Does the fish-infecting *Trypanosoma micropteri* belong to *Trypanosoma carassii*?

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Abstract: Recently, based on a limited morphological characterisation and partial 18S rRNA gene sequence, Jiang et al. (2019) described *Trypanosoma micropteri* Jiang, Lu, Du, Wang, Hu, Su et Li, 2019 as a new pathogen of farmed fish. Here we provide evidence based on the expanded sequence dataset, morphology and experimental infections that this trypanosome does not warrant the establishment as a new species, because it is conspecific with the long-term known *Trypanosoma carassii* Mitrophanow, 1883, a common haemoflagellate parasite of freshwater fish. The former taxon thus becomes a new junior synonym of *T. carassii*.

Key words: Trypanosomes, 18S rRNA gene, morphological data, host ranges, fish, classification, synonymy

The taxonomy of fish trypanosomes has long been considered complex or even controversial, since most of the species were named mainly based on the new-host new-trypanosome paradigm (Fantham et al. 1942, Mackerras and Mackerras 1961, Lom 1979, Joshi 1982). Therefore, it was suggested already a long time ago that many species of fish trypanosomes might be synonymous (Baker 1960). As a matter of fact, the fast growing sequence data support this notion.

In 2019, an outbreak of trypanosomiasis was recorded in farmed largemouth bass *Micropterus salmoides* (Lacépède) in southern China. The pathogen was reported as a new trypanosome species, namely *Trypanosoma micropteri* Jiang, Lu, Du, Wang, Hu, Su et Li, 2019, with its description based on a limited morphological characterisation and partial 18S rRNA gene sequence (Jiang et al. 2019). However, for reasons detailed below, the establishment of this species, primarily based on morphological features, seems to be unsustainable.

Jiang et al. (2019) performed a comparative analysis of *T. micropteri* with just three species of fish trypanosomes available in Gu et al. (2007) and Grybchuk-Jeremenko et al. (2014). One of the previously described species, *Trypanosoma* sp. pseudobagri (Gu et al. 2007), has a 99.8% sequence identity of its 18S rRNA sequence with *T. micropteri*. Such a very high level of sequence similarity calls for a detailed comparison of morphological features between both flagellates, yet this was not performed. Another closely related fish trypanosome, the MARV strain of *Trypanosoma carassii* Mitrophanow, 1883 (see Gibson et al. 2005), was also evaluated only on the level of the 18S rRNA sequence. Moreover, we have identified two likely erroneous calculations in Jiang et al. (2019); namely, the nucleus width (mean of 1.2 µm derived from a range from 0.7 to 1.8 µm; mean of 1.8 µm, derived from a range from 0.7 to 0.9 µm), and a reversely defined flagellar index.

A freshwater fish trypanosome isolated from *M. salmoides* was maintained in our laboratory (Chen et al. 2022). Tilapia juveniles were bought from Tilapia Breeding Farm of Guangdong Province (Guangzhou, China). Fishes were kept in fish tanks for two weeks before any experiment. Blood from each fish was also examined by microscopy to further confirm they were free from any trypanosome infection. Infected blood was collected and inoculated into healthy fish through a syringe injection into the pericardial cavity. An infection was confirmed by the presence of parasitemia on day 10 post-injection. Biometric data of trypanosomes were conducted using Giemsa-stained smears, and approximately 200 randomly selected flagellates were...
### Table 1. Biometric data from the discussed strains and species of freshwater fish trypanosomes (Trypanosoma spp.).

| Isolates | T. carassii (Woo 1981) | T. micropteri – this study | T. carassii – Gu et al. (2007) | T. carassii – Jiang et al. (2019) | T. micropteri – Letch and Ball (1979) | T. micropteri – Gu et al. (2007) | T. micropteri – Jiang et al. (2019) | T. micropteri – Needham (1969) |
|----------|-----------------------|---------------------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-------------------------------|
| BW       | 3.5–9.5               | 2.4 ± 0.2                 | 0.7–1.1                       | 1.4 ± 0.1                      | 1.2 ± 0.2                       | 1.1 ± 0.0                        | 0.4–2.3                         | 0.8–2.3                        |
| NW       | 4.3–13.5              | 4.5–12.6                  | 3.5–9.5                       | 3.1–9.5                        | 4.1–12.6                       | 3.0–12.6                        | 3.5–12.6                        | 3.2–12.6                       |
| NL       | 3.5–9.5               | 3.3–9.5                   | 2.5–9.5                       | 3.1–9.5                        | 3.1–12.6                       | 2.2–12.6                        | 3.0–12.6                        | 3.2–12.6                       |
| IB       | 3.5–9.5               | 3.3–9.5                   | 2.5–9.5                       | 3.1–9.5                        | 3.1–12.6                       | 2.2–12.6                        | 3.0–12.6                        | 3.2–12.6                       |
| FL       | 3.5–9.5               | 3.3–9.5                   | 2.5–9.5                       | 3.1–9.5                        | 3.1–12.6                       | 2.2–12.6                        | 3.0–12.6                        | 3.2–12.6                       |

### Table 2. The comparison of 18S rRNA sequences among freshwater fish trypanosomes (Trypanosoma spp.), expressed as sequence identities (%), bottom left) and uncorrected pair-wise distances (top right).

| Seq No. | Zhang et al.: T. micropteri MARV | Zhang et al.: T. carassii TRCa | Zhang et al.: (This study) T. carassii | Gu et al. (2007) T. micropteri | Jiang et al. (2019) T. micropteri | Letch and Ball (1979) T. micropteri | Gu et al. (2007) T. micropteri | Needham (1969) T. micropteri |
|---------|---------------------------------|-------------------------------|----------------------------------|-------------------------------|---------------------------------|----------------------------------|-------------------------------|-----------------------------|
| 1        | 1.00                            | 1.00                          | 1.00                             | 0.95                           | 0.92                             | 0.91                              | 0.92                            | 0.90                        |
| 2        | 0.95                            | 1.00                          | 0.99                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 3        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 4        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 5        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 6        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 7        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 8        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 9        | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
| 10       | 0.94                            | 0.99                          | 1.00                             | 0.95                           | 0.93                             | 0.92                              | 0.93                            | 0.91                        |
measured as described previously (Su et al. 2022). Significance was assessed with Z-test at $P < 0.05$, using the following formula:

$$Z = \frac{\text{ABS}(\text{Mean A} - \text{Mean B})}{\sqrt{\text{Standard error A}^2 + \text{Standard error B}^2}},$$

$$P = (1 - \text{NORMSDIST}(Z)) \times 2$$

Total DNA was extracted using the phenol-chloroform method as described elsewhere (Su et al. 2022). The full-length 18S rRNA gene was amplified using the forward (5'-GACTTTGCTTCTCTATTG-3') and reverse primers (5'-CATATGCTTGTTTCAAGGAC-3'). PCR reactions were conducted using the Phanta Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing, China) according to the manufacturer's protocol. PCR cycling parameters were as follows: initial denaturation at 94°C for 3 min followed by 35 cycles at 95°C for 15 s, 61°C for 15 s, 72°C for 2 min, and a final extension at 72°C for 5 min. PCR amplicons were resolved in 1% agarose gel and sequenced by Thermo Fisher Scientific, Guangzhou, China, while additional 18S rRNA gene sequences of freshwater fish trypanosomes were obtained from the GenBank database. Sequences were aligned using Clustal X (Thompson et al. 1997), using default settings and with final manual adjustments. In order to determine the evolutionary distances among the 18S rRNA genes of freshwater fish trypanosomes, we calculated the sequence identities and p-distance by BLAST+ 2.8.1 and MEGA VII.

The neighbor-joining (NJ) and Maximum likelihood (ML) methods were used to create phylogenetic trees by MEGA VII (Kumar et al. 2016) with Kimura 2-parameter model, pairwise deletion for gaps and bootstrap of 1,000 replicates.

It is worth mentioning that the trypanosome in question is a severe pathogen of farmed fish and its correct classification is therefore of importance for the aquaculture industry. Hence, to shed more light on the problem at hand, we have critically reviewed the published data and further investigated the identity of the MARV strain. Since only a 1.5 kb-long region of its 18S rRNA gene is available in the GenBank database (AJ620549), we have completely resequenced the entire gene in question. The newly obtained full-size 2,057 nt-long 18S rRNA gene sequence of the MARV strain (OL963935) allowed us to perform a thorough phylogenetic analysis. Alignment with the original sequence of Jiang et al. (2019) revealed 99.67% identity, the only difference represented by a five nucleotides deletion. A further extended alignment with other 18S rRNA sequences available in the public domain for *T. carassii* (Fig. 1) showed that such deletion was confined to MARV clone 11 (AJ620549) and might likely be an artifact restricted to this clone. Hence, we argue that the newly ob-

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**Fig. 1.** Partial comparison of 18S rRNA sequences from freshwater fish trypanosomes. Left panel: 1 – *Trypanosoma carassii* MARV clone 11 (AJ620549) with a five-nucleotide deletion at position 979; 2 – *T. carassii* MARV (OL963935, this study); 3 – *T. carassii* TrCa (OL963934, this study); 4 – *Trypanosoma micropteri* (OM397104, this study); 5 – *T. micropteri* (EF375883, Gu et al. 2007); 6 – *T. micropteri* (MH635421, Jiang et al. 2019); Right panel: Corresponding sequencing profile from Abi technique for *T. carassii* MARV (OL963935).

**Fig. 2.** Phylogenetic analysis of 18S rRNA gene sequences from fish trypanosomes. 18S rRNA-based phylogenetic tree. Bootstrap values were shown at the nodes with 500 replicates. The numbers at nodes refer to Maximum likelihood/Neighbor joining support values derived from 500 replicates, bar represents 0.005 substitutions per site.
Table 3. Natural and experimental hosts of freshwater fish trypanosomes (*Trypanosoma* spp.).

| Species     | Reference                  | Cypriniformes                  | Perciformes                     | Siluriformes | Esociformes Centrarchiformes Anabantiformes |
|-------------|----------------------------|--------------------------------|---------------------------------|--------------|-------------------------------------------|
| *T. carassii* TrCa | Woo and Black (1984)       | Carassius auratus, Pethia conchonius, Cotostomus commersonii, Loxilus cornutus | Ethostoma caeruleum               | Ameurus nebulosus Esox lucius -            |
| Trypanosoma micropteri | This study                | Carassius auratus*, Misgurnus anguillicaudatus* | -                              | -                                        | Micropterus salmoides Channa argus* |
| *T. micropteri* | Gu et al. (2007)           | -                              | Tachysurus fulvidraco           | -            | -                                         |
| *T. micropteri* | Jiang et al. (2019)        | -                              | -                              | -            | -                                         |
| *T. carassii* EL-CP Gibson et al. (2005) | -                         | -                              | -                              | -            | -                                         |
| Trypanosoma cobitis | Letch et al. (1979)       | Barbatula barbatula, Phoxinus phoxinus, Gobio gobio | -                              | -            | -                                         |
| Trypanosoma sp. ex *Scardinius erythrophthalmus* | Grybchuk-Ieremenko et al. (2014) | -                              | -                              | -            | -                                         |
| Trypanosoma tincae | Needham (1969)            | Tinca tinca, Carassius carassius | -                              | -            | -                                         |

* Experimental host

Morphologically similar full-length 18S rRNA sequence is superior and shall solely be used for further analyses.

Phylogenetic analysis that includes this complete 18S rRNA gene sequence allowed us to identify a 99.95% sequence identity between two strains of *T. carassii*, namely TrCa isolated from *Carassius carassius* (Linnaeus), previously extensively used in experimental infections, and the MARV strain mentioned above (Suppl. Fig. 1) (Woo 1981, Bienek et al. 2002, Kovacevic and Belosevic 2015).

Next, we compared the published data on the morphology of *T. micropteri* and *T. carassii* TrCa, revealing differences in the posterior end to kinetoplast distance, as well as in the body width size and the kinetoplast index (Table 1). However, when subjected to the Z-test, these differences turned out to be statistically insignificant. This is not unexpected when one considers the previously proposed influence of the host on morphological characteristics of fish trypanosomes (Lom 1979, Woo and Black 1984) and the general morphological flexibility of members of the genus *Trypanosoma* Gruby, 1843 (Baker 1960).

Therefore, for comparative purposes, we have isolated a trypanosome from a diseased largemouth bass specimen captured in Foshan, Guangdong Province, China, and sequenced its 18S rRNA gene, which turned out to be 100% identical with *T. micropteri* of Jiang et al. (2019). Morphology of this newly isolated trypanosome (here temporarily called *T. micropteri*) (Table 1) was carried out in a recently developed Nile tilapia infection model (Chen et al. 2022).

Morphological differences in the length of the free flagellum and total cell length were within the ranges provided in previous descriptions of this species (p < 0.05) (Gu et al. 2007, Jiang et al. 2019). Consequently, the slight morphological differences between *T. micropteri* and *T. carassii* (TrCa) can be attributed to different hosts, from which they have been isolated.

It is worth noting that the genetic distances between *T. micropteri* on one side and *T. carassii* TrCa or MARV strains on the other side are smaller than the distances separating different strains of *T. carassii*, namely TrCa and MARV, as well as EL-CP and Ts-Cc-SP isolated from pike *Esox lucius* (Linnaeus) and common carp (*Cyprinus carpio* Linnaeus), respectively (Fig. 2; Table 2). Based on the available 18S rRNA sequences, we have generated Neighbor-joining and Maximum likelihood phylogenetic trees of fish trypanosomes.

Individual strains of *T. carassii* fall into three distinct clades, labeled A, B and C, in accordance with Gibson et al. (2005), where they are mixed with the non-*T. carassii* trypanosomes (Fig. 2). For example, in clade B, *T. carassii* EL-CP branches robustly with another species, namely *Trypanosoma cobitis* Mitrophanov, 1883 (AJ009143), as their 18S rRNAs share 99.9% identity (Table 2). A similar situation occurs in clade A, where *T. carassii* Ts-Cc-SP forms a sister branch to *Trypanosoma tincae* Laveran et Mesnil, 1904 (AJ620553), reflecting their 99.95% sequence identity, while an unnamed *Trypanosoma* sp. ex *Scardinius erythrophthalmus* (Linnaeus) of Grybchuk-Ieremenko (2014) (KJ601720) is their next closest relative.

Since all clades contain at least one strain affiliated with *T. carassii*, we conclude that the analysed dataset makes this species paraphyletic. Indeed, the available data are consistent with the conclusion that *T. carassii* is a umbrella species that actually lumps together several distinct fish trypanosomes. Moreover, the genetic distances between *T. carassii* TrCa and MARV in the clade C, and those between the clades A and B, range from 1.9 to 2.3%, whereas the genetic distance between *T. carassii* TrCa/MARV and *T. micropteri* is only 0.9%.

Smit et al. (2020) proposed a 3% sequence difference in 18S rRNA (only 300 nt-long region covering the hypervariable V7 region was included) as a genetic distance sufficient to distinguish two different genotypes of fish trypanosomes. However, Diaz et al. (2020) used for the same purpose a 1% difference criterion (using a 1.4 kb-long region of 18S rRNA and full-size GAPDH), arguing that the same genetic distance distinguishes strains of *Trypanosoma cruzi* Chagas, 1909. Therefore, we propose that *T. micropteri* is not a valid species but rather a strain (and a new synonym) of *T. carassii*.  

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Another important criterion for the identification of a new fish trypanosome species is its host range. *Trypanosoma micropteri* was recorded from *Tachysurus fulvidraco* Richardson (Siluriformes) and *M. salmoides* Lacépède (Centrarchiformes) (Gu et al. 2007, Jiang et al. 2019), which are not closely related with the wide range of cypriniform fish parasitised by *T. carassii*. Thus, we have performed a set of experimental infections in the laboratory, in which we succeeded in introducing *micropteri* from *M. salmoides* (Centrarchiformes) into *Carassius auratus* (Cypriniformes) (Table 3). Therefore, it is quite likely that the host ranges of *micropteri* and *carassii* overlap in nature, further supporting the notion that the former is in fact a strain of *carassii* known to have a wide host range. An extensive parasitological examination and set of experimental infection will be needed to confirm or disprove this scenario.

In conclusion, since several lines of evidence questioned the species status of *micropteri*, we consider it highly likely that this is actually a strain of *carassii*. Moreover, we have also shown that *carassii*, which is likely cosmopolitan and infects a wide range of freshwater fish hosts, seems to represent a complex of more species and its taxonomy will require an extensive revision.

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