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High prevalence of trypanosome co-infections in freshwater fishes

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Abstract: One thousand three hundred seventy three fish specimens of eight different species from the vicinity of Kyiv, Ukraine, were examined for the presence of trypanosomes and 921 individuals were found to be infected. The prevalence of infection ranged from 24% in freshwater bream, *Abramis brama* (Linnaeus), to 100 % in spined loach, *Cobitis* '*taenia*' Linnaeus. The level of parasitaemia also varied significantly between generally mild infections in pikeperch, *Sander lucioperca* (Linnaeus), and heavy ones in *C.* '*taenia*'. In most cases the infections with trypanosomes were asymptomatic. Cases of co-infection with species of *Trypanoplasma* Laveran et Mesnil, 1901 were documented for five out of eight examined host species. Molecular analysis of the 18S rDNA sequences revealed that four hosts, namely northern pike, *Esox lucius* Linnaeus, freshwater bream, spined loach and European perch, *Perca fluviatilis* Linnaeus, were simultaneously infected with two different trypanosome species. Our findings advocate the view that to avoid the risk posed by mixed infections, subsequent molecular taxonomic studies should be performed on clonal lines derived from laboratory cultures of fish trypanosomes.

Keywords: *Trypanosoma*, blood parasites, mixed infections, phylogeny, 18S rRNA, teleosts

Flagellates of the genus *Trypanosoma* Gruby, 1843 are common blood parasites of vertebrates. Some species of this genus such as *Trypanosoma brucei* Plimmer et Bradford, 1899 and *T. cruzi* Chagas, 1909 are of special medical importance because they cause severe diseases in humans (Holmes 2013, Nunes et al. 2013). Therefore, main research efforts have been focused on these parasites. Species affecting livestock [e.g. *T. vivax* Ziemann, 1905, *T. equiperdum* Doflein, 1901 and *T. evansi* (Steel, 1885) causing nagana in cattle, dourine in equines and surra in camels, respectively] account for significant economic losses and therefore were also studied rather extensively (Holzmuller et al. 2013).

Meanwhile, much less attention was devoted to trypanosomes parasitising fish and birds, whereas those found in other host groups, such as amphibians and reptiles, can be considered as neglected. Historically, fish trypanosomes were among the first described species of this genus (Lom 1979). They are of practical importance as many of them infect farmed fish and have noticeable economic impact (Woo 2006), as is the case of infections reaching 75 to 100% prevalence in juvenile farmed carp (Overath et al. 1998). It was demonstrated that due to the stressful conditions, fish cultivated in farms or laboratory specimens are more susceptible to infections and have higher mortality level compared to those living under natural conditions (Lom 1979). Pathological manifestations of *Trypanosoma* infections in wild populations are relatively mild and usually limited to hypocholesterolemia, anemia and anorexia (Tandon and Chandra 1977, Khan 1985).

Despite the long history of studying piscine trypanosomes, the taxonomy of these leech-transmitted parasites remains obsolete. Similar to monoxenous trypanosomatids (Maslov et al. 2013), these flagellates were assumed to be host-specific. This led to descriptions of numerous trypanosome species from different hosts on the basis of the 'one host – one parasite' paradigm. According to the "Catalogue of world fauna of Trypanosomatidae", there were 177 described species as well as 35 undescribed isolates of fish trypanosomes by 1990 (Podlipaev 1990). Since then this number did not change significantly. A series of elegant cross-infection experiments refuted the concept of strict host specificity for fish parasites leading

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to the conclusion that the number of species of these flagellates is greatly overestimated (Lom 1973, Letch 1979, Woo and Black 1984).

Traditional taxonomy of the Trypanosomatidae Doflein, 1901 used to rely on two criteria: (a) morphological features, such as cell shape, relative positions of the nucleus and kinetoplast (= mitochondrial DNA), etc.; and (b) host specificity (Hoare 1966, Wallace 1966). The issue of host specificity in fish trypanosomes was discussed above. As for the morphological characteristics, these were also considered to be of limited reliability because of extensive pleomorphism, i.e. changes of many morphological characters during development (Letch 1979, Gibson et al. 2005). While this phenomenon significantly complicates the comparison of species by morphological traits only, molecular taxonomy has a potential to solve this problem.

Different molecular markers such as conserved region(s) of kinetoplast minicircles (Baron et al. 1995, Kolesnikov et al. 1995, Yurchenko et al. 2000), as well as genes encoding 12S rRNA (Figueroa et al. 1999), 18S rRNA (Stevens et al. 2001, Gibson et al. 2005) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) (Hamilton et al. 2004) were considered suitable for this purpose. However, since the 18S rRNA gene proved to produce best results in terms of tree resolution, it became the gold standard for phylogenetic inference in studies of piscine trypanosomes. In these reconstructions, they represent a monophyletic group that is further subdivided into two clades corresponding to the freshwater and marine habitats of their hosts (Gibson et al. 2005).

Trypanosomes of other aquatic vertebrates that are also transmitted by leeches represent their closest relatives (Jakes et al. 2001, Martin et al. 2002). Another molecular marker routinely used for inferring relationships between closely related species of the Trypanosomatidae is the variable region of the spliced leader (SL) RNA gene (Yurchenko et al. 2006a, 2008, 2014, Jirků et al. 2012, Votýpka et al. 2012). In contrast to the subfamily Leishmaniinae Maslov et Lukeš, 2012, in trypanosomes this marker was found to be too variable for informative comparison (Gibson et al. 2000).

Cases of co-infections with fish trypanosomes and parasitic parabodonids of the genus *Trypanoplasma* Laveran et Mesnil, 1901 are well documented (Lom 1979, Burreson 2007). Usually, infections with *Trypanoplasma borreli* Laveran et Mesnil, 1901 reach higher levels of parasitaemia, as compared to infections with *Trypanosoma carassii* Mitrophanow, 1883. Interestingly, in mixed infections by these flagellates, parasitaemia was reduced and the survival rate was higher as compared to the infections with *T. borreli* alone. This unexpected phenomenon was explained by the production of the cross-reactive protective antibodies (Joerink et al. 2007). To date nothing is known about fish co-infected with two or more different *Trypanosoma* species. Two genotypes (potentially two different species) of trypanosomes were detected in samples originated from the mochokid catfish *Synodontis nigromaculatus* Boulenger captured in Botswana. Yet only monospecific infections were observed in individual hosts (Davies et al. 2005).

In this work we investigated the diversity and host-parasite relationships of trypanosomes of freshwater fishes in eastern Europe and uncovered several cases of mixed infections. We conclude that to obtain a reliable and trustworthy data on trypanosomatid parasites of fish, researchers should establish clonal cell lines and avoid working with populations potentially containing more than one *Trypanosoma* species.

MATERIALS AND METHODS

Trypanosomes from eight freshwater fish species, northern pike *Esox lucius* Linnaeus, crucian carp *Carassius carassius* (Linnaeus), freshwater bream *Abramis brama* (Linnaeus), common rudd *Scardinius erythrophthalmus* (Linnaeus), spined loach *Cobitis* '*taenia'* Linnaeus, wels catfish *Silurus glanis* Linnaeus, pikeperch *Sander lucioperca* (Linnaeus) and European perch *Perca fluviatilis* Linnaeus collected in the vicinity of Kyiv, Ukraine, were analysed (Table 1). Fish specimens were between 0.5 and 5 years old. The presence of trypanosomes in the blood and the level of parasitaemia were established on smears and recalculated per millilitre of blood as described before (Losev and Ovcharenko 2004). The 'seasonal' parasitaemia and infection prevalence were assayed in the following conditions: water temperature rising from +5 °C to +17 °C (spring); water temperature between +17 °C and +28 °C (summer); water temperature falling from +17 °C to +7 °C (autumn). The smears containing trypanosomes were fixed with methanol, stained with Giemsa and used for subsequent morphological study of these parasites as described elsewhere (Yurchenko et al. 2008). All measurements were taken on Giemsa-stained samples and are expressed in micrometres (μm).

For molecular phylogenetic studies we selected several trypanosome-positive host specimens with medium-to-high parasitaemia levels (in most cases, one representative for each fish species). Parasites were isolated from fresh blood using a protocol described elsewhere (Losev and Ovcharenko 2004). Concentrated cells were stored in SDS-EDTA solution (Votýpka et al. 2013). Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany).

PCR, cloning and sequencing

Two pairs of trypanosomatid-specific primers producing PCR amplicons that represent almost the whole 18S rRNA gene and have ~600 bp overlap, thus preventing chimera formation, were used. The first pair of primers was SSUdir (5'-CATATGCTTGTTTCAAGGAC-3') and A757 (5'-GCGAACGTACTCCCCCCTGA-3'), the second one consisted of oligonucleotides 883F (5'-GACTTGAATTAGMAAGCATGGGA-3') and SSUrev (5'-GACTTTTGCTTCCTCTAWTG-3'). The amplification conditions were adopted from the previously published work (Maslov et al. 1996) with the following modifications of the cycling parameters: 94 °C for 15 s, at 55 °C for 20 s and at 72 °C for 1 min 30 s. All PCR products were cloned using InsTAclone PCR Cloning Kit (Thermo Fisher Scientific,

Table 1. Summarised parameters of fish infections with trypanosomes

Fish host	Years collected	GPS of localities	Prevalence	Parasitaemia per ml
*Esox lucius** Linnaeus (n = 133)	2006–2013	50°39'04"N; 30°40'09"E 50°36'44"N; 30°37'59"E 50°28'09"N; 30°32'40"E	73 %	3 740 ± 1 075 (1 020–15 640)
*Carassius carassius** (Linnaeus) (n = 157)	2009–2013	50°53'24"N; 30°25'45"E 50°28'09"N; 30°32'40"E	82 %	3 966 ± 1 257 (1 020–13 940)
*Abramis brama** (Linnaeus) (n = 172)	2009–2013	50°53'24"N; 30°25'45"E 50°06'47"N; 30°52'18"E	24 %	3 910 ± 724 (680–7 820)
*Scardinius erythrophthalmus** (Linnaeus) (n = 226)	2006–2013	50°57'38"N; 30°50'06"E 50°39'04"N; 30°40'09"E 50°36'44"N; 30°37'59"E	38 %	1 408 ± 614 (340–3 740)
Cobitis '*taenia*'* Linnaeus (n = 398)	2006–2013	51°03'12"N; 30°51'18"E 50°36'44"N; 30°37'59"E 50°28'09"N; 30°32'40"E 51°09'06"N; 30°54'26"E	100 %	6 905 ± 2 442 (1 700–34 000)
Silurus glanis Linnaeus (n = 41)	2013	50°53'24"N; 30°25'45"E	42 %	1 335 ± 300 (680–3 060)
Sander lucioperca (Linnaeus) (n = 28)	2013	50°53'24"N; 30°25'45"E 50°06'47"N; 30°52'18"E	32 %	453 ± 160 (340–680)
Perca fluviatilis Linnaeus (n = 218)	2007–2013	50°39'04"N; 30°40'09"E 50°36'44"N; 30°37'59"E 50°28'09"N; 30°32'40"E	67 %	4 055 ± 1 007 (1 700–13 600)

* found co-infected with *Trypanoplasma* sp.

Waltham, USA) and sequenced. The GenBank accession numbers for the new sequences determined in the course of this work are: KJ601712 [*Trypanosoma* sp. from *Abramis brama* (1-1)], KJ601713 [*Trypanosoma* sp. from *A. brama* (1-2)], KJ601714 [*Trypanosoma* sp. from *Esox lucius*), KJ601715 (*Trypanosoma* sp. from *Carassius carassius*), KJ601716 [*Trypanosoma* sp. from *A. brama* (2-1)], KJ601717 [*Trypanosoma* sp. from *A. brama* (2-2)], KJ601718 (*Trypanosoma* sp. from *Scardinius erythrophthalmus*), KJ601719 [*Trypanosoma* sp. from *Cobitis* '*taenia*' (1)], KJ601720 [*Trypanosoma* sp. from *C.* '*taenia'* (2)], KJ601721 [*Trypanosoma* sp. from *Silurus glanis* (1)], KJ601722 [*Trypanosoma* sp. from *S. glanis* (2)], KJ601723 (*Trypanosoma* sp. from *Sander lucioperca*), KJ601724 [*Trypanosoma* sp. from *Perca fluviatilis* (1)], KJ601724 [*Trypanosoma* sp. from *P. fluviatilis* (2)].

The obtained 18S rDNA sequences together with those taken from Genbank were aligned using Muscle 3.8.31 (Edgar 2004) and following manual refinement using the BioEdit sequence alignment editor (Hall 1999), ambiguously aligned positions were removed. The resulting alignment of 54 sequences contained 1 419 nucleotide positions. Akaike information criterion was used to select evolutionary model for this dataset in jModeltest 2.1.4 (Darriba et al. 2012). The selected model (TIM3ef + I + G) with 5 gamma categories was then applied for inferring maximum likelihood in PhyML 3.1 (Guindon et al. 2010). Heuristic search was performed using SPR branch swapping algorithm. Statistical support of bipartitions was assessed with the use of bootstrap resampling (1 000 replicas). Bayesian inference of phylogeny was accomplished in MrBayes 3.2.2 (Ronquist et al. 2012) with analysis run for 5 million generations under GTR + I + G model (5 categories) and sampling every 1 000 generation. Other parameters were left in their default states.

RESULTS

Morphology and infection of fish trypanosomes

While we believe that the morphology of the Trypanosomatidae should not be used as a sole taxonomical feature (Yurchenko et al. 2006b, 2008, 2009, Maslov et al. 2010, Votýpka et al. 2010), to comply with established practices, here we present a brief morphological description of the most prevalent morphotypes detected in different fish species. Detailed size characteristics are provided in Table 2. We do this with the understanding that accounts provided below may not correlate with the trypanosomes present as minor fractions in the mixed infection (see below). We are also convinced that without clonal isolates available, their relationships with other species cannot be established with any certainty.

Trypanosoma sp. from *Esox lucius* Fig. 1A

Morphological description. Cell body narrow with pointed ends, lined by well-developed undulating membrane; its width comparable to that of cell body. Kinetoplast prominent in Giemsa stained smears. Nucleus oval, stretched along longitudinal body axis and located in anterior half of cell. Nucleus length approximately twice as much as its width. Free flagellum usually not longer than quarter of body length.

Infection rate. The prevalence of infection was 73% (83%, 66% and 86% in the spring, summer, and autumn, respectively). The degree of parasitaemia ranges between 1 020 and 15 640 cells/ml of blood. Co-infections with trypanoplasmes were detected in 80–90% cases. In the blood smears, the ratio between trypanosomes and trypanoplasmes varied between approximately 3 : 1 in the spring and 3 : 2 in the autumn. Both males and females were found to be infected at about the same level.

Remark. Morphological description of *Trypanosoma* sp. from *E. lucius* broadly corresponds to that of *Trypanosoma remaki* Laveran et Mesnil, 1901 from the same host species.

Table 2. Morphometric characteristics of studied piscine trypanosomes, all measurements in micrometres (μm).

Parasite	Length	Width	N–A*	K–N*	P–K*	Free flagellum
Trypanosoma sp. ex *Esox lucius* (n = 76)	31.9 ± 3.7 (28.7−39.7)	2.2 ± 0.4 (1.4−3.9)	12.7 ± 2.1 (8.9−18.4)	16.4 ± 1.9 (13.9−20.5)	1.6 ± 0.4 (1.2−1.8)	7.5 ± 2.3 (2.8−14.0)
Trypanosoma sp. ex *Carassius carassius* (n = 332)	27.6 ± 1.3 (20.7−31.5)	1.6 ± 0.1 (1.4−1.7)	13.5 ± 0.8 (11.9−15.0)	12.9 ± 0.3 (7.5−15.4)	1.3 ± 0.1 (1.1−1.5)	8.4 ± 0.7 (7.7−22.0)
Trypanosoma sp. ex *Abramis brama* (n = 116)	27.6 ± 1.7 (20.2−32.1)	1.5 ± 0.1 (1.4−2.4)	13.6 ± 0.7 (10.2−16.0)	12.4 ± 0.5 (9.3−16.5)	1.4 ± 0.5 (0.7−2.2)	9.1 ± 0.9 (7.7−18.0)
Trypanosoma sp. ex *Scardinius erythrophthalmus* (n = 132)	27.8 ± 1.3 (22.9−30.0)	1.6 ± 0.2 (1.4−1.8)	13.5 ± 0.8 (9.6−14.9)	12.9 ± 0.4 (10.2−13.6)	1.4 ± 0.1 (1.1−1.5)	8.4 ± 0.7 (4.5−11.4)
Trypanosoma sp. ex *Cobitis* '*taenia*' (n = 500)	35.1 ± 2.6 (26.4−39.7)	2.0 ± 0.3 (1.1−2.4)	18 ± 2.9 (11.9−23.2)	15.5 ± 1.7 (10.0−17.5)	1.5 ± 0.5 (1.0−2.0)	8.4 ± 3.5 (10.7−21.2)
Trypanosoma sp. ex *Silurus glanis* (n = 107)	37.2 ± 0.4 (26.5−48.2)	1.3 ± 0.4 (1.2−1.5)	14.8 ± 0.5 (14.1−25.3)	16.7 ± 1.1 (12.3−23.4)	1.0 ± 0.2 (0.7−1.4)	3.2 ± 0.7 (2.0−4.5)
Trypanosoma sp. ex *Sander lucioperca* (n = 28)	26.8 ± 4.3 (18.4−33.8)	3.5 ± 1.8 (1.5−5.5)	12.1 ± 1.3 (7.1−15.4)	13.8 ± 2.5 (11.0−16.9)	0.8 ± 0.4 (0.3−1.5)	4.3 ± 2.0 (0−10.4)
Trypanosoma sp. ex *Perca fluviatilis* (slender) (n = 150)	31.3 ± 3.1 (27.7−34.5)	3.5±3.5 (3.1−7.0)	14.6± 2.4 (12.9−15.5)	17.2 ± 1.6 (13.9−22.1)	1.4 ± 0.5 (1.7−1.9)	8.2 ± 3.2 (0−11.5)
Trypanosoma sp. ex *Perca fluviatilis* (stumpy) (n = 157)	31.2 ± 3.3 (26.9−35.5)	6.2 ± 2.0 (4.8−9.6)	12.3 ± 2.8 (9.0−15.2)	14.7 ± 2.0 (12.5−17.3)	1.7 ± 1.2 (0.4−3.0)	4.3 ± 2.5 (0−6.8)

* N–A – a distance between the nucleus and the anterior end of the cell; K–N – a distance between the nucleus and the kinetoplast; P–K – a distance between the kinetoplast and the posterior end of the cell.

Trypanosoma* sp. from *Carassius carassius Fig. 1B

Morphological description. Cell body slim, without conspicuous thickening, often twisted. Posterior end pointed, anterior thinned out. Undulating membrane well developed; its width equal or wider than that of cell body. Kinetoplast well stained and situated close to anterior end. Nucleus oval, located in central part of cell, oriented lengthwise. Free flagellum extends to about half of cell body length.

Infection rate. The prevalence of infection 81% (45%, 67% and 92% in the spring, summer, and autumn, respectively). The degree of parasitaemia varies between 1 020 and 13 940 cells/ml of blood. Co-infections with trypanoplasmes were detected in up to 90% cases. The ratio between trypanosomes and trypanoplasmes varied between approximately 3 : 1 and 6 : 1. Both males and females were found to be infected at about the same level.

Remark. Morphological description of *Trypanosoma* sp. from *C. carassius* broadly corresponds to that of *Trypanosoma carassii* from the same host species.

Trypanosoma* sp. from *Abramis brama Fig. 1C

Morphological description. Cell body elongated, twisted without pronounced thickening. Kinetoplast located close to anterior end of cell. Undulating membrane well developed, but narrower compared to cell body. Nucleus oval, situated in middle of cell. Free flagellum of same length or slightly longer than cell body.

Infection rate. The prevalence was relatively low, reaching 24% (22%, 23% and 25% in the spring, summer and autumn, respectively). The degree of parasitaemia varies between 680 and 7 820 cells/ml of blood. In *Abramis brama*, co-infections with trypanoplasmes were detected in about 20–25% cases and the ratio between these flagellates varied between 1 : 6 and 1 : 8. Co-infected specimens were significantly smaller in size compared to those of the same age but free of infection or specimen positive only for trypanosomes (data not shown). No differences concerning sex ratio imbalance were observed.

Remark. Morphological description of *Trypanosoma* sp. from *A. brama* broadly corresponds to that of *Trypanosoma abramidis* Laveran et Mesnil 1904 from the same host species.

Trypanosoma* sp. from *Scardinius erythrophthalmus Fig. 1D

Morphological description. Cell body elongated with no visible thickening, pointed at both ends. Kinetoplast located close to anterior end, whereas oval nucleus stretched along longitudinal axis of cell body. Width of prominent undulating membrane comparable to that of cell. Length of free flagellum varies from one third to one half of cell length.

Infection rate. The prevalence of infection is 38% (19%, 25% and 47% in the spring, summer and autumn, respectively). As compared to other host species, degree of parasitaemia is low, varying between 340 and 3 740 cells/ml. Co-infections of trypanosomes and trypanoplasmes (approximate ratio of 2 : 3) were encountered in up to 90% of specimens. Both males and females were found to be infected at about the same level.

Remark. Morphological description of *Trypanosoma* sp. from *S. erythrophthalmus* broadly corresponds to that of *Trypanosoma scardinii* Brumpt, 1906 from the same host species.

***Trypanosoma* sp. from *Cobitis* '*taenia*'** Fig. 1E

Morphological description. Cell body twisted with no visible widening, tapered at posterior and narrowed at anterior end. Cytoplasm intensely stained. Undulating

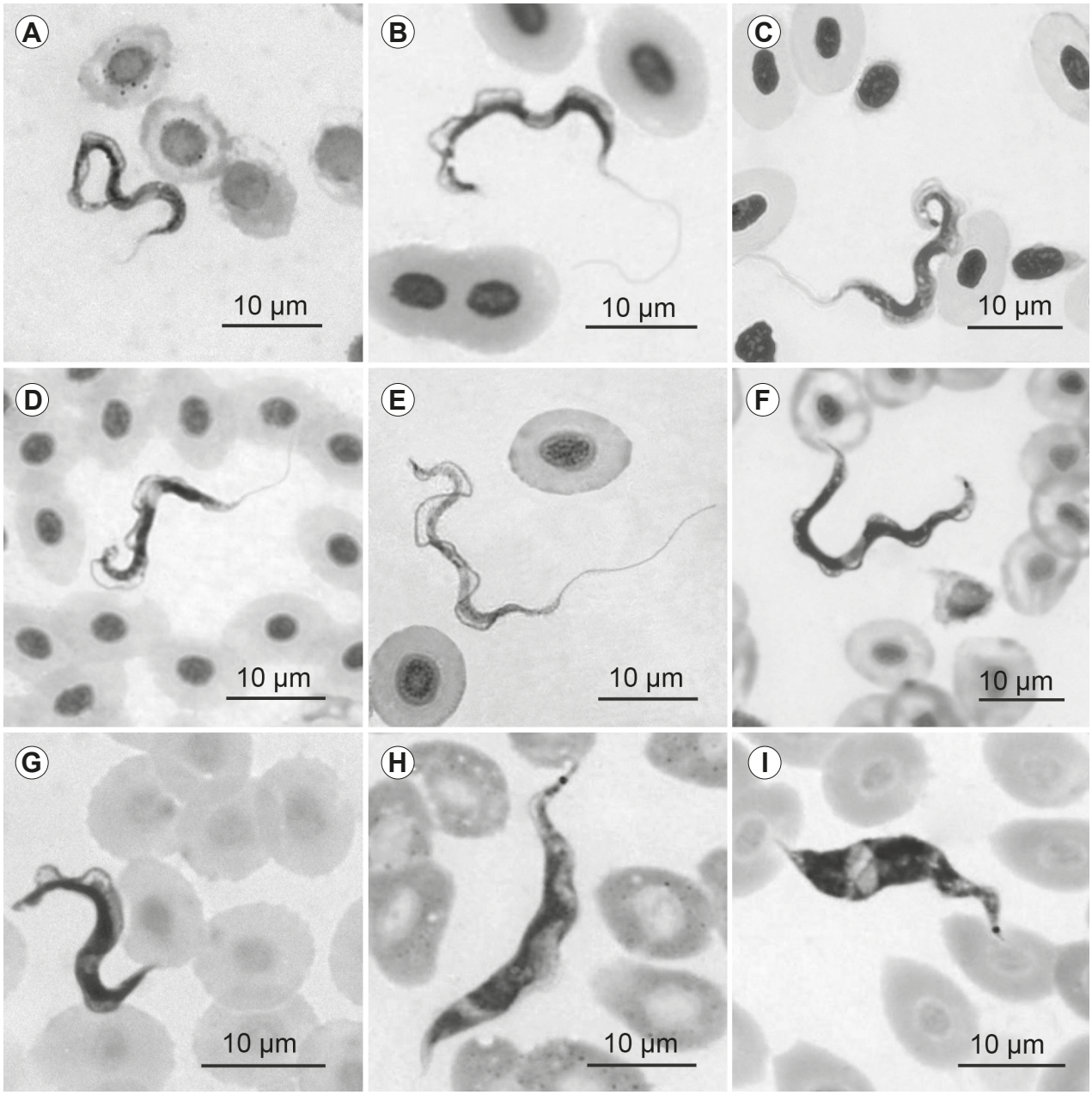

Fig. 1. Major morphotypes of piscine trypanosomes. **A** – *Trypanosoma* sp. from *Esox lucius*; **B** – *Trypanosoma* sp. from *Carassius carassius*; **C** – *Trypanosoma* sp. from *Abramis brama*; **D** – *Trypanosoma* sp. from *Scardinius erythrophthalmus*; **E** – *Trypanosoma* sp. from *Cobitis* '*taenia*'; **F** – *Trypanosoma* sp. from *Silurus glanis*; **G** – *Trypanosoma* sp. from *Sander lucioperca*; **H** – *Trypanosoma* sp. from *Perca fluviatilis* (slender); **I** – *Trypanosoma* sp. from *Perca fluviatilis* (stumpy).

membrane conspicuous, wider than cell body. Kinetoplast relatively small, situated close to anterior end. Nucleus oval, located in posterior part of cell and occupies its full width. Free flagellum reaches half of cell body length.

Infection rate. All examined spined loach were infected, with usually extremely high parasitaemia, varying from 1 700 to over 34 000 cells/ml. All specimens were co-infected with trypanoplasmes, present in the ratio of approximately 1 : 10, with no differences noted between host sexes.

Remark. Morphological description of *Trypanosoma* sp. from *C.* '*taenia*' broadly corresponds to that of *Trypanosoma cobitis* Mitrophanov, 1883 (syn. *T. barbatulae* Léger, 1904) from the same host species. However, these data must be considered cautiously because previous identifications of *C.* '*taenia*' were often incorrect as other morphologically similar species occur in Europe.

Trypanosoma* sp. from *Silurus glanis Fig. 1F

Morphological description. Cell body narrow with cytoplasm always well stained with Giemsa. Posterior end sharply pointed, anterior one blunt. Undulating membrane well discernible, but narrow. Kinetoplast of medium size found near anterior end of cell. Nucleus quite large, elongated, with length/width ratio of 2.5 : 1. Free flagellum very short.

Infection rate. The prevalence of infection reached 41%, with most parasitaemias being low (680 and 3 060 cells/ml). Both males and females found to be infected at about the same level.

Remark. Morphological description of *Trypanosoma* sp. from *S. glanis* broadly corresponds to that of *Trypanosoma markewitschi* Salewskaja-Schapowal, 1950 from the same host species.

Trypanosoma* sp. from *Sander lucioperca Fig. 1G

Morphological description. Cell body elongated, broadened in central and anterior half, with anterior end rounded and posterior one tapered. Cytoplasm intensely stained. Kinetoplast very close to anterior end, round nucleus located in posterior part of cell. Free flagellum very short undulating membrane moderately developed, in some cells both hardly discernible.

Infection. The infection was found in 32% zanders, and was characterised by very low parasitaemia, varying from 340 to 680 cells/ml. The rate of infection was similar for both sexes. No co-infection with trypanoplasmes was documented.

Remark. Morphological description of *Trypanosoma* sp. from *S. lucioperca* broadly corresponds to that of *Trypanosoma lucioperca* Nikitin, 1929 from the same host species.

Trypanosoma* sp. from *Perca fluviatilis Fig. 1H,I

Morphological description. There were two different morphotypes with same type of kinetoplast, found alongside in blood, easily distinguishable based on cell morphology. First morphotype (stumpy) characterised by sacciform cells, significantly widened in middle and posterior parts, tapered at both ends and often twisted in anterior half. Undulating membrane not discernible in most cases. Nucleus oval, but unlike that in other trypanosome species oriented transversally. Free flagellum very short and sometimes indiscernible. Second morphotype (slender) represented by fusiform cells with pointed ends. Nucleus round, situated in posterior half of cell. Undulating membrane of short free flagellum narrow and hardly visible.

Infection rate. The prevalence of infection ranged from moderate to high, reaching 67% (88%, 57% and 94% in the spring, summer and autumn, respectively) and so is degree of parasitaemia, which ranges from 1 700 to 13 600 cells/ml. No bias in the sex of the host was observed. Interestingly, the ratio between different morphotypes was seasonal. Sacciform trypanosomes were dominant in the spring, representing 70% to 90% of flagellates, while the fusiform-shaped cells predominated (85–95%) in the autumn. We could not determine whether these two morphotypes corresponded to two different *Trypanosoma* spp. (also see below) or were two non-overlapping pleomorphic forms of the same species. No co-infection with trypanoplasmes was documented.

Remark. Morphological description of *Trypanosoma* sp. from *P. fluviatilis* broadly corresponds to that of *Trypanosoma percae* Brumpt, 1906 from the same host species.

In our analysis we noticed variability of the infection prevalence ranging from 24 % in freshwater bream to 100 % in spined loach. With a single exception of spined loach, the infection prevalence was seasonal, peaking in the autumn, and age-dependent, reaching maximum in the young (1–2 years old) fishes. The level of parasitaemia also varied significantly between generally mild infections in pikeperch and heavy ones in spined loach.

Phylogenetic analysis

The trees inferred using both maximum likelihood and Bayesian approaches were generally congruent, with minor differences in groups with low support (Fig. 2). We were able to recover all the main clades (e.g. freshwater fish-, marine fish-, amphibian- and 'other aquatic' trypanosomes) described in previous reports (Gibson et al. 2005, Hamilton et al. 2005), although now in an extended form. Amphibian trypanosomes appeared to be paraphyletic in our trees due to the distinct position of *T. chattoni* Mathis et Léger, 1911, although this grouping was not statistically significant. Other clades including the rest of amphibian parasites had high support in both phylogenetic reconstructions.

Freshwater fish trypanosomes investigated herein appeared to be a sister clade of trypanosomes parasitising marine fish. This branching was supported by high posterior probability and moderate bootstrap values. Meanwhile, most subgroups within this clade had no significant statistical support, probably due to the small genetic distances and short length of some sequences used in phylogenetic reconstructions.

For each of the eight putatively different *Trypanosoma* spp., one to two clones of the 18S rRNA gene were sequenced. Surprisingly, none of them matched any of the previously reported ones. The smallest distance between two sequences in our set was 0.16%, which is still more than the distance between *T. brucei brucei* and its closely related species *T. evansi* and *T. equiperdum* (see Stevens et al. 1999). Therefore, it is reasonable to treat all 18S rRNA sequences obtained in our study as representatives of separate species of fish trypanosomes. Importantly, in the case of *A. brama*, four putative species were encountered, as the differences between their sequences ranged from 0.17 to 3.17% from two independent biological samples.

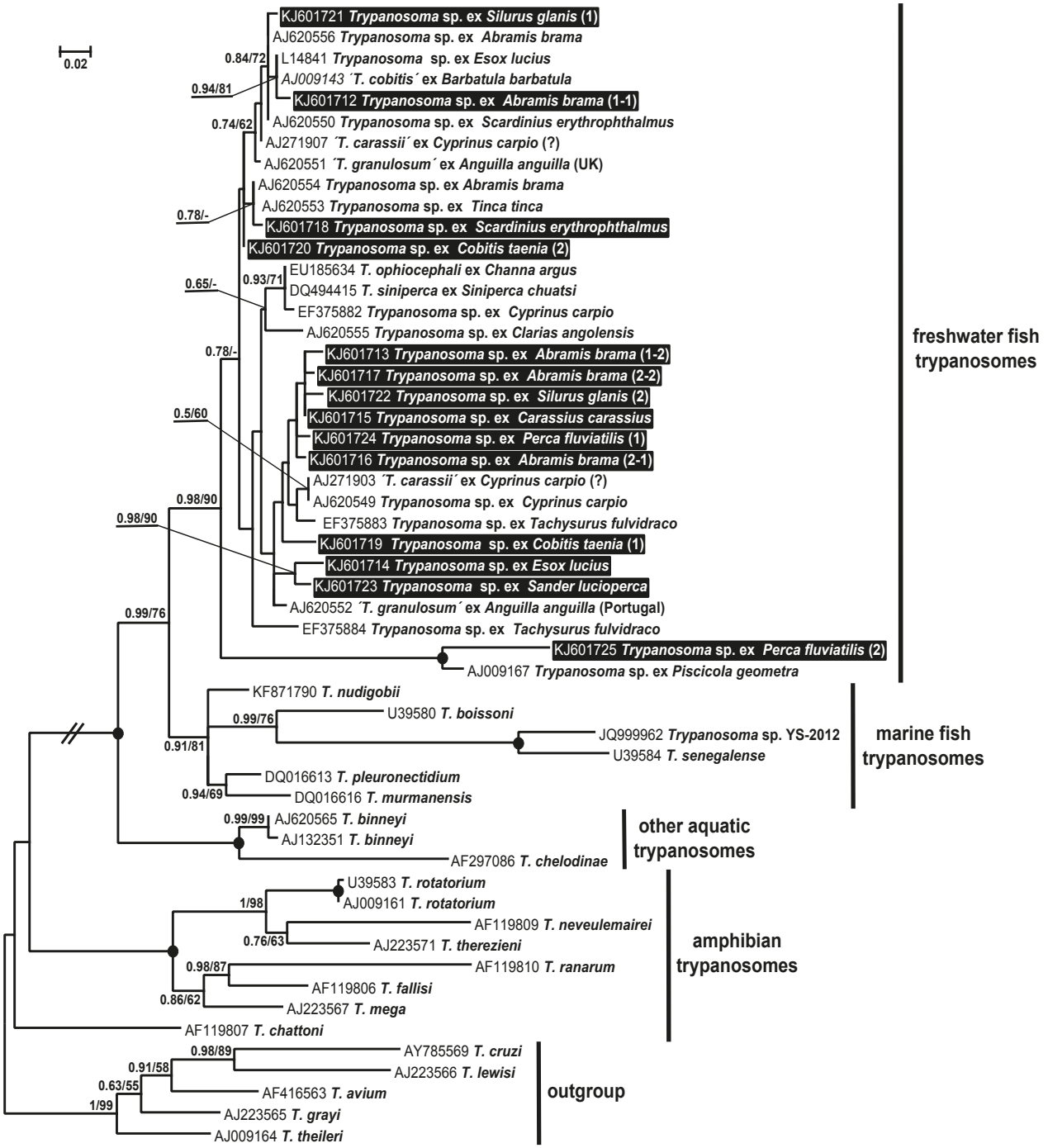

Fig. 2. 18S rRNA-based Bayesian phylogenetic tree of piscine trypanosomes. Sequences determined in the present paper are highlighted. Names of isolates and their respective GenBank accession numbers are indicated for all sequences used. Bootstrap values from Bayesian posterior probabilities (5 million generations) and maximum likelihood (1 000 replicates) are shown at the nodes. Dashes indicate bootstrap support below 50% or Bayesian posterior probability below 0.5 or different topology. Black dots represent 100% bootstrap support and Bayesian posterior probability of 1.0. The tree was rooted with five *Trypanosoma* spp. sequences. The scale bar denotes the number of substitutions per site. Sequence alignment is available from authors upon request. Single numbers in parentheses correspond to molecular clones. In the cases of numbers separated by dashes, they indicate samples and molecular clones, respectively.

One of these species (*Trypanosoma* sp. from *A. brama* 1-1) reliably grouped together with trypanosomes from *E. lucius* and *Barbatula barbatula* Linnaeus, while the others (*Trypanosoma* sp. from *A. brama* 1-2, 2-1 and 2-2) formed a monophyletic group with flagellates from

S. glanis, *C. carassius* and *P. fluviatilis* (Fig. 2). However, the statistical support for this clade was low.

One *Trypanosoma* species from the European perch (*Trypanosoma* sp. from *P. fluviatilis* 2) deserves special attention. Phylogenetic analysis revealed it to be the most distant representative of the investigated set (10.8% difference) it consistently grouped with *Trypanosoma* sp. isolated from the leech *Piscicola geometra* Linnaeus, 1761, thus constituting basal branch of all freshwater fish trypanosomes (Stevens et al. 1999).

Interestingly, we noticed that one of the sequences deposited in GenBank (accession number EF375884) represents a chimera of the gene fragments originating from *Trypanosoma* sp. and *Trypanoplasma* sp. of the yellow catfish *Tachysurus fulvidraco* (Richardson) (Gu et al. 2007). In the tree presented in Fig. 2 we used only its part apparently derived from *Trypanosoma* sp. As noted above, given relatively high frequency of co-infections of trypanosomes and trypanoplasmes in freshwater fish (Saeij et al. 2002), special attention is needed to avoid such artefacts.

DISCUSSION

Thus far all attempts to investigate the diversity of piscine trypanosomes were performed either by (re)-description of particular species on the basis of morphological and limited molecular data, or by a sequence-only based approach, which completely ignored morphological traits (Davies et al. 2005, Gibson et al. 2005, Gu et al. 2007). In the current study we intended to avoid limits of the above-mentioned approaches by complementing morphological descriptions of trypanosomes in *ex vivo* blood smears with molecular data on a large scale. In doing so, we have faced a rather unanticipated situation: four out of nine of examined samples contained more than one trypanosome 18S rRNA haplotype, which strongly indicates mixed infections.

To the best of our knowledge, infections of fish with more than one trypanosome species have not been recognised so far. In most cases, the authors chose to explain morphological differences by implying (extensive) pleomorphism, a feature typical for almost all kinetoplastid flagellates (Woo 2006). Due to unambiguous morphological differences, the simultaneous presence of trypanosomes and trypanoplasmes in fish blood was recognised long time ago (Lom 1979) and we encountered it in some samples as well (Table 1). However, having in mind the inherent variability of trypanosomes, the co-existence of two or more different trypanosome species within one fish host, proposed solely on morphological basis (Khaibulaev and Shulman 1984), remained a pure speculation.

In four cases (*Trypanosoma* spp. from *S. glanis*, *A. brama*, *C.* '*taenia*' and *P. fluviatilis*), the 18S rRNA sequences derived from a single blood sample unambiguously contained two different trypanosome species, as the obtained sequence differences exceeded those among other well-defined *Trypanosoma* spp. parasitising warm-blooded vertebrates (Stevens et al. 1999). However, in the absence of cross-infection experiments, as well as deeper knowledge about the 18S rRNA sequence variation in piscine trypanosomes, this assignment still remains somewhat arbitrary. As often in research on trypanosomatids, morphology proved not to be of much help (Yurchenko et al. 2008, Schmid-Hempel and Tognazzo 2010, Votýpka et al. 2010, Teixeira et al. 2011, Borghesan et al. 2013, Maslov et al. 2013). With the notable exception of trypanosomes from perch, we were not able to identify more than one trypanosome morphotype per sample. It is possible that this was due to the overlapping variability of some trypanosome species or because some of them were present only rarely, being detectable by sensitive molecular approaches only. Hence, we decided to avoid the formal description of putative novel species until more information is available.

Although no mixed trypanosome infections were known from fish to date, many such cases have been reported from mammals (Kayang et al. 1997, Jamonneau et al. 2004, Ramirez et al. 2010). Furthermore, co-infections of trypanosomes with monoxenous trypanosomatids of insects have recently been encountered in fleas (Siphonaptera) collected from mammals and birds (Votýpka et al. 2013).

The microscopic examination of 1 373 fish specimens belonging to eight different species captured in the vicinity of Kyiv revealed an unexpectedly high prevalence of trypanosome infections. In the case of spined loach, all specimens were found to be infected. Such a high prevalence, likely due to the same widespread leach vector, creates circumstances under which mixed infections of different piscine *Trypanosoma* species can hardly be considered surprising. However, their documented presence, combined with their likely broad host specificity and cell pleomorphism creates problems regarding the taxonomic assignment of the studied isolates. The delimitation into species (or correlation with the previously described species) can be considered only preliminary, as more sequence information from these parasites is necessary. It seems imperative that molecular studies are performed on clonal lines derived from laboratory cultures of these trypanosomes, which would eliminate the risks posed by mixed infections.

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REFERENCES

Baron L.I., Yurchenko V., Kolesnikov A.A. 1995: [Structure of minicircle kinetoplast DNA from a river perch trypanosome]. Mol. Biol. (Moscow) 29: 1341–1348. (In Russian.)

Borghesan T.C., Ferreira R.C., Takata C.S., Campaner M., Borda C.C., Paiva F., Milder R.V., Teixeira M.M., Camargo E.P. 2013: Molecular phylogenetic redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. Protist 164: 129–152.

Burreson E.M. 2007: Hemoflagellates of Oregon marine fishes with the description of new species of *Trypanosoma* and *Trypanoplasma*. J. Parasitol. 93: 1442–1451.

Darriba D., Taboada G.L., Doallo R., Posada D. 2012: jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9: 772.

Davies A.J., Gibson W., Ferris V., Basson L., Smit N.J. 2005: Two genotypic groups of morphologically similar fish trypanosomes from the Okavango Delta, Botswana. Dis. Aquat. Org. 66: 215–220.

Edgar R.C. 2004: MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl. Acids Res. 32: 1792–1797.

Figueroa F., Mayer W.E., Lom J., Dyková I., Weller M., Pecková H., Klein J. 1999: Fish trypanosomes: their position in kinetoplastid phylogeny and variability as determined from 12S rRNA kinetoplast sequences. J. Euk. Microbiol. 46: 473–481.

Gibson W., Bingle L., Blendeman W., Brown J., Wood J., Stevens J. 2000: Structure and sequence variation of the trypanosome spliced leader transcript. Mol. Biochem. Parasitol. 107: 269–277.

Gibson W.C., Lom J., Pecková H., Ferris V.R., Hamilton P.B. 2005: Phylogenetic analysis of freshwater fish trypanosomes from Europe using ssu rRNA gene sequences and random amplification of polymorphic DNA. Parasitology 130: 405–412.

Gu Z., Wang J., Li M., Zhang J., Ke X., Gong X. 2007: Morphological and genetic differences of *Trypanosoma* in some Chinese freshwater fishes: difficulties of species identification. Parasitol Res 101: 723–730.

Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W., Gascuel O. 2010: New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59: 307–321.

Hall T.A. 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41: 95–98.

Hamilton P.B., Stevens J.R., Gaunt M.W., Gidley J., Gibson W.C. 2004: Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int. J. Parasitol. 34: 1393–1404.

Hamilton P.B., Stevens J.R., Gidley J., Holz P., Gibson W.C. 2005: A new lineage of trypanosomes from Australian vertebrates and terrestrial bloodsucking leeches (Haemadipsidae). Int. J. Parasitol. 35: 431–443.

Hoare C.A. 1966: The classification of mammalian trypanosomes. Ergeb. Mikrobiol. Immunitätsforsch. Exp. Ther. 39: 43–57.

Holmes P. 2013: Tsetse-transmitted trypanosomes – their biology, disease impact and control. J. Invertebr. Pathol. 112: S11–S14.

Holzmuller P., Grebaut P., Semballa S., Gonzatti M.I., Geiger A. 2013: Proteomics: a new way to improve human African trypanosomiasis diagnosis? Expert Rev. Proteomics 10: 289–301.

Jakes K.A., O'Donoghue P.J., Adlard R.D. 2001: Phylogenetic relationships of *Trypanosoma chelodina* and *Trypanosoma binneyi* from Australian tortoises and platypuses inferred from small subunit rRNA analyses. Parasitology 123: 483–487.

Jamonneau V., Ravel S., Koffi M., Kaba D., Zeze D.G., Ndri L., Sane B., Coulibaly B., Cuny G., Solano P. 2004: Mixed infections of trypanosomes in tsetse and pigs and their epidemiological significance in a sleeping sickness focus of Cote d'Ivoire. Parasitology 129: 693–702.

Jirků M., Yurchenko V.Y., Lukeš J., Maslov D.A. 2012: New species of insect trypanosomatids from Costa Rica and the proposal for a new subfamily within the Trypanosomatidae. J. Euk. Microbiol. 59: 537–547.

Joerink M., Groeneveld A., Ducro B., Savelkoul H.F., Wiegertjes G.F. 2007: Mixed infection with *Trypanoplasma borreli* and *Trypanosoma carassii* induces protection: involvement of cross-reactive antibodies. Dev. Comp. Immunol. 31: 903–915.

Kayang B.B., Bosompem K.M., Assoku R.K., Awumbila B. 1997: Detection of *Trypanosoma brucei*, *T. congolense* and *T. vivax* infections in cattle, sheep and goats using latex agglutination. Int. J. Parasitol. 27: 83–87.

Khaibulaev K.K., Shulman S.S. 1984: [Key to Parasites of Freshwater Fish]. Zoologicheskii Institut AN SSSR, Leningrad, 431 pp. (In Russian.)

Khan R.A. 1985: Pathogenesis of *Trypanosoma murmanensis* in marine fish of the northwestern Atlantic following experimental transmission. Can. J. Zool. 63: 2141–2144.

Kolesnikov A., Jirků M., Pecková H., Polák A., Maslov D.A., Lukeš J. 1995: Analysis of kinetoplast DNA of freshwater fish trypanosomes. Folia Parasitol. 42: 251–254.

Letch C.A. 1979: Host restriction, morphology and isoenzymes among trypanosomes of some British freshwater fishes. Parasitology 79: 107–117.

Lom J. 1973: Experimental infections of freshwater fishes with blood flagellates. J. Protozool. 20: 537.

Lom J. 1979: Biology of the trypanosomes and trypanoplasms of fish. In: W.H.R. Lumsden and D.A. Evans (Eds.), Biology of the Kinetoplastida, Academic Press, London, pp. 269–337.

Losev A.A., Ovcharenko N.A. 2004: Fundamentals of methods for studying fish blood parasites. Hydrobiol. J. 40: 97–105.

Martin D.S., Wright A.D., Barta J.R., Desser S.S. 2002: Phylogenetic position of the giant anuran trypanosomes *Trypanosoma chattoni*, *Trypanosoma fallisi*, *Trypanosoma mega*, *Trypanosoma neveulemairei*, and *Trypanosoma ranarum* inferred from 18S rRNA gene sequences. J. Parasitol. 88: 566–571.

Maslov D.A., Lukeš J., Jirků M., Simpson L. 1996: Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasitol. 75: 197–205.

Maslov D.A., Votýpka J., Yurchenko V., Lukeš J. 2013: Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol. 29: 43–52.

Maslov D.A., Yurchenko V.Y., Jirků M., Lukeš J. 2010: Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. J. Euk. Microbiol. 57: 177–188.

Nunes M.C., Dones W., Morillo C.A., Encina J.J., Ribeiro A.L. 2013: Chagas disease: an overview of clinical and epidemiological aspects. J. Am. Coll. Cardiol. 62: 767–776.

Overath P., Ruoff J., Stierhof Y.D., Haag J., Tichy H., Dyková I., Lom J. 1998: Cultivation of bloodstream forms of *Trypanosoma carassii*, a common parasite of freshwater fish. Parasitol. Res. 84: 343–347.

Podlipaev S.A. 1990: [Catalogue of World Fauna of Trypanosomatidae (Protozoa)]. Zoologicheskii Institut AN SSSR, Leningrad, 178 pp. (in Russian.)

Ramirez J.D., Guhl F., Rendon L.M., Rosas F., Marin-Neto J.A., Morillo C.A. 2010: Chagas cardiomyopathy manifestations and *Trypanosoma cruzi* genotypes circulating in chronic Chagasic patients. PLoS Negl. Trop. Dis. 4: e899.

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Hohna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. 2012: MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 539–542.

Saeij J.P., Van Muiswinkel W.B., Groeneveld A., Wiegertjes G.F. 2002: Immune modulation by fish kinetoplastid parasites: a role for nitric oxide. Parasitology 124: 77–86.

Schmid-Hempel R., Tognazzo M. 2010: Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Euk. Microbiol. 57: 337–345.

Stevens J.R., Noyes H.A., Dover G.A., Gibson W.C. 1999: The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*. Parasitology 118: 107–116.

Stevens J.R., Noyes H.A., Schofield C.J., Gibson W. 2001: The molecular evolution of Trypanosomatidae. Adv. Parasitol. 48: 1–56.

Tandon R.S., Chandra S. 1977: Studies on ecophysiology of fish parasites: effect of trypanosome infection on the serum cholesterol levels of fishes. Z. Parasitenkd. 52: 199–202.

Teixeira M.M., Borghesan T.C., Ferreira R.C., Santos M.A., Takata C.S., Campaner M., Nunes V.L., Milder R.V., de Souza W., Camargo E.P. 2011: Phylogenetic validation of the genera *Angomonas* and *Strigomonas* of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. Protist 162: 503–524.

Votýpka J., Klepetková H., Yurchenko V.Y., Horák A., Lukeš J., Maslov D.A. 2012: Cosmopolitan distribution of a trypanosomatid *Leptomonas pyrrhocoris*. Protist 163: 616–631.

Votýpka J., Maslov D.A., Yurchenko V., Jirků M., Kment P., Lun Z.R., Lukeš J. 2010: Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal. Mol. Phylogenet. Evol. 54: 243–253.

Votýpka J., Suková E., Kraeva N., Ishemgulova A., Duží I., Lukeš J., Yurchenko V. 2013: Diversity of trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus *Blechomonas* gen. n. Protist 164: 763–781.

Wallace F.G. 1966: The trypanosomatid parasites of insects and arachnids. Exp. Parasitol. 18: 124–193.

Woo P.T.K. 2006: Fish Diseases and Disorders, Volume 1: Protozoan and Metazoan Infections, 2nd ed., CAB International, Wallingford, 800 pp.

Woo P.T., Black G.A. 1984: *Trypanosoma danilewskyi*: host specificity and host's effect on morphometrics. J. Parasitol. 70: 788–793.

Yurchenko V., Kolesnikov A.A., Lukeš J. 2000: Phylogenetic analysis of Trypanosomatina (Protozoa: Kinetoplastida) based on minicircle conserved regions. Folia Parasitol. 47: 1–5.

Yurchenko V., Lukeš J., Jirků M., Maslov D.A. 2009: Selective recovery of the cultivation-prone components from mixed trypanosomatid infections: a case of several novel species isolated from Neotropical Heteroptera. Int. J. Syst. Evol. Microbiol. 59: 893–909.

Yurchenko V., Lukeš J., Jirků M., Zeledon R., Maslov D.A. 2006a: *Leptomonas costaricensis* sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus *Leishmania*. Parasitology 133: 537–546.

Yurchenko V., Lukeš J., Tesařová M., Jirků M., Maslov D.A. 2008: Morphological discordance of the new trypanosomatid species phylogenetically associated with the genus *Crithidia*. Protist 159: 99–114.

Yurchenko V., Lukeš J., Xu X., Maslov D.A. 2006b: An integrated morphological and molecular approach to a new species description in the Trypanosomatidae: the case of *Leptomonas podlipaevi* n. sp., a parasite of *Boisea rubrolineata* (Hemiptera: Rhopalidae). J. Euk. Microbiol. 53: 103–111.

Yurchenko V., Votýpka J., Tesařová M., Klepetková H., Kraeva N., Jirků M., Lukeš J. 2014: Ultrastructure and molecular phylogeny of four new species of monoxenous trypanosomatids from flies (Diptera: Brachycera) with redefinition of the genus *Wallaceina*. Folia Parasitol. 61: 97–112.

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