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# Molecular characterisation and infection dynamics of *Dentitruncus truttae* from trout (*Salmo trutta* and *Oncorhynchus mykiss*) in Krka River, Croatia

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ABSTRACT

*Dentitruncus truttae* (Acanthocephala, Palaeacanthocephala) is an intestinal parasite of fish that can cause extensive damage to the host digestive tract, yet little is known about its epidemiology and genetic variability. It is a member of the Illiosentidae family with a worldwide distribution restricted to parts of southeast Europe. Its usual host is brown trout (*Salmo trutta*), but we report here the first detection in the intestine of rainbow trout (*Oncorhynchus mykiss*). We examined the physiology of *D. truttae*-infected *S. trutta* and *O. mykiss*, seasonal and spatial variability of *D. truttae* infections, and genetic variability of the parasite population in Krka River, Croatia. *D. truttae* was more abundant in both trout populations in the autumn, with no seasonal variation in prevalence. The parasite was more abundant in male than female trout ($n = 75$, $p < 0.01$). Analysis of the spatial distribution of the parasite across various sampling sites along the river showed the lowest prevalence and abundance of parasitic infections at the most downstream sampling site, which may reflect the predominance of female fish there and/or the smaller population of intermediate hosts. To provide the first molecular insights into *D. truttae*, we analysed sequences at three marker loci: the 18S rRNA gene, the cytochrome c oxidase subunit 1 (COI) gene and the internal transcribed spacer region. Phylogenetic analysis based on 18S rRNA confirmed the taxonomic grouping of *D. truttae* in the Illiosentidae family, first made more than 50 years ago based on morphology. The COI haplotype network did not show discrete genetic clusters corresponding to the different sampling sites, suggesting a stable population. These insights into *D. truttae* haplotype frequency distribution and intrapopulation genetic variation revealed minimal genetic variability, compared to the other acanthocephalan species.



## 1. Introduction

Members of the phylum Acanthocephala are a relatively homogeneous group of intestinal parasites that use an arthropod as intermediate host and a vertebrate, primarily fish, as definitive host. Some acanthocephalans are

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found worldwide and tolerate a broad range of hosts, while others are confined to limited geographic areas and show strong host specificity (Kennedy, 2006). *D. truttae* is the only member of the genus *Dentitruncus* in the acanthocephalan family Illiosentidae. It has been observed only in limited areas in Bosnia and Herzegovina (Šinžar, 1956; Ćorić, 1963; Čanković et al., 1968), Italy (Manilla et al., 1976; Dezfuli et al., 2008b) and Croatia (Topić-Popović et al., 1999). Its most commonly reported definitive host is brown trout, although it has also been found in Adriatic trout (Ćorić, 1963; Čanković et al., 1968), grayling (Ćorić, 1963), powan (Dezfuli et al., 2009) and European eel (Dezfuli et al., 2012). *D. truttae* can cause serious intestinal damage to fish (Dezfuli et al., 2008a), but little is known about its epidemiology and we are unaware of any molecular information on its intra- and interpopulation genetic variation.

The aim of the present study was to examine the genetic variability of *D. truttae* in salmonids in the Krka River in Croatia, fish physiology during infection, and temporal and spatial variability in these infections. To describe the dynamics of the *D. truttae* population, parasite prevalence was recorded from 2005 to 2008, and abundance was measured in trout during three seasons (summer and autumn 2007, spring 2008) at four sites along the Krka River. The sequences of three genes evolving at different rates (nuclear-encoded ribosomal 18S rDNA gene, ribosomal internal transcribed spacers ITS1 and ITS2, and mitochondrial cytochrome c oxidase subunit I gene) were analysed in order to gain the first molecular insights into *D. truttae* phylogeny and genetic variability.

## 2. Materials and methods

### 2.1. Sampling

Parasitological examination of *D. truttae* specimens was performed on 286 brown trouts (*S. trutta*) and 23 rainbow trouts (*O. mykiss*) sampled with an electrofishing device on the Krka River (Table 1) according to the Croatian standard protocol HRN EN 14011 (2005). The Krka River flows 72.5 km to its mouth in the Adriatic Sea. The upper 49 km of the course are purely freshwater, while the lower 23.5 km are brackish. The river shows longitudinal zonation featuring a simple, coldwater trout assemblage (Salmonidae) upstream and a more diverse warmwater fish assemblage comprising mainly chub (Cyprinidae) downstream (Teskeredžić et al., 2011). Our sampling sites were in the freshwater part of the river and included localities containing exclusively trout (Krčić and Butišnica, sites 1 and 2) and localities containing trout, chub and other warmwater species (Brljan and Roški, sites 3 and 4). Sampling was carried out from April 2005 to April 2008. Prevalence was determined for all samplings, while abundance was determined for the last three in July and November 2007 and in April 2008. These three samplings were taken to be representative, respectively, of summer, autumn and spring.

Prevalence and abundance of *D. truttae* infections in Krka River trout were calculated according to Bush et al. (1997). Standard length, weight and sex of trouts were determined and Fulton's condition index (FCI) was calculated (Nash et al., 2006). Fish were dissected under sterile conditions and *D. truttae* were collected, fixed in 75% ethanol or formalin for morphological determination and stored at −80 °C for molecular analysis. Fixed *D. truttae* species were cleared in lactophenol or stained in Semichon's acetocarmine (Heil, 2009) and identified at genus level by light microscopy (Dezfuli et al., 2008b; Moravec, 2004). Specimens from *O. mykiss* were deposited in the Aschelminthes collection of the Croatian Natural History Museum under registration number 226.

### 2.2. Molecular analysis

Three sequence markers were analysed: two nuclear, 18S rRNA and the internal transcribed spacer (ITS) 1-5.8S rRNA-ITS2; and one mitochondrial, cytochrome c oxidase subunit 1 (COI) gene. Total DNA was extracted from an individual acanthocephalan using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR amplifications (50 μl) were carried out using 1x PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 20 pmol of each primer, 1 U of Taq Polymerase (Applied Biosystems), 50 ng of total DNA and molecular biology-grade water (Sigma). Primers used to amplify 18S rRNA, ITS and COI regions were described previously (Perrot-Minnot, 2004). Reaction conditions were as follows: 10 min at 94 °C (initial denaturation), 35 cycles of 30 s at 94 °C (denaturation), 45 s at 56 °C (annealing) and 1 min at 72 °C (extension), with a final elongation step of 10 min at 72 °C. PCR amplicons were purified using the Gel Extraction Kit (Qiagen) and sequenced commercially (Macrogen, Netherlands). Sequences were deposited in GenBank under accession numbers JX460859–JX460903.

Sequences were analysed using the GenBank BLAST program and aligned with previously characterised 18S rRNA, ITS and COI sequences of the closest acanthocephalans using ClustalX (Thompson et al., 1997). Three Rotifera 18S rDNA sequences (accession numbers AF092434, AF154566 and AF154568) and two Rotifera COI sequences (HQ444174 and AF416995) were included as outgroups. Only unique sequences were used to construct phylogenetic trees, which were inferred by Bayesian inference using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) and by maximum parsimony and maximum likelihood using MEGA version 5 (Tamura et al., 2011). A best fit model of nucleotide substitution was selected using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) in MEGA 5 (Tamura et al., 2011). The following parameters were used for phylogenetic analysis of 18S rDNA sequences: DNA data type, a 4 × 4 nucleotide model, Nst = 6 with a Dirichlet prior, no covariation, four states with frequencies of a Dirichlet prior and an invariable gamma. A single run with four chains (one cold and three heated) was performed for 1 million generations, with sampling every 100 generations and 2500 trees discarded as burning. For phylogenetic analysis of COI sequences, the invertebrate mitochondrial code was set and data were partitioned by codon positions with the evolutionary models assigned to a defined partition based on the AIC and BIC in MEGA 5 (Nst = 6 for codon positions 1 and 2; Nst = 2 for codon position 3; unlink rate heterogeneity model = gamma for all partitions). Dirichlet priors were