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Characterization of a trypanosome from large yellow croaker (*Larimichthys crocea*), cage-cultured in seawater, in China

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ABSTRACT

A significant outbreak of trypanosomosis occurred in farmed large yellow croaker (*Larimichthys crocea*) in Southeast China resulting in daily mortality rates, over 70 days, of up to 1 % with cumulative losses nearing 60 %. Despite the severity of the outbreak, details of the specific trypanosomes infecting large yellow croaker remain unclear. In this study, samples of fish were collected from the fish farmer during the outbreak. A high prevalence of trypanosomes, confirmed by microscopy and PCR analysis, was associated with pathology. Sequence identification and phylogenetic analysis indicated that the isolated trypanosome had 99.27 % sequence identity with *Trypanosoma carassii* MARV, a typical freshwater fish trypanosome, and clustered within the *T. carassii* complex C group. Morphological characterization, using Giemsa staining, further supported the similarity between the trypanosomes infecting large yellow croaker and the *T. carassii* complex C group. Additionally, a broader host range was established as it also infected Nile tilapia (*Oreochromis niloticus*), koi (*Cyprinus carpio*) and largemouth bass (*Micropterus salmoides*), leading to its designation as a subspecies within the *T. carassii* complex, named *T. carassii spectrum*. This represents the first documented instance of *T. carassii*, a typical freshwater fish trypanosome, infecting a marine fish whilst highlighting its severe pathogenicity and the urgent need for effective control measures. Furthermore, *in vitro* cultivation was successfully established, providing a foundation for future research on prevention and management strategies.

1. Introduction

Large yellow croaker (*Larimichthys crocea*) is a traditional popular marine fish species in China, known for hundreds of years as a well-known Chinese dish, due to its good taste and rich resources (Shi et al., 2022). Over the past three decades, it has become one of the most economically important species in China's mariculture industry, with production reaching 280,997 tons in 2023 (Liu et al., 2024). However, demand and farming practices have led to a rapid scale up of fish growth and density which in turn has led to increases in the prevalence of different diseases and has threatened the sustainability of the industry (Li et al., 2020; Qin et al., 2024; Qu et al., 2022; Shan et al., 2005; Wang et al., 2022). Among these diseases, trypanosomosis recently was recorded, caused by hemoflagellates in the genus *Trypanosoma*, has led to significant mortality in the cultured large yellow croaker in Southeast China (Qin et al., 2024). During these outbreaks, daily mortality rates could reach up to 1 %, with a cumulative mortality rate close to 60 % within 70 days.

Trypanosoma is a genus within the Kinetoplastea, a monophyletic group of unicellular parasitic protozoa (Hamilton et al., 2004). Members of this genus infect a variety of vertebrate hosts, including fish, reptiles, amphibians, birds, and mammals. They cause severe trypanosomosis including surra and Nagana, important diseases in domestic or wild mammals as well as African sleeping sickness and Chagas' disease in

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humans (Grybchuk-Ieremenko et al., 2014; Hoare, 1972; Lom and Dyková, 1992). Over 280 species of trypanosomes have been described from fish, mostly from freshwater environments (Gupta and Gupta, 2012; Zhang et al., 2023). Recent outbreaks of fish trypanosomosis have been reported in both wild and farmed species across South America and Asia, including largemouth bass, Amazon sailfin catfish, grouper, barramundi, Nile tilapia, blood parrot cichlids, and large yellow croaker (de Souza and Corrêa, 2019; Jesus et al., 2018; Jiang et al., 2019; Luo et al., 2019; Qin et al., 2024; Wang et al., 2015; Zhou et al., 2024). Fish infected with trypanosomes exhibit some typical clinical changes such as anorexia, anemia, lethargy, hypoglycemia and splenomegaly, often leading to death and substantial economic losses (Islam and Woo, 1992; Jiang et al., 2019; Qin et al., 2024; Zhou et al., 2024) and without effective treatment as far as we know.

The outbreaks of trypanosomosis, particularly in large yellow croaker, in Southeast China, underscore the urgent need for effective management and control strategies to mitigate the impact of this trypanosomosis on aquaculture. Despite the severity of these outbreaks, little is known about the biological characteristics of the pathogen infecting large yellow croaker, highlighting a key knowledge gap. In this study, we report that we have isolated the trypanosome from infecting large yellow croaker and successfully established cultivation *in vitro*. Results from morphological and genetic analyses as well as host range assessments strong indicate that this trypanosome belongs to the C group of the *Trypanosoma carassii* (syn. *T. danilewskyi*) complex. While this group has long been considered a typical freshwater fish trypanosome and is distributed worldwide (Overath et al., 1998), it is shown here to also be an important pathogen of marine fish.

2. Materials and methods

2.1. Ethics statement

All animal procedures were approved by the Laboratory Animal Use and Care Committee of Sun Yat-Sen University (license number 32170470).

2.2. Trypanosome isolation and morphological examination

Large yellow croakers (lengths ranging from 17.8 to 22.1 cm) were collected from a cage farm during a deadly trypanosomosis outbreak on October 11, 2023, in Sanduao Bay, Ningde, Fujian Province, China (Fig. 1A). The water conditions were as follows: salinity 32.1 ‰, temperature 20.2°C, and pH 8.10. Blood (approximately 300 μL per fish) was collected from the hearts of fish with sodium heparin and a 2-μL wet mount was examined under a microscope. The blood was diluted 100-fold with PBS, and the parasitemia was quantified using a haemocytometer. Blood smears were made from the positive samples and stained with Giemsa for morphological observation. Morphological parameters of 200 trypanosomes from the sample with the highest parasitemia were measured using ImageJ software (Schneider et al., 2012). Parameters included body length (BL), total length including flagellum (TL), free flagellum length (FF), cell maximum body width (BW), center to center distances of posterior end to nucleus (PN), posterior end to kinetoplast (PK), kinetoplast to nucleus (KN), and anterior end to nucleus (NA). Additionally, nucleus index (NI = PN/NA), kinetoplast index (KI = PN/KN), and flagellum index (FI = FF/BL) were calculated to represent the relative positions of the nucleus, kinetoplast, and free flagellum (Hoare, 1972). Data and figures were analyzed or plotted using GraphPad 10.3 (GraphPad Software, CA, USA) or R scripts.

2.3. DNA extraction and PCR amplification

Total DNA from infected fish blood or cultured trypanosomes was extracted using the phenol-chloroform method (Green and Sambrook, 2017). PCR amplifications of three genes including small subunit ribosomal DNA (18S rDNA), internal transcribed spacer 1 (ITS1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were performed using Super-Fidelity DNA Polymerase (P525–01, Vazyme International LLC, China). The 18S rDNA was amplified with the primers 5′-GACTTTTGCTTCCTCTATTG-3′ and 5′-CATATGCTTGTTTCAAGGAC-3′ (Maslov et al., 1996), under the following PCR conditions: 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 48°C for 15 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. GAPDH

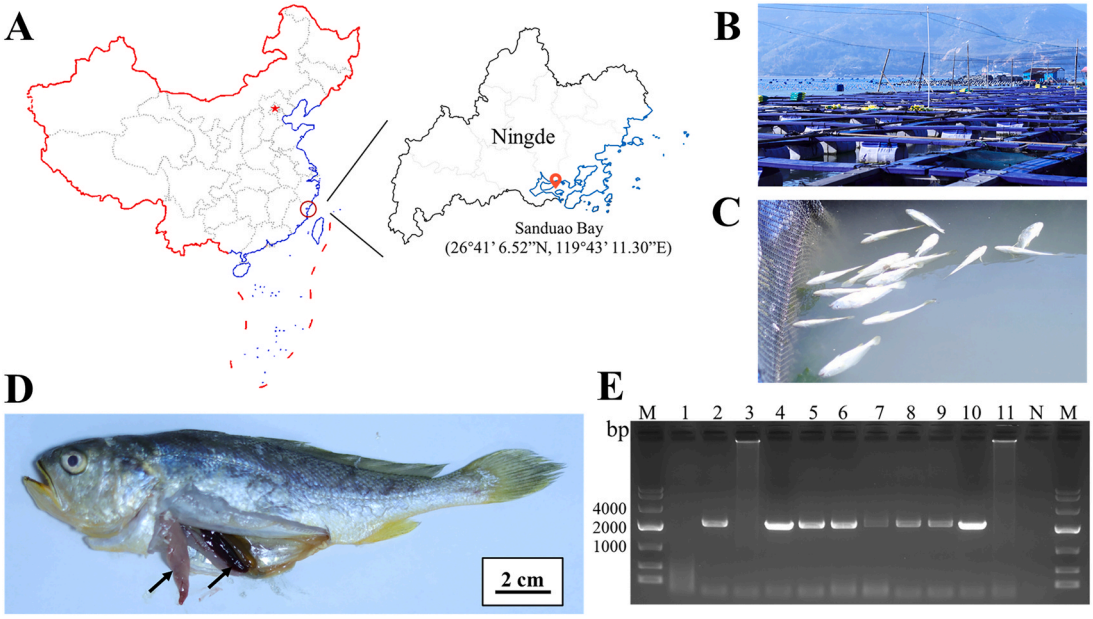

Fig. 1. Sampling site and macroscopic internal changes of infected fish. (A) Location of the sampling site in Sanduao Bay, Ningde, Fujian Province, China. (B) Picture showing the high-density environmental conditions of the sampling site. (C) Example of the death of large yellow croaker at the sampling location. (D) Macroscopic internal changes observed in infected large yellow croaker, highlighting a pale liver (left arrow) and the dark spleen (right arrow). (E) Gel electrophoresis of PCR amplicons of 18S rDNA, indicating the presence of parasites. Lanes 1–11, DNA extracted from large yellow croaker samples; N, a negative control; M, DL10000 DNA marker.

(5′-GTGAAGGCGCAGCGCAAC-3′ and 5′-CCGAGGATGYCCTTCATG-3′) and ITS1 (5′-GCTGTAGGTGAACCTGCAGCAGCTGGATCATT-3′ and 5′-GC GGGTAGTCCTGCCAAACACTCAGGTCTG-3′) were amplified (Maia Da Silva et al., 2004; Zhang et al., 2023), with an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. Amplicons were separated on 1 % agarose gels and visualized under UV light. Amplicons were sequenced commercially (Sangon Biotech Co., Ltd. China) and analyzed using BLAST (Camacho et al., 2009).

2.4. Phylogenetic and genetic distance analysis

The 18S rDNA, GAPDH, and ITS1 sequences of the *T. carassii* complex were obtained from GenBank and Zhang et al. (2023). Sequences were aligned using MAFFT with default settings and manually adjusted as needed (Katoh, 2002). Phylogenetic trees were constructed using FastTree2 and visualized with the ITOL web server (Letunic and Bork, 2024; Price et al., 2010). Genetic distances of 18S rDNA between the trypanosome from large yellow croaker (TFL) and the *T. carassii* complex were calculated using MEGA7 (Kumar et al., 2016).

2.5. Infection of fish with trypanosomes and in vitro cultivation

Specific pathogen-free (SPF) Nile tilapia (*Oreochromis niloticus*) with lengths ranging from 14 to 19 cm were sourced from the Tilapia Breeding Farm in Guangdong Province, China. Koi (*Cyprinus carpio*) with lengths of 14–15 cm and largemouth bass (*Micropterus salmoides*) with lengths of approximately 12 cm were obtained from Huadiwan Flower, Fish, Bird Market, also located in Guangdong Province, China. The fish were monitored in laboratory tanks over a period of two weeks, with blood samples screened via microscopy to ensure the absence of haemoflagellate infections.

Fifty microliters of trypanosome infected large yellow croaker blood was diluted 100-fold with PBS and inoculated into tilapia (n = 10), koi (n = 3), or largemouth bass (n = 5) via the pericardial cavity respectively (Chen et al., 2022). Each fish received 1×10^6 trypanosomes, after which they were kept in isolation and monitored twice daily. Parasitemia checks started on day 5 post-inoculation and repeated every two days. Two microliters of blood from the fish caudal vein were diluted 100-fold with PBS, and the parasitemia was quantified as mentioned above.

For *in vitro* cultivation, 100 μL blood from trypanosome infected large yellow croaker (approx. 10^8/ml) were diluted tenfold with SDM79 medium and centrifuged at 50 ×g for 10 min to remove the majority of the erythrocytes. The supernatant was then added into 4 ml of SDM79 medium supplemented with 10 % FBS and 1 % Penicillin-Streptomycin, and incubated at 27 °C (Zhou et al., 2024). Trypanosomes were subsequently cloned in the same medium, and the resulting populations were cryopreserved in culture medium with 10 % glycerol in liquid nitrogen for future use.

2.6. Electron microscopy

Culture samples for electron microscopic observations were prepared as previously described (Wan et al., 2023; Zhang et al., 2019). For scanning electron microscopic observations, approximately 10^7 trypanosomes were washed with PBS and placed onto coverslips, then fixed with 2.5 % glutaraldehyde in PBS for 24 hours at 4 °C in the dark. After rinsing with PBS, the samples were dehydrated in a graded ethanol series (30 %, 50 %, 60 %, 70 %, 80 %, 90 % and 100 %), each for 15 minutes. The coverslips were subsequently critical-point dried, coated with an 8 nm layer of gold nanoparticles, and imaged using Apreo 2 scanning electron microscope (Thermo Fisher, USA).

For transmission electron microscopy, approximately 10^7 trypanosomes were fixed overnight at 4 °C in a PBS-based solution containing 2 % paraformaldehyde and 3 % glutaraldehyde. After washing with PBS, they were fixed in 1 % osmium tetroxide in PBS at 4 °C for 1 hour. The samples were washed, dehydrated through an ethanol series (30 %, 50 %, 70 %, 80 % and 95 %), and treated with acetone before embedding in Spurr. The samples were sectioned at 100 nm using a Leica ultramicrotome (Tousimis, USA). Ultrathin sections were collected on grids and imaged using JEOL transmission electron microscopy (JEM1400FLASH, Japan).

3. Results

3.1. The disease associated with trypanosomes in large yellow croaker

On October 11, 2023, eleven large yellow croakers were collected from a farm in Sanduao Bay, Ningde, Fujian Province, China (26°41′6.52″N, 119°43′11.30″E) (Fig. 1A-B). It was reported that approximately 750 kg of large yellow croaker were dying daily in the farm where the fish were collected. The fish showed some abnormal changes, including anorexia, anemia, abnormal swimming, pale body coloration, spleen enlargement with black discoloration, and a visibly pale liver (Fig. 1C-D). Eleven fish samples were collected, with 54.5 % (6/11) examined positive under light microscopy, exhibiting parasitemia levels of around 10^8/ml. Sample no. 8 reached nearly 10^9/ml of trypanosomes (video 1) and showed a similar density to the erythrocytes. PCR results confirmed trypanosome presence in 8 out of 11 samples (Fig. 1E) and all the DNA sequences obtained are identical to previous trypanosomes isolated from large yellow croaker (GenBank no. OR934688). Surprisingly, these sequences displayed up to 99.27 % similarity (2050/2065) to *Trypanosoma carassii* MARV (GenBank no. OL963935), a typical freshwater trypanosome (Gibson et al., 2005).

3.2. Phylogenetic analyses on trypanosomes from large yellow croaker

Results from current publication by Zhang and colleagues provided a comprehensive classification framework for the *T. carassii* complex (Zhang et al., 2023). We conducted phylogenetic analyses to determine the precise positioning of TFL within the *T. carassii* complex. Our analysis of 18S rDNA indicated that TFL clusters with the *T. carassii* group C (Fig. 2A), with genetic distances ranging from 0.002 to 0.006, notably smaller than the typical intra-group C range of 0–0.0093. These results demonstrated a close genetic relationship between the TFL and the *T. carassii* isolates from freshwater fish. The GAPDH sequence analysis further confirmed the genetic similarity between TFL and the *T. carassii* group C (Fig. 2B). Moreover, the ITS1 sequence of TFL, obtained via PCR amplification, exhibited an amplicon size of approximately 400 bp, consistent with sequences observed in group C (Figure S1). Sequence alignment of ITS1 further validated a high degree of similarity between TFL and *T. carassii* group C. Collectively, all these results indicated a strong genetic linkage between TFL and the members of the *T. carassii* complex.

3.3. Morphology of trypanosomes from large yellow croaker

Morphological analysis was conducted following Giemsa staining. TFL were identified as trypomastigotes, distinguished by a prominent nucleus and a rounded kinetoplast, located in the anterior region and near the posterior end of the body, respectively (Fig. 3A). Detailed morphological data are presented in Table S1 and Fig. 3B-F. The total length of the trypanosomes ranged from 19.5 μm to 42.3 μm, with an average of 31.8 μm. The Flagellum Index is approximately 0.7. These parameters are consistent with those observed among the four isolates of the *T. carassii* group C. Most parameters including PN, NA, KN, KI, NI and FI are also similar, supporting the molecular identification that these trypanosomes should belong to the same species and are classified alongside the C group of *T. carassii*. Interestingly, TFL exhibit a significantly larger body width and PK distance (Fig. 3D), but still partially overlap with the four controls.

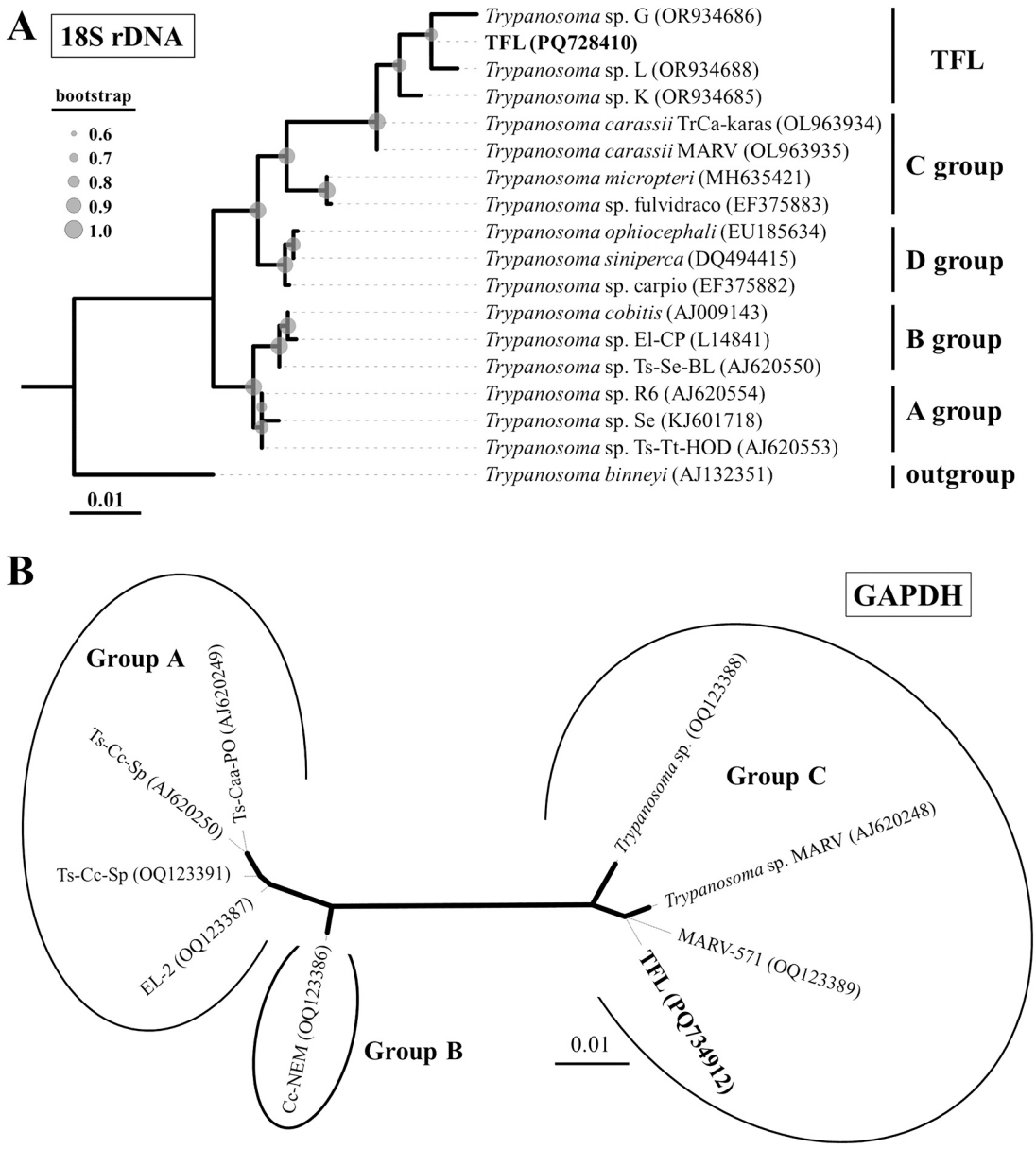

Fig. 2. Phylogenetic relationships among the trypanosomes from large yellow croaker and the *T. carassii* complex. Maximum likelihood trees were constructed based on 18S rDNA (A) and GAPDH (B) gene sequences. Bootstrap values at the nodes were derived from 1000 repetitions, with branch lengths scaled to represent evolutionary distances.

The trypanosomes were examined using both scanning electron microscopy and transmission electron microscopy. Typical trypomastigote shapes were observed, with the flagellum emerging from a flagellar pocket and extending along the cell body, forming well-developed undulating membranes and with the anterior tip projecting as a free flagellum (Fig. 3G). The trypanosomes exhibited an asymmetric longitudinal division pattern, with the new cells being noticeably smaller than the older ones, and similar variations observed in the flagella. Typical disc-shaped kinetoplasts were identified next to the base of the flagellum (Fig. 3H). A prominent nucleus was located near the anterior end of the cell, along with visible mitochondria and numerous vesicles.

3.4. Growth status of TFL in koi, largemouth bass, and Nile tilapia

To access the infectivity of TFL in freshwater fish, the koi, largemouth bass, and the euryhaline species Nile tilapia were inoculated with the trypanosome infected blood from large yellow croaker, respectively (Fig. 4A & B). Parasitaemias were observed in all tilapia on 5–7 days post inoculation, with a peak range between 5×10^7 and 2×10^8 on around day 10. Parasitaemias were detected in two out of three koi with a peak around 1×10^7 during the detection period. Notably, all inoculated largemouth bass exhibited parasitaemia, with peak levels reaching approximately 2×10^9.

3.5. Cultivation of trypanosomes from large yellow croaker

We have successfully established a culture system with SDM79 medium for the TFL. When employing a high seeding inoculum of 5×10^5/ml, the trypanosomes proliferated to 6×10^6/ml on day 9, and died within 3 days without subculture (Fig. 4C). A low seeding inoculum of 1×10^5/ml also showed similar proliferation rates, reaching about six-fold growth during the first four days, albeit with a peak at 2×10^6/ml (Fig. 4C-D). These findings suggest that SDM79 medium is suitable

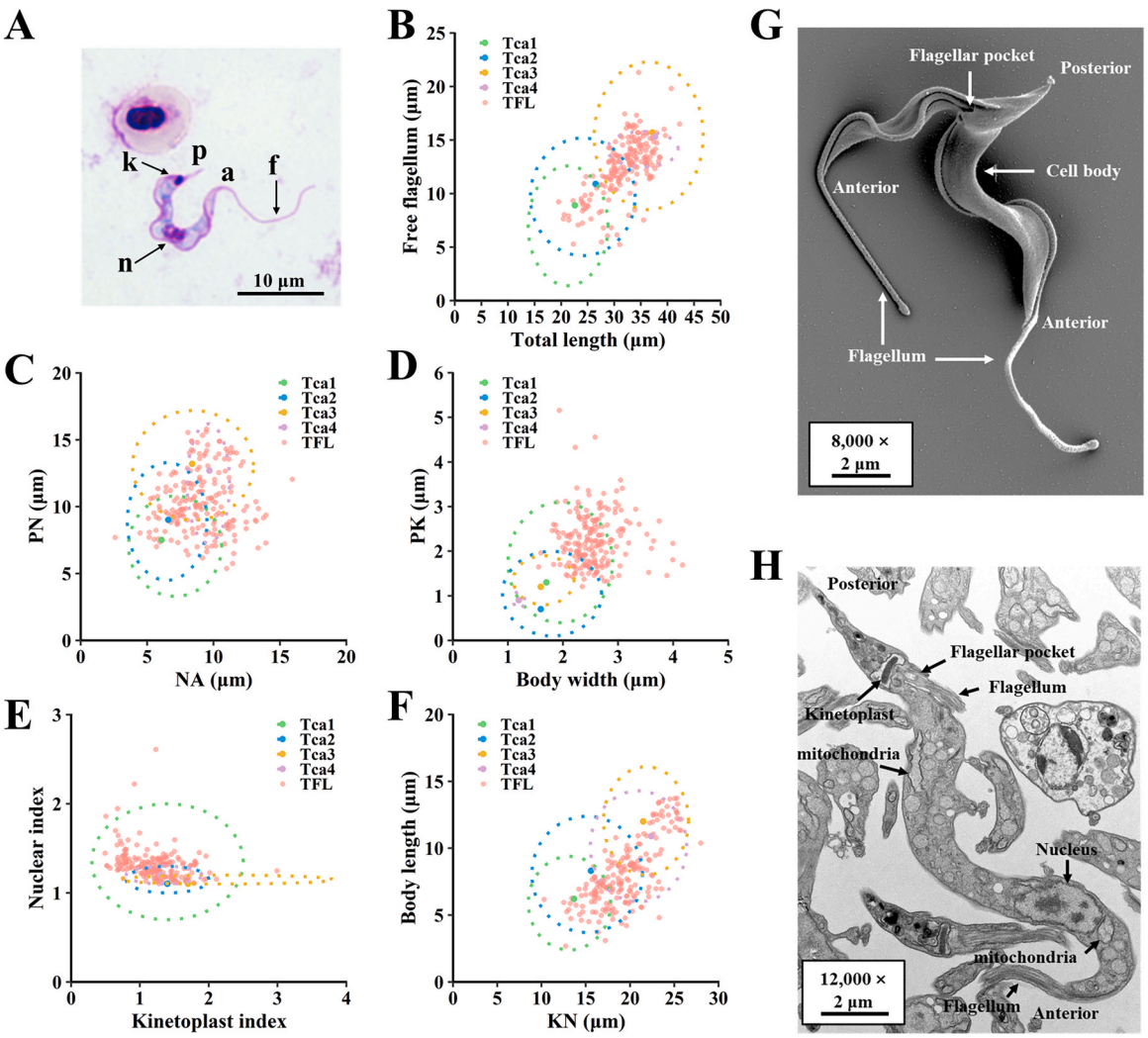

Fig. 3. Morphological characterization of Giemsa-stained and electron microscopy images of TFL. (A) Graph depicting Giemsa-stained trypanosomes from infected large yellow croaker. a, anterior; p, posterior; n, nucleus; k, kinetoplast; f, flagellum. (B-F) Morphological indices of center-to-center distance were measured for TFL, n = 200. Mean values from four isolates of *T. carassii* group C are indicated in distinct colors. Tca1, *T. carassii danilewskyi* described by Zhou et al. (2024); Tca2, *T. micropteri* described by Zhang et al. (2022); Tca3, *T. micropteri* described by Jiang et al. (2019); Tca4, *T.* sp. ex *P. fulvidraco* described by Gu et al. (2007). PN, posterior end to mid-nucleus; NA, anterior end to nucleus; KN, kinetoplast to mid-nucleus; PK, posterior end to kinetoplast. Scanning (G) and transmission (H) electron microscopy images of TFL highlighting common cellular structures with arrows.

for the proliferation of this trypanosome species. In addition, we have also successfully established more than a dozen cloned populations of TFL using this system.

4. Discussion

Accurate identification of parasite species is crucial for pathogen biology studies and subsequent development of control strategies. Recent outbreaks of trypanosomosis in large yellow croaker in Southeast China may link to significant economic losses, yet knowledge regarding the pathogen remains limited (Qin et al., 2024).

Recently, the TFL was nominated as *Trypanosoma larimichthysi*, a new species of fish trypanosome, based only on morphological characteristics and differences in the highly variable region of the 18S rDNA sequence (Yang et al., 2025). However, we found several significant errors regarding both the morphological description and criteria for nomenclature in this paper. According to our DNA sequence alignments, results demonstrate that the trypanosomes reported by Qin et al. (2024), Yang et al. (2025) and our current trypanosome samples are belong to the same species, which belongs to Group C of the *T. carassii* complex typically found in freshwater fish. The SPECIES concept is usually defined by independent lineages that are reproductively isolated (Biological Species Concept) or are monophyletic and diagnosably distinct lineages (Phylogenetic Species Concept). Due to the difficulty and almost impossibility of testing hybrids in these trypanosomes, taken alongside the limited differences in test genes, we are not able to claim solid evidence of a new species within the *T. carassii* complex. This might be overcome, in the future, by using genomic sequencing to survey a wider variety of taxonomic markers. Although genome sequencing offers a more precise method for species delimitation, it also faces technical limitations and accessibility challenges, particularly in terms of sample quality, budget and time. In the case of the *T. carassii* complex, though there may be cryptic species, based on the current data, we prefer to consider it as one complex species and propose to name some subspecies. Given the 18S rDNA similarity between TFL and *T. carassii* MARV, if *T. carassii* MARV is confirmed as belonging to *T. carassii*, then TFL should also be considered as *T. carassii*. Importantly, considering that the host is so distinct, we would rather name it as a subspecies calling *Trypanosoma carassii spectrum*. Defining if it is a new trypanosome species would only be appropriate after assessing the phylogeny using genome data.

Documented cases of *T. carassii*-induced trypanosomosis have led to

A

Fish	Positive rate	peak parasitemia
Nile tilapia	100% (10/10)	5×10^7 - 2×10^8
Koi	66.7% (2/3)	1×10^7
Largemouth bass	100% (5/5)	2×10^9

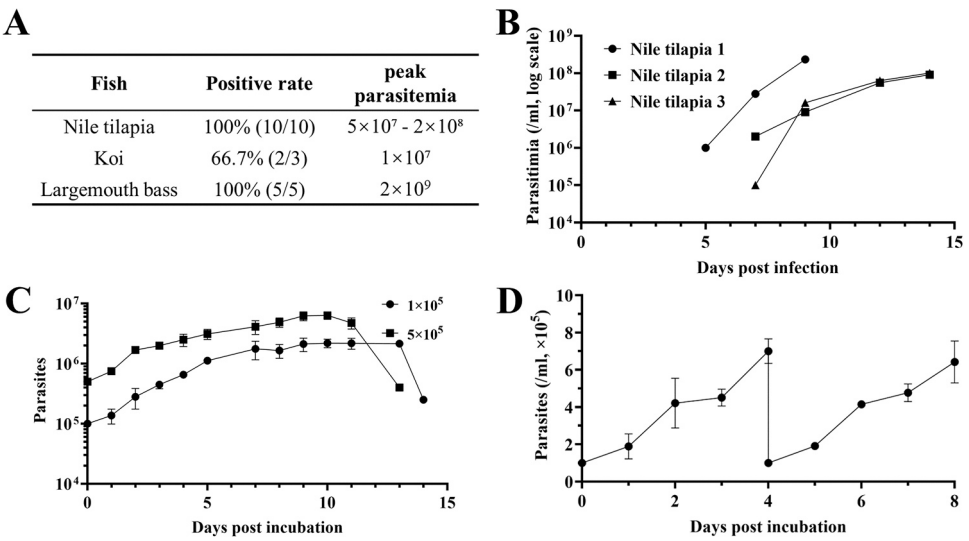

Fig. 4. Growth status of TFL in three species of fish and in *in vitro* cultivation in SDM79 medium. A shows the positive rates and peak parasitemias in Nile tilapia, koi and largemouth bass which were inoculated with the blood collected from naturally infected large yellow croaker. B indicates the growth curves illustrating the temporal changes in parasitemia among three individual Nile tilapia specimens infected with trypanosomes. C presents the growth curves of TFL and cultured in SDM79 medium, initiated at two different densities: 1×10^5 cells/ml and 5×10^5 cells/ml. D displays the growth curves of a trypanosome isolate in SDM79 medium, starting from an initial density of 1×10^5 /ml, with subculture every 4 days.

significant economic losses across many freshwater species of fish in various regions (Chen et al., 2022; Luo et al., 2019; Zhou et al., 2024). These trypanosomes negatively affect the health of the fish, causing lethargy, anemia, splenomegaly, hematopoietic damage, and weight loss. Given the significant aquaculture losses in the fish farms experiencing outbreaks, greater attention is required by local governments to combat the impact of trypanosomosis occurring in this economically important fish species.

Trypanosoma carassii was first observed and described in 1883, from the blood of crucian carp (*Carassius carassius*) (Mitrophanow, 1883). It was subsequently identified as the causative agent of trypanosomosis in carp and many other cyprinid species, as well as in several non-cyprinid freshwater fish (Laveran and Mesnil, 1904; Lom and Dyková, 1992). Several fish species were reported to be susceptible to *T. carassii* (as its previous name *T. danilewskyi*) including *Carassius auratus*, *Barbus conchus*, *Danio malabaricus*, *Catostomus commersoni*, *Notropis cornutus*, *Etheostoma caeruleum* and *Ictalurus nebulosus*, (Woo and Black, 1984). Moreover, some strains in the *T. carassii* Group C (namely *Trypanosoma micropteri* syn. at that time) were found in hosts such as *C. auratus*, *Misgurnus anguillicaudatus*, *M. salmoides*, *Channa argus* and *Tachysurus fulvidraco* (Gu et al., 2007; Jiang et al., 2019; Zhang et al., 2022). In addition, strains in the *T. carassii* Group A/B (namely *Trypanosoma cobitis* syn. at that time) were also found in various fish hosts, including *Barbatula barbatula*, *Phoxinus phoxinus*, *Gobio gobio*, *Cottus gobio*, *Gasterosteus aculeatus* and *Pungitius pungitius* (Letch and Ball, 1979). Additionally, Grybchuk-Ieremenko et al. (2014) reported that *T. carassii* was detected from several species, including *Abramis brama*, *C. carassius*, *Silurus glanis*, *Perca fluviatilis*, *Sander lucioperca* and *Scardinius erythrophthalmus*. Based on these references, to our surprise, the broad host range of *T. carassii* involves several Orders of fish such as the Cypriniformes, Perciformes, Siluriformes, which all are freshwater species of fish (Zhang et al., 2022). The identification of TFL as a new isolate within the *T. carassii* complex marks the first record of this freshwater fish trypanosome infecting marine fish.

Given that the establishment of an infection model in this study makes use of Nile tilapia, which can inhabit both freshwater and brackish environments, it indicates that there may no strict barrier between the *in vivo* environments of marine and freshwater fish, at least for some fish trypanosomes. This opinion is also supported by the fact that Nile tilapia is susceptible to infection with both *T. carassii* and *Trypanosoma epinepheli* as shown in our previous study (Chen et al., 2022). This finding obviously expands the recorded host range of *T. carassii* and implies that other trypanosomes from marine fish, particularly those from inshore fish, where molecular studies are lacking, might also belong to *T. carassii*. However, there are a number of possible origins of these trypanosomes including that the *T. carassii* found in large yellow croaker actually originates from marine fish or alternatively is just a case of adaption of this freshwater fish trypanosome to infect some marine fish.

Blood-sucking leeches are well-known vectors for fish trypanosomes (Khan, 1977; Qadri, 1962). Qadri (1962) studied the life cycle of *T. carassii* (as *T. danilewskyi*) in the freshwater leech *Hemiclepsis marginata*. Khan (1977) reported the transmission of the marine fish trypanosome (*Trypanosoma murmanensis*) from Atlantic cod (*Gadus morhua*) to other fish species, including pleuronectiform, perciform, anguilliform and gadiform fish, through the marine leech *Johonssonia* sp. A similar transmission mechanism may also occur in TFL while it should be confirmed by evidence from the finding of natural trypanosome infected vector or experimental results. More information can be found in the book edited by Prof. P. Woo (Woo, 2006), which reviews the investigations on the role of transmission of fish trypanosomes by freshwater and marine leeches. For this outbreak of large yellow croaker, we consider that infected wild marine fish and infected marine leeches are highly likely the sources. Besides the leeches, some other blood sucking invertebrates or some parasitic turbellarian such as *Pseudograffilla* sp. (Guo et al., 2006), may also involve in the transmission. Although there is no report yet of trypanosomes being transmitted by parasitic turbellarians, they are present in the same water regions and have been recorded on the same fish species on the branchial filaments (Wang et al., 2006). Someone may ask when the large yellow croaker fish are infected with the trypanosome? It can be sure that the possibilities of infection during the fry (juvenile) stage can be excluded. This is because the fry were purchased from companies where the fry were breed in the cement ponds with seawater at a salt content of 3.0 %. Identifying the specific vector and routes responsible for the infection is crucial for understanding the epidemiology and implementing control measures against this trypanosome.

From an evolutionary perspective, we believe that the trypanosome may have originated from infected brackish fish and leeches. This assumption is mainly based on the close relationship between TFL

(assuming this is a specific trypanosome strain) and other classical *T. carassii* strains. Since certain euryhaline fish, such as Nile tilapia, are susceptible to infection by both freshwater and marine fish trypanosomes, both under natural and experimental conditions (Chen et al., 2022; Jesus et al., 2018) and thus it is clear that there is a strong possibility that it may come into contact with both freshwater and marine leeches or other parasites. In the process, they can transmit the trypanosome to the vectors and, as a consequence, to other fish species. Identifying the specific vector responsible for the infection is crucial for understanding the epidemiology and implementing control measures against this trypanosome.

It is highly notable that, *T. carassii* exhibits considerable morphological diversity. Studies have shown that *T. carassii* isolated from *C. auratus*, *B. conhus*, *C. commersoni* and *E. caeruleum* display distinct morphological variations. While samples from the same host exhibit consistent traits, notable variations are observed between hosts (Woo and Black, 1984). Likewise, *T. carassii* isolated from tilapia and largemouth bass exhibited notable morphological differences (Jiang et al., 2019; Zhang et al., 2022). *T. carassii* from blood parrot cichlids were found to be significantly smaller than those from other fish, especially in the KN, PN, FF, and BL values (Zhou et al., 2024). In this study, TFL showed greater body width and PK distance, which may be attributed to the different of host (freshwater and marine host). These findings suggest that relying solely on host and morphological traits is insufficient for classification of trypanosomes in fish and that molecular evidence needs to be incorporated. We also noticed a complex history in nomenclature and synonyms of *T. carassii*, which was first identified from crucian carps in Moscow by Mitrophanow in 1883 (Mitrophanow, 1883), and should be required redescription.

Currently, treatment and prevention of fish trypanosomosis is not available due to the lack of effective drugs or vaccines. Therefore, as we mentioned previously, the most economical and effective strategy, especially for mass-cultured fish, is transmission blocking to prevent pathogen transmission (Zhou et al., 2024). To achieve this, detailed large-scale studies particularly the natural vector(s) and the origine of the pathogen are required in regions where severe outbreaks occur. Important questions need to be addressed which include the identification of potential vectors of transmission and the range of fish species which can be infected or could act as reservoir hosts.

CRediT authorship contribution statement

Zhao-Rong Lun: Writing – review & editing, Visualization, Supervision, Funding acquisition, Formal analysis. **Ju-Feng Wang:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Xin-Tao Li:** Writing – review & editing, Formal analysis, Data curation. **Peng Zhang:** Writing – review & editing, Formal analysis, Data curation. **Li-Wen Xu:** Writing – review & editing, Resources. **Jin-Yong Zhang:** Writing – review & editing, Resources. **Geoff Hide:** Writing – review & editing, Visualization, Formal analysis, Funding acquisition. **De-Hua Lai:** Writing – review & editing, Visualization, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102868.

Data availability

Data will be made available on request.

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