The neglected diversity: Description and molecular characterisation of *Trypanosoma haploblephari* Yeld and Smit, 2006 from endemic catsharks (Scyliorhinidae) in South Africa, the first trypanosome sequence data from sharks globally

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ABSTRACT

With over 200 species of sharks reported from South African waters, the potential of discovering new blood parasites is very high. Unfortunately, this remains a poorly explored area of research, particularly in this biogeographical region. To date, only a single trypanosome species, *Trypanosoma haploblephari* Yeld and Smit, 2006, has been described from elasmobranchs off the coast of South Africa infecting the catsharks *Haploblepharus pictus* (Müller & Henle) and *Haploblepharus edwardsii* (Schinz). With only a single trypanosome species described and absence of molecular information, a study was conducted to provide further morphological and molecular information on *T. haploblephari*, a species considered not to demonstrate any pleomorphism. Thin blood smears were prepared, and blood was collected in molecular-grade ethanol from the caudal vein of two shark species, *H. pictus* and *Poroderma pantherinum* (Müller & Henle). Trypanosomes were morphologically described and molecularly characterised based on analysis of fragments of the 18S ribosomal gene. The presence of *T. haploblephari* in *H. pictus* was confirmed using the original description based on morphology, type host and locality, which allowed for the molecular characterisation of the species. In addition, this species was found parasitising *P. pantherinum*, its morphology considerably different in this host species as compared to that in the species of *Haploblepharus*, demonstrating that *T. haploblephari* may show extreme pleomorphism. This paper provides both morphological and molecular data for both morphotypes of *T. haploblephari*, with molecular comparisons to the only two other elasmobranch species of trypanosome for which sequence data is available. To elucidate the relationship of trypanosomes from aquatic hosts in general, more efforts need to be placed on elasmobranchs, as current phylogenetic studies are predominantly focused on trypanosomes infecting freshwater fishes.

1. Introduction

Southern Africa is known as one of the most biodiverse regions for chondrichthyans worldwide with over 200 reported species (Ebert and van Hees, 2015). The chondrichthyan diversity also encompasses catsharks of the family Scyliorhinidae, most of which are endemic to southern Africa. With such a high diversity and endemicity (i.e. 13 %) of shark species, the potential for discovering new parasites is large (Schaeffner and Smit, 2019). If each fish species from cartilaginous to bony fishes harbours at least one unique parasite taxon (Adlard et al., 2015; Smit and Hadfield, 2015), there is a large number of parasites still awaiting discovery (Schaeffner and Smit, 2019; Smit and Hadfield, 2015). This is especially true for blood parasites, as at present, only two species of trypanosomes have been recorded from marine hosts in South Africa.

Trypanosomes (Kinetoplastida: Trypanosomatidae) are obligate, endoparasitic protozoans that can be found in almost every vertebrate and invertebrate class in both aquatic and terrestrial environments

(Barta et al., 2012). They belong to the class Mastigophora within the phylum Euglenozoa and are transmitted by haematophagous vectors such as leeches and biting flies. Even though species of *Trypanosoma* Gruby, 1843 have been well studied in mammals, especially the causative agents of African trypanosomiasis (sleeping sickness), due to their veterinary and medical importance, the biodiversity and pathogenicity of this genus in fish and elasmobranch hosts remains poorly explored (Ferreira and Avenant-Oldewage, 2013; Smit et al., 2020). The only two species currently known from marine hosts in South Africa are *Trypanosoma nudigobii* Fantham, 1919 from various intertidal teleost fishes (Hayes et al., 2014) and *Trypanosoma haploblephari* (Yeld and Smit, 2006) infecting elasmobranchs (Yeld and Smit, 2006). To date, only 12 species of elasmobranch trypanosomes have been described worldwide (see Table 1 in Yeld and Smit, 2006) and of these, the majority have been described from skates and rays (Bacigalupo and de la Plaza, 1948; Burreson, 1989; Laird, 1951; Neumann, 1909; Yeld and Smit, 2006). In South Africa, *T. haploblephari* was described from the dark shyshark, *Haploblepharus pictus* (Müller & Henle), and the puffadder shyshark, *Haploblepharus edwardsii* (Schinz). Only two other trypanosomes have been described infecting sharks in the family Scyliorhinidae, namely *Trypanosoma humboldti* Morillas, George-Nascimentoo, Valeria and Khan, 1987 from the Chilean catshark *Schroederichthys chilensis* Guichenot and *Trypanosoma scylliumi* (Laveran and Mesnil, 1902) from the small spotted catshark *Scyliorhinus stellaris* (L.). The present study reports on the findings of a survey on trypanosome infections in near-shore scyliorhinids off South Africa, adding to the known description of *T. haploblephari* by providing molecular data for this species, as well as a new host and locality record. Furthermore, novel information is provided on this species morphological plasticity, and its close genetic relationship with another elasmobranch trypanosome of European origin, *Trypanosoma rajae* (Laveran and Mesnil, 1902) is discussed.

2. Materials and methods

2.1. Host collection, blood smear preparation and screening for blood protozoans

A total of 61 sharks of two different species, *H. pictus* (47 individuals) and *P. pantherinum* (14 individuals) were collected at both Granger Bay in Cape Town (33°54′2.31″S, 18°24′56.38″E) as well as Hermanus (34°25′15.76″S, 19°14′37.56″E) along the Western Cape coast of South Africa (Fig. 1). Sharks were collected either by hand, using a handline from the shore, or using baited longlines. The present study received the relevant ethical approval from the North-West University's AnimCare Research Ethics Committee (ethics approval nos: NWU-00065-19-A5 and NWU-00372-16-A5) and research permits from the South African Department of Environmental Affairs (permit nos. RES 2018-58 and RES 2019-61 issued to Mrs. M. McCord, South African Shark Conservancy; and RES 2019-105 issued to BCS). As part of a larger parasitological study, 29 individuals of *H. pictus* and 9 individuals of *P. pantherinum* were euthanised and 18 individuals of *H. pictus* and 5 individuals of *P. pantherinum* were released at the capture site following bloodletting. Ectoparasites (e.g., leeches) present on sharks were removed and placed in either 70% ethanol or formalin for identification and further life-cycle evaluations. A maximum of 0.1 ml of blood was drawn from the caudal vein between the pelvic and caudal fin with a 21-gauge sterile, hypodermic needle fitted to a 1 ml syringe. Thin blood smears were prepared

and fixed with absolute methanol upon being air-dried completely. Remaining blood was placed in a tube containing 70% molecular-grade ethanol for subsequent molecular analysis. Microscope slides were stained with a dilution of 10% Giemsa-stain (Sigma-Aldrich, Steinheim, Germany) for 20 min and screened for parasites using a Nikon Eclipse N*i* (Nikon, Amsterdam, Netherlands) at 1000× magnification, and images captured using the accompanying NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo, Japan). Trypanosome stages were measured according to Hayes et al. (2014). Measurements are given in μm, unless otherwise indicated, and include midnucleus to anterior region (MA); midnucleus to posterior region (MP); midnucleus to kinetoplast (MK); posterior region to kinetoplast (PK); nuclear length (NL); nuclear index (NI) which is calculated MP/MA; body width at nucleus [BW(N)]; body width with undulating membrane [BW(UM)]; total body length (TBL) and flagellum length (FL).

2.2. Molecular and phylogenetic analysis

Genomic DNA was extracted from all individuals identified as positive for trypanosomes during screening by microscopy using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) following manufacturer's instructions for animal blood. The resulting supernatant was used as a template for PCR using 18S rRNA trypanosome-specific primers listed in Table 1. The conditions of the PCR with external primers are as follows: initial denaturation cycle of 95 °C for 5 min, 50 °C for 2 min, 72 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min 20 s, a final extension of 72 °C for 7 min. The conditions of the PCR with internal primers are as follows: initial denaturation cycle of 95 °C for 5 min, 50 °C for 2 min, 72 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min 20 s, a final extension of 72 °C for 7 min. All PCR reactions were performed with volumes of 25 μl, using 12.5 μl Thermo Scientific DreamTaq PCR master mix (2 ×) (2 × DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl$_2$), 1.25 μl of each primer (10 μM), and at least 25 ng of DNA. PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up the final reaction volume. Reactions were undertaken in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore). A 1% agarose gel electrophoresis was produced, and the results visualised under ultraviolet light to determine whether DNA amplicons were obtained. PCR products were then sent to Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) for purification and sequencing.

The quality of resultant sequences was assessed using Geneious ver. 11.1.4 (http://www.geneious.com, Kearse et al., 2012) before consensus sequences were generated from both forward and reverse sequence reads. Sequences obtained were deposited in the NCBI GenBank database under the following accession numbers (GenBank: MZ061638, MZ061640). Resultant sequences were identified using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and an additional 23 comparative sequences of *Trypanosoma* were selected with *Trypanosoma avium* Danilewsky, 1885 (KT728402) as the outgroup following Hayes et al. (2014). Comparative sequences were aligned using the ClustalW alignment tool available on Geneious ver. 11.1.4. A model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion using jModelTest 2.1.4 (Guindon and Gascuel, 2003; Darriba et al., 2012). The best model for the alignment was the general time-reversible model incorporating invariant sites and gamma distributed among site-rate variations (GTR + I + G). A Bayesian Inference tree was constructed in Geneious ver. 11.1.4 using the MrBayes parameter with a four category Gamma distribution. A maximum likelihood tree was constructed using PhyML 3.0 (Guindon et al., 2010) and run on the ATGC bioinformatics platform (available from http://www.atgc-montpellier.fr/phyml/, Guindon et al., 2010) with support using 1000 rapid bootstrap inferences. Phylogenetic trees were visualised using FigTree v. 1.4.4 software (Rambaut, 2012), the p-distance was calculated using MEGA 7

Table 1
PCR primers used for amplification and sequencing of the 18S rRNA gene region.

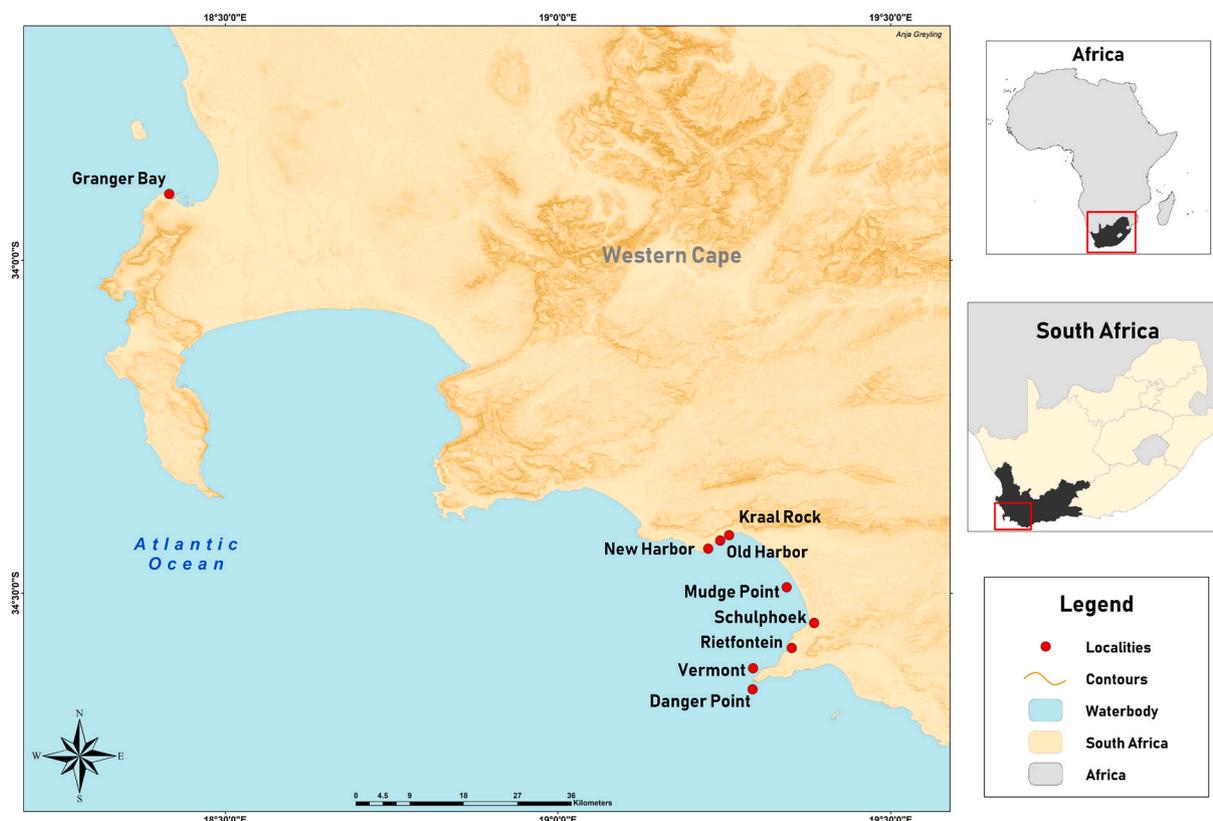| Primer		Sequence	Reference
External	SLF	5′-GCTTGTTTCAAGGACTTAGC-3′	McInnes et al. (2009)
External	S762	5′-GACTTTTGCTTCCTCTAATG-3′	Maslov et al. (1996)
Internal	B	5′-CGAACAACTGCCCTATCAGC-3′	Hayes et al. (2014)
Internal	I	5′-GACTACAATGGTCTCTAATC-3′	Hayes et al. (2014)

Fig. 1. Map of sampling sites on the south coast of South Africa.

([Kumar et al., 2015](#)) and the number of base pair differences was calculated using Geneious ver. 11.1.4.

3. Results

3.1. General observations of trypanosomes in the blood of sharks

Of the 61 individuals of two species examined, 93% were infected with trypanosomes. The metrical information, prevalence, and average numbers of trypanosomes per blood smear are recorded in Table 2. Of the 47 specimens of *H. pictus* screened, 91% were parasitised with a trypanosome species morphologically similar to *T. haploblephari*. All size classes were infected. A total of 14 *P. pantherinum* were screened, with 100% prevalence of a morphotype of the trypanosome species found in *H. pictus* in this study. Two of the *P. pantherinum* individuals and four of the *H. pictus* individuals had leeches on various external surfaces not limited to a specific area on the host. Table 3 provides the morphometrics of *T. haploblephari* observed in this study in *H. pictus* and that of the original description in Yeld and Smit (2006), as well as the morphotype parasitising *P. pantherinum* in the current study, and measurements provided by the original descriptions of *T. humboldti* from *Schroederichthys chilensis* and *T. scylliumi* infecting *Scyliorhinus stellaris* (Laveran and Mesnil, 1912; Pulsford, 1984; Morillas et al., 1987). The following diagnosis and description of *T. haploblephari* will provide for a

detailed description of trypanosome morphotypes in both *H. pictus* and *P. pantherinum* respectively.

3.2. Description and diagnosis of stages found in the blood for T. haploblephari

Kinetoplastea (Honigberg, 1963) Vickerman, 1976.
Trypanosomatida (Kent, 1880) Hollande, 1952.
Trypanosomatidae (Doflein, 1901) Grobben, 1905.
Trypanosoma haploblephari Yeld and Smit, 2006.
Restricted synonymy: Yeld and Smit (2006): 829–833, Figs. 1 and 2;
Hayes et al., (2006): 241; Hayes et al., (2014): 2; Smit and Hadfield (2015): 84; Schaeffner and Smit (2019): 2, 7, 16.
Type host: *Haploblepharus pictus* (Müller & Henle) (Chondrichthyes: Scyliorhinidae).
Other hosts: *Haploblepharus edwardsii* (Schinz), *Poroderma pantherinum* (Müller & Henle) (Chondrichthyes: Scyliorhinidae).
Type locality: Granger Bay, Western Cape, South Africa (33°52′S, 18°24′E).
Other localities: Hermanus, Western Cape, South Africa (34°25′15.76″S, 19°14′37.56″ E).

3.2.1. Material studied in H. pictus (morphotype A)

Locality: Granger Bay, Cape Town (33°54′2.31″S, 18°24′56.38″E) and Hermanus (34°25′15.76″S, 19°14′37.56″ E), Western Cape, South Africa.
Site in host: Peripheral blood.
Prevalence: 91% (43/47).
Vector: Unknown. Possibly leech found on sharks preliminarily identified as *Pontobdella* sp. Leach, 1815 (Prof. E. Burreson, Virginia Institute of Marine Science, USA; pers. comm.).
Representative DNA sequence(s): Three partial sequences of the 18S rRNA gene; 1740 bp, 1155 bp and 920 bp in length respectively (GenBank accession numbers: MZ061638, MZ061640, MZ061642).

Table 2
Information on elasmobranch hosts, including prevalence of peripheral blood trypanosomes.

Sharks			Trypanosomes
Species	N	ML ± SD (range) in mm	Prevalence
Haploblepharus pictus	47	435.6 ± 101.6 (260–614)	91% (43/47)
Poroderma pantherinum	14	511.4 ± 95.5 (363–725)	100% (14/14)

N, number; ML, mean length; SD, standard deviation.

Table 3

Morphometrics of trypanosomes measured from the shark species examined and measurements provided by Morillas et al. (1987) and Pulsford (1984) for *Trypanosoma humboldti* Morillas, George-Nascimento, Valeria and Khan, 1987 and *Trypanosoma scylliumi* (Laveran and Mesnil, 1902) respectively. Measurements have been rounded to the nearest whole number.

Species	*T. haploblephari (P. pantherinum)*			*T. haploblephari (H. pictus)*					*T. humboldti*		*T. scylliumi*	
Study	Present study			Present study			Yeld and Smit (2006)		Morillas et al. (1987)		Laveran and Mesnil (1902)	
	N	Range	ML±SD	N	Range	ML±SD	Range	ML±SD	Range	ML±SD	ML (SF)	ML (LF)
MA	87	6–34	20 ± 6	126	15–60	30 ± 7	26–46	35 ± 4	22–30	26 ± 2	28	25
MP	87	13–69	31 ± 12	126	16–64	38 ± 8	–	–	47–64	55 ± 4	39	42
MK	87	8–52	24 ± 8	126	8–45	25 ± 6	–	19	31–46	37 ± 4	–	–
PK	87	1–24	7 ± 5	126	2–34	13 ± 6	7–46	17 ± 5	16–25	19 ± 2	9	10
NL	87	1–7	4 ± 1	126	3–8	6 ± 1	5–9	7 ± 1	5–6	5 ± 0	4	5
NI	87	0–3	2 ± 1	126	0–3	1 ± 0	–	–	2–3	2 ± 0	1	2
BW(N)	87	2–11	5 ± 2	126	–	–	–	–	–	–	4	6
BW(UM)	87	2–16	6 ± 3	126	5–20	12 ± 4	13–24	17 ± 3	4–10	7 ± 2	6	10
TBL	87	20–93	52 ± 16	126	38–112	68 ± 14	54–99	70 ± 9	78–93	87 ± 4	54	59
FL	87	0–11	5 ± 3	126	–	–	–	–	5–11	7 ± 2	14	12

N, number; SD, standard deviation; ML, mean length; MA, mid-nucleus to anterior region; MP, mid-nucleus to posterior region; MK, mid-nucleus to kinetoplast; PK, posterior region to kinetoplast; NL, nuclear length (MP/MA); NI, nuclear index, BW(N), body width at nucleus; BW(UM), body width with undulating membrane; TBL, total body length; FL, flagellum length; SF, small form; LF, large form.
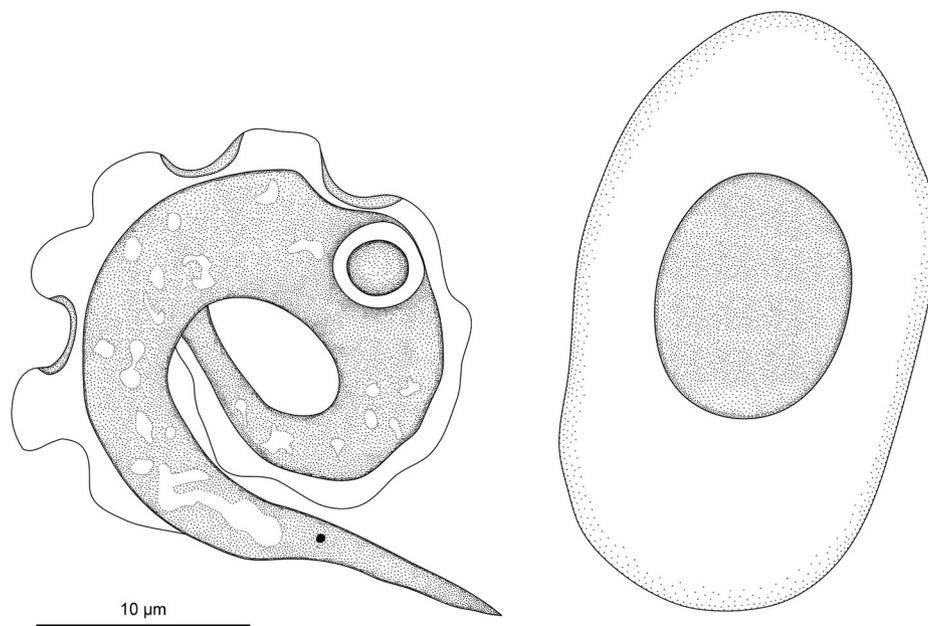

10 µm

Fig. 2. Line drawing of *Trypanosoma haploblephari* (Yeld and Smit, 2006) from the host *Poroderma pantherinum* (Slide HE18-18) next to a drawing of a red blood cell.

Diagnosis: Present specimens of *T. haploblephari* stained a deep blue with a wide and long body, and a distinct undulating membrane. The mean length of the trypanosome stages 68.1 ± 13.5 (38.2–112.2) (Table 3) correspond to the mean length of *T. haploblephari*; 70.4 ± 9.4 (53.7–99.4) reported by Yeld and Smit (2006). The karyosome is prominent within the nucleus, the latter situated in the anterior half of the parasite. Longitudinal striations were observed on larger specimens as reported for type specimens of *T. haploblephari* (see Yeld and Smit, 2006).

Description: Chromatic granules visible in cytoplasm, which stain a deep purple along with the kinetoplast and nucleolus. Karyosome visible in the nucleus, a similar finding to that of Yeld and Smit (2006). Kinetoplast distinct and located close to posterior region; 12.8 ± 6.1 (1.8–33.5 or 18.8% of body length – this study) or 16.8 ± 4.5 (6.9–45.6 – or 23.9% of body length – Yeld and Smit, 2006). Similar to Yeld and Smit (2006), the flagellum can be observed but is not easily stained or measured. In smaller stages, the posterior end is more slender and pointed in comparison to the larger stages that often have a blunt and rounded end (Yeld and Smit, 2006).

Remarks: The morphometrics of the trypanosome species observed from *H. pictus* in this study, closely resemble the data provided by Yeld and Smit (2006) for *T. haploblephari*. In addition to samples of the current study being collected from the type host and type locality, the species identification of *T. haploblephari* is further supported. This species of *Trypanosoma* was also found infecting *H. pictus* off the coast of Hermanus, a previously unknown distribution area of *T. haploblephari*, thus expanding the known biogeographical distribution of *T. haploblephari* to the southern Western Cape coast. Infection rates or parasitaemia vary among individuals of *H. pictus*, ranging from 0 to 200 trypanosomes per blood smear (45 trypanosomes on average), which is higher than Yeld and Smit's (2006) finding of 11 trypanosomes on average. As in Yeld and Smit (2006), all host size classes were infected, but in this study, prevalence was slightly lower at 91% than the 100% reported by Yeld and Smit (2006).

3.2.2. Material studied in P. pantherinum (morphotype B)

Voucher material: Slide HE18-18, one blood film with 150 trypanosomes deposited in the National Museum, Bloemfontein, South

Africa (accession number: NMB P 793).

Locality: Hermanus, Western Cape, South Africa (34°25′15.76″S, 19°14′37.56″E).

Site in host: Peripheral blood.

Prevalence: 100% (14/14).

Representative DNA sequence(s): Partial sequence of the 18S rRNA gene; 1477 bp in length (GenBank accession number: MZ061641).

Diagnosis: A slender, blue-purple staining trypanosome (51.7 ± 16.0 in body length) with a short free flagellum (4.9). Body is slender (5.4 ± 2.0) and distinct kinetoplast is visible located on average 7.3 from the posterior region. Usually found curling in on itself in form of a doughnut (Fig. 2).

Description: Cytoplasm, kinetoplast and nucleolus are basophilic and stain deep blue-purple with numerous chromatic granules visible in the cytoplasm. The karyosome can be seen within the nucleus (Fig. 3 D, E) and a distinct kinetoplast, located close to the posterior part of the body, is visible; 7.3 ± 5.1 (1.1–23.6) from posterior region to kinetoplast or 10.7% of the body length (Fig. 3 F). The undulating membrane is well developed with several undulations (Fig. 3 F) and a distinct, free flagellum is present; FL = 4.9 ± 3.1 (0–11.3) (Fig. 3 F). In more deeply stained individuals, longitudinal striations are visible, often seen across the nucleus and anterior region. The location of the nucleus from the anterior end is 20.7 ± 6.2 (6.2–33.9), or 40% of the body length, thus lies just anterior to the midpoint of the body for the majority of specimens examined, with a nuclear index of 1.5 ± 0.7 (0.4–3.0).

Remarks: Trypomastigote stages found in the blood of *P. pantherinum* differ both in morphology and size to *T. haploblephari* morphotype A (Fig. 3) (Table 3). On average, morphotype B has a shorter body (51.7 ± 16) as compared to *T. haploblephari* morphotype A in both the present and Yeld and Smit's (2006) study (68.1 ± 13.5; 70.4 ± 9.4; respectively). A distinct, free flagellum (4.9 ± 3.1) is present as compared to morphotype A of *T. haploblephari* where the flagellum is

longer and not easily seen in the present material (6.9). Furthermore, morphotype B is a slender trypanosome (5.4 ± 2.0), shown in both immature and mature stages, as compared to the characteristically wide form of *T. haploblephari* morphotype A [11.6 ± 3.5 (4.6–20.2)]. The undulating membrane is clearly visible in morphotype B with a body width including the undulating membrane measuring on average 6.8 (2.3–15.9) (N = 79), with the width of the undulating membrane approximately 1.9, in comparison to a body width of 13.5 (6.4–21.1) (N = 111) for *T. haploblephari* morphotype A (present study). In the original description, measurements of the trypanosome including the undulating membrane were 17.4 ± 2.6 (12.6–24.3), with the width of the undulating membrane at between 1 and 4. The location of the nucleus of morphotype B is 20.7 from the anterior end, or 40% of the body length, compared to 34.7 or 49.3% of total body length of *T. haploblephari* morphotype A. The kinetoplast of morphotype B is located close to the posterior part of the body (PK = 7.3 ± 5.1) or 14.1% of the body length and 23.8 to the nucleus, or 46% of the total body length, as compared to morphotype A at 16.8 (23.9%) and 18.9 (26.9%), respectively. Additionally, nuclear length of morphotype B is much shorter (3.9 ± 1.3) than that of morphotype A [6.5 in Yeld and Smit (2006), 5.5 in present study]. Morphotype B exhibits a unique characteristic by curling in on itself or appearing coiled, appearing almost circular in a doughnut-shape, with anterior and posterior ends situated closely together. This was not mentioned as a unique feature in either the immature or mature stages of *T. haploblephari* in the original description. Yeld and Smit (2006) also observed dividing forms of *T. haploblephari* in the peripheral blood of *H. pictus* and *H. edwardsii*, however in this study, no dividing forms were observed in either *T. haploblephari* morphotype A or B. It was also proposed in the original description that *T. haploblephari* could be an endemic species, due to the restricted geographic distribution and high level of endemicity of the host species (Yeld and Smit, 2006). Even though both *H. pictus* and *P. pantherinum* collected from
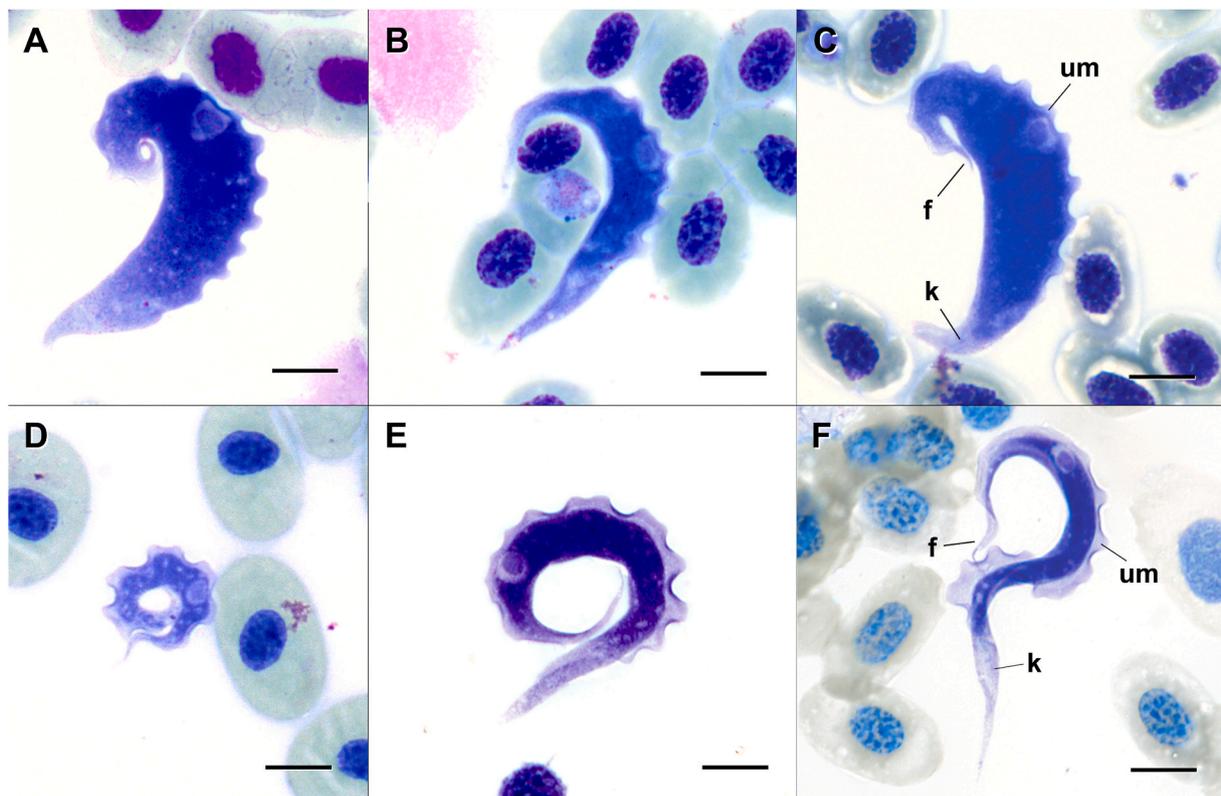
Fig. 3. Micrographs of *Trypanosoma haploblephari* (Yeld and Smit, 2006) morphotype A (A–C) and *T. haploblephari* morphotype B (D–F) in Giemsa-stained blood films of *Haploblepharus pictus* and *Poroderma pantherinum*, respectively. Blood stage with kinetoplast (k) and undulating membrane (μm) visible (A–C); slender forms (B, E); presence of a flagellum (f) in deeply stained individuals (C, F). Scale bar: 10 μm.

Hermanus were infected with trypanosomes, those in *H. pictus* always resembled morphotype A as in the original description of *T. haploblephari*, with no stages resembling the morphotype isolated from *P. pantherinum.*. Likewise, no trypanosome stages in *P. pantherinum* resembled morphotype A. Although pleomorphism is known to occur in species of *Trypanosoma*, this was not observed in *T. haploblephari*, even between the two species of hosts, *H. pictus* and *H. edwardsii*, infected with this trypanosome in the original description (Yeld and Smit, 2006). As such, the now apparent pleomorphism may potentially be the result of the difference in host genus.

A trypanosome species *T. humboldti* was described in another cat-shark species, *S. chilensis*, off the Pacific coast of Chile (Morillas et al., 1987). Even though morphotype B in *P. pantherinum* does show 'C' and 'S' shaped forms when trypomastigotes are larger, as does *T. humboldti*, the latter is much larger (87 ± 3.8; including free flagellum) than that of *T. haploblephari* morphotype B (~56.6 including the free flagellum) (Table 3). Furthermore, *T. haploblephari* morphotype B does not conform with regards to nuclear position as compared to *T. humboldti* (NI = 1.5 vs. 2.1, respectively). Morphologically, especially with regards to the shape, *T. haploblephari* morphotype B conforms closely to *T. scylliumi*, found in the dogfish *Scyliorhinus canicula* (L.) and *Sc. stellaris* off Roscoff, France (Pulsford, 1984). *Trypanosoma scylliumi* was later reported from *Sc. canicula* from British waters (Henry, 1910; Coles, 1914), and Pulsford (1984) provided additional measurements of this parasite from *Sc. canicula* from both Plymouth and the type locality Roscoff. Similarly, *T. scylliumi* and *T. haploblephari* morphotype B show trypanosome stages that are coiled when smaller or 'S' shaped when larger. *Trypanosoma haploblephari* morphotype B also conforms closely to *T. scylliumi* in length (51.7 ± 16.0; 54.1–58.6, respectively), nuclear length (3.9 ± 1.3; 3.7–5.0, respectively) and nuclear position (NI = 1.5; 1.4–1.7 respectively). However, *T. haploblephari* morphotype B differs considerably in flagellum length (4.9 ± 3.1) as compared to that of *T. scylliumi* (12.0–13.5) (Table 3).

Furthermore, with regards to trypanosome infections, geographical proximity is often given priority (Khan, 1977; Morillas et al., 1987). In this case, the distance between type localities of *T. haploblephari* and *T. scylliumi* are so distant that the potential of these species being conspecific is considered extremely low. However, this should be investigated molecularly in future.

3.3. Molecular phylogeny

The sequences of *T. haploblephari* morphotype A isolated from *H. pictus* were approximately 800bp long for both internal and external primers, respectively, and a consensus sequence was constructed of 1740bp. Similarly, sequences of 800bp were obtained for morphotype B from *P. pantherinum*, where three amplicons were used to construct a consensus sequence of 1477bp. The alignment consisted of 25 trypanosome sequences (Table 4) with a final alignment length of 1417bp. *Trypanosoma haploblephari* morphotype A and B showed a divergence of 0.5% (p = 0.005) with 10 base pair differences in the phylogenetic alignment. *Trypanosoma haploblephari* morphotype A and B fall within the marine fish trypanosome clade (Fig. 4), showing a close relationship with *T. rajae* (p = 0.006 and p = 0.007; a divergence of 0.6 and 0.7%, respectively – Table 5), described from various species of skates (*Raja*).

4. Discussion

To date, there is a lack of studies and knowledge on the trypanosomes of elasmobranchs, with only 12 reported species (Yeld and Smit, 2006) compared to marine bony fishes of which at least 30 are known globally (Woo, 1994). Most of the species infecting elasmobranchs have been recorded from skates and rays (Yeld and Smit, 2006), while only five of the twelve species have been described from sharks. This paucity in knowledge of shark trypanosomes is particularly noticeable in elasmobranchs off the shores of southern Africa. Up until now, only a single species of *Trypanosoma* has been described infecting sharks from this region, *T. haploblephari*, which was described over a decade ago by Yeld and Smit (2006); this as compared to regions in the Mediterranean and Northern Atlantic from which four species have been described (Yeld and Smit, 2006). Since the study by Yeld and Smit (2006), research on southern African elasmobranch trypanosomes has been neglected. Furthermore, this species was described solely on morphological characteristics. Given the minor morphological differences between trypanosome species and the tendency for pleomorphism in forms of a single species, morphological differentiation of species of trypanosomes can be challenging.

Yeld (2009) highlighted this with reference to *Trypanosoma gargantua* (Laird, 1951), *T. giganteum* (Neumann, 1909), *T. rajae* and *T. murmanense* Nikitin, 1927, all well-known examples of species in which pleomorphism occurs. This, in the past, has produced a false

Table 4
Species of *Trypanosoma* Gruby, 1843 implemented in the phylogenetic analysis.

Trypanosoma species	Host	Country	Reference	Accession number
Trypanosoma avium	*Anthochaera phrygia*	Australia	Šlapeta et al. (2016)	KT728402
Trypanosoma binneyi	*Ornithorhynchus anatinus*	Australia	Paparini et al. (2014)	KJ867148
Trypanosoma boissoni	*Zanobatus atlanticus*	Senegal	Maslov et al. (1996)	U39580
Trypanosoma chelodinae	*Emydura signata*	Australia	Jakes et al. (2001)	AF297086
Trypanosoma cobitis	*Noemacheilus barbatulus*	England	Stevens et al. (1999)	AJ009143
Trypanosoma fulvidraco	*Pseudobagras fulvidraco*	China	Gu et al. (2007b)	EF375883
Trypanosoma granulosum	*Anguila anguila*	Portugal	Unpublished	AJ620552
Trypanosoma granulosum	*Anguila anguila*	United Kingdom	Unpublished	AJ620551
Trypanosoma micropteri	*Micropterus salmoides*	China	Jiang et al. (2019)	MH635421
Trypanosoma murmanense	*Hippoglossus hippoglossus*	Norway	Karlsbakk and Nylund (2006)	DQ016616
Trypanosoma ophiocephali	*Channa argus*	China	Gu et al. (2010)	EU185634
Trypanosoma pleuronectidium	*Melanogrammus aeglefinus*	Norway	Karlsbakk and Nylund (2006)	DQ016618
Trypanosoma rajae	*Raja* spp.	–	Unpublished	MG878996
Trypanosoma siniperca	*Siniperca chuatsi*	China	Gu et al. (2007a)	DQ494415
Trypanosoma sp.	*Carassius carassius*	Ukraine	Grybchuk-Ieremenko et al. (2014)	KJ601715
Trypanosoma sp.	*Scardinius erythrophthalmus*	Ukraine	Grybchuk-Ieremenko et al. (2014)	KJ601718
Trypanosoma sp. carpio	*Cyprinus carpio*	China	Gu et al. (2007b)	EF375882
Trypanosoma sp. CLAR	*Clarias angelensis*	Africa	Hamilton et al. (2004)	AJ620555
Trypanosoma sp. EL-CP	*Esox lucius*	Czech Republic	Maslov et al. (1996)	L14841
Trypanosoma sp. Marv	*Cyprinus carpio*	–	Unpublished	AJ620549
Trypanosoma sp. R6	*Abramis brama*	Poland	Unpublished	AJ620554
Trypanosoma sp. Ts-Ab-TB	*Abramis brama*	Czech Republic	Unpublished	AJ620556
Trypanosoma triglae	*Trigla lineata*	Senegal	Maslov et al. (1996)	U39584
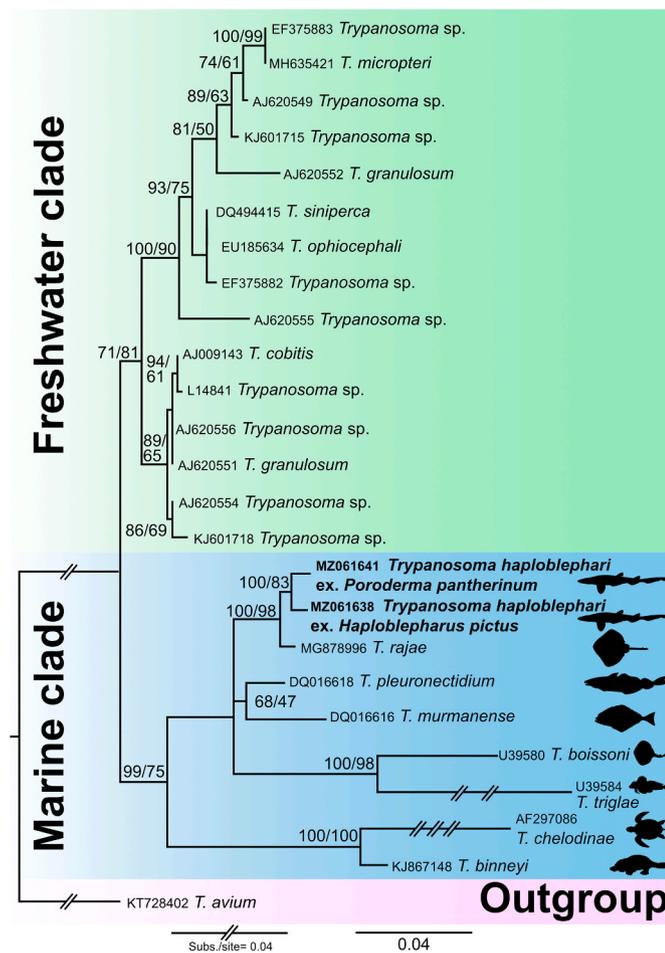
Fig. 4. Bayesian Inference (BI)/Maximum Likelihood (ML) analysis showing the phylogenetic position of *Trypanosoma haploblephari* (Yeld and Smit, 2006) genotypes representing morphotypes A and B inferred from partial 18S rRNA gene sequences. Comparative sequences representing known *Trypanosoma* species, with *Trypanosoma avium* (KT728402) as outgroup, were obtained from GenBank. Tree topologies for both the BI and ML trees were identical; the nodal support values (BI/ML) are represented on the BI tree. Some branches have been shortened with each //= 0.04 substitutions per site.

characters used in trypanosome descriptions are not standardised leading to further challenges. Such challenges were experienced during the current study, but given the distinctive characteristics of *T. haploblephari* such as the species' body width, length and shape, as well as the collection of current samples from the type host and locality, current samples of *T. haploblephari* were easily identified and unequivocally assigned to this species.

During the current study, a trypanosome was found infecting *P. pantherinum* that was morphologically distinguishable from the original description of *T. haploblephari*, as well as to the specimens of *T. haploblephari* described from *H. pictus* during the current study. Molecular data, however, indicated that the trypanosome found parasitising *P. pantherinum* was closely related to *T. haploblephari* with a divergence of 0.5%, well below the 3% threshold considered necessary to differentiate between separate species (see Smit et al., 2020). Yeld and Smit (2006) described *T. haploblephari* as a trypanosome species in which pleomorphism does not appear to occur. However, the current study indicates that this is not the case, and that *T. haploblephari* demonstrates extreme pleomorphism, particularly between the two sympatric genera of catsharks.

To date, the best-known example of extreme pleomorphism shown in a single species of marine trypanosome is *T. rajae* (see Yeld and Smit, 2006). Phylogenetic analysis places both genotypes of *T. haploblephari* in the same clade as *T. rajae*, with a divergence of less than 1%, which strongly suggests that *T. haploblephari* and *T. rajae* are the same species. In the original description of *T. rajae*, authors collected this species off the coast of Roscoff, France, in two species of skates *Raja asterias* Delaroche (syn. *R. punctata* Risso) and *Raja undulata* Lacepède (syn. *R. mosaica* Lacepède) (Laveran and Mesnil, 1912). Both species of skates have an Eastern Atlantic distribution, from the Mediterranean to possibly the coasts off Mauritania and Senegal respectively (www.fishbase.org, Froese and Pauly, 2020). Even though these two host species have not been reported off the coasts of southern Africa, they may overlap in distribution with species that do occur in this region. At least nine species of skate occurring off the coasts of southern Africa have a distribution range which spans to the Eastern Atlantic, or may have a distribution which is bipolar in the former and latter regions (Compagno and Ebert, 2009). As such, the potential for a multi-host species of trypanosome with an extensive distribution range cannot be excluded, and this may be the case with *T. rajae*.

As mentioned previously, geographical proximity is often given priority when attempting to differentiate between species, but this would not be applicable in a multi-host species with a wide distribution range. The *P. pantherinum* morphotype of *T. haploblephari* compared very closely to *T. scylliumi,* described from sharks of the same family (Scyliorhinidae) off the coasts of Roscoff. Given this and the possibility that *T. haploblephari* is a genotype of *T. rajae*, the extreme pleomorphism of the latter, and the same type locality of *T. rajae* and *T. scylliumi*, it calls into question whether *T. scylliumi* is yet another morphotype of *T. rajae*. It also questions then the reliability of geographical proximity for differentiating between morphologically similar parasites. Unfortunately, the above remains hypothetical at present, as the study linked to

representation of the true trypanosome biodiversity. Modern molecular techniques have proven useful in differentiating between closely related or even morphologically similar species (Borges et al., 2016; Davies et al., 2005). At the same time, these techniques have revealed the high levels of genetic diversity that can occur in a single species of trypanosome (Davies et al., 2005; Smit et al., 2020). Currently, most of the trypanosome diversity is described on morphometric data alone, particularly for elasmobranch trypanosomes (Laird, 1951; Morillas et al., 1987; Yeld, 2009). Furthermore, the set of morphometric

Table 5
Evolutionary differences of species of *Trypanosoma* Gruby, 1843 isolated from the 18S rRNA gene region of marine organisms included in the phylogenetic analysis presented in Fig. 4, expressed as percent similarity (bottom left) and uncorrected pair-wise distance (p-distance) (top right).

	Accession number	*Trypanosoma* species	Host	1	2	3	4	5	6	7	8	9
1	MH635421	*Trypanosoma micropteri*	*Micropterus salmoides*		0.055	0.044	0.068	0.026	0.033	0.029	0.031	0.031
2	KT728402	*Trypanosoma avium*	*Anthochaera phrygia*	93%		0.071	0.086	0.059	0.06	0.056	0.06	0.058
3	U39580	*Trypanosoma boissoni*	*Zanobatus schoenleinii*	94%	92%		0.059	0.035	0.034	0.038	0.038	0.038
4	U39584	*Trypanosoma triglae*	*Trigla lineata*	89%	88%	91%		0.063	0.065	0.064	0.067	0.065
5	DQ016618	*Trypanosoma pleuronectidium*	*Melanogrammus aeglefinus*	96%	93%	95%	90%		0.017	0.014	0.017	0.017
6	DQ016616	*Trypanosoma murmanense*	*Hippoglossus hippoglossus*	95%	93%	95%	90%	98%		0.02	0.019	0.021
7	MG878996	*Trypanosoma rajae*	*Raja* spp.	96%	93%	95%	90%	99%	98%		0.007	0.006
8	MZ061641	*Trypanosoma haploblephari*	*Poroderma pantherinum*	95%	93%	95%	89%	98%	98%	99%		0.005
9	MZ061638	*Trypanosoma haploblephari*	*Haploblepharus pictus*	95%	93%	95%	89%	98%	98%	99%	99%	

both sequences of *T. rajae* (MG878996, MG878995) in GenBank has, as of yet, not been published, and as such, it is not possible to be certain that these sequences are in fact representative of *T. rajae* without the diagnosis that should accompany them.

Based on the morphological findings, it would be easy to describe the two morphotypes of *T. haploblephari* as separate species, as was done in the study by Sehgal et al. (2015) in which a new species of avian trypanosome was described based on distinct morphology. This new species also showed an 18S sequence divergence of under 1%, particularly when compared to other sympatric trypanosome species, which had been described from the same species of host. Attempts at the reconstruction of a phylogenetic tree were not possible by these authors as the sequence data lacked adequate variation. A similar issue was encountered during the study by Smit et al. (2020) on the freshwater fish trypanosome *Trypanosoma mukasai* Hoare, 1932. When molecularly characterising *T. mukasai* from various fish hosts of different genera and species as well as the probable leech vector, sequence variation was too low to allow for any definitive conclusions regarding the specific relationships between the taxa within these clades. According to these authors, this was accounted for by the close relationship of the sequences, all showing a divergence under the 3% threshold, suggesting that they may not be separate species parasitising the different genera and species of host, but more likely a species of multi-host trypanosome which shows a high level of intraspecific genetic diversity. Similarly, an extensive molecular study on the trypanosome lineages of bats, did not differentiate between species or operational taxonomic units (OUT) when divergence was below 1% (Clément et al., 2019).

As such, with our present knowledge on the trypanosomes of elasmobranchs, it would be best to be cautious and not describe the distinct morphotype of *T. haploblephari* as a new species. It is possible that both these morphotypes together with the probable *T. rajae* represent a single species with an extensive distribution range, such as the multi-host *T. mukasai,* which is considered to have a pan African distribution. As mentioned above *T. mukasai* demonstrates a high level of intraspecific genetic variation, with potentially emerging host-specific lineages (Davies et al., 2005; Smit et al., 2020). If the sequences included in the phylogenetic analysis of the current study do represent *T. rajae*, the current *T. haploblephari* genotypes could represent two of these host-specific lineages. Even though this cannot be determined at present without the diagnosis of these sequences as *T. rajae*, this study does highlight the lack of molecular phylogenetic effort given to elasmobranch trypanosomes. Apart from *T. rajae*, only one other elasmobranch trypanosome has molecular data available, *T. boissoni* (U39580)*,* isolated from *Zanobatus atlanticus* off the coast of Senegal, this species showing an above threshold, but still low 3.8% divergence from the *T. haploblephari* and *T. rajae* clade.

Both *T. haploblephari* from *H. pictus* and *P. pantherinum*, as well as the probable *T. rajae* fell within the marine fish *Trypanosoma* clade. *Trypanosoma binneyi* (KJ867148)*,* described from a platypus, *Ornithorhynchus anatinus* Shaw as well as *T. chelodinae* (AF297086) from a turtle, *Emydura signata* Ahl, forms a subclade within the marine *Trypanosoma* clade. The same configuration was observed in other phylogenetic analyses including that of Hayes et al. (2014), Karlsbakk and Nylund (2006) as well as Gu et al. (2010). A possible reason for this occurrence could be due to insufficient taxon sampling and a lack of additional sequences of trypanosomes infecting other aquatic tetrapods. Only with additional survey efforts to characterise more trypanosome species infecting marine organisms as well as aquatic tetrapods on a molecular basis, can evolutionary histories be explained and more conclusive answers on the true phylogenies of aquatic trypanosomes be provided. *Trypanosoma haploblephari*, *T. rajae* and *T. boissoni* occupy a basal position, which could suggest that the trypanosomes from elasmobranchs are evolutionarily older than those parasitising other marine vertebrates.

It is difficult to determine if and to what degree trypanosome infections affect sharks, as there is no agreement in the literature on how to assess these impacts (Yeld, 2009). In several species of amphibians,

birds and reptiles, trypanosomes have been known to cause disease, however, in contrast it appears as if these parasites rarely cause any pathogenicity in fishes, especially marine cartilaginous or bony fishes (Pulsford, 1984; Yeld, 2009). Little information is known on the effect of trypanosomes on elasmobranchs, and it may be suggested that due to the long co-evolutionary time, the pathogenicity of trypanosomes seen in other vertebrate groups, might be absent in elasmobranchs. Parasitaemia in the blood of both *H. pictus* as well as *P. pantherinum* were notably high, a similar finding to that of Yeld and Smit (2006). It has been suggested that the high parasitaemia present in the blood could be attributed to the benthic-orientated and more sedentary behaviour of the shark hosts. This increases their exposure to marine leeches, the suggested vectors of these blood parasites. In contrast to other studies where trypanosomes were found infecting only hosts that are larger, and ultimately older (Aragort et al., 2005; Pulsford (1984)), this study, along with that of Yeld and Smit (2006) found that sharks from all size classes were infected with trypanosomes. The infection rates of trypanosomes were high with an average of 43 trypanosomes per blood smear of *H. pictus* and 48 trypanosomes per blood smear of *P. pantherinum* in comparison to the low numbers reported by Aragort et al. (2005) (0–2), Pulsford (1984) (1–4) and Yeld and Smit (2006) (average of 11). It has also been suggested by Negm-Eldin (1998) that some trypanosomes might rather be vector-specific than vertebrate host-specific. This was concluded following the transmission experiments where the freshwater teleost infecting *T. mukasai* was successfully transmitted to eight different fish species using its vector *Batracobdelloides tricarinata* Blanchard. A similar finding was also observed for the marine teleost infecting *Trypanosoma cobitis* Mitrophanow 1883 and *T. murmanense*, both demonstrating a specificity to their vectors, *Hemiclepsis marginata* Müller and *Johanssonia arctica* Johansson, respectively (Negm-Eldin, 1998). To date, life-cycle data of trypanosomes infecting marine fishes are scarce and studies on leeches infesting South African catsharks are entirely absent. As such, future work should include further research into identifying the leeches found on these sharks to species level and whether these invertebrates can act as vectors to these trypanosome species.

5. Conclusion

Many species of trypanosomes are known for their pleomorphism that, in the past, has created a false sense of their true biodiversity. This though appears to be a continuing dilemma. The current study draws attention to the need to be cautious in describing new trypanosome taxa based on new host and/or geographical distributions, as well as descriptions based on unique morphology or a combination of all these factors. This is particularly applicable to elasmobranch trypanosomes for which there is, at this time, too few molecular studies to begin to fully understand the phylogenetic relationships and taxonomy of this group of trypanosomes. More extensive sampling and molecular characterization of described species from elasmobranchs needs to occur before the degree of pleomorphism, as well as factors such as host-specificity, potential for mixed-infections, and distribution ranges can begin to be clearly understood. A further limit to unravelling the biodiversity and taxonomy of these parasites includes the use of one genetic marker, when likely it would be beneficial to apply multiple markers (Lemos et al., 2015; Clément et al., 2019; Smit et al., 2020). The above concerns may not only apply to elasmobranch or other aquatic species of trypanosome, such as those parasitising bony fishes, but to species of trypanosome in general.

Regardless, more effort needs to be placed in acquiring more data on trypanosomes from sharks, as until now there has been no sequence data on trypanosomes of sharks in general and from South Africa in particular. As South African waters present such a high diversity of elasmobranchs, the potential of finding additional parasite species and revealing host-specific lineages of these is high, particularly with increased survey efforts. This study represents the first account on the

molecular characterisation of trypanosomes parasitising sharks and the first screening of *P. pantherinum* for trypanosomes from South African waters.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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