

Host specificity, pathogenicity, and mixed infections of trypanoplasms from freshwater fishes

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Abstract This work summarizes the results of the 8-year study focused on *Trypanoplasma* sp. parasitizing freshwater fishes in the vicinity of Kyiv, Ukraine. Out of 570 fish specimens of 2 different species analyzed, 440 individuals were found to be infected. The prevalence of infection ranged from 24 % in *Abramis brama* Linnaeus (freshwater bream) to 100 % in *Cobitis taenia* Linnaeus (spined loach). The level of parasitemia also varied between moderate in freshwater bream and very high in spined loach. Interestingly, no clinical manifestations of trypanoplasmosis were observed even in extremely heavily infected *C. taenia*. We hypothesize that different species may differ in evolutionary timing allowing for reciprocal adaptation of the members of the “host-parasite” system. Molecular analysis of the 18S rRNA sequences

revealed that several specimens were simultaneously infected with at least two different trypanoplasma species. To the best of our knowledge, this is the first report of the mixed infection with fish trypanoplasms.

Keywords *Trypanoplasma* · Freshwater fish · Mixed infections · Phylogeny · 18S rRNA

Introduction

The diversity and taxonomy of kinetoplastid flagellates (class Kinetoplastea Honigberg, 1963) remains underinvestigated despite the significance of this group encompassing several independently originated parasitic lineages (Lukeš et al. 2014). At present, the class Kinetoplastea is subdivided into five orders: Prokinetoplastida Vickerman, 2004; Eubodonida Vickerman, 2004; Parabodonida Vickerman, 2004; Neobodonida Vickerman, 2004; and Trypanosomatida Kent, 1880 (Moreira et al. 2004). Out of these, only the order Trypanosomatida is being scrutinized since this taxon-rich group includes numerous human pathogens (Stevens et al. 2001; McCall and McKerrow 2014). Other kinetoplastids, even parasitic ones, are generally neglected because of their less practical significance.

Parabodonids contain both free-living and parasitic species characterized by anterolateral cytostome and prominent preoral ridge, with their mitochondrial DNA in pankinetoplastic or eukinetoplastic arrangement (Lukeš et al. 2002). These flagellates are subdivided into four genera: *Cryptobia* Leidy, 1846; *Parabodo* Skuja, 1939; *Procryptobia* Vickerman, 1978; and *Trypanoplasma* Laveran et Mesnil, 1901. The names *Cryptobia* and *Trypanoplasma* are often considered synonymous (Crawley 1909; Kozloff 1948; Newman 1978; Khan et al. 2001). We

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share the opinion of Lom (1979) and reserve the name *Cryptobia* for non-hematozoic parasites (the type species is *Cryptobia helici* Leidy, 1846 from the snail *Neohelix albolabris albolabris*) and the name *Trypanoplasma* for hematozoic leech-transmitted parabodonids (the type species is *Trypanoplasma borreli* Laveran et Mesnil, 1901 first isolated from the common rudd *Scardinius erythrophthalmus*). Our view is justified by the molecular data showing these two genera to be phylogenetically separated, with their parasitic life style likely originated independently (Doležel et al. 2000; Moreira et al. 2004; von der Heyden and Cavalier-Smith 2005).

Trypanoplasms are the causative agents of trypanoplasmosis (cryptobiosis) in fishes. Clinical manifestations of the disease include splenomegaly, anemia, anorexia, and edema. Affected specimens have reduced metabolism resulting in, for example, significantly slower swimming rate. Infection intensity, prevalence, and mortality rates vary between different species and even populations of the same species (Woo 2003). Trypanoplasms may cause heavy losses in economically important fishes and thus more efforts should be devoted to understanding biology and diversity of these parasites (Makeyeva 1956; Bower and Margolis 1984).

Unfortunately, as with their trypanosomatid kins, the taxonomy of trypanoplasms is rather confusing. Morphological plasticity of these flagellates along with scarcity of distinctive traits resulted in numerous unresolved taxonomical issues. By the end of the last century, 52 *Trypanoplasma* (*Cryptobia*) spp. were formally described (Woo 1994). Most of them were initially characterized as parasites of particular fish species, but later, recognized host ranges for many trypanoplasms were expanded. It was inevitable since many authors have encountered similar forms in related fish taxa and therefore considered the specificity of these flagellates to be wide. That was subsequently confirmed by several successful cross-infection experiments with parasites of carps and goldfish (Lom 1979). Meanwhile, some researchers documented the occasional presence of more than one *Trypanoplasma* sp. in one host species (Khaibulaev and Shulman 1984). However, due to the difficulties of species discrimination, the concern of mixed infection was never raised. In summary, the situation with the taxonomy of these flagellates proved to be quite complicated and it could not be clarified using classical approaches. In the

neighboring order Trypanosomatida, these complex relationships between the species of parasites and hosts (one-to-many and many-to-one) were recently demonstrated in various taxa using molecular methods (Votýpka et al. 2012; Týč et al. 2013; Grybchuk-Ieremenko et al. 2014). To date, the true diversity of trypanoplasms has not been investigated in such minutiae, and sequences of only four species of these flagellates were available in the GenBank prior to this work.

The “gold standard” for molecular phylogenetic studies of kinetoplastids is the 18S ribosomal RNA gene (von der Heyden and Cavalier-Smith 2005; Yurchenko et al. 2006; Kostygov et al. 2014). In particular, this marker was the only one used for inferring phylogenetic affinities of trypanoplasms (Wright et al. 1999; Doležel et al. 2000; Moreira et al. 2004). Other genes routinely used in molecular taxonomy of Trypanosomatida (e.g., glycosomal glyceraldehyde-3-phosphate dehydrogenase or spliced leader RNA) have not yet found their way into the field of trypanoplasm research (Wiemer et al. 1995; Hannaert et al. 1998).

In this study, we analyzed host specificity and prevalence of trypanoplasms in a large sample of freshwater fish from Ukraine using molecular methods.

Materials and methods

Sample isolation, light microscopy, and DNA extraction

Trypanoplasms from two freshwater fish species—*Abramis brama* Linnaeus, 1758 (freshwater bream) and *Cobitis taenia* Linnaeus, 1758 (spined loach) collected in the vicinity of Kyiv, Ukraine—were analyzed (Table 1). The presence of trypanoplasms in fish blood and the level of parasitemia were established on smears and recalculated per milliliter of blood as described before (Grybchuk-Ieremenko et al. 2014). The seasonal parasitemia and infection prevalence were assayed in the spring (water temperature rising from +5 to +17 °C), summer (water temperature between +17 and +28 °C), and fall (water temperature falling from +17 to +7 °C). The smears containing trypanoplasms were fixed with methanol, stained with Giemsa, and used for subsequent analysis of morphology of these parasites as described elsewhere (Yurchenko et al.

Table 1 Summarized parameters of fish infections with trypanoplasms

Fish host	Years collected	GPS localities	Infection prevalence (%)	Parasitemia per ml
<i>C. taenia</i> (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	69,050±24,420 (17,000–340,000)
<i>A. brama</i> (n=172)	2009–2013	50°53'24"N, 30°25'45"E 50°06'47"N, 30°52'18"E	24.4	27,370±5100 (4760–54,740)

2008). All measurements were taken on Giemsa-stained samples and are expressed in micrometers (μm) (Table 2). We firmly believe that morphology should not be used as a sole basis for the taxonomy of Kinetoplastida since it may be misleading (Yurchenko et al. 2009; Maslov et al. 2010; Votýpka et al. 2010). However, morphology is an integral part of any systematic study and, in most cases, the only means to compare our results with those of our predecessors. Trypanoplasms were isolated from fresh infected fish blood using a protocol described elsewhere (Losev and Ovcharenko 2004). Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany).

PCR, cloning, and sequencing

Four samples (two from *A. brama* and two from *C. taenia*) were subjected to molecular phylogenetic analyses. Two pairs of trypanosomatid-specific primers producing PCR amplicons that represent almost whole 18S rRNA gene and have ~600 bp overlap thus preventing chimera formation were used. The first pair of primers was SSUdir (5'-catatgcttgttcaaggac-3') and A757 (5'-gcgaacgtactccccctga-3'), the second one consisted of primers 883F (5'-gacttgaattagmaagcatggga-3') and SSUrev (5'-gactttgtctctctawtg-3'). The amplification conditions were adopted from the previously published work (Maslov et al. 1996) with the following modifications of the cycling parameters: at 94 °C for 15 s, at 55 °C for 20 s, and at 72 °C for 1 min 30 s. We successfully amplified both 18S rRNA gene fragments from all four samples. PCR products were cloned using InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, Waltham, USA), and 12 clones in total were sequenced. The GenBank accession numbers for the new sequences determined in the course of this work are KP054302–KP054313.

Phylogenetic analyses

18S rRNA gene sequences of trypanoplasms obtained in this work along with those of all species of Parabodonida present in GenBank and three species of Eubodonida (serving as an outgroup) were aligned using Muscle 3.8.3.1 (Edgar 2004). Next, manual refinement was performed using the BioEdit sequence alignment editor (Hall 1999), and ambiguously aligned positions were removed from non-trypanoplasm sequences to ensure elimination of noise as well as preservation of differences between trypanoplasms' haplotypes. The resulting alignment of 23 sequences contained 1992 nucleotide positions. Model testing in jModeltest 2.1.4 using the Akaike information criterion showed GTR+I+G to be the best model (Darriba et al. 2012). Maximum likelihood phylogenetic inference was performed using RAxML v 8.0 with the selected model and 1000 "thorough" bootstrap replicates (Stamatakis 2014). Phylogenetic analysis in the Bayesian

Table 2 Size characteristics of studied piscine trypanoplasms (μm)

#	Parasite	Length	Width	Nucleus length	Nucleus width	A-K ^a	Anterior flagellum	Posterior flagellum
1	<i>Trypanoplasma</i> sp. ex <i>C. taenia</i> (n=100)	24.8±7.1 (18.8–34.0)	19.0±6.7 (16.1–25.8)	6.3±2.0 (5.0–8.0)	2.1±1.2 (1.0–3.6)	3.5±2.0 (0.9–5.3)	15.3±6.4 (11.1–23.7)	11.9±10.0 (5.7–24.2)
2	<i>Trypanoplasma</i> sp. ex <i>A. brama</i> (v. 1) (n=50)	26.2±4.9 (15.9–31.8)	10.5±4.1 (7.4–16.4)	5.8±1.4 (4.3–7.3)	2.4±0.5 (1.3–3.8)	4.4±2.2 (3.1–6.2)	14.7±6.0 (11.9–22.9)	2.9±2.4 (1.7–6.2)
3	<i>Trypanoplasma</i> sp. ex <i>A. brama</i> (v. 2) (n=50)	25.0±5.4 (17.2–32.5)	11.0±3.7 (8.1–15.4)	6.4±1.7 (5.9–8.2)	2.9±0.9 (1.2–4.1)	2.6±1.9 (1.0–4.1)	16.3±5.2 (12.1–25.2)	13.9±2.5 (10.7–19.2)

^a A-K is the distance between the anterior end of the cell and the kinetoplast

framework was accomplished in MrBayes 3.2.2 with the following parameters: 5 million generations under the GTR+I+G model (five categories) and sampling every 1000 generations (Ronquist et al. 2012). Other parameters were left in their default states.

Results

Morphology and infection of fish trypanoplasms

We present a brief morphological description of the prevalent morphotypes detected in analyzed fish species. Detailed size characteristics are provided in Table 2. We do this with the understanding that the accounts provided here may reflect mostly those species of trypanoplasms that composed major fractions of mixed infections (see below). We are also

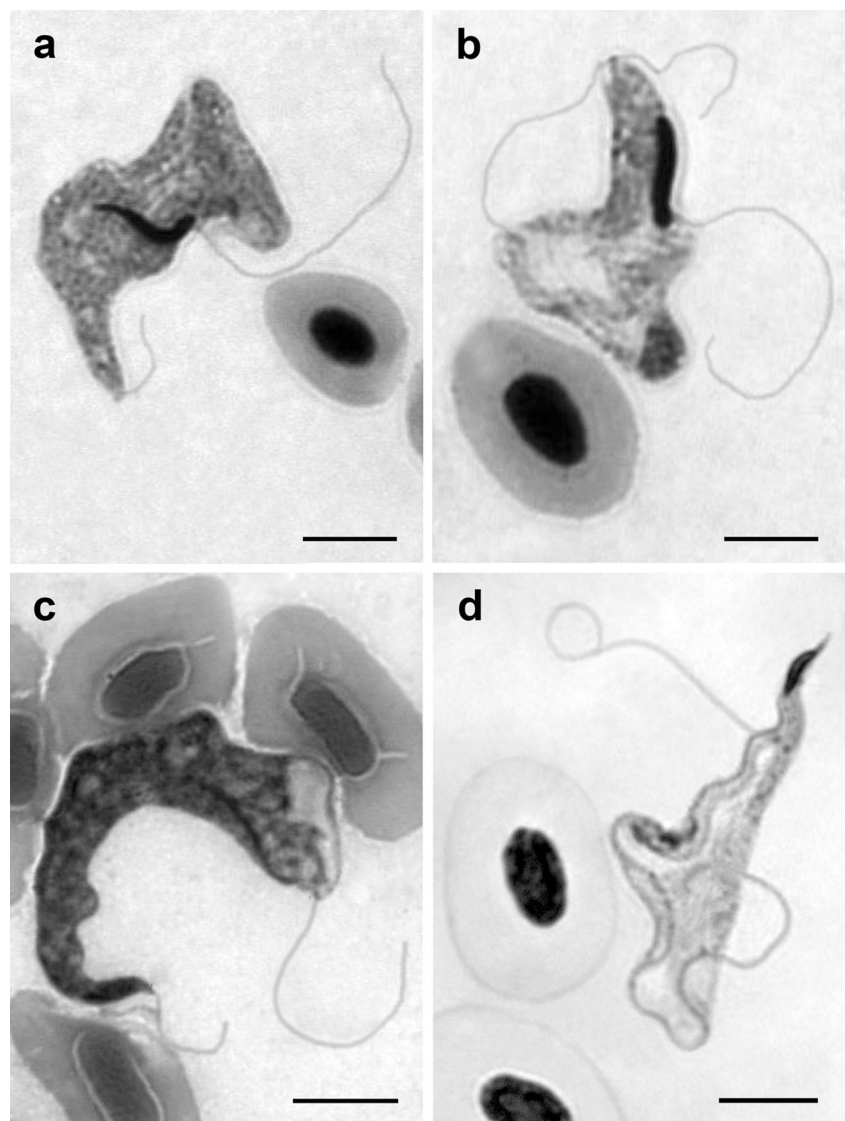
convinced that without clonal isolates available their relationships with other species cannot be established with any certainty (Pecková and Lom 1990).

Trypanoplasma sp. ex *C. taenia* (Fig. 1a, b)

Infection: All examined spined loaches were infected, usually with extremely high parasitemia, varying from 17,000 to over 340,000 parasites/ml of blood. No seasonal changes were observed. All specimens were co-infected with trypanosomes, present in the ratio of approximately 1:10, with no differences noted between host sexes.

Pathology: Despite extremely high levels of parasitemia, trypanoplasms did not aggregate in the kidney blood vessels and nephrons. Infected specimens showed no apparent clinical manifestations of trypanoplasmosis.

Fig. 1 Major morphotypes of piscine trypanoplasms. **a, b** *Trypanoplasma* sp. ex *C. taenia* (*T. varium* species group); **c** *Trypanoplasma* sp. ex *A. brama* (*T. abramidis* species group); **d** *Trypanoplasma* sp. ex *A. brama* (*T. borreli* species group). Scale bar is 5 μ m



Morphological description: Cell morphology is variable ranging from a twisted sickle-like to amoeba-like shape. The anterior flagellum is usually longer compared to its posterior counterpart, but the ratio is flexible. A large oval nucleus locates in the anterior part of the cell. The large kinetoplast is rod-shaped. The cytoplasm is intensely stained and granular.

Note: Morphological description of *Trypanoplasma* sp. ex *C. taenia* broadly corresponds to that of *Trypanoplasma varium* Leger, 1904 from the same host species.

Trypanoplasma spp. ex *A. brama* (Fig. 1c, d)

Infection: The prevalence is moderate and stable over seasons (22, 23, and 25 % in the spring, summer, and fall, respectively). The degree of parasitemia ranges between 4760 and 54,740 parasites/ml of blood. In *A. brama*, co-infections with trypanosomes were detected in about 25 % of cases, and the ratio between these flagellates varied between 6:1 and 8:1. No differences concerning sex ratio imbalance were noticed.

Pathology: The clinical manifestations of the infected fishes included splenomegaly, pale fins, and anemia. Infested specimens were markedly smaller compared to their healthy kins of the same age. Swelling, petechial hemorrhages of the kidney and liver, and exophthalmia were observed in heavily infected fishes. These pathological outcomes are consistent with previous reports (Bunnajirakul et al. 2000; Rudat et al. 2000; Woo 2003). All clinical signs correlated with the presence of trypanoplasms, whereas *Trypanosoma*-only infected specimens were mainly asymptomatic.

Morphological description: There were two distinct morphotypes encountered in the blood of *A. brama*. Morphotype 1 (Fig. 1c): Cell body is twisted, enlarged at the anterior, and narrowed at the posterior end. The anterior flagellum is significantly longer compared to the posterior one. The anteriorly located nucleus is lightly stained. The kinetoplast is large.

Morphotype 2 (Fig. 1d): Often twisted cell body is sickle-like narrowing towards the posterior end. The anterior flagellum is usually longer than the posterior counterpart, but this difference is not as well pronounced as in morphotype 1. The nucleus is elongated and is usually located on the dorsal side of the cell. The rod-shaped kinetoplast seems to be smaller.

Note: The morphological description of the first variant of trypanoplasma cells from *A. brama* broadly corresponds to that of *Trypanoplasma abramidis* Brumpt, 1906 from the common bream (Fig. 1c). As for the second morphotype, it appears similar to *T. borreli* Laveran et Mesnil, 1901, a parasite with a presumed wide host specificity that was originally characterized from the common rudd (Fig. 1d). The main distinctive

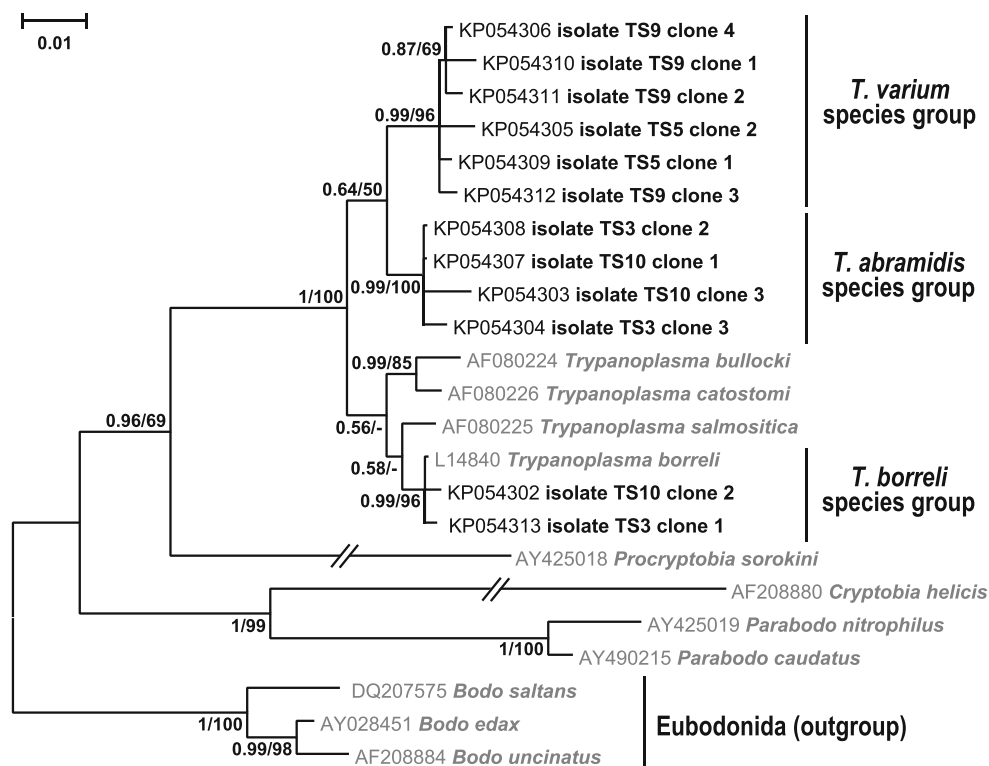
morphological trait for correct species identification in this case is a ratio of the anterior and posterior flagella lengths (Khaibulaev and Shulman 1984).

Phylogenetic analysis

The trees inferred using both maximum likelihood and Bayesian approaches were mostly congruent, with minor differences in groups with low support (Fig. 2). They were also topologically concordant with the previously published phylogenetic reconstructions (Doležel et al. 2000; Moreira et al. 2004). Using Eubodonida as an outgroup, we recovered a clade comprising the hematozoic leech-transmitted parasites (*Trypanoplasma* spp.) with the free-living *Procrystobia sorokini* as a sister taxon and the non-hematozoic *Cryptobia* clustered together with the two species of free-living flagellates of the genus *Parabodo* (Fig. 2). All the clades mentioned above showed high statistical support except the bootstrap value for the *Procrystobia*+*Trypanoplasma* group that was only moderate.

For each of the samples used in molecular analysis, 2–4 clones of the 18S rRNA gene were sequenced. Surprisingly, they were all distinct, and none of them matched any of the previously reported sequences. Since the level of intraspecific variability of the 18S rRNA gene sequences for this genus has not been estimated to date, it is difficult to determine whether they all represented different species. To avoid potential confusion, we decided to refer to them as haplotypes hereafter. The sequences of trypanoplasms from our samples formed three well-supported monophyletic groups: (1) all sequences from the *C. taenia* hosts, (2) four haplotypes from the *A. brama* hosts, and (3) two haplotypes from the *A. brama* plus *T. borreli* sequence from the GenBank. Phylogenetic relationships between these clades as well as their relationships with three other species of *Trypanoplasma* whose 18S rRNA sequences were published before (*Trypanoplasma catostomi*, *Trypanoplasma bullocki*, and *Trypanoplasma salmositica*) could not be reliably determined as demonstrated by the relatively low statistical supports on the presented tree (Fig. 2). Sequence divergence within the three clades reached 1.94, 0.86, and 0.4 %, correspondingly. Given these numbers, we propose that each of these clades encompasses more than one species and designate them as “species groups” with nominative species selected according to our morphological data and phylogenetic affinity. Thus, the *C. taenia*-derived parasites were designated as “*Trypanoplasma varium* species group”, parasites from *A. brama* related to *T. borreli* as “*Trypanoplasma borreli* species group”, and the remaining clade as “*Trypanoplasma abramidis* species group”. Importantly, each of the two samples from the bream harbored parasites belonging to the *T. borreli*

Fig. 2 18S rRNA-based Bayesian phylogenetic tree of piscine trypanoplasms. Sequences determined in the present paper are in *black*. 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 (1000 replicates) are shown at the nodes. Dashes indicate bootstrap support below 50 % or different topology. The tree was rooted with three Eubodonida sequences. The scale bar denotes the number of substitutions per site. Double-crossed branches are at 50 % of their original lengths. Sequence alignment is available from authors upon request



and *T. abramidis* species groups that unequivocally indicates mixed infections. Though both samples from the spined loach enclosed only flagellates of the *T. varium* species group, the differences between the haplotypes originated from one sample may also be interspecific (ranged from 0.76 to 1.1 %).

Discussion

Our results highlight three important features of *Trypanoplasma* spp. infection: (1) a wide range of clinical manifestations (from asymptomatic to acute), (2) co-infection by representatives of different species, and (3) apparent host specificity. Because all these characteristics are of practical significance, we will discuss them in detail.

Diverse clinical outcomes may reflect (i) different degrees of virulence of different *Trypanoplasma* spp. or strains, (ii) disparate levels of host immune response to parasite infection, or (iii) a combination of abovementioned factors. The questions related to fish immunity have been extensively studied (Saeij et al. 2003a; Alvarez-Pellitero 2008). Both adaptive and innate immune responses play a role here and rely on pathogen-recognizing receptors, phagocytosis and phagocyte activity (including oxidative mechanisms), complement activity, and production of parasite-specific antibodies and other immune mediators, such as cytokines (IL-1, IL-8, TNF, IFN),

chemokines (CXC, CC) and small molecules (iNO) (Saeij et al. 2002; Saeij et al. 2003b). Despite all these efforts, it is still not clear why certain host species (exemplified in the current study by *C. taenia*) can tolerate heavy trypanoplasmosis fairly well, while others are highly susceptible to the disease. We speculate that the difference in evolutionary timing allowed for reciprocal adaptation of the members of the “host-parasite” system (Lukeš et al. 2014).

The question of host specificity is also quite important. All haplotypes recovered in the current study clustered in accordance with their host species, and those groups did not overlap. This observation is somewhat counterintuitive as there are very few leech vector species implicated in *Trypanoplasma* spp. transmission (Kruse et al. 1989; Woo 2003; Bureson 2007). We explain this result by the fact that host species used in the current study populate different ecological habitats. The freshwater breams are bottom feeders. They can tolerate lower oxygen levels and usually spend winter in deeper water. In contrast, the spined loaches prefer shallow habitats dominated by submerged vegetation, which may be important for spawning.

Finally, in the current work, we are reporting a case of simultaneous co-infection of *A. brama* by different trypanoplasms. To our knowledge, this is the first record of mixed infections involving *Trypanoplasma* spp. In our opinion, it is important to apprehend such a possibility for future studies because different parasites might have different (potentially, cumulative) effects on their hosts' physiology.

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