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Molecular and morphological characterisation of one known and three new species of fish parasitic *Trypanosoma* Gruby, 1843 from the South Coast of South Africa.

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Abstract

The genus *Trypanosoma* Gruby, 1843 encompasses unique, flagellate haemoparasites infecting all vertebrate classes globally (excluding Antarctica). While trypanosomes in terrestrial mammals are well-studied due to their medical and veterinary significance, those in fishes remain largely unexplored, with limited data on their life cycles and ecological roles. Furthermore, the phylogenetic relationships of numerous aquatic species are unresolved. This gap is notable in South Africa, a region with high marine fish biodiversity, yet only two documented marine trypanosome species, one in teleosts and one in elasmobranchs are known. Our research aims to bridge this knowledge gap for marine fish trypanosomes along South Africa's southern coast. Blood samples were collected from 246 fishes spanning 23 species at Chintsa East, Tsitsikamma (Garden Route National Park), Boknes, Kariega River Estuary, and Groot River West Estuary from 2020 to 2023. Giemsa-stained blood smears were screened for trypanosomes, which were morphologically characterised. Molecular analyses targeting the 18S rRNA gene region were conducted on blood samples positive for trypanosomes. Combined morphological and molecular evidence identified four *Trypanosoma* species: one known species, *Trypanosoma nudigobii* from the klipfish (*Clinus superciliosus*), and three new species: *Trypanosoma* sp. A from the prison goby (*Caffrogobius gilchristi*), *Trypanosoma bakana* n. sp. from the white steenbras (*Lithognathus lithognathus*), and *Trypanosoma bokkom* n. sp. from five mullet species [the grooved mullet (*Chelon dumerili*), South African mullet (*Chelon richardsonii*), striped mullet (*Chelon tricuspidens*), flathead grey mullet (*Mugil cephalus*) and the freshwater mullet (*Pseudomyxus capensis*)].

Keywords: blood parasites, marine fish, molecular, morphology, trypanosomiasis, life below water

Introduction

Marine trypanosomes are single-celled eukaryotic organisms belonging to the phylum Euglenozoa (Kinetoplastida). Often overshadowed by their terrestrial counterparts, such as the well-known *Trypanosoma brucei* (Plimmer and Bradford, 1899), which causes human sleeping sickness, marine trypanosomes have recently emerged as an intriguing topic of scientific inquiry (Hoare, 1972; Karlsbakk *et al*., 2005; Hayes *et al*., 2006; Yeld and Smit, 2006; Hayes *et al*., 2014; Su *et al*., 2014; Pretorius *et al*., 2021). These parasites have been documented in various marine environments worldwide, ranging from coastal regions to the open ocean (Burreson, 2007; Hayes *et al*., 2014). Understanding their ecological roles and potential impact on marine ecosystems is essential to advance our knowledge of marine parasitology and ecosystem health. Although most trypanosome life cycles are unknown, leeches have been identified as the invertebrate hosts or vectors for some marine and freshwater fish trypanosomes (Hayes *et al*., 2014; Smit *et al*., 2020). Leal *et al*. (2009) proposed that the dissonance in the characterisation of species of *Trypanosoma* Gruby, 1843 is due to the extreme polymorphism that may be found in a single species, as well as individual species being able to infect numerous vertebrate and invertebrate host species. Additionally, by restudying trypanosomes using fresh material from type hosts and utilising modern methods such as molecular data, additional inferences on species identification can be obtained. This approach may help infer likely synonymies of species described in different hosts or geographical regions. (Leal *et al*., 2009; Hayes *et al*., 2014). The inclusion of non-human trypanosome molecular data in phylogentic analyses is important for better understanding of evolutionary and ecological relationships of all the host species infected with trypanosomes. The most significant division in the phylogenetic relationships of non-human trypanosomes is between the species infecting terrestrial and aquatic vertebrates. Based on phylogenetic evidence of 18S rRNA, amphibian and fish trypanosomes are the origin from which all other trypanosomes are derived (Hamilton *et al*., 2007; Fermino *et al*., 2019). Despite the value of the division of the clades, the utilisation of subgenera (see Kostygov *et al*., 2021) has been largely disregarded, possibly due to the expectation of conflicts between morphology- and phylogeny-based systems that did not complement each other. The subgenus *Haematomonas*

(Mitrophanow, 1883 emend. Votýpka and Kostygov, 2021) comprises leech-transmitted parasites that infect aquatic vertebrates, and its classification is based on phylogenetic analyses using the 18S rRNA gene region. Mitrophanow (1883) was the first to describe two monoflagellates from freshwater fish, placing them in the genus *Haemotomonas* (*Haematomonas cobitis* Mitrofanov, 1883 and *Haematomonas carassii* Mitrofanov, 1883), but Doflein (1901) later reassigned them to *Trypanosoma*. Following Mitrophanow (1883)'s description of the first fish trypanosomes, more than 190 trypanosome species have been described from both marine and freshwater aquatic hosts including amphibians, teleost, elasmobranchs, crocodilians, turtles, and the platypus (Woo, 2006). Of these, 29 are known from marine teleost fish and 12 from elasmobranchs, of which only two have been reported from the coast of South Africa. The first record of marine trypanosomes from South Africa was by Fantham (1919), who described two new species: *Trypanosoma capigobii* Fantham, 1919 and *Trypanosoma nudigobii* Fantham, 1919, from the heart blood of a barehead goby, *Caffrogobius nudiceps* (Valenciennes) at Kalk Bay on the southern coast. Later, *Trypanosoma blenniclini* Fantham, 1930 was recorded from the blenniid, *Parablennius cornutus* (Linnaeus) and the clinid *Blennophis anguillaris* (Valenciennes). Fantham (1930) also reported larger forms of *T. capigobii* from *Ca. nudiceps* collected close to Kalk Bay, at St James. However, following a redescription and molecular analysis of the trypanosomes of the Blenniidae and Clinidae, Hayes *et al*. (2014) proposed that the three species described by Fantham (1919, 1930) are a single pleomorphic species, retaining the name *T. nudigobii* and considering *T. capigobii* and *T. blenniclini* as junior synonyms. The second species of marine trypanosome from South Africa is *Trypanosoma haploblephari* Yeld and Smit, 2006, originally described from the dark shyshark, *Haploblepharus pictus* (Müller and Henle), and the puffadder shyshark, *Haploblepharus edwardsii* (Schinz) from False Bay and Granger Bay, and more recently molecularly characterised from *H. pictus* and the leopard catshark, *Poroderma pantherinum* (Smith), also from Granger Bay (Yeld and Smit, 2006; Pretorius *et al*., 2021). South Africa is known for hosting more than 1900 marine fishes, including many endemic species (Smit and Hadfield, 2015). Surprisingly, as mentioned above, only two trypanosomes have been described across this diverse range of fish hosts. Thus, the current study aimed to increase the known diversity of marine fish trypanosomes by collecting and screening blood from different species of teleost hosts from selected locations along the south coast of South Africa. All trypanosomes found were described using morphological and morphometrical characterisation and molecular analyses.

Materials and methods

Collection of research specimens

Nearshore fish species, mainly of the family Sparidae, were collected using rod and line fishing, intertidal species of the Clinidae and Gobiidae were caught using baited traps, and estuarine species of the Gobiidae and Mugilidae were collected using sein and cast nets. Fish collections took place on the south coast of South Africa at the coastal towns of Chintsa East (32°50'12"S, 28°7'1"E), Kenton-on-Sea (Kariega River Estuary) (33°36'32"S, 26°39'16"E) and Boknes (33°43'32"S, 26°34'60"E) (Fig. 1). Sampling was also done at two sites in the Garden Route National Park, Tsitsikamma Storms River Mouth (34°1'15"S, 23°52'43"E) and in the Groot River West Estuary, Natures Valley (33°58'49"S, 23°34'2"E) (Fig. 1). Following collection, fishes were identified using the keys in Smith's Sea Fishes (Smith and Heemstra, 1986). As mullet species are particularly difficult to identify on morphology alone, DNA from fin clips of each sampled specimen was barcoded using the Cytochrome c oxidase subunit I (COI) marker following Thieme *et al*. (2022), and resulting sequences were compared to available sequences in GenBank using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The fish used in this study formed part of a larger project on the parasites of South African marine fishes (see Vermaak *et al*., 2023), wherein fish were humanely killed using the Standard Operating Procedure (SOP no. NWU-00267-17-A5) of blunt force trauma followed by cervical transection. A maximum of 0.4 ml of blood was drawn from the caudal vein using a hypodermic needle (Ethics nos. NWU-00372-16-A5, NWU-01265-23-A9). Thin blood smears were prepared. Each blood smear was air-dried, then fixed with absolute methanol and allowed to air-dry and subsequently stained for 20 minutes in a 10% dilution of Giemsa stain (Sigma-Aldrich, Steinheim, Germany). For genetic analysis, the remainder of the blood drawn was preserved in a 2 mL microtube containing 96% molecular-grade ethanol (Pretorius *et al*., 2021).

Morphological characterisation

Giemsa-stained blood smears were screened for blood parasites using a Nikon Eclipse N*i* microscope (Nikon, Amsterdam, Netherlands) at 100–400× magnification and immersion oil was added for photography and detailed study at 1000× magnification. When trypanosomes were observed, they were examined, photographed, and measured with a fixed digital camera (DS-Fi3) using the NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo, Japan). Measurements included the distance from the mid-nucleus to the posterior tip of the flagellate (MP), the distance from the mid-nucleus to the anterior end (MA), the nuclear length (NL) and the distance from the posterior tip of the flagellate to the kinetoplast (PK) following Hayes *et al*. (2014) and Pretorius *et al*. (2021). Additionally, the nuclear index (NI) was calculated by dividing MP by MA (Dias and de Freitas, 1943). Body width, with the undulating membrane (BW(UM)) and at the nucleus (BW(N)), flagellum length (FL) and total body length (TBL) were measured following Pretorius *et al*. (2021). Measurements are presented in micrometres (µm) as range (mean ± standard deviation). The aim was to measure at least 20 trypanosome specimens per host species, however, if less than 20 in total were on the slides, all of them were measured.

A non-metric multidimensional scaling (nMDS) biplot (Clarke and Gorley, 2015; PRIMER v7) was constructed to illustrate the correlation of trypanosome measurements across different species. The data matrix was compiled, with rows representing species and columns representing quantitative measurements. The stress value indicates whether the data was compressed or manipulated to show a specific result. Stress values close to 1 suggest a reduction in plotting accuracy, while values closer to 0 demonstrate that the dataset was not compressed or manipulated. A distance matrix was calculated from this data using Euclidean metric to quantify dissimilarities. The nMDS analysis utilised the distance matrix to reduce dimensionality, typically to two dimensions for visualisation. The coordinates from the nMDS output were extracted and plotted on a scatter plot to generate the biplot. This visualisation showed the spatial relationships between species based on their measurements, allowing for the assessment of similarities and differences. Additionally, features or vectors were highlighted to indicate the correlation of specific measurements with the dimensions of the nMDS plot, and the stress value was evaluated to ensure the adequacy of the dimensional reduction. A stress value below 0.10 is ideal, showing a reliable representation, while values between 0.10 and 0.20 are acceptable but may have some distortion. Values above 0.20 suggest a poor fit, indicating that the nMDS may not adequately capture the data's relationships.

DNA extraction, amplification, and sequencing

Whole blood of hosts with trypanosome-positive blood smears were used for DNA extraction using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions specific for animal blood. The 18S rRNA gene was selected as the target because it is widely used for classifying ectotherm trypanosomes and has the highest number of reference sequences currently available of species in the genus *Trypanosoma* (Jordaan *et al.*, 2023). For the Polymerase Chain Reaction (PCR), the resulting supernatant obtained from the DNA extraction served as the template. PCR reactions were carried out in final volumes of 25 µl. The reaction mixture consisted of 12.5 µl of Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$), 1.25 µl of each primer (10 µM), and at least 25 ng of DNA. PCR-grade nuclease-free water was used to adjust the final reaction volume. A nested PCR was followed, which utilised primary PCR primers SLF (5'-GCTTGTTTCAAGGACTTAGC-3') and S762.2 (5'-GACTTTTGCTTCCTCTAATG-3') and for the secondary PCR, two rounds were conducted using different primer sets, namely B (5'-CGAACAACTGCCCTATCAGC-3') and I (5'-GACTACAATGGTCTCTAATC-3'), and S825 (5'-ACCGTTTCGGCTTTTGTTGG-3') and SLIR (5'-ACATTGTAGTGCGCGTGTC-3'). The thermal cycling conditions were as follows: for the primary PCR, an initial single cycle (×1) of 95°C denaturation for 5 min., 50°C annealing for 2 min., 72°C extension for 4 min.; followed by 35 cycles of 94°C denaturation for 30 sec., 52°C annealing for 30 sec., 72°C extension for 2 min. 20 sec., followed by a final 72°C extension for 7 min. For the secondary PCR (B & I) the same thermal profile was followed, but with an annealing temperature of 60°C instead of 52°C. Finally with the last set of primers (S825 & SLIR) an initial single cycle (×1) of 95°C annealing for 3 min.; followed by 35 cycles of 95°C denaturation for 30 sec., 57°C annealing for 30 sec., 72°C extension for 1 min., followed by a final 72°C extension for 7 min. To verify the presence of DNA amplicons visualisation was done using a 1% agarose gel electrophoresis. Positive PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd for purification and sequencing.

Phylogenetic analysis

Sequence data for each isolate were assembled, and chromatogram-based contigs were generated and trimmed (1278–1506 bp) using Geneious Prime® 2024.0.7. (Kearse *et al*., 2012). To identify the closest congeners to the sequences obtained in the present study, the sequences were subjected to a BLAST search (Altschul *et al*., 1990) followed by an alignment with congeners using the MUSCLE (Edgar 2004) alignment tool in Geneious Prime®. Relevant information regarding the country in which each *Trypanosoma* has been found, the host it was recorded in, and an accession number for each trypanosome, respectively are provided in Table 3. The uncorrected p-distance and number of base differences per site between sequences were calculated under the "pairwise deletion" option using MEGA11 (Supplementary Table 1) (Tamura *et al*., 2021). This analysis included 83 unique nucleotide sequences and the most suitable nucleotide substitution model (GTR + I + G) was determined using jModelTest (Darriba *et al*., 2012; Guindon and Gascuel, 2003). A Bayesian Inference (BI) analysis of the alignment (2,564 bp including gaps) was done using MrBayes 3.2.7 (Ronquist *et al*., 2012) through the online computational resource CIPRES (Miller *et al*., 2010) with parameters with four category Gamma distribution. Markov Chain Monte Carlo (MCMC) chains were run for 10,000,000 generations, sampling every 100 generations. The 'burn-in' parameter was set at 25% discarded. A Maximum Likelihood (ML) analysis was implemented using PhyML 3.0

(Guindon *et al*., 2010), and 1000 rapid bootstrap inferences were run on the ATGC Montpellier Bioinformatics Platform (available from http://www.atgc-montpellier.fr/phyml/, Guindon *et al*., 2010). Phylogenetic trees obtained from the respective analyses was visualized using FigTree v. 1.4.4 (Rambaut, 2012).

Results

General observations of trypanosomes in the fish blood

A total of 246 fishes belonging to 23 species in nine families were collected and screened for trypanosomes. Individuals of eight of the 23 species were infected with trypanosomes (Table 1). The infected species were the klipfish [*Clinus superciliosus*) (Linnaeus)], prison goby [*Caffrogobius gilchristi* (Boulenger)], white steenbras [*Lithognathus lithognathus* (Cuvier)], South African mullet [*Chelon richardsonii* (Smith)], grooved mullet [*Chelon dumerili* (Steindachner)], striped mullet [*Chelon tricuspidens* (Smith)], flathead grey mullet (*Mugil cephalus* Linnaeus) and freshwater mullet [*Pseudomyxus capensis* (Valenciennes)]. All eight infected species had unidentified leeches in their mouth or on their gills that could potentially serve as vectors of these trypanosomes.

Description and diagnosis of stages found in the fish blood

Diagnosis

Phylum: Euglenozoa Cavalier-Smith, 1981

Class: Kinetoplastea Honigberg, 1963, emend. Vickerman, 1976

Subclass: Metakinetoplastina Vickerman, 2004

Order: Trypanosomatida Kent, 1880

Family: Trypanosomatidae Doflein, 1951

Genus: *Trypanosoma* Gruby, 1843

Trypanosoma nudigobii *Fantham 1919*

Host from the present study: *Clinus superciliosus* (Linnaeus)

Other hosts: *Caffrogobius nudiceps* (type host), *Blennophis anguillaris*, *Clinus agilis*, *Clinus cottoides*, *Clinus taurus*, *Parablennius cornutus* (Hayes *et al*., 2014).

Localities from the present study: Chintsa East, Eastern Cape, South Africa (32°50'12"S, 28°7'1"E) and Tsitsikamma Storms River Mouth, Eastern Cape, South Africa (34°1'15"S, 23°52'43"E).

Other localities: Kalk Bay (type locality), St. James (Fantham, 1919), Tsitsikamma and Koppie Alleen (Hayes *et al*., 2014).

Site of infection: Peripheral blood

Vector: The leech, *Zeylanicobdella arugamensis* Silva (see Hayes *et al*. 2006, 2014)

Present study

Voucher material: Three peripheral blood smears deposited in the parasite collection of the National Museum, Bloemfontein, South Africa, under accession numbers NMB P XXX, NMB P XXX and NMB P XXX.

Representative DNA sequences: The sequence data specifically associated with *T. nudigobii* have been submitted to GenBank: nuclear 18S rRNA (nu 18S) partial sequence XXX, XXX and XXX.

Morphology: Twenty-eight trypanosomes were measured: total body length 57.2 ± 11.6 (29.2 –69.0); body width 5.8 ± 1.9 (2.0–8.7); nucleus length 3.5 ± 0.9 (1.6–5.2); nucleus width 1.4 ± 0.4 (1.0–2.2); mid-nucleus-to-anterior-body-end distance 32.8 ± 8.6 (13.8–47.7); mid-nucleus-to-posterior-body-end distance 25.6 ± 6.4 (16.8–34.3); undulating membrane width 2.2 ± 0.8 (1.2–4.5); number of undulations 15.7 ± 2.1 (13–18) and kinetoplast width 0.9 ± 0.1 (0.7–1.2). Free flagellum length 7.3 ± 1.6 (4.5–9.7). Body index 10.4 ± 2.1 (7.4–14.3); nucleus index 0.8 ± 0.2 and nucleus position as a percentage 56.7 ± 7.1 (47.3–69.2%) (Table 2). The body stains blueish purple in colour with uniform density (Fig. 2A–F). The undulating membrane stains lighter purple with a colourless and transparent outer edge. The nucleus stains light pink and is oval-shaped, extending the width of the body. It is positioned parallel to the body and present in the posterior half. The kinetoplast is distinct, stains dark purple in colour and is typically positioned close to the posterior end, on the edge of the body and posterior to the undulating membrane. The flagellum is visible in most of the individuals. The individuals are mostly positioned in a curly manner with the posterior and anterior ends bent.

Remarks

Consequently, and due to the identical nucleotide sequences at both locations, we are clearly dealing with the same species of trypanosome from both these localities and the same host species. The present trypanosome from *Cl. superciliosus* at both sites agrees with the original morphological description of *T. nudigobii* by Fantham (1930) as well as the redescription provided by Hayes *et al*. (2014). The latter is expected as it also includes specimens from *Cl. superciliosus* collected from Tsitsikamma. In general, it shares common features such as a long, slender body, a free flagellum, and an undulating membrane. Although the *T. nudigobii* described by Fantham (1930) is larger in size, our specimens are proportionality relative to the dimensions presented in the redescription of this species by Hayes *et al*. (2014) (73.3 ± 11.0 vs. 57.2 ± 11.6). Our trypanosomes are the same length as the small trypanosomes (25.2–46.3) redescribed from *Clinus cottoides* Valenciennes, but not as large as the big trypanosomes (55.1–97.7) from *Cl. supercilious* reported in Hayes *et al*. (2014) (Fig. 2A and D). Free flagella, especially in large form, were challenging to stain, with some trypanosomes, especially the large ones, appearing to be without a free flagellum (Fig. 2A). However, when comparing the body width and nucleus length and orientation, it's the same as the description by Hayes *et al*. (2014), the posterior region appeared thicker than the anterior, and both extremities were attenuated, often reflexed, or curled. The kinetoplast, referred to as the parabasal body by Fantham (1930), is consistently positioned away from the posterior end with long PK regions (6–9µm for *T. blenniclini*, and 10µm for *T. capigobii*). This is one of the differences between the originally described *T. nudigobii* and the current redescription by Hayes *et al.* (2014). Additionally, the posterior ends of the trypanosomes did not taper and were rounded. The undulating membrane was generally well developed (Fig. 2B and C), with larger trypanosomes exhibiting eight to ten waves close to the body. The nucleus of normal trypomastigote forms of *T. nudigobii*, as described by Fantham (1930), is rounded or oval. Parasitaemia in the blood of *Cl. superciliosus* was relatively high, a similar finding to that of Hayes *et al*. (2014), with five to six trypanosome individuals per slide.

Molecular characterisation

DNA was extracted from eight samples of infected fish. Following PCR, five samples were positive with the correct amplicons, yielding a success rate of 63%. All the trypanosome sequences were from the host *Cl. superciliosus*, on which the morphological characterisation was based. The sequences generated from this host were 99% to 100% identical to each other. According to GenBank, the best match for these sequences was *Trypanosoma pleoronectidium* (Karlsbakk and Nylund, 2006) with a similarity of 97.97%, and *Trypanosoma rajae* (Kefil and Grellier, 2018) with a similarity of 97.95%.

Description of Trypanosoma *sp. A*

Host: *Caffrogobius gilchristi* (Boulenger) (syn. *Gobius gilchristi*)

Voucher material: Three peripheral blood smear deposited in the parasite collection of the National Museum, Bloemfontein, South Africa under accession number NMB P XXX, NMB P XXX and NMB P XXX. Under Article 73.3.2 of the ICZN.

Locality: Groot River West Estuary, Natures Valley, Eastern Cape, South Africa (34°1'15"S, 23°52'43"E).

Site of infection: Peripheral blood

Vector: Unknown

Representative DNA sequences: The sequence data associated with *Trypanosoma* sp. A have been submitted to GenBank: nuclear 18S rRNA (nu 18S) partial sequence XXX, XXXX and XXXX.

Description

Eighteen trypanosomes were measured, yielding the following results: total body length 63.1 ± 6.3 (52.2–70.8); body width 6.2 ± 1.0 (7.8–5.2); nucleus length 3.0 ± 0.6 (2.4–4.0); nucleus width 2.2 ± 1.4 (1.2–2.1); mid-nucleus-to-anterior-body-end distance 30.8 ± 4.8 (22.2–36.9) and mid-nucleus-to-posterior-body-end distance 34.4 ± 2.4 (31.5–38.7) and kinetoplast width 1.1 ± 0.5 (0.8–2.3). Body index 10.4 ± 2.3 (8.0–13.1); nucleus index 1.1 ± 0.2 and nucleus position as a percentage 48.6 ± 3.8 (42.4–53.7%) (Table 2). The body stains purple in colour with striae in the cytoplasm of varying density (Fig. 3A–F). The undulating membrane stains lighter purple with a colourless and transparent outer edge, which is difficult to visualise in some of the individuals. The nucleus stains light pink and is narrow and elongated. It is typically oriented with its long axis transversely, running across the body of the organism and present in the middle of the body. The kinetoplast has a round form and stains dark purple in colour and is positioned on the posterior end of the body with a kinetoplast index of 1.3 and PK distance of 3.3 ± 0.7 (2.8–4.4). The free flagellum is a short, thin extension of the anterior end and may be absent in some of the specimens.

Remarks

Trypanosoma sp. A is characterised by having a half-moon shape body. Morphologically the closest trypanosome described in relation to

Trypanosoma sp. A is the species previously reported and described from the *Caffrogobius* species in South Africa, namely *T. nudigobii* and *T. capigobii* (Table 4). Fantham (1919) described *T. nudigobii* from *Caffrogobius nudiceps*. This species is 60–85 µm long and has a body width of 6.6–7.5 µm. This is similar to *Trypanosoma* sp. A, with a total body length ranging from 52.2 to 70.8 µm and a body width ranging from 5.2 to 7.8 µm. *Trypanosoma capigobii* is smaller than *Trypanosoma* sp. A, with a body length ranging from 42 to 60 µm and body width of 2–4.4 µm. Both *Trypanosoma* sp. A and *T. nudigobii* have short flagellums, their membrane is close to their body and the nuclei are oval. However, this species is not similar to *T. nudigobii* redescribed by Hayes *et al.* 2014. Individuals of *Trypanosoma* sp. A differ from those of *T. nudigobii* (redescribed by Hayes et al. 2014) in their overall elongated body with a small and oval-sized nucleus positioned centrally. *Trypanosoma* sp. A can also be differentiated from other described marine species by being overall stretched out and half-moon shaped (Fig. 3A and E) compared to the curled shape of *T. nudigobii* (Fig. 2C). The undulating membrane is not clearly visible which makes counting of the waves challenging. Further, *Trypanosoma* sp. A has an overall larger body size than *T. nudigobii* (63.1 ± 6.3 (52.2–70.8) vs. 57.2 ± 11.6 (29.2–69.0). No discernible difference in stain colour or density between the anterior and posterior regions was observed. Based on the above morphological comparison, it now seems quite possible that this species (*Trypanosoma* sp. A) represents the true *T. nudigobii*, and that the synonymising of *T. nudigobii*, *T. capigobii* and *T. blenniclini* by Hayes *et al.* (2014) was erroneous. However, in order to make a conclusive recommendation regarding the synonymy proposed by Hayes *et al.* (2014) molecular data from the type hosts and type localities of all three the original species described by Fantham (1919) is needed. In order not to create more confusion regarding these three synonymised species by introducing a fourth name, we refrain from naming the species infecting *Ca. gilchristi* here until such genetic data from these trypanosomes become available. Parasitaemia in the blood of *Ca. gilchristi* was relatively low, with three to four trypanosome individuals per slide.

Molecular characterisation

Molecular data were obtained from DNA extracted from seven samples of infected fish. Of these, four samples were positive following PCR for the correct amplicons, resulting in a success rate of 57%. All trypanosome sequences were derived from the host *Ca. gilchristi*, which was the basis for the morphological characterisation. The sequences generated from the four infected *Ca. gilchristi* were 100% identical. According to GenBank, the closest match for these sequences was *Trypanosoma pleoronectidium* (Karlsbakk and Nylund, 2006), with a similarity of 97.09%, and *Trypanosoma rajae* (Kefil and Grellier, 2018), with a similarity of 96.94%.

Description of Trypanosoma bakana *n. sp.*

Type host: *Lithognathus lithognathus* (Cuvier) (syn. *Pagrus lithognathus*)

Type material: Hapantotype, 1× peripheral blood smear deposited in the parasite collection of the National Museum, Bloemfontein, South Africa under accession number NMB P XXX. Other voucher material, 2× peripheral blood smears NMB P XXX and NMB P XXX. Under Article 73.3.2 of the ICZN.

Type locality: Boknes, Eastern Cape, South Africa (33°43'32"S, 26°34'60"E).

Site of infection: Peripheral blood

Vector: Unknown

Representative DNA sequences: The sequence data specifically associated with *T. bakana* n. sp. have been submitted to GenBank: nuclear 18S rRNA (nu 18S) partial sequence XXX, XXX and XXX.

ZooBank registration: n. sp.: urn:lsid:zoobank.org:act:0808EDA4-84A4-474F-9AE6-297E66CA9BC5

Etymology: The name “Bakana” is derived from the Khoekhoen language indigenous to the area and means “father’s river”. The Bakana river flows into Boknes where the hosts of this trypanosome were collected.

Description

Twenty-six trypanosome specimens were measured: total body length 38.6 ± 4.1 (31.8–44.7); body width 2.8 ± 0.4 (2.1–3.3); nucleus length 3.1 ± 0.7 (2.4–4.9); nucleus width 2.3 ± 1.5 (1.2–2.3); mid-nucleus-to-anterior-body-end distance 19.1 ± 4.8 (10.9–25.6) and mid-nucleus-to-posterior-body-end distance 20.1 ± 5.8 (11.0–26.2); undulating membrane width 2.4 ± 0.6 (1.2–2.8); number of undulations 2.5 ± 0.6 (1–3) and kinetoplast width 1.6 ± 0.9 (0.7–2.5). Free flagellum length 3.7 ± 0.4 (2.1–3.3). Body index 14.2 ± 3.1 (10.8–21.6); nucleus index 1.1 ± 0.6 and nucleus position as a percentage 50.0 ± 13.8 (30.9–69.3%) (Table 2). The body is stained purple in colour with uniform density and has white dots (Fig. 4A–F). The undulating membrane stains lighter purple with a darker purple outer edge. The nucleus is oval or round and stains light pink. It is positioned parallel to the body and present in the anterior half. The kinetoplast is distinct, big, stains deep pink in colour and is typically positioned on the posterior end (Fig. 4E). The flagellum is short and visible in most of the individuals.

Remarks

Trypanosoma bakana n. sp. is elongated with an S-shape body (Fig. 4B), has a large round or oval nucleus that is positioned towards the anterior end (Fig. 4A) and the kinetoplast is located at the tip of the posterior region (Fig. 4E). *Trypanosoma bakana* n. sp. is large and clear in all the images (Fig. 4A–F). The posterior region is more likely to present curled while the anterior region does not curl, flagellum is curved (Fig. 4D). The undulating membrane composed of 3 to 4 waves from the anterior region to just past the nucleus, is not as clear as in *T. nudigobii* but stains light purple and is more visible when compared to that of *Trypanosoma* sp. A. The posterior region of *T. bakana* n. sp. is thicker and darker than its anterior region. According to our knowledge, this is the first marine teleost trypanosome described from a host of the Sparidae, however, Fantham (1919) reported and described a *Herpetomonas denticis* Fantham, 1919 from *Argyrozona argyrozona* (Valenciennes) in South Africa, which could potentially be an early form of a trypanosome. When compared to South African species *Trypanosoma bakana* n. sp. is overall smaller than both *Trypanosoma* sp. A and *T. nudigobii* with a total length of 38.6 ± 4.1 (31.8–44.7). The closest non-South African marine teleost trypanosome species to *T. bakana* n. sp., based on measurements and an average body length of 39 µm, is *Trypanosoma yakimovi* (Yakimov, 1911), which was described from the greater pipefish *Syngnathus acus* Linnaeus in the Gulf of Naples, Italy (Table 4). *Trypanosoma yakimovi* has a similar nucleus length (2.8 µm) but is smaller in body length (31.2–35.5 µm) compared to *T. bakana* n. sp. Additionally, *T. yakimovi* exhibits a curly body shape with a long flagellum (4.3–9.9 µm) and a distinctive undulating membrane. Furthermore, *T. yakimovi* has a distinctive kinetoplast positioned further away from the posterior end (KI = 1.28) than the kinetoplast of *T. bakana* n. sp. (see Karlsbakk and

Nylund, 2006) and has a more centrally positioned nucleus. In contrast, *T. bakana* n. sp. has a long, S-shaped body with a less distinctive undulating membrane, while its nucleus is located toward the anterior end of the body, and the kinetoplast is large, distinctive, and situated at the posterior end (Fig. 4). The thickened flagellar end makes *T. yakimovi* unique among marine fish trypanosomes, but its size and proportions are otherwise similar to *T. platessae* (Karlsbakk and Nylund, 2006). *Trypanosoma pulchra* (Mackerras and Mackerras, 1925), described in Sydney, Australia, from the white-ear scalyfin *Parma microlepis* Günther, is larger in size (40.8–57.1 µm) and has a long free flagellum (7.3 µm), but has a similar body width (3.5 µm) compared to *T. bakana* n. sp. The nucleus positions differ, and the undulating membrane is more visible than in *T. bakana* n. sp. Parasitaemia in the blood of *L. lithognathus* was relatively low, with five to six trypanosome individuals per slide.

Molecular characterisation

DNA was extracted from the whole blood of six infected fish for PCR analysis. Of these, three samples tested positive for the correct amplicons, resulting in a success rate of 50%. All the trypanosome sequences originated from the same host specimens, which was used for the morphological characterisation. These sequences showed 97.8% identity with those of *Trypanosoma* sp. A. The closest matches in GenBank were *Trypanosoma pleoronectidium* (Karlsbakk and Nylund, 2006), with a 97.55% identity, and *Trypanosoma murmanense* (Karlsbakk and Nylund, 2006), with a 97.35% identity.

Description of Trypanosoma bokkom *n. sp.*

Type host: *Chelon richardsonii* (Smith) (syn. *Mugil richardsonii*)

Other hosts: *Chelon dumerili* (Steindachner), *Chelon tricuspidens* (Smith), *Mugil cephalus* Linnaeus and *Pseudomyxus capensis* (Valenciennes)

Type material: Hapantotype, 1× peripheral blood smear deposited in the parasite collection of the National Museum, Bloemfontein, South Africa under accession number NMB P XXX. Other voucher material, 2× peripheral blood smears NMB P XXX and NMB P XXX. Under Article 73.3.2 of the ICZN.

Type locality: Tsitsikamma Storms River Mouth, Eastern Cape, South Africa (34°1'15"S, 23°52'43"E)

Other localities: Chintsa East, Eastern Cape, South Africa (32°50'12"S, 28°7'1"E), Boknes, Eastern Cape, South Africa (33°43'32" S, 26°34'60"E) and Kariega River, Eastern Cape, South Africa (33°36'32" S, 26°39'16"E).

Site of infection: Peripheral blood

Vector: No vector data

DNA sequences: The nuclear 18S rRNA (nu 18S) partial sequence data from all the different hosts and localities of *T. bokkom* n. sp. have been submitted to GenBank: Sequences from trypanosomes from: *Ch. richardsonii* (PV344723; PV344724), *Ch. dumerili* (PV344722), *Ch. tricuspidens* (PV344726), *M. cephalus* (PV344727) and *P. capensis* (PV344728).

ZooBank registration: n. sp.: urn:lsid:zoobank.org:act:BDF2BA09-E6AC-4794-91B0-4F85FD22D97C

Etymology: The name "bokkom" refers to whole, salted, and dried mullet, a well-known delicacy from South Africa.

Description

In total 75 specimens were measured from all five hosts including 45 specimens infecting the type host. Measurements of specimens from the type host: Total body length 43.1 ± 5.0 (29.2–52.3); body width 2.5 ± 0.4 (1.9–3.3); nucleus length 3.4 ± 0.6 (2.1–4.4); nucleus width 2.5 ± 1.6 (1.2–3.1); mid-nucleus-to-anterior-body-end distance 15.1 ± 3.7 (10.0–23.3) and mid-nucleus-to-posterior-body-end distance 28.7 ± 3.7 (17.2–35.4); undulating membrane width 1.3 ± 0.5 (0.7–1.9); number of undulations 1.8 ± 0.4 (1–10) and kinetoplast width 1.0 ± 0.5 (0.9–1.7). Free flagellum length 3.4 ± 0.6 (2.4–4.8). Body index 17.5 ± 3.2 (13.2–27.7); nucleus index 1.9 ± 0.5 and nucleus position as a percentage 33.9 ± 6.0 (23.9–48.9%) (Table 2). The body stains purple or blue in colour with uniform density (Fig. 5A–L). The undulating membrane stains light purple with a colourless and transparent outer edge. The nucleus stains light pink and is stretched. It is positioned parallel to the body and present in the anterior half. The kinetoplast is prominent, large, stains deep pink in colour and is positioned on the posterior end. The flagellum is long (Fig. 5 A, C, K).

Remarks

Consequently, and due to the identical nucleotide sequences at all the locations, the morphometrics of specimens from these areas were amalgamated in Table 2 as all the trypanosomes of the different mullet species are here considered to be of the same species. *Trypanosoma bokkom* n. sp. is characterised by having a long, thin, and wavy body shape where the anterior and posterior regions tend to curl more than the middle part of the body (Fig. 5D). The undulating membrane is not clear and almost absent (Fig. 5A and C). The posterior and anterior regions stained lighter with a prominent pink kinetoplast at the very tip of the posterior region. The nucleus is oval or sometimes elongated more in the anterior region and the flagellum is mostly invisible (Fig. 5K and L). *Trypanosoma bokkom* n. sp. is easily distinguishable from *T. bakana* n. sp., *Trypanosoma* sp. A and *T. nudigobii* in the following morphological features. Overall *T. bokkom* n. sp. is longer in total body length compared to *T. bakana* n. sp.; further, *T. bokkom* n. sp. has a wavy body shape, compared to the half-moon shape of *Trypanosoma* sp. A. The undulating membrane of *T. bokkom* n. sp. is not as visible as seen in *T. nudigobii*. To our knowledge, three trypanosome species have been described from mullets of the genus *Mugil*: *Trypanosoma froesi* Lima, 1976; *Trypanosoma mugilicola* Becker and Overstreet, 1979; and *Trypanosoma platanusi* Ribeiro, Ranzani-paiva, Ishikawa, Lopes, Satake and Carraro, 1996. *Trypanosoma froesi* was first recorded by Lima (1976) in *Mugil platanus* (later changed to *Mugil liza* Valenciennes) in Brazil. A morphologically similar parasite, *T. mugilicola*, was described from *M. cephalus* captured in the Gulf of Mexico by Becker and Overstreet (1979), and *T. platanusi*, which also resembles *T. froesi* morphologically, was recorded from *M. liza* (reported as *M. platanus*) by Ribeiro *et al*. (1996) in Brazil. The latter authors were either not aware or did not accept the proposal of Eiras *et al*. (1995), who indicated that *T. mugilicola* and *T. platanusi*, based on their morphological similarity and with reference to Conroy and Conroy (1984), are the same species as *T. froesi*. Revisiting the morphology and morphometrics of these three species, we agree with Eiras *et al.* (1995) and consider *T. mugilicola* and *T. platanusi* junior synonyms of *Trypanosoma froesi*. *Trypanosoma bokkom* n. sp. can be distinguished from *T. froesi* in that the latter has a long free flagellum (10.0–12.0 µm) (Table 4), while T. *bokkom* n. sp. has a shorter flagellum (2.4–4.8 µm), which is more similar in size to the flagellum of *T. platanusi* (mean 4.87 µm). Furthermore, *Trypanosoma bokkom* n. sp. differs from *T. froesi* (reported as *T. mugilicola*) in the position of the kinetoplast that is at the posterior end in *T. bokkom* n. sp. and the nucleus that is closer to the anterior end. Other measurements that differ from those of *T. bokkom* n. sp. include the following from *T. froesi* (reported as *T. platanusi*) total body length (27.0–43.5 µm); free flagellum length (2.0–11.8 µm); mid-nucleus to posterior end (17.0–25.0 µm); kinetoplast diameter (0.5–1.2 µm); and kinetoplast to posterior end (1.1–3.0 µm) (see Ribeiro *et al*., 1996). Parasitaemia in the blood of all the parasitised mullet hosts was notably high, with 10 to 15 trypanosome individuals per slide.

Molecular characterisation

DNA was extracted from 30 samples of infected fish for PCR analysis as part of the molecular data collection. Of these, 23 samples were positive for the correct amplicons, yielding a success rate of 77%. All trypanosome sequences were derived from the same host specimens that served as the basis for the morphological characterisation. The sequences obtained from these hosts showed 99.9% to 100% identity. The closest matches in GenBank were *Trypanosoma pleoronectidium* (Karlsbakk and Nylund, 2006), with a similarity of 97.08% , and *Trypanosoma rajae* (Kefil and Grellier, 2018), with a similarity of 96.94% identity.

Swimming behaviour

Another new observation in the description of these above trypanosomes is their swimming behaviour. This behaviour was observed during the screening of a live thick blood smear. The swimming behaviour observed for *T. nudigobii* during the present study is considered to fall within the intermediate swimming behaviour category as described in Doro *et al*. (2019), showing a combination of directional and non-directional movements (see Supplementary Video 1). The swimming behaviour of *Trypanosoma* sp. A can be defined as being persistent, where parasites travel in a straight line over significant distances (Doro *et al*. 2019). This represents another clear difference between *Trypanosoma* sp. A and *T. nudigobii*, an intermediate swimmer (see Supplementary Video 2). *Trypanosoma bakana* n. sp. is categorised as a tumbler, with non-directional movement or swimming, travelling no further than their body length (Doro *et al*. 2019) (see Supplementary Video 3). This swimming behaviour represents an additional clear difference between *T. nudigobii*, an intermediate swimmer, and *Trypanosoma* sp. A, a persistent swimmer. According to Doro *et al*. (2019), this swimming behaviour is classified as tumbling. Tumbling behaviour in trypanosomes, as detailed by Doro *et al*. (2019), is characterised by erratic, non-directional movement where the parasites move only short distances, generally not exceeding their body length (see Supplementary Video 4). This highlights a distinct difference between *T. nudigobii*, an intermediate swimmer, and *Trypanosoma* sp. A, which displays persistent swimming behaviour but shares the same tumbling behaviour as *T. bakana* n. sp. These live appearances of trypanosomes may vary between host individuals. This may potentially also depend on host species, host immune status, nutrients in the blood and stage of trypanosomes and on inapparent plesiomorphy.

Phylogenetic analysis

A final alignment of the 18S rRNA spanning 2,581 bp (including gaps) contained 83 unique sequences of various trypanosome species from a range of vertebrate hosts including amphibians, reptiles and freshwater fishes (Table 3). The BI and ML trees obtained during the analyses have similar topologies. Trypanosome species from marine hosts were recovered as sister to all trypanosome species isolated from clawed frogs, freshwater turtles and terrapins from South Africa, a platypus from Australia and freshwater turtles from Sub-Saharan Africa. All representative sequences of *T. nudigobii* form a well-supported clade. The isolate *T. nudigobii* [KF871790], previously reported by Hayes *et al*. (2014) from *Cl. superciliosus* and *Z. arugamensis* from Tsitsikamma National Park, nested between two subclades from isolates in the current study. The intraspecific genetic divergence calculated as uncorrected p-distances among *T. nudigobii* isolates ranged from 0.0%–0.9% and 0–13 bp differences (1316 bp compared; Fig. 7; Supplementary Table 1). The two sequences obtained of *Trypanosoma* sp. A were identical and formed a sister taxon to *T. bakana* n. sp., which is its closest relative. The genetic divergence between *Trypanosoma* sp. A and *T. bakana* n. sp. was 1.6%, corresponding to a 21 bp difference (1303 bp compared; see Supplementary Table 1). The relatedness of these two species could be due to *L. lithognathus* and *Ca*. *gilchristi* utilising estuaries as nursery grounds, where the hosts are exposed to similar vectors (Froese and Pauly, 2024) (Fig. 7). This could potentially suggest past host switching, which may explain the close genetic similarity observed between the species. The interspecific divergence between *T. nudigobii* and *T. bakana* n. sp. ranged from 2.4% to 3.7%, with 48–52 bp differences (1314 bp compared; see Supplementary Table 1). There were 44–47 bp differences between *T. bokkom* n. sp. and *T. bakana* n. sp., with an uncorrected p-distance ranging from 3.1% to 3.5% (1498 bp compared). All six isolates of *T. bokkom* n. sp. formed a sister clade to *T. bakana* n. sp. and *Trypanosoma* sp. A (Fig. 7). Isolates 1 and 3–6 was identical to each other (see Supplementary Table 1), but isolates 3–5 were not identical to isolate 2 sequenced from *Ch. richardsonii* from Kariega River, with an uncorrected p-distance of 0.07% and 1 base pair difference. All these mullets occur in the same geographical regions and habitat types, suggesting that they are exposed to similar vectors, explaining the monophyletic clade. *Trypanosoma bokkom* n. sp. forms part of the marine clade within the aquatic clade and forms a sister clade with *T. bakana* n. sp. and

Trypanosoma sp. A. The sister group to this clade consists of trypanosomes parasitising marine fish from the Eastern Atlantic [U39580; U39584] and Indo-Pacific [JQ999962] regions.

Discussion

The current study enriched knowledge of marine fish trypanosomes by proposing four distinct species from eight host species of which three are new to science. According to Hayes *et al*. (2014), *T. nudigobii* infects a wide range of intertidal fishes (Fantham, 1930; Hayes *et al*., 2014), with the type host being *Caffarogobius nudiceps*. In the present study, the *Cl. superciliosus* was collected inside and outside intertidal zones, from two localities and their morphological and molecular characterisation revealed that the species parasitising *Cl. superciliosus* was morphologically and genetically similar to *T. nudigobii*. Interestingly *Ca. nudiceps* (the type host of *T. nudigobii*) were collected from intertidal pools in Tsitsikamma, but none were found to be infected. (see Table 1). This was unexpected, as *T. nudigobii* was previously reported to infect fish collected by Hayes *et al*. (2014) from Tsitsikamma National Park. Furthermore, a congeneric host, *Ca. gilchristi* were found to be infected with *Trypanosoma* sp. A, from an estuary, the Groot River West Estuary, which leads us to believe that despite belonging to the same host genus, the difference in habitat exposes hosts to different vectors and consequently to distinct trypanosome species. The third species reported here is *T. bakana* n. sp. (ex. *L. lithognathus*) collected from Boknes, and the fourth species, *T. bokkom* n. sp. collected from five mullet species. Morphological and morphometric analyses revealed differences among the four trypanosome species, as illustrated in Fig. 6. Notably, the trypanosomes in *Ca. gilchristi* (*Trypanosoma* sp. A) was the largest. These findings advance our understanding of these parasites, particularly when considering early 20th-century research, which followed a strict host-specificity paradigm. This earlier view suggested that each newly infected host species represented a distinct haemoflagellate species (Lom, 1979; Karlsbakk and Nylund, 2006). Recent studies, particularly by Karlsbakk and Nylund (2006), using leech vectors, have shown that a single haemoflagellate species can infect multiple fish species. Furthermore, the concept of host specificity among fish trypanosomes has been further challenged by pairwise genetic distance analyses of 12S rRNA gene sequences from freshwater fish trypanosomes in Europe (Figueroa *et al*., 1999), which have failed to support the classification of distinct species for trypanosomes isolated from various host species. The comprehensive review by Karlsbakk and Nylund (2006) on marine fish trypanosomes in the North Atlantic has led to the synonymising of several species based on consistent size measurements. In some cases, various trypanosome species were delineated within a single host species based solely on minor size variations (Lom, 1979). Karlsbakk and Nylund (2006) emphasise the importance of focusing on individual infections across multiple host species, a practice applied in the case of the three novel species described in this study. The nMDS is usually used to show similarity between individuals, however, here differences are shown between the morphometric data of the trypanosome species reported from various hosts. The plot depicted in Fig. 6 strongly supports the similarity in morphometrics between individual trypanosomes within a specific host family, but the dissimilarity of trypanosomes between host families, reinforces the morphological evidence of three distinct species (Dexter *et al*., 2018).

It is essential to recognise the challenges associated with earlier descriptions, as historical accounts were often brief. However, current knowledge regarding inter- and intra-specific morphological variation among trypanosomes remains incomplete. Variations in fixation, staining, microscopy, and measurement tools further complicate these challenges. The morphological description of distinct trypanosome species, or the claim of novel species, remains difficult due to the extensive descriptions based on morphological characterisations across different species and pleomorphism (Kunz, 2002; Hayes *et al*., 2014; Lemos *et al*., 2015; Zhang *et al*., 2023). Consequently, researchers have increasingly turned to DNA sequencing to clarify species designations and confidently differentiate between species. Both ML and BI phylogenies define marine fish trypanosomes as a distinct clade (Fig. 7). While the overall structure of the ML and BI phylogenetic trees shows some variation, the mullet species are grouped closely in both analyses. All sequences from the present study are part of the marine fish, shark, and ray clade. Additionally, the sequences from *Trypanosoma* sp. A from *Ca. gilchristi* and *T. bakana* n. sp. from *L. lithognathus* were recovered as sister taxa. Each species—*Trypanosoma bakana* n. sp., *Trypanosoma bokkom* n. sp. and *Trypanosoma* sp. A—isolated from *L. lithognathus*, *Ca. gilchristi*, and all mullet hosts, respectively, formed their own monophyletic clade. These hosts inhabit estuaries as adults or at some point in their life cycle (Whitfield, 2023). In contrast, *Cl. superciliosus*, infected with *T. nudigobii*, does not inhabit estuaries, suggesting that the vector for this trypanosome species may differ. The majority of sequences from *Cl. superciliosus* (*T. nudigobii*) cluster together, with one *T. nudigobii* sequence from Tsitsikamma and another from Chintsa East clustering below the known sequence of *T. nudigobii*, showing a bootstrap value of 84%, indicative of high genetic diversity within the host species. However, these sequences are sister to *T. nudigobii* [GenBank: KF871790], isolated from *Cl. superciliosus* and the leech *Zeylanicobdella arugamensis* Silva, 1963. It is important to note that both ML and BI trees exhibit similar branch lengths, with only a few nodes having bootstrap values below 50. This highlights the close relationships among putative trypanosome species infecting marine fish, as evidenced by the 18S rRNA gene region. The combination of morphological and molecular evidence supports the identification of three new trypanosome species, indicating a relationship to previously known marine trypanosomes in southern Africa. These findings enhance the knowledge of marine trypanosome diversity within the region.

Understanding the three different swimming behaviours described by Doro *et al*. (2019)—intermediate swimmers, persistent swimmers, and tumblers—adds value to comprehending the dynamics of newly described trypanosome species. While limitations exist, such as the influence of thick blood smears and the timing of screenings, information was still obtained to distinguish between species and warrants further investigation. Modern molecular techniques facilitate the differentiation of closely related or morphologically similar species, and these techniques have also been employed to assess the high levels of pleomorphism found within the same trypanosome species (Pretorius *et al*., 2021).

Supplementary material.

The supplementary material for this article can be found at XXX

Supplementary Table 1: The proportion of nucleotide sites with sequence differences (bottom diagonal) and the base pair differences used in the phylogenetic analyses (top diagonal).

Supplementary Video 1: Swimming behaviour of *Trypanosoma nudigobii* Fantham, 1919 from *Clinus superciliosus*.

Supplementary Video 2: Swimming behaviour of *Trypanosoma* sp. A from *Caffrogobius gilchristi*.

Supplementary Video 3: Swimming behaviour of *Trypanosoma bakana* n. sp. from *Lithognathus lithognathus*.

Supplementary Video 4: Swimming behaviour of *Trypanosoma bokkom* n. sp. from *Chelon richardsonii*.

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Author's contribution.

C. le Roux, N. J. Smit conceived and designed the study. C. le Roux, N. J. Smit, C. A. Cook and M. Truter conducted data gathering and analyses thereof. C. le Roux, N. J. Smit, C. A. Cook, M. Truter and E. C. Netherlands wrote the article.

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Competing interests.

None.

Ethical standards.

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Table 1. Study sites on the south coast of South Africa with year of collection, details of the fish species samples, number and size of collected fishes, as well as the prevalence of trypanosome infections (No = number of fishes sampled; TL= Total Length; SD= Standard deviation)

Site and date		**Fishes**		**Prevalence**
	Species	**No**	**TL ± SD (range) in cm**	
Chintsa East (2020; 2022)	*Acroteriobatus annulatus*	2	51.5 ± 7.1 (47–57)	0/2 (0%)
Chintsa East (2020; 2022)	*Amblyrhynchote honckenii*	15	10.6 ± 4.5 (9–28)	0/15 (0%)
Tsitsikamma (2022)	*Arothron immaculatus*	1	28	0/1 (0%)
Chintsa East (2020)	*Boopsoidea inornata*	1	13.3	0/1 (0%)
Nature's Valley (2023)	*Caffrogobius gilchristi*	8	10.6 ± 1.6 (9.5–13)	5/8 (63%)
Tsitsikamma (2024)	*Caffrogobius nudiceps*	5	12.6 ± 1.8 (9.6–17.8)	0/5 (0%)
Chintsa East and Kariega river (2020; 2023)	*Chelon dumerilli*	14	18.6 ± 9.8 (11.9–36.5)	11/14 (79%)
Chintsa East and Tsitsikamma (2020; 2022; 2023)	*Chelon richardsonii*	50	23.6 ± 12.5 (8–40.5)	44/50 (88%)
Boknes (2023)	*Chelon tricuspidens*	1	37	1/1 (100%)
Tsitsikamma and Chintsa East (2020; 2022; 2023)	*Clinus superciliosus*	37	14.4 ± 1.8 (11–18)	30/37 (81%)
Chintsa East; Tsitsikamma and Boknes (2020; 2021; 2022; 2023)	*Diplodus capensis*	40	25.7 ± 6.6 (12–34)	0/40 (0%)
Chintsa (2020; 2022)	*Diplodus hottentotus*	6	19.6 ± 2.4 (15.5–22)	0/6 (0%)
Chintsa (2022)	*Galeichthys feliceps*	1	21,5	0/1 (0%)

Boknes (2023)	*Glossogobius callidus*	12	9.4 ± 1.8 (5.2−11.7)	0/12 (0%)
Chintsa East and Boknes (2022; 2023)	*Lithognathus lithognathus*	9	25 ± 9.5 (13.5−36)	5/9 (56%)
Tsitsikamma (2020; 2021; 2022)	*Lithognathus mormyrus*	4	24 ± 6.4 (14.5−28.5)	0/4 (0%)
Tsitsikamma and Boknes (2020; 2023)	*Monodactylus falciformis*	4	13.6 ± 3.7 (9.5−17)	0/4 (0%)
Boknes (2023)	*Mugil cephalus*	3	48	1/3 (33%)
Chintsa East (2022)	*Pachymetopon grande*	2	33.5 ± 7.8 (28−39)	0/2 (0%)
Chintsa East (2022)	*Pavoclinus* (*Clinus* spp.) *mentalis*	1	23	0/1 (0%)
Chintsa East (2020)	*Pomadasys olivaceus*	8	10.3 ± 2.3 (7.3−14)	0/8 (0%)
Boknes (2023)	*Pseudomyxus capensis*	18	28.3 ± 2.5 (25−34)	10/18 (56%)
Tsitsikamma and Boknes (2022; 2023)	*Sparodon durbanensis*	4	37 ± 21.9 (17.5−68)	0/4 (0%)
TOTAL		**246**		**107/246 (44%)**

Table 2. Morphometrics of all the trypanosomes collected from various host fishes at the study sites (No = number measured; MA = mid-nucleus to anterior region; MP = mid-nucleus to posterior region; MK = mid-nucleus to kinetoplast; PK = posterior region to kinetoplast; BW = body width; TBL = total body length; nuclear index (NI) = MP/MA; kinetoplast index (KI) = MP/MK)

Fish hosts by species	**Number measured and morphometrics of fish trypanosomes**									
	No	**MA**	**MP**	**MK**	**PK**	**NL**	**BW**	**TBL**	**KI**	**NI**
Trypanosoma nudigobii	28*	32.8 ± 8.6	25.6 ± 6.4	21.7 ± 4.2	3.3 ± 0.7	3.5 ± 0.9	5.8 ± 1.9	57.2 ± 11.6	1.2	0.8
(*Clinus superciliosus*)		(13.8–47.7)	(16.8–34.3)	(10.2–25.9)	(2.8–4.4)	(1.6–5.2)	(2.0–8.7)	(29.2–69.0)		
Trypanosoma sp. A	18	30.8 ± 4.8	34.4 ± 2.4	29.4 ± 5.2	4.7 ± 1.3	3.0 ± 0.6	6.2 ± 1.0	63.1 ± 6.3	1.2	1.1
(*Caffrogobius gilchristi*)		(22.2–36.9)	(31.5–38.7)	(28.3–34.0)	(2.6–5.4)	(2.4–4.0)	(5.2–7.8)	(52.2–70.8)		
Trypanosoma bakana n. sp.	26	19.1 ± 4.8	20.1 ± 5.8	19.4 ± 3.2	0.7 ± 1.3	3.1 ± 0.7	2.8 ± 0.4	38.6 ± 4.1	1.0	1.1
(*Lithognathus lithognathus*)		(10.9–25.6)	(11.0–26.2)	(10.8–25.2)	(0.5–1.3)	(2.4–4.9)	(2.1–3.3)	(31.8–44.7)		
Trypanosoma bokkom n. sp.	30	15.1 ± 3.7	28.7 ± 3.7	27.9 ± 5.0	0.9 ± 0.4	3.4 ± 0.6	2.5 ± 0.4	43.1 ± 5.0	1.0	1.9
(*Chelon richardsonii*)		(10.0–23.3)	(17.2–35.4)	(15.5–34.3)	(0.4–1.5)	(2.1–4.4)	(1.9–3.3)	(29.2–52.3)		
Trypanosoma bokkom n. sp.	12	14.6 ± 1.7	30.2 ± 4.0	28.9 ± 2.7	0.8 ± 0.6	3.7 ± 0.8	2.8 ± 0.5	46.5 ± 4.8	1.0	2.2
(*Chelon dumerilli*)		(12.0–17.5)	(23.0–36.7)	(22.2–35.3)	(0.3–1.6)	(2.6–5.6)	(2.2–3.5)	(34.0–51.6)		
Trypanosoma bokkom n. sp.	11	15.2 ± 2.5	27.3 ± 3.5	25.3 ± 5.1	1.6 ± 0.4	3.5 ± 0.7	2.6 ± 0.3	44.2 ± 5.1	1.1	1.9
(*Chelon tricuspidens*)		(10.4–24.2)	(17.4–32.2)	(16.9–31.6)	(0.9–1.8)	(2.0–4.1)	(1.8–3.0)	(29.0–51.2)		
Trypanosoma bokkom n. sp.	10	14.8 ± 2.8	27.4 ± 3.8	26.1 ± 3.4	1.5 ± 0.9	3.3 ± 0.5	2.7 ± 0.6	43.3 ± 5.2	1.0	2.0

(*Mugil cephalus*)		(12.3–18.1)	(20.4–33.2)	(18.1–32.8)	(0.9–1.7)	(2.3–5.3)	(2.3–3.2)	(34.1–50.0)		
Trypanosoma bokkom n. sp. (*Pseudomyxus capensis*)	12	15.0 ± 3.4 (13.3–17.7)	28.4 ± 4.1 (25.2–35.9)	27.5 ± 2.9 (24.4–35.1)	1.0 ± 1.2 (0.6–1.3)	3.4 ± 0.6 (2.4–5.5)	2.5 ± 0.4 (2.0–3.5)	44.1 ± 4.9 (34.3–49.8)	1.0	2.1

*Include specimens from fish collected at Tsitsikamma and Chintsa East.

Table 3. List of marine *Trypanosoma* species used in the phylogenetic analyses of this study, with associated host, country, references and GenBank accession numbers

Host clades	***Trypanosoma* species**	**Host**	**Location**	**Reference**	**Accession number**
●	*Trypanosoma bakana* n. sp.	*Lithognathus lithognathus*	South Africa	Present study	PV344721
●	*Trypanosoma boissoni*	*Zanobatus atlanticus*	Senegal	Maslov *et al*. (1996)	U39580
●	*Trypanosoma bokkom* n. sp.	*Chelon dumerili*	South Africa	Present study	PV344722
●	*Trypanosoma bokkom* n. sp.	*Chelon richardsonii*	South Africa	Present study	PV344723
●	*Trypanosoma bokkom* n. sp.	*Chelon richardsonii*	South Africa	Present study	PV344724
●	*Trypanosoma bokkom* n. sp.	*Chelon tricuspidens*	South Africa	Present study	PV344726
●	*Trypanosoma bokkom* n. sp.	*Mugil cephalus*	South Africa	Present study	PV344727
●	*Trypanosoma bokkom* n. sp.	*Pseudomyxus capensis*	South Africa	Present study	PV344728
●	*Trypanosoma epinepheli*	*Epinephelus fuscoguttatus*	China	Su *et al*. (2013)	JQ999962
●	*Trypanosoma haploblephari*	*Poroderma pantherinum*	South Africa	Pretorius *et al*. (2021)	MZ061641
●	*Trypanosoma* sp. A	*Caffrogobius gilchristi*	South Africa	Present study	PV344729
●	*Trypanosoma* sp. A	*Caffrogobius gilchristi*	South Africa	Present study	PV344730
●	*Trypanosoma murmanense*	*Gadus morhua*	Norway	Karlsbakk and Nylund (2006)	DQ016616
●	*Trypanosoma nudigobii*	*Clinus superciliosus* / *Zeylanicobdella arugamensis*	South Africa	Hayes *et al*. (2014)	KF871790

●	*Trypanosoma pleuronectidium*	*Gadus morhua*	Norway	Karlsbakk and Nylund (2006)	DQ016613
●	*Trypanosoma rajae*	*Raja* spp.	Algeria	Unpublished (Kefil and Grellier, 2018)	MG878995
●	*Trypanosoma rajae*	*Raja* spp.	Algeria	Unpublished (Kefil and Grellier, 2018)	MG878996
●	*Trypanosoma triglae*	*Chelidonichthys lastoviza*	USA	Maslov *et al*. (1996)	U39584

Table 4. Comprehensive list of all 32 known marine fish *Trypanosoma* species described location, reference, vectors and measurements where available (BL = body length; FL = flagellum length; BW = body width; NL = nucleus length; NW = nucleus width)

***Trypanosoma* species**	**Host species**	**Location**	**Reference**	**Vector**	**BL**	**FL**	**BW**	**NL**	**NW**
Trypanosoma aulopi Mackerras and Mackerras, 1925	*Latropiscis purpurissatus* (Richardson)	Sydney, N.S.W.	Mackerras and Mackerras, 1925	Not known	29.1–57.1	N/A	N/A	N/A	N/A
Trypanosoma bakana n. sp.	*Lithognathus lithognathus* (Cuvier)	Boknes, Eastern Cape, South Africa	Present study	Not known	31.8–44.7	2.1–3.3	2.1–3.3	2.4–4.9	1.2–2.3
Trypanosoma balistes Saunders, 1959	*Balistes capriscus* Gmelin	Waters around Key Largo, Florida	Saunders, 1959	Not known	55.5	12.0	3.0	1.2	0.6
Trypanosoma bokkom n. sp.	*Chelon richardsonii* (Smith)	Tsitsikamma, Eastern Cape, South Africa	Present study	Not known	29.2–52.3	2.4–4.8	1.9–3.3	2.1–4.4	1.2–3.1
Trypanosoma callionymi Brumpt and Lebailly, 1904	*Callionymus lyra* Linnaeus	Luc-sur-Mer, France	Karlsbakk and Nylund, 2006	Not known	65–70	N/A	5	3–3.5	N/A
Trypanosoma capigobii Fantham, 1919	*Caffrogobius nudiceps* (Valenciennes)	Kalk Bay	Fantham, 1919	Not known	42–60	N/A	2–4.4	N/A	N/A
Trypanosoma caulopsettae Laird, 1951	*Arnoglossus scapha* (Forster) and *Rhombosolea plebeia* (Richardson)	Cape Campbell, Cook Strait	Laird, 1951	Not known	26.3–94.8	21.7	1.1–8.4	1.7–2.3	0.8–1.1
Trypanosoma cephalacanthi Ranque, 1973	*Dactylopterus volitans* (Linnaeus)	Not known	Karlsbakk, 2006	Not known	N/A	N/A	N/A	N/A	N/A
Trypanosoma coelorhynchi Laird, 1951	*Coelorinchus australis* (Richardson) and *Pseudophycis bachus* (Forster)	Cape Campbell, Cook Strait	Laird, 1951	Not known	61.5–70.1	9.4–13.0	2.0–4.2	2.7–4.0	1.9–3.0
Trypanosoma congiopodi Laird, 1951	*Congiopodus leucopaecilus* (Richardson)	Cape Campbell, Cook Strait	Laird, 1951	Not known	41.9–45.8	N/A	4.3–4.7	N/A	N/A

Trypanosoma cotti Brumpt and Lebailly, 1904	*Taurulus bubalis* (Euphrasen)	Luc-sur-Mer, France	Karlsbakk and Nylund, 2006	*Calliobdella punctata* Beneden and Hesse, 1863	48.7 ± 5.0	8.3 ± 1.3	3.6 ± 0.6	4.5	3.5
Trypanosoma delagei Brumpt and Lebailly, 1904	*Lipophrys pholis* (Linnaeus)	Roscoff, France	Karlsbakk and Nylund, 2006	Not known	21	12	2.5	N/A	N/A
Trypanosoma dohrni Yakimov, 1911	*Monochirus hispidus* Rafinesque	Gulf of Naples, Italy	Karlsbakk and Nylund, 2006	Not known	36.9	4.3	4.3	N/A	N/A
Trypanosoma epinepheli	*Epinephelus fuscoguttatus* (Forsskål)	Xincun Bay, province of Hainan, South China Sea	Su *et al.* 2014	Not known	17.6–25.9	7.4–13.3	1.3–2.0	2.2–4.1	1.0–1.6
Trypanosoma froesi Lima, 1976 (syn. *Trypanosoma mugilicolum* Becker and Overstreet, 1979; *Trypanosoma platanusi* Ribeiro *et al.* 1996)	*Mugil liza* Valenciennes	Brazil; Mississippi Sound, Gulf of Mexico; Estuarine-lagunar region of Cananéia, State of São Paulo, Brazil	Eiras *et al.* 1995; Becker and Overstreet, 1979; Ribeiro *et al.* 1996	Not known	31.3–37.5 28–36 30.8	10.0–12.0 14–20 0–4.9	3.5 N/A 1.9	2.8–3.2 2.5–3.6 2.6	N/A 1.1–2.6 1.5
Trypanosoma gobii Brumpt and Lebailly, 1904	*Gobius niger* Linnaeus	Luc-sur-Mer, France	Karlsbakk and Nylund, 2006	Not known	56	4–5	5–5.5	N/A	N/A
Trypanosoma khani Burreson, 2007	*Microstomus pacificus* (Lockington)	Pacific Ocean, 10 km off Newport, Oregon	Burreson, 2007	Not known	N/A	N/A	N/A	N/A	N/A
Trypanosoma laternae Lebailly, 1904	*Arnoglossus laterna* (Walbaum)	Luc-sur-Mer, France	Karlsbakk and Nylund, 2006	Not known	44	11	4–5	N/A	3.5–4 x 3
Trypanosoma murmanense Nikitin, 1927 (syn. *Trypanosoma myoxocephali* Fantham, Porter and Richardson, 1942)	*Gadus morhua* Linnaeus	Aleksandrovsk (Polyarny), Kola Fjord, Russia	Karlsbakk and Nylund, 2006	*Johanssonia arctica* (Johansson, 1898)	59.5–75	6–13.5	2–3.5	2–5	2–3
Trypanosoma nudigobii Fantham, 1919	*Caffrogobius nudiceps* (Valenciennes)	Kalk Bay	Hayes *et al.* 2014	*Zeylanicobdella arugamensis* Silva, 1963	60–85	N/A	6.6–7.5	N/A	N/A

Trypanosoma pacifica Burreson and Pratt, 1972	*Parophrys vetulus* Girard	Pacific Ocean, 5–10 km off Newport, Oregon	Burreson and Pratt, 1972	Not known	N/A	N/A	N/A	N/A	N/A
Trypanosoma parapercis Laird, 1951	*Parapercis colias* (Forster)	Cannibal Cove, Marlborough Sounds	Laird, 1951	Not known	50.8–53.8	N/A	2.2–2.3	3.3–3.6	2.3–2.5
Trypanosoma platessae Lebailly, 1904 (syn. *Trypanosoma bothi* Lebailly, 1905; *Trypanosoma flesi* Lebailly, 1904; *Trypanosoma limandae* Brumpt and Lebailly, 1904)	*Pleuronectes platessa* Linnaeus	Luc-sur-Mer, France	Karlsbakk and Nylund, 2006	Not known	35.8–38.4	10.0–15.2	3–3.5	1.6–2.2	2.0–3.2
Trypanosoma pleuronectidium Robertson	*Pleuronectes platessa* Linnaeus	Millport, Scotland	Karlsbakk and Nylund, 2006	*Calliobdella nodulifera* (Malm, 1863)	40–74	0–14.5	2.5–7	2.5–7.5	2–6
Trypanosoma pulchra Mackerras and Mackerras, 1925	*Parma microlepis* Günther	Sydney and Broken Bay, N.S.W.	Mackerras and Mackerras, 1925	Not known	40.8–57.1	7.3	3.5	N/A	N/A
Trypanosoma scorpaenae Neumann, 1909	*Scorpaena notata* Rafinesque	Gulf of Naples, Italy	Karlsbakk and Nylund, 2006	Not known	58–65	5–7	2–2.5	N/A	4 x 2.5
Trypanosoma senegalense Ranque, 1973 (syn. *Trypanosoma triglae senegalensis*, Ranque, 1973)	*Chelidonichthys lucerna*(Linnaeus)	Senegal	Su *et al*. 2014 / Karlsbakk and Nylund, 2006	Not known	39	11.6	2.2	2.1	1.5
Trypanosoma soleae Laveran and Mesnil, 1901	*Solea solea* (Linnaeus)	St. Martin, French Channel Area	Karlsbakk and Nylund, 2006	*Hemibdella soleae* (van Beneden and Hesse, 1863)	32–35	4–5	N/A	N/A	N/A
Trypanosoma sp. A	*Caffrogobius gilchristi* (Boulenger)	Groot River West Estuary, Natures Valley, Eastern Cape, South Africa	Present study	Not known	52.2–70.8	N/A	7.8–5.2	2.4–4.0	1.2–2.1

Trypanosoma triglae R.O.Neumann, 1909	*Chelidonichthys lucerna* (Linnaeus)	Gulf of Naples, Italy	Karlsbakk and Nylund, 2006	Not known	45	c.15	8	N/A	5 x 4
Trypanosoma tripterygium Laird, 1951	*Forsterygion varium* (Forster) and *Bellapiscis medius* (Günther)	Poisoning rock pools at, Island Bay, Wellington	Laird, 1951	Not known	36.3–58.1	3.3–16.8	1.3–7.4	2.4–2.4	1.1–1.7
Trypanosoma yakimovi Yakimov, 1911	*Syngnathus acus* Linnaeus	Gulf of Naples, Italy	Karlsbakk and Nylund, 2006	Not known	31.2–35.5	4.3–9.9	2.8	2.8	N/A

Figure legends

Figure 1. Map showing the sampling locations. Chintsa East, Tsitsikamma Storms River Mouth, Boknes, Kariega River Estuary and Groot River West Estuary.

Figure 2. Photomicrographs of *Trypanosoma nudigobii* Fantham, 1919 from *Clinus superciliosus* (Linnaeus). Scale bar = 10 µm. (N: Nucleus; UM: Undulating Membrane; K: Kinetoplast; F: Free Flagellum).

Figure 3. Photomicrographs of *Trypanosoma* sp. A from *Caffrogobius gilchristi* (Boulenger). Scale bar = 10 µm. (N: Nucleus; UM: Undulating Membrane; K: Kinetoplast; F: Free Flagellum).

Figure 4. Photomicrographs of *Trypanosoma bakana* n. sp. from *Lithognathus lithognathus* (Cuvier). Scale bar = 10 µm. (N: Nucleus; UM: Undulating Membrane; K: Kinetoplast; F: Free Flagellum).

Figure 5. Photomicrographs of *Trypanosoma bokkom* n. sp. from five mullet species. (A–C) *Chelon richardsonii* (Smith); (D–F) *Chelon dumerili* (Steindachner); (G–H) *Chelon tricuspidens* (Smith); (I–J) *Mugil cephalus* Linnaeus; (K–L) *Pseudomyxus capensis* (Valenciennes). Scale bar = 10 µm. (N: Nucleus; UM: Undulating Membrane; K: Kinetoplast; F: Free Flagellum).

Figure 6. The nMDS biplot indicates the differences in trypanosome measurements across different species.

Figure 7. Phylogenetic position of fish trypanosomes from Chintsa East, Tsitsikamma Storms River Mouth, Boknes and Kariega River inferred from the partial 18S rRNA gene region. The outgroups used are *Trypanosoma cruzi* ex. *Homo sapiens* and *Trypanosoma dionisii* ex. *Pipistrellus pipistrellus*. Pink: Trypanosome sequences from frogs and toads. Green: Trypanosome sequences from frogs, lizards and toads. Black: Trypanosome sequences from marine fish. Blue: Trypanosome sequences from clawed frogs, freshwater

turtles, platypus and terrapins. Yellow: Trypanosome sequences from caiman, freshwater fish and a freshwater leech. Red: Trypanosome sequences from geckos, lizards and monitors. Purple: Trypanosome sequences from caiman and a tsetse fly.

Figure 8. Line drawings of (A) *Trypanosoma* sp. A from *Caffrogobius gilchristi* (Boulenger); (B) *Trypanosoma nudigobii* from *Clinus superciliosus* (Linnaeus); (C) *Trypanosoma bakana* n. sp. from *Lithognathus lithognathus* (Cuvier); (D) *Trypanosoma bokkom* n. sp. from *Chelon richardsonii* (Smith). Scale bars = 10 µm.