A re-evaluation of conflicting taxonomic structures of Eurasian *Triaenophorus* spp. (Cestoda, Bothriocephalidea: Triaenophoridae) based on partial *cox1* mtDNA gene sequences

Pavel G. Vlasenko[1*], Sergey G. Sokolov[2], Evgeny P. Ieshko[3], Evgeniy V. Frolov[4], Alexander P. Kalmykov[5], Aleksey N. Parshukov[3], Yulia K. Chugunova[6], Elena N. Kashinskaya[1,7], Anastasiya V. Shokurova[1], Nikolai A. Bochkarev[1], Karl B. Andree[8], Mikhail M. Solovyev[1,9]

[1]*Institute of Systematics and Ecology of Animals SB RAS, Novosibirsk, Russia*
[2]*A N Severtsov Institute of Ecology and Evolution RAS, Moscow, Russia*
[3]*FSBIS Karelian Research Centre RAS, Petrozavodsk, Russia*
[4]*Sakhalin Branch of the Russian Federal Research Institute of Fisheries and Oceanography, Uzno-Sahalinsk, Russia*
[5]*Astrakhan Nature Reserve, Astrakhan, RU*
[6]*Krasnoyarsk Branch of Russian Federal Research Institute of Fisheries and Oceanography, Krasnoyarsk, Russia*
[7]*I D Papanin Institute of Biology of Inland Waters RAS, Borok, Russia*
[8]*Instituto de Investigación y Tecnología Agroalimentarias San Carlos de la Rapita, Tarragona, Spain*
[9]*National Research Tomsk State University, Tomsk, Russia*

***Corresponding author:** vlasenkopg@gmail.com

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Abstract

Cestodes of the genus *Triaenophorus* are one of the most common parasites of esocids, percids, salmonids and fish of a number of other fish families in the Holarctic. Taxonomic models of different authors, based on morphological and ecological-biogeographic characters, suggest the presence of two to five species of this genus in Eurasia. The genetic variation of Eurasian *Triaenophorus* spp. was evaluated using DNA barcoding (*cox1* gene). It confirmed the validity of five *Triaenophorus* species: *T. amurensis, T. crassus, T. meridionalis, T. nodulosus* and *T. orientalis*. Through this analysis, we have demonstrated concordance between traditional taxonomic criteria and DNA sequence data, while at the same time raising new hypotheses to be tested. Phylogenetic reconstructions support the monophyletic origin of the group of species with a long basal plate of the scolex hook (*T. crassus*, *T. meridionalis* and *T. orientalis*). *T. crassus* is represented by two haplogroups, associated to Siberia and North-Western Russia. Our results show differences between *T. nodulosus*, *T. amurensis* and *T crassus* in terms of the haplotype diversity level, which are probably related to the Quaternary history of the development of their ranges, as well as the degree of euryxeny to the second intermediate host.

Introduction

The genus *Triaenophorus* Rudolphi, 1793 constitutes the cestodes of the order Bothriocephalidea having a scolex with two shallow bothria and four trident-shaped hooks, and strobila devoid of external segmentation (Protasova, 1977; Bray et al., 1994; Kuchta et al., 2008). The geographic range of this genus spans northern Eurasia and North America. The life cycle of *Triaenophorus* includes copepods as the first intermediate host and various species of omnivorous and planktivorous fishes as the second intermediate host (Kuperman, 1973). Adult specimens of the Eurasian species of *Triaenophorus* are specific parasites of esocid fish (Kuperman, 1973).

Triaenophorus spp. are one of the most common helminthes of freshwater fish in the boreal climate zone. For example, infestation of pike with adult specimens of *Triaenophorus nodulosus* (Pallas, 1781) in the desalinated part of the Gulf of Bothnia may reach 93% (Valtonen et al., 1989). According to Dieterich and Eckmann, (2000), plerocercoids of this species occur in more than 80% of perch aged one year and older in some waters of Germany. Extensive literature is devoted to the study of various aspects of *Triaenophorus* spp. biology, including pathogenicity for its hosts (Kuperman, 1973; Rosen and Dick, 1984; Shostak and Dick, 1986; Valtonen et al., 1989; Ieshko and Evseeva, 1989; Pronin, 1990; Evseeva, 1994; Pasternak et al., 1999; Izvekova, 2001; Rusinek and Kuznedelov, 2001; Dezfuli et al., 2014; Schaufler et al., 2014; Izvekova and Solovyev, 2012, 2013, 2016; Borvinskaya et al., 2019; Kashinskaya et al., 2021).

The systematics of *Triaenophorus* has undergone a number of fundamental changes over the past 60 years. Until the late 1960s, only two valid species of this genus were recorded in Eurasia: *T. nodulosus* and *T. crassus* Forel, 1868. However, a detailed study of *Triaenophorus* undertaken by Kuperman (1968) allowed the description of three other species, *T. amurensis* Kuperman, 1968, *T. orientalis* Kuperman, 1968 and *T. meridionalis* Kuperman, 1968, two of which inhabit the Amur transitional zoogeographic region and the third is typical of the waterbodies of southern European Russia. This work was preceded by publications by Dubinina

(1964) and Kuperman himself (1965), indicating significant differences in morphology and host specificity (in the phase of the plerocercoid) between *Triaenophorus* from the Amur River basin, Siberia, southeast Europe and other European water bodies. According to Kuperman (1968, 1973), morphological differences among the five Eurasian species of this genus are primarily related to the size and shape of the scolex hooks.

Dubinina (1987) had contrasting views on the systematics of *Triaenophorus*. According to this author, in Eurasia this genus is represented by only two species, *T. nodulosus* and *T. crassus*, each of which includes two subspecies: *T. crassus crassus, T. crassus orientalis*, *T. nodulosus nodulosus* and *T. nodulosus amurensis*. Kuchta et al. (2007) studied the material on *Triaenophorus* spp. from B.I. Kuperman's collection kept at the Zoological Institute of the Russian Academy of Sciences. According to these authors, the ranges of variation of morphological features of *T. amurensis*, *T. orientalis* and *T. meridionalis* are, in fact, much wider than those indicated by Kuperman (1968, 1973). Kuchta et al. (2007) came to the conclusion that reliable identification of the species described by B.I. Kuperman is impossible; therefore, they synonymize *T. amurensis* with *T. nodulosus*, and *T. orientalis* and *T. meridionalis* with *T. crassus*, thereby reducing the diversity of the Eurasian group of *Triaenophorus* to only two species. Meanwhile, when describing new species, Kuperman (1968) relied not only on morphological features, but also on the set of second intermediate hosts, embryogenesis terms, and other biological parameters, which was not taken into account by Kuchta et al. (2007).

As a result, three competing taxonomic models exist in the literature for description of the Eurasian species of *Triaenophorus* and classical morphological methods of research do not provide an adequate solution in favor of any of them. Therefore, it is necessary to verify the most correct model from among these using molecular genetic data. The *cox1* mtDNA gene is one of the most widely used markers for the taxonomy of species of parasitic flatworms (Vilas et al., 2005). The aim of this paper is to identify the species composition of *Triaenophorus* in Eurasia using partial *cox1* gene sequences obtained from regional fish hosts.

Materials and methods

Study area and sampling

Gravid and subadult specimens or plerocercoids of five species of *Triaenophorus*, *T. amurensis*, *T. crassus*, *T. meridionalis*, T. *nodulosus* and *T. orientalis*, were collected in the course of a parasitological investigation of fishes caught in waterbodies in the European and Asian parts of Russia (Suppl). Parasites were fixed in 96% ethanol and stored at –18 °C.

The species affiliation of the cestodes was annotated in accordance with identification keys using characters described by Kuperman (1968, 1973): the width of the basal plate of scolex hooks, the host and locality. The scolex hooks were measured on the squashed scolices mounted in Berlese's medium (isolates from the Ob and Yenesei river basins) or the isolated hooks extracted from the bodies using needles followed by treatment with 60 µg/ml proteinase K (all other isolates).

DNA extraction, amplification and sequencing

Before DNA extraction, samples fixed in ethanol were washed in water. Total DNA was extracted from single plerocercoids following the manufacturer's protocols for the extraction kit (DNA-sorb B kit, Central Research Institute of Epidemiology, Russia). To reconstruct phylogenetic relationships within the genus *Triaenophorus* partial sequences of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*) were used. The amplification by PCR was conducted using the primers Dice 1F and Dice 11R as described previously (Steenkiste et al., 2015), and the conditions described therein. Briefly, the PCR conditions consisted of XX ng of DNA per XXX µL reaction with a thermal profile that consisted of a touch-down PCR step of 5 cycles (dropping 1 °C per cycle from 50 °C to 46 °C for 40 s of primer annealing at each

cycle), then followed by 35 cycles of 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min. The double-stranded DNA was amplified using the reagent mix provided in the BioMaster HS-Taq PCR-Color (2x) kit (Novosibirsk, Russia), and prepared according to the manufacturer's instructions (http://biolabmix.ru/products/klassicheskaja_pcr/biomaster_hs-taq_pcr-color__2_/). The PCR products were purified by adsorption on Agencourt Ampure XP (Beckman Coulter, Indianapolis, IN, USA) columns and subjected to Sanger sequencing using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) with subsequent unincorporated dye removal by the Sephadex G-50 gel filtration (GE Healthcare, Chicago, IL, USA). The Sanger products were analysed on an ABI 3130XL Genetic Analyzer (Applied Biosystems). The purification and sequencing of PCR products were performed in SB RAS Genomics Core Facility (Novosibirsk, Russia). The sequences were manually aligned, edited and checked for unexpected stop codons in MEGA 7 (Kumar et al., 2016). Newly obtained sequences were deposited into the GenBank database (Suppl).

Phylogenetic analysis

Analysis of genetic distances was conducted in MEGA 7 (Kumar et al., 2016). The number of haplotypes and levels of DNA polymorphism were calculated using the program DNASP 6 (Rozas et al., 2017). The best model of nucleotide substitutions was determined using MEGA 7. Phylogenetic reconstruction within the genus *Triaenophorus* was performed using the Maximum Likelihood (ML) and the Bayesian inference (BI) approaches. For the ML approach implemented in MEGA 7, the HKY+G model of nucleotide substitutions was used. Statistical support of the test of phylogeny was performed using the Bootstrap method with 1000 replications. Bayesian analysis was performed with MrBayes v.3.2.1 using the same model as the previous approach. Two simultaneous runs with four Markov chains each were run for 1 × 106

generations and sampled every 500 generations. The first 25% of generations were discarded as burn-in. The sequence of *Dibothriocephalus latus* (Linnaeus, 1758) (Diphyllobothriidae) with accession number AB269325.1 from the GenBank database was included in the phylogenetic analyses as an outgroup. Popart 1.7 software (https://popart.otago.ac.nz) was used to calculate and visualize the median-joining network of phylogenetic relationships among haplotypes (Bandelt et al. 1999).

Results

Species identification

A total of 63 specimens of *Triaenophorus* spp. from different fish species and waterbodies of Eurasia were examined (Suppl). All representatives of *Triaenophorus* collected were initially assigned to five species based on the width of the basal plate of scolex hooks, the host and locality: *T. amurensis, T. crassus, T. meridionalis,* T. *nodulosus*, and *T. orientalis*.

Worms with the scolex hooks having a basal plate width 63–84 µm and parasitizing in the intestines of *Esox reicherti* Dybowski, 1869 (adult of subadult cestodes) or liver of cyprinids (plerocercoids) from Sakhalin Island and Primorsky Region of Russia were referred to as *T. amurensis* (Fig. 1A).

Representatives of *Triaenophorus* with the scolex hooks having a basal plate width of 90–185 µm and parasitizing in the intestines of *Esox lucius* Linnaeus, 1758 (adult of subadult cestodes) and liver of cottid, lotid or percid fish (plerocercoids) from Siberia or European part of Russia were referred to as *T. nodulosus* (Fig. 1B).

Cestodes with the scolex hooks having a basal plate width 221–411 µm and parasitizing in the intestines of *E. lucius* (adult of subadult cestodes) and muscles of *Coregonus* spp. (plerocercoids) from Siberia or European part of Russia were referred to as *T. crassus* (Fig. 2A).

Specimens with the scolex hooks having a basal plate width 196–228 µm and parasitizing in the intestines of *E. lucius* from the Volga delta were referred to as *T. meridionalis* (Fig. 2B).

Worms with the scolex hooks having a basal plate width 119–133 μm and parasitizing in the intestines of *E. reicherti* from Sakhalin Island were referred to as *T. orientalis* (Fig. 2C).

Phylogeny and genetic diversity

The fragments of the *cox1* gene with a length of 586 bp were amplifed and sequenced from 63 specimens of *Triaenophorus* spp. previously identified by traditional taxonomical methods (see above). The species-level clades of the 5 studied cestode specimens are shown in figure 3.

The topology of the trees constructed by ML and BI approaches were identical, excluding clustering of the samples within the species-level clades. The posterior probability values for all species-level clades obtained by the BI method were not less than 0.95. Bootstrap support for the ML tree was generally weaker: for three species-level clades it was 99% (*T. orientalis*, *T. meridionalis*, and *T. nodulosus*), in two other cases it was 90% (*T. crassus*) and 78% (*T. amurensis*).

Triaenophorus meridionalis, *T. crassus* and *T. orientalis* form a highly supported group, in which *T. crassus* and *T. orientalis* appear as a poorly supported sister species. *Triaenophorus nodulosus* appears as a poorly supported sister taxon to the *T. meridionalis* + (*T. crassus* + *T. orientalis*) group, and *T. amurensis* occupies a sister position to the clade uniting all mentioned *Triaenophorus* spp.

Only 10 haplotypes with 19 polymorphic sites were identified among 24 specimens of *T. crassus*. The haplotype and nucleotide diversity for this species was 0.667±0.109 and 0.00539, respectively. Twenty haplotypes with 35 polymorphic sites were presented in 22 studied specimens of *T. nodulosus*. This species was characterized by the highest levels of haplotype (0.991±0.017) and nucleotide (0.01248) diversity. Eight haplotypes with 10 polymorphic sites were found among nine specimens of *T. amurensis*. The haplotype and nucleotide diversity for this species was 0.972±0.064 and 0.00626, respectively. We did not take into account this parameter for *T. meridionalis* and *T. orientalis* due to the low numbers of analyzed specimens.

In general, the level of intraspecific variability of the portion of the *cox1* genes that were sequenced was much lower than their level of interspecific variability. Within the species-level clades, the mean p-distance values for *T. crassus, T. orientalis, T. meridionalis, T. nodulosus* and *T. amurensis* were 0.54±0.13%, 0.17±0.12%, 0.57±0.22%, 1.25±0.24%, and 0.63±0.20% respectively. The mean p-distance between these clades varied in range from 10.9±1.3% (*T. crassus* by *T. orientalis*) to 18.0±1.6 (*T. meridionalis* by *T. amurensis*).

The species-level haplogroups are distinctly separated in the haplotype network (Figures 4, 5). For some sampling sites only a single haplotype was identified. The geographically specific haplogroups were found only in one widespread species, *T crassus.* We identified two haplogroups with different geographic distribution in this species. One of the mentioned haplogroups of *T. crassus* was recorded in Siberia. The most common haplotype of this haplogroup was found in Lake Teletskoye and the rivers Khatanga and Yenisei (Fig. 5). Another geographically specific haplogroup of this species was found in North-Western Russia. We were unable to identify the differentiation of haplotypes in terms of frequency of occurrence in this haplogroup, due to the small number of samples from North-Western Russia.

Discussion

This is the first analysis that includes a traditional taxonomic approach together with DNA sequence analyses for evolutionary reconstruction of *Triaenophorus* spp. The results of the phylogenetic analyses, based on partial sequences of *cox1* mtDNA, confirm the hypothesis of Kuperman (1968, 1973); there are five species within the genus *Triaenophorus* parasitizing fishes of Eurasia. According to Kuchta et al. (2007), the ranges of values of the width of the basal plate of the scolex hooks designated by B. I. Kuperman inadequately describes the variability of this character in each of these five species. Two species from our material, *T. crassus* and *T. nodulosus* (Suppl), had the scolex hooks in which minimum width of the base was

229 smaller than that indicated by Kuperman (1968; 1973). In turn, the minimum values for the
230 width of the basal plate of scolex hooks in these species overlap (or almost coincide) with the
231 maximum values in *T. meridionalis* and *T. amurensis* respectively, which is consistent with the
232 data of Kuchta et al. (2007). Nevertheless, primary identification of individual specimens of *T.*
233 *amurensis*, *T. crassus*, *T. meridionalis* and *T. nodulosus* became possible on the basis of
234 ecological-biogeographic characteristics.

235 According to Kuperman (1969, 1973), *T. crassus* and *T. nodulosus* are the most ancient
236 species of the genus *Triaenophorus* and share the most recent common ancestor, but *T.*
237 *nodulosus* is evolutionarily closer to the ancestor than *T. crassus*. All the rest of the Eurasian
238 species originate from *T. nodulosus* (*T. amurensis*) and *T. crassus* (*T. orientalis* and *T.*
239 *meridionalis*) respectively. Meanwhile, Petkevičiūtė and Ieshko (1991) presented a hypothesis
240 about the plesiomorphic organization of the chromosome set in *T. crassus* and, thus, greater
241 closeness of this species to the ancestral form. According to the present results, *T. amurensis* has
242 the most basal position on the trees (based on *cox1* mtDNA) and this species is apparently closer
243 to the ancestral form. Our data show that the group of *Triaenophorus* spp. with a long basal plate
244 of the scolex hook (*T. crassus, T. meridionalis* and *T. orientalis*) has a monophyletic origin.
245 However, the poor support of nodes that unite *T. crassus* with *T. orientalis*, and *T. nodulosus*
246 with *T. meridionalis* + (*T. crassus* + *T. orientalis*) clade in our phylograms, as well as absence of
247 phylogenetic data for North American *T. stizostedionis* Miller, 1945, does not allow us to discuss
248 phylogenetic relationships among *Triaenophorus* spp. in a more global context.

249 The present results have shown that the level of haplotype diversity was the lowest for *T.*
250 *crassus*, higher for *T. amurensis* and the highest for *T. nodulosus*. The difference in the level of
251 haplotype diversity between *T. amurensis* and *T. nodulosus* was not so obvious if compared to *T.*
252 *amurensis* and *T. crassus*. Both *T. nodulosus* and *T. crassus* use the same fish species as
253 definitive hosts (*E. lucius*) and have a similar transholarctic range, whereas *T. amurensis* uses
254 another esocid fish – *E. reicherti*, as a definitive host (Kuperman, 1973). This fish species is

endemic to the Amur River basin and adjacent rivers that were once part of the paleo-Amur system. Hence, we may assume that the differences found are based on the levels of haplotype diversity between *T. nodulosus* and *T. crassus*, as well as between *T. nodulosus* / *T. crassus* and *T. amurensis* on account of different mechanisms.

It is known that the genetic diversity of a species depends on the effects of demographic processes in populations (Nei, 1987). Taking into account the dramatic glacial events of the quaternary period on the territory of North Eurasia, we may assume that the modern structure of genetic diversity of Eurasian isolates (*T. crassus* and *T. nodulosus*) was formed under the pressure of a genetic bottleneck. At the same time we may expect different genetic bottleneck pressures for these cestodes due to the different frequency of dramatic events in various parts of their paleo-areas, different population sizes in the refugia, depletion of some host species in different refugia, etc. All of this could affect both their differences in the level of haplotype diversity and the presence/absence of the geographically specific haplogroups. However, we assume the effect of host-specificity as an additional factor that contributes to the level of haplotype differences between *T. nodulosus* and *T. crassus*. According to Nadler's hypothesis (Nadler, 1995; Martinů et al., 2018), the parasites with a low degree of host-specificity should possess a higher level of genetic diversity than those species that are strictly host-specific. For the studied pair of cestode species, *T. nodulosus* is characterized by a lower degree of host-specificity to its second intermediate host, and, consequently, more various fish species are infected by this cestode (Kuperman, 1973). The effect of the second intermediate host on genetic diversity of these species of *Triaenophorus* is clearly seen in the example of *T. crassus* population from Teletskoye Lake, where this parasite infests sympatric whitefishes, *Coregonus lavaretus pidschian* (Gmelin, 1789) and *C. l. pravdinellus* Dulkeit, 1949 (Kashinskaya et al., 2021). Here, both *T. crassus* and *Coregonus* spp. are characterized by a star-like shape of the network of haplotypes (Fig.5; Bochkarev et al., 2018). This fact suggests that during some period of time the host and its parasite were subjected to similar evolutionary processes.

Triaenophorus amurensis is characterized by the largest difference (among the studied *Trienophorus* spp.) between levels of haplotype and nucleotide diversity. It is known that such differences (relatively high haplotype and low nucleotide diversities) are found in fast growing populations that originated from a low number of founders (Avise, 2000). The studied specimens of *T. amurensis* were collected from two regions (Suppl), but mostly in Sakhalin Island. The Amur species of fishes in Sakhalin Island belong to relict populations that have been surviving after disruption of the paleo-Amur system under the effect of Quaternary transgressions (Lindberg, 1972; Bogatov et al., 2006). We hypothesize that the revealed structure of *T. amurensis* genetic diversity has been determined by the descendants of ancient populations with a small effective size.

CONCLUSION

This study has provided new data on the evolution of the genus *Triaenophorus* in Eurasia. From this work it can be concluded that there are significant genetic differences among the five species of the genus *Triaenophorus* which are taken into account by the taxonomic model of Kuperman (1968): *T. amurensis, T. crassus, T. meridionalis, T. nodulosus* and *T. orientalis*. Thus, these five species previously described, in accordance with the genetic analyses from this study, are recognized as valid.

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Titles

Fig.1. Scolex hooks of *Triaenophorus amurensis* (A) and *T. nodulosus* (B) at the same scale. Scale bar 10 μm.

Fig. 2. Scolex hooks of *Triaenophorus crassus* (A), *T. meridionalis* (B) and *T. orientalis* (C) at the same scale. Scale bar 11 μm.

Fig. 3. Phylogenetic relationships of *Triaenophorus* spp. reconstructed by Maximum Likelihood (left tree) and Bayesian Inference (right tree) analyses of *coxI* gene sequences. Sample numbers are displayed at branch tips. Bootstrap values (ML) and posterior probabilities (BA) are displayed at the branch nodes.

Fig. 4. Geographical distribution of *Triaenophorus* spp. haplotypes across the sampling points (Russian Federation). Circle – *T. crassus*, rhombus – *T. nodulosus*, square – *T. amurensis*, hexagon – *T. orientalis*, triangle – *T. meridionalis*. The single haplotypes are marked by different colors within each pikeworm species symbol.

Fig. 5. Median networks of *Triaenophorus* spp. haplotypes from studied sample points. Black circle - undetected or extinct hypothesized haplotypes. Numbers above circles designate the number of haplotypes. Numbers above connections designate the number of substitutions among studied cestodes. Diameter of circles is proportional to haplotype frequency.

SUPPLEMENT. Sample information for *Triaenophorus* spp. from different fish hosts and waterbodies of Eurasia.