosome genotypes in the Okavango Delta. The first genotype occurred in the cichlids and three families of catfishes, with the sequences Davies et al. (2005) referred to as Group 1 closely representing that of European fish parasite *Trypanosoma cobitis* Mitrophanow, 1883. The second genotype (Group 2) was found parasitising two types of catfishes, the sequences showing similarity with trypanosomes infecting *Clarias angolensis* Steindachner, 1866, another African catfish. Davies et al. (2005) thus concluded that based on their molecular results there are potentially two species of trypanosomes in the Okavango Delta, although they did not name these species. Molecular screening through PCR amplification was shown to be more sensitive as compared to traditional light microscopy screening of fresh and stained material alone. However, to date, despite the utility of molecular approaches in studying the diversity and systematics of trypanosomes, the classification of the pan Africa *T. mukasai* remains unresolved.

In addition to the lack of molecular characterization, another major deficit in the knowledge of fish trypanosomes is the paucity in the understanding of their transmission and development in potential vectors. To date, leeches have been considered as the main vector of

fish and other aquatic vertebrate trypanosomes, including frogs, turtles and crocodiles (Hayes et al., 2014, Attias et al., 2016). However, only a few studies have been able to elucidate the full life cycle of fish trypanosomes in both vertebrate and invertebrate hosts. In Burreson's (2006) review on the Hirudinea as vectors, only three species of freshwater leeches were listed known to transmit six different trypanosomes to their respective fish hosts. These records are all based on either the presence of trypanosome development stages in the leech vector (Letch, 1980) or experimental infection (Negm-Eldin, 1997). Recently in two studies, one marine in South Africa (Hayes et al., 2014) and the other freshwater in Brazil (Lemos et al., 2015), morphologically characterized trypanosome stages found in leech vectors were in both instances molecularly linked to those found in the blood of the fish hosts that the leeches had fed on. These results provided valuable insight not only into the life cycle of fish trypanosomes, but also the phylogeny of the *Trypanosoma*.

Therefore, the aims of the present study were to (i) elucidate the life cycle of a freshwater fish trypanosome from the Phongolo River, South Africa, using an integrated approach which incorporated morphological and molecular characterization of the parasites from both the fish host and leech vector and (ii) to provide further insight into the phylogeny of specifically the aquatic clade of the *Trypanosoma*.

2. Material and methods

2.1. Fish collection and blood sampling

To locate trypanosome stages, fishes were captured in the lower Phongolo River situated within the Ndumo Game Reserve, South Africa (26°52'58"S, 32°18'41"E) (Fig. 1), using previously published methods (see Svitin et al., 2019). Following capture, fishes were identified using Skelton (2001), total length (TL) measured, screened for leeches and other external parasites and kept in aerated aquaria containing fresh river water until bloodletting. Sampling was carried out under the

permit OP 899/2016 and bloodletting followed the standard operating procedure described by Hayes et al. (2014) and approved by the North-West University AnimCare Animal Research Ethics Committee (NWU-00372-16-A5).

When present on a fish, leeches were carefully removed with a fine brush, identified using the key in Oosthuizen (1991) and transferred to a 50 ml bottle with fresh river water. All leeches were kept alive, single or in groups according to hosts until ten days' post feeding in order for the blood meal to digest and potential development of trypanosomes to occur. After ten days, Giemsa stained squashes of the leeches were prepared following Hayes et al. (2014).

Fish heart blood smears were also Giemsa-stained and together with leech squashes screened for trypanosomes under a 100x objective on a Nikon Eclipse Ni compound photomicroscope (Nikon, Tokyo, Japan). Micrographs were captured by digital camera attached to the microscope and measured using NIS-Elements BR Camera analysis software. Standard box plots (box-and-whiskers plot) were constructed in R version 3.6.1 (R Core Team, 2019) and used to graphically present data on the Total Length (TL) and Nuclear Index (NI) of trypomastigote stages in fish species. In addition to the blood films, approximately 1ml whole blood samples of each fish were fixed in 70% molecular grade ethanol and frozen at -20^{0}C for molecular analysis.

2.2. DNA extraction, PCR and sequence analysis

To determine whether trypanosome stages in fishes and leeches were of the same genotype, DNA was extracted from whole blood samples and leech squashes positive for trypanosome stages, using a Qiagen DNeasy blood and tissue kit (QIAGEN Ltd., UK) according to the manufacturers' instructions. Fragments of the 18S rDNA gene were amplified using the trypanosome-specific 18S rRNA gene primers, D (5'-ACCGTTTCGGCTTTTGTTGG-3') and I (5'-GACTACAATGGTCTCTAATC-3'), known to amplify a region of high phylogenetic signal from Davies et al. (2005). These primers generated sequences of approximately 330 bp (PCR conditions were as follows: initial

denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 50–52°C for 1 min and 72°C for 2 min, and a final extension time of 10 min at 72°C). Sequencing reactions were performed directly using an Applied Biosystems BigDye Kit version 1.1 and run on an Applied Biosystems 3730 DNA Analyzer. Resultant sequences were viewed and edited in Bioedit 7.5.0.2 (Hall, 1999) and identified as trypanosomes using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.3. Phylogenetic analysis and sequence divergence analyses

The trypanosome sequences generated from fish blood (*Synodontis zambezensis*, n=2) and a leech (*Batracobdelloides tricarinata*, n=1) in this study were aligned with published trypanosome 18S rDNA sequences representing 31 trypanosome species which parasitize freshwater and marine animals, including fishes, reptiles and a monotreme mammal, the duck-billed platypus, which were retrieved from GenBank (http:// www. Ncbi.nlm.nih.gov/blast/Blast.cgi). An additional four trypanosome 18S rDNA sequences representing two trypanosome genotypes identified from different fish species from the Okavango Delta, retrieved from Davies et al. (2005), were also included in the analysis. As the trypanosome sequences obtained from the two individual *S. zambezensis* were identical, only a single sequence was used to represent the parasite during phylogenetic reconstruction.

Alignments were performed using the MUSCLE sequence alignment tool (http://www.ebi.ac.uk) and visualised in Bioedit, where any final minor adjustments were performed by eye. Phylogenetic analyses were undertaken using MEGAX (Kumar et al., 2018). Maximum parsimony (MP) and maximum likelihood (ML) phylogenies were constructed with a final alignment of 311 bp. The MP analysis was performed using the Subtree-Pruning-Regrafting (SPR) algorithm, while the ML analysis was performed under the conditions of the K2P model with a four category gamma (G) distribution, as determined using the model test function also in

MEGAX (Kumar et al., 2018). Bootstrap analysis was undertaken with 500 replicates and only those values >50 were shown. In all the analyses *Trypanosoma lewisi* [GenBank: AJ223566], *Trypanosoma theileri* [GenBank: AB007814] and *Trypanosoma avium* [GenBank: U39578] sequences were used as the outgroup.

To estimate the evolutionary divergence between the partial 18S rDNA freshwater fish and leech trypanosome sequences from the Phongolo River, those from the Okavango Delta (Samples 1, 5, 30 and 31, Davies et al., 2005) and closely related trypanosomes from Eurasian freshwater fishes (KJ601714, KJ601723, EF375884), uncorrected *p*-distances were calculated in MEGA5 (Tamura et al., 2011).

3. RESULTS

3.1 Parasite prevalence and morphometric description of trypanosomes in fish and leech hosts

Trypanosomes were detected in the blood of four of the 11 fish species studied (Table 1), namely *Coptodon rendalli* (Boulenger, 1897) (Fig. 2A), *Oreochromis mossambicus* (Peters, 1852) (Fig. 2B), *Clarias gariepinus* (Burchell, 1822) (Fig. 2C) and *Synodontis zambezensis* (Peters, 1852) (Fig. 2D), belonging to the families Cichlidae (the first two), Clariidae and Mochokidae respectively. No other blood parasites were observed and none of the Alestidae, Anguillidae, Cyprinidae, Gobiidae or Schilbeidae appeared infected.

Table 1: Identity, number and length of fishes captured in the Phongolo River, and trypanosome and leech prevalences.

Species	No	TL ± SD (range) in mm	Prevalence of trypanosomes in fishes	Prevalence of fishes with leeches
Alestidae				
Brycinus imberi	3	142 ± 15 (126–155)	0/3 (0%)	0/3 (0%)
Anguillidae				
Anguilles mossambicus	1	605	0/1 (0%)	0/1 (0%)
Cichlidae				

Coptodon rendalli	12	258,75 ± 108 (110–360)	7/12 (58%)	0/12 (0%)
Oreochromis mossambicus	2	370, 350	2/2 (100%)	0/2 (0%)
Oreochromis placidus	2	132, 132	0/2 (0%)	0/2 (0%)
Clariidae				
Clarias gariepinus	5	626 ± 168 (350–800)	4/5 (80%)	1/5 (20%)
Cyprinidae				
Labeo rosae	2	112, 220	0/2 (0%)	0/2 (0%)
Gobiidae				
Glossogobius callidus	2	45, 50	0/2 (0%)	0/2 (0%)
Glossogobius giuris	3	185 ± 58 (120–230)	0/3 (0%)	0/3 (0%)
Mochokidae				
Synodontis zambezensis	8	175,62 ± 34 (150–240)	7/8 (87%)	2/8 (25%)
Schilbeidae				
Schilbe intermedius	8	132,25 ± 66 (70–230)	0/8 (0%)	0/8 (0%)

Three leeches collected, one from *C. gariepinus* and two from *S. zambezensis*, all three with trypanosomes; TL, total length; SD, standard deviation.

The morphological and morphometric analyses of the trypanosomes from the two cichlids, *C. redalli* (Fig. 3A), *O. mossambicus* (Fig. 3B) and the clariid, *C. gariepinus* (Fig. 3C) illustrated the parasites to be similar in size, with measurements of 37.07±5.16µm (25.66–47.12µm) (n=24), 42.14±6.58µm (33.00–50.10µm) (n=7) and 39.84±6.96µm (30.25–46.91µm) (n=6) respectively (Fig. 4). Nuclear index (NI) values were >1 for all species of fish (1.4, 1.3, 1.2 respectively, and 1.6 for *S. zambezensis*). On average the mochokid, *S. zambezensis* (Fig. 3D, E) trypanosomes were smaller at 29.27±5.25µm (17.18–49.47µm) (n=115), the range, however, included the mean values of the other three species.

Leeches identified as *Batracobdelloides tricarinata* (Blanchard, 1897) were collected from three individual fishes, one *C. gariepinus* and two *S. zambezensis.* All three leeches were infected with trypanosomes but squashes from only one of the leeches that had fed on an infected *S. zambezensis* revealed all the different trypanosome developmental stages. These included rounded amastigote measuring 5.3±0.68µm (3.81–6.51µm) x 4.28±0.57µm (2.92–5.02µm) (n=12) (Fig. 3F), ovoid and pear-shaped sphaeromastigote measuring 5.76±0.58µm (4.86–6.48µm) x

4.05±0.78µm (3.01–5.03µm) (n=10), with a free flagellum measuring 3.98±1.06µm (2.72–5µm)) (n=4) (Fig. 3G), short or stumpy epimastigote measuring 8.36±1.23µm (5.98–9.84µm) long x 2.19±0.55 (1.59–3.16µm) wide (n=13), with a free flagellum measuring 4.23±2.1 (2.21–9.14µm)) (n=12) and longer thick epimastigote measuring 13.07±1.1µm (11.26–15.5µm) long x 2.33±0.48µm (1.8–3.46µm) wide (n=16), with a free flagellum measuring 2.92±1.02µm (1.5–4.25µm)) (n=15) (Fig. 3H), possible division (Fig. 3I), and a slender epimastigote measuring 12.55µm long x 1.61µm wide) (n=1) (Fig. 3J) stages.

3.2. Phylogenetic analysis and divergence estimates of trypanosomes of African freshwater fish

Both the ML and MP phylogenies provided the same overall topologies, showing the freshwater trypanosomes to be polyphyletic comprising of two distinct clades (Freshwater clade 1 and 2) which phylogenetically bracketed the marine trypanosome species (Fig. 5). Within the ML phylogeny the trypanosome sequence from *B. tricarinata* clustered more closely with sequences generated from *Oreochromis andersonii* (Castelnau, 1861), denoted as Sample 1 and 5 from Davies et al. (2005), whereas in the MP the leech derived sequence clustered more closely with trypanosomes found in catfish species. However, in both ML and MP analyses the trypanosome sequence from *S. zambezensis* clustered with other trypanosome species that were also found to parasitise different species of catfish, including *S. nigromaculatus* Boulenger, 1905 (sample 30 and 31) and *Clarias* species (AJ620555 and CLAR) forming a distinct clade of trypanosomes infecting African catfish.

Based on uncorrected pairwise differences, divergence between the freshwater fish trypanosomes from the Phongolo River and Okavango Delta was extremely low, ranging from 0.4–2.2% with 0.7% seen between Phongolo sequences, and 0.4–2.2% in the Okavango. Sequences from the trypanosomes of *S. zambezensis* from the Phongolo appeared to have lowest level of divergence between sequences from samples 30 and 31, referred to as trypanosome group 2 by

Davies et al (2005), of 0.4–0.7% relative to those from group 1, sample 1 and 5, with a divergence of 1.8–2.2% (Table 2). This pattern of divergence was also reflected when the parasite sequence from *B. tricarinata* was compared to group 1 with a divergence of 0.4–0.7% relative to a divergence of 1.1–1.5% when compared to group 2. However, when the African freshwater trypanosomes were compared to sequences of trypanosomes from Eurasian freshwater fish species *Pseudobagri* (EF375884), *Esox lucius* Linnaeus, 1758 (KJ601714) and *Sander lucioperca* (Linnaeus, 1758) (KJ601723), the divergence was also extremely low ranging from 0.4–4%. The trypanosomes of *S. zambezensis* from the Phongolo had the lowest divergence of 1.1 % with sequences from *S. lucioperca* (KJ601723). A lower divergence of 0.4% was also seen when *S. lucioperca* (KJ601723) was compared to the trypanosome sequence from *B. tricarinata.*

Table 2: Uncorrected p-distances of the partial 18S rDNA freshwater fish and leech trypanosome sequences from the Phongolo River compared with fish trypanosome sequences from the Okavango Delta (Davies et al. 2005)[a], and closely related trypanosomes from Eurasian freshwater fishes (Gu et al., 2007; Grybchuk-Ieremenko et al., 2014)[b, c]

	AJ620555 CLAR [c]	Phongolo S. zam	Sample30 S. nigro [a]	Sample 31 S. nigro [a]	Sample 5 O. anders [a]	Sample 1 O. anders [a]	Phongolo B. tri	KJ601714 *Esox lucius* [c]	EF375884 *pseudobagri* [b]
Phongolo S. zam	0.015								
Sample 30	0.022	0.007							
Sample 31	0.018	0.004	0.004						
Sample 5	0.026	0.018	0.018	0.015					
Sample 1	0.029	0.022	0.022	0.018	0.004				
Phongolo B. tri	0.022	0.007	0.007	0.004	0.011	0.015			
KJ601714	0.033	0.018	0.018	0.015	0.022	0.026	0.011		
EF375884	0.040	0.026	0.026	0.022	0.022	0.026	0.018	0.029	
KJ601723	0.026	0.011	0.011	0.007	0.015	0.018	0.004	0.015	0.022

Phongolo S. zam**,** *Synodontis zambezensis*; S. nigro, *Synodontis nigromaculatus*; O. anders; *Oreochromis andersonii*; Phongolo B. tri, *Batracobdelloides tricarinata*

4. DISCUSSION

4.1. Host-vector and parasite relationships

In the present study four of the 11 fish screened harboured trypanosomes. Both the clariid *Clarias gariepinus* and cichlid *Coptodon redalli* are known hosts for trypanosomes (McHugh et al. 2016), however this is the first report of trypanosome infections from the cichlid *O. mossambicus* and the mochokid *S. zambezensis* although not from this locality. Discovering trypanosomes in a member of *Synodontis* is not surprising as at least three other species of *Synodontis* from the Okavango Delta, Botswana and Lake Liambezi, Namibia have been reported as hosts (Smit et al., 2004; McHugh et al. 2016). Only one of the seven trypanosome negative fish species from the present study has previously been found to harbour trypanosomes. Smit et al. (2000) reported a trypanosome infection in one of 12 *Schilbe intermedius* collected from the Okavango Delta and later Smit et al. (2004) reported another two infected *S. intermedius* from 25 screened from the same locality. It thus appears that *S. intermedius* in general have a low prevalence of infection of less than 10% and therefore with only eight fish sampled in the present study it cannot at this stage be ruled out that at least some *S. intermedius* from the Phongolo River are parasitised by trypanosomes.

The African fish leech, *Batracobdelloides tricarinata* is, according to Hadfield and Smit (2018), currently the only confirmed parasitic leech of freshwater fishes in Africa and has been recorded feeding on more than five different fish hosts including *C. gariepinus*, as was also found in the present study. This is most probably an underestimation of its potential host range and thus the record here of *B. tricarinata* feeding on S. *zambezensis* constitutes a new host record.

The trypanosomes seen parasitising all four fish host species resemble *Trypanosoma mukasai* in both size and morphology. Furthermore, the NI values for the trypomastigotes in all four species of fish are >1, which is suggestive of *T. mukasai* (Baker, 1960; Smit et al., 2004; Davies et al., 2005). In this study, however, it would appear that only smaller forms of this trypanosome were present, average length falling within the small form range of *T. mukasai* (22–44µm long) (see Baker, 1960). The small forms may be representative of a recent infection, particularly in the

272 case of *S. zambezensis*. Khan (1976) did note a progressive increase in size in trypomastigotes as
273 the infection aged when studying the life cycle of another piscine trypanosome species
274 *Trypanosoma murmanensis*. This was also observed by Negm-Eldin (1997, 1998) for *T. mukasai*
275 infecting nine fish species. This increase in length of trypomastigotes with age has caused
276 difficulties when trying to identify and differentiate species of trypanosomes, which can be further
277 complicated by the existence of pleomorphism in some infections (Smit et al., 2004; Davies et al.,
278 2005).

279 Based on the size ranges and morphology of the trypanosomes found in this study, it is
280 probable that they are further records of *T. mukasai*, a species of problematic identity (Davies et
281 al., 2005). The species has been reported parasitising a broad range of fish hosts from across
282 Africa, with reports even from India (Davies et al., 2005), one of these reports being as recently as
283 2013 (Shahi et al., 2013). It is understood that the recent latter report was from wild-caught fishes
284 from the Kashmir Himalaya region. Four species of fish were screened for blood parasites in the
285 study by Shahi et al. (2013), all of Cypriniformes, and three of these species were found to be
286 parasitised with what was identified as *T. mukasai*. No infection was observed in the common carp
287 *Cyprinus carpio* Linnaeus, 1758. If the trypanosome infection is indeed *T. mukasai*, it is not the first
288 report of no observable infection in *C. carpio*. Ferreira and Avenant-Oldewage (2013), during their
289 study on the occurrence of *Trypanosoma* species in freshwater fishes of South Africa, reported no
290 infection in this species of fish from localities where *T. mukasai* was present, in fact *T. mukasai*
291 was not found parasitising any of the four species of Cyprinidae screened in these authors' study.
292 It would appear that *T. mukasai* has, to date, not been reported as a natural infection in cyprinids
293 in Africa (Negm-Eldin, 1997, 1998; Scholtz et al., 2018). Equally, if the species observed in this
294 current study is *T. mukasai*, it was not observed parasitising the cyprinid *Labeo rosae*
295 Steindachner, 1894. Experimentally, Negm-Eldin (1998) was successful at transmitting this parasite
296 to African cyprinid hosts via inoculation by the biological vector *B. tricarinata*. Even so, he did note

297 that regardless of these fish hosts being readily accessible to the vectors, they were not the
298 preferred hosts, which would ultimately explain the apparent lack of recorded infections of *T.*
299 *mukasai* in cyprinids in Africa. Furthermore, Negm-Eldin (1998) mentioned that *T. mukasai*
300 showed a greater specificity to its vector than to its potential fish hosts, which would be of interest
301 to explore in future given the past and present global translocation of fish for recreation and food;
302 as well as potentially providing an avenue that may be helpful in the differentiation of piscine
303 trypanosome species.
304 One leech collected from an infected *S. zambezensis* revealed trypanosome developmental
305 stages similar to that noted by Negm-Eldin (1997) in his observations of *T. mukasai* in the leech
306 host *B. tricarinata*. Negm-Eldin (1997) observed 2 days post feeding (dpf) dividing stumpy
307 epimastigote stages (measuring on average ~13.3µm long x 2.4µm wide, with a free flagellum of
308 1.9µm). In the current material, short or stumpy epimastigote stages were also observed, one
309 short (8.4µm long x 2.2µm wide, with a free flagellum of 4.2µm) and one longer stage (13.1µm
310 long x 2.3µm wide with a free flagellum of 2.9µm); divisional stages were also observed. At 6 dpf,
311 Negm-Eldin (1997) noted sphaeromastigote forms (~4µm in diameter, free flagellum 6µm) as well
312 as transitional forms between stumpy epimastigotes and asphaeromastigotes. It may be that the
313 shorter stumpy epimastigote form in the current material represents one of these transitional
314 forms, particularly as the longer epimastigote forms in the current material conform closely to
315 what was described by Negm-Eldin (1997) as the shorter stumpy forms. At 6 dpf Negm-Eldin
316 (1997) also noted individual and dividing amastigotes (~7µm in diameter), slightly larger than the
317 current material measuring ~5.3µm long x 4.3µm wide; no division was seen in the current
318 material. Also noted in his study at this time were numerous longer epimastigote stages (~16.6µm
319 long x 2.5µm wide, free flagellum of 1.9µm), which appeared to, through multiple fission, give rise
320 to rosettes of 14 epimastigotes. A slender epimastigote stage (12.55µm long x 1.61µm wide) was
321 also observed in the current material, but was much shorter and half the width of that observed

by Negm-Eldin (1997). However, as only one was observed in the current material, we cannot determine if this was an anomaly. No rosettes were seen. Negm-Eldin (1997) notes that later infection stages were observed; at 6 dpf promastigotes were noted, and at 7–8 dpf these stages began to gradually lengthen until at 9–11 dpf they developed into long, slender and attenuated trypomastigotes (~42.3µm long x 1µm wide, free flagellum of 1.3µm).

Since, rosettes and later stages were never observed in the current material, it might be suggested that the infection in the leech was a recent one. However, as the leech in the present study was collected in the field, it cannot be supposed that the developmental stages found were as a direct result of the infection noted in the *S. zambezensis*, nor can we be certain as to the sequence of the developmental stages. It is worth noting, nonetheless, that if the stages in the leech are representative of *T. mukasai*, these stages are similarly on the smaller scale as are the blood stages in *S. zambezensis*, and as such may indicate a morphotype of *T. mukasai*, a trypanosome species which based on previous research already appears to show complexity in its genetic diversity (Davies et al., 2005).

4.2. Phylogenetic analysis and divergence estimates

All trypanosomes from the Phongolo and Okavango Delta nested within freshwater clade 2 and despite the ML and MP phylogenies having subtle topological differences the 18S trypanosome sequences generated from *S. zambezensis* and *B. tricarinata* did not resolve as sister taxa (Fig. 5). Within the ML phylogeny the trypanosome sequences from *B. tricarinata* clustered more closely with sequences generated from *Oreochromis andersonii*, denoted as Sample 1 and 5 from Davies et al. (2005), whereas in the MP the leech derived sequence cluster more closely with trypanosomes found in catfish species. Interestingly, despite the lack of resolution as sister taxa there appears to be only 0.7% divergence between the parasite sequences from the *S. zambezensis* and *B. tricarinata*, substantially below the standard 3% divergent threshold used to

distinguish between protist species or at least operational taxonomic units (OTU) (Stoeck et al., 2009; Schulz et al., 2019). This is similar to the findings by Davies et al. (2005) where two distinct genotypes of parasites were found but also illustrates the potential of high levels of genetic diversity within a single trypanosome species. In fact, comparisons of all the sequences including sample 1, 5, 30 and 31, those from Phongolo and those denoted as CLAR all show a lack of divergence above 3% indicating that they may not be separate species but rather a genetically diverse species of trypanosome able to infect multiple hosts. This is a pattern that is seen in many multi host parasite species often with host specific lineages emerging (Ganz and Ebert, 2010; Cole and Viney, 2018). This close relationship between the trypanosome sequences would account not only for inability to resolve the position of the parasite sequenced from *B. tricarinata* but also account for the low nodal support values seen in both trees. However, in order to disentangle the phylogenetic relationships within this group of trypanosomes it is clear that phylogenetic studies using full nuclear and/or kinetoplast genome, or even multiple gene data would aid in resolving the evolutionary history and radiation of these parasites.

Also, in both ML and MP analyses the trypanosome sequence from *S. zambezensis* clustered with other trypanosome species that were also found to parasitize different species of catfish including *S. nigromaculatus* (sample 30 and 31) and *Clarias* species (AJ620555 and CLAR) forming a distinct clade of trypanosomes infecting African catfish. Potentially supporting the emergence of host specific lineages as discussed above but at least providing further evidence of the close evolutionary relationship between trypanosome parasites and their hosts. The emergence of a distinct catfish lineage of trypanosomes could represent evidence of a radiation of these parasites which followed the speciation and diversification of catfish throughout Africa. However, further extensive sampling from across Africa would need to be able to completely disentangle this relationship. It is also important to note that in both phylogenies that the 18S trypanosome sequences generated from *S. zambezensis* appeared to have a close association with

freshwater trypanosomes from the Eurasian fish species *Esox lucius* (KJ601714) and *Sander lucioperca* (KJ601723) with divergence levels well below the 3% threshold unable to categorically distinguish them as distinct species.

Currently, it is not completely clear why there would be such low divergence between African and European fish trypanosomes. However, one potential explanation could be as a result of cross transmission of trypanosomes from African species of catfish within the genus *Clarias* which are now invasive across Europe and Asia. These fish were originally introduced to establish aquacultural stocks and eventually escaped into the wild. The original stock could have also been carrying trypanosomes and, with the abundance of freshwater leech species found across Africa and Asia, establishing sustainable life cycles. The promiscuous host feeding habits of freshwater leeches could subsequently have led to transmission of these African freshwater trypanosomes to native fish which would include the pike *E. lucius* and the zander *S. lucioperca*. Although this latter statement is highly hypothetical, and considerable work would be needed to resolve this issue, it does illustrate the paucity of understanding of fish trypanosomes and the requirement for further sampling, deeper sequencing and monitoring in order to better understand the diversity and distribution of these parasites, especially as they have been shown to be highly pathogenic emerging infections of wild life and aquaculture (Jesus et al., 2018).

4.4. Concluding remarks

From morphological and molecular data presented here it is clear the trypanosomes from Phongolo are closely related to those of the Okavango. Development stages of trypanosomes found in the leech *B. tricarinata* supports its status as the vector and the molecular evidence shows the genetic relationship between the trypanosome in the fish and leech but also the exceptional diversity of a single species of trypanosome between different host species. The work

presented here provides us with a solid foundation to make further steps in resolving the taxonomy and systematics of African freshwater fish trypanosomes.

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Legends to figures

Fig. 1. Map indicating the study site in the Phongolo River, South Africa.

Fig. 2. Photographs of the four fish species infected with trypanosomes in the Phongolo River, South Africa. A – *Coptodon rendalli*; B – *Oreochromis mossambicus*; C – *Clarias gariepinus*; D – *Synodontis zambezensis.* Photos not to scale.

Fig. 3. Photomicrographs of Giemsa-stained trypanosomes from the four infected fishes and trypanosome developmental stages from a squash of a leech, *Batracobdelloides tricarinata* that fed on an infected *Synodontis zambezensis.* A – *Coptodon rendalli* trypanosome; B – *Oreochromis mossambicus* trypanosome; C – *Clarias garipienus* trypanosome; D – Small (young) trypanosome from *Synodontis zambezensis*; E – Large (adult) trypanosome from *S. zambezensis*; F – amastigote; G – sphaeromastigote; H – short (bottom) and longer (top) thick epimastigote; I – possible division; J – slender epimastigote. Scale bar: A-E – 10µm; F-J – 20µm.

Fig. 4. Box-plot illustrating the size (Total Body Length – TBL) and the Nuclear Index (NI) of trypanosomes in the peripheral blood of four fish species from the Phongolo River, South Africa. A – Total body length (µm); B – Nuclear index, of trypanosomes found in *Coptodon rendalli*, *Oreochromis mossambicus*, *Clarias gariepinus* and *Synodontis zambezensis*. Bold centre line denotes the median value (50^{th} percentile), with the box containing the 25^{th} to 75^{th} percentiles of the dataset. The black whiskers mark the 5^{th} and 95^{th} percentiles, with circles above and below denoting values considered as outliers.

Fig. 5. Phylogenetic position of fish trypanosomes from the Phongolo River and Okavango Delta inferred from partial 18S rDNA gene sequences. A) Maximum Likelihood (ML) analysis using the K2P model with a four category gamma (G) distribution. B) Maximum parsimony using the Subtree-Pruning-Regrafting (SPR) algorithm. For both phylogenetic representations nodal support was calculated using 500 bootstrap replicates with only values higher than 50% presented. Overall, the trees share a similar topology with the South African trypanosomes falling within the same clade and fresh water trypanosomes being split into two separate clades