**Phylogeny and genetic variability of Rotifer’s closest relatives Acanthocephala: an example from Croatia**

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**Abstract**

The high intraspecific variability of the morphological characters of Acanthocephala can complicate their taxonomic classification. To solve this problem, molecular markers are used. Here we present the identification and determination of genetic variability of four species of the Acanthocephala genera *Pomphorhynchus*, *Acanthocephalus* and *Dentitruncus*, obligate endoparasites of freshwater fishes. The DNA sequences of the three markers that evolve at different rates (18S rDNA, COI and ITS) were analysed. To put the genetic position of the Croatian Acanthocephala specimens in a broader context, the COI and ITS sequences of the other European specimens from the NCBI GenBank were used. Genetic structuring at the local level was minor or not visible at all, but in the context of European phylogeographic structuring, the Croatian *P. laevis* showed a clear grouping to the geographical subcluster Ponto-Caspian Balkans. Only two *P. laevis* Danubian haplotypes, which were not analysed morphologically, were assigned to the Ponto-Caspian Danube subclade together with the recently identified *P. bosniacus* from Austria. The haplotypes of *P. tereticollis* from Croatia were clustered within the two main European clades. In connection with the phylogeographic distribution of Acanthocephala and their hosts, we hypothesised possible phylogeographic patterns.

**Keywords:** *Pomphorhynchus*, *Acanthocephalus*, *Dentitruncus*, nucleotide diversity, haplotype diversity, Croatia

**Introduction**

Acanthocephala or thorny headed worms are a group of obligate endoparasites of the Mandibulata and Gnathostomata, which together with the Rotifera form a monophyletic group called Syndermata (Herlyn et al., 2003). Most Acanthocephala (62.7%) infect aquatic animals of which fish are the most common final hosts (Kennedy, 2006). Fish-parasitizing species infect a wide range of definitive and intermediate hosts (usually Crustacea) with complex life cycles and provide an excellent model for various types of studies on their ecological associations (Perrot Minnot et al., 2023). In the river basins of continental Europe, the most common fish-parasitizing acanthocephalans belong to the class Palaeacanthocephala, order Echinorhynchida, members of the family Echinorhynchidae and Pomphorhynchidae, with 22 recognized species (Gibson et al., 2014; Reier et al., 2020). Only one member of the class Eoacanthocephala, order Neoechinorhynchida, family Neoechinorhynchidae, namely *Neoechinorhynchus rutili* (Müller, 1780), is also widely distributed throughout Europe (Moravec, 2004). In Croatian freshwater systems, members of the families Echinorhynchidae, Pomphorhynchidae, Neoechinorhynchidae and Illiosentidae have been publishedwith at least 5 genera since their first record in 1935 (Babić, 1935): *Pomphorhynchus*, *Acanthocephalus*, *Echinorhynchus*, *Neoechinorhynchus* and *Dentitruncus* (Šinžar, 1955; Topić-Popović et al., 1999; Mladineo et al, 2009; Filipović Marijić et al, 2013; Vardić Smrzlić et al, 2013; Filipović Marijić et al, 2014; Vardić Smrzlić et al, 2015, Mijošek et al, 2022).

One of the main problems in studying the species distribution of Acanthocephala is their complex taxonomy, which can and/or has led to misidentifications in the past (Perrot Minnot et al., 2023). This complexity is due to their sparse morphological characters and high intraspecific variation (Kennedy, 2006). To address these issues, molecular markers such as ribosomal DNA (small 18S and large 28S subunits and internal transcribed spacers 1 and 2, ITS) and mitochondrial (mt) cytochrome c oxidase subunit 1 (COI) are used (Perrot-Minnot, 2004; Steinauer et al, 2007; Špakulová et al., 2011; Rosas-Valdez et al, 2012, 2020; Wayland et al, 2015; Perrot-Minnot et al, 2018; Pinacho-Pinacho et al, 2018; Lewisch et al, 2020; Reier et al, 2020; García-Varela et al, 2023). Morphological and genetic variants of species from the genera *Pomphorhynchus*, *Echinorhynchus* and *Acanthocephalus* in Europe have already been published (Král'ová-Hromadová et al., 2003; O'Mahony et al., 2004; Perrot-Minnot, 2004; Benesh et al., 2006; Špakulová et al., 2011; Wayland et al., 2015; David et al., 2017; Hohenadler et al., 2018; Perrot-Minnot et al., 2018; Nedić et al., 2019; Reier et al., 2019; Amin et al., 2019; Reier et al., 2020; Ros et al., 2020). The genus *Pomphorhynchus*, which has a worldwide distribution and is very common in Europe (Kennedy 2006, Hohenadler et al., 2018), currently includes the three main European species: *P. laevis* sensu stricto (Zoega in Mueller, 1776), *P. tereticollis* (Rudolphi, 1809) and *P. bosniacus* (Kiskároly and Čanković, 1967) (Reier et al., 2019; Ros et al., 2020). In the past, *Pomphorhynchus* species with certain morphological characters were usually identified as *P. laevis* (Hohenadler et al., 2018), while recent studies report a significant and underestimated distribution of *P. tereticollis* (Emde et al., 2012; Hohenadler et al., 2018; Reier et al., 2019.; Ros et al., 2020) and *P. bosniacus* (Reier et al., 2019) in Central and Western Europe. Morphological differences between these sister species are further confirmed by molecular methods (Špakulová et al. 2011; Perrot-Minnot et al., 2018; Reier et al., 2019). In Croatia, *P. laevis* was studied in the Sava River, where it was reported as a new strain distinct from the other continental European haplotypes (Vardić Smrzlić et al., 2015). Another comprehensive study by Perrot-Minnot et al. (2018) distinguished five genetic lineages of *P. laevis* in Europe, some of which correspond to several major biogeographical regions of the European riverine fish fauna: Central Peri-Mediterranean, Eastern Peri-Mediterranean, Ponto-Caspian Sea, Central Europe and Western Europe, while *P. tereticollis* showed a weak geographical and genetic structuring. Recent studies have highlighted the importance of combined morphological and molecular studies of the genus *Pomphorhynchus* in Europe (Reier et al., 2019; Ros et al., 2020). *Dentitruncus truttae* (Šinzar, 1955) is an endemic species and only one study on genetic intrapopulation variability in the Krka River in Croatia has been published so far (Vardić Smrzlić et al., 2013). The genus *Acanthocephalus*, although widely distributed in Europe, has been little studied at the level of genetic variability (Benesh et al., 2006; Amin et al., 2019; Reier et al., 2020). Studies on this genus are also lacking in Croatia.

In addition to complex taxonomy, changes in the distribution of intermediate and definitive hosts may also influence the species diversity of Acanthocephala (Lagrue, 2017; Giari et al., 2020; Ros et al. 2020; Vogel and Taraschewski, 2023). Invasive species of intermediate and final hosts of Acanthocephala could migrate and transfer non-native species of Acanthocephala (e.g. Ponto-Caspian species across the Danube), thus changing their biodiversity (Hohenadler et al. 2018; Hohenadler et al., 2019; Vogel and Taraschewski, 2023). Croatia is located on the Balkan Peninsula and its continental biogeographical region is bounded by the rivers of the Black Sea basin (Danube drainage), while the rivers of the Mediterranean biogeographical region have direct (e.g. Krka) or underground (e.g. Lika) connections to the Adriatic Sea (Žganec et al., 2020). The Balkan Peninsula is considered a hotspot in the evolution of many European species (Žganec et al., 2016) and this is also reflected in the diversity of intermediate and final fish hosts of Acanthocephala. However, the increase of alien and invasive species that can serve as intermediate (Kralj et al., 2022) and final hosts (Ćaleta et al., 2019) of Acanthocephala in Croatian freshwaters could change species diversity.

Therefore, the current study is a continuation of our efforts to understand the genetic diversity of the most common Acanthocephala in this part of south-eastern Europe and to investigate the geographical distribution of their nuclear and mt sequence variation through extensive sampling at different geographical sites (12 rivers of the Black Sea and 2 of the Adriatic Sea basin) and definitive fish hosts (7 species). In particular, our aim was to: 1) identify the genetic variability of species from three Acanthocephala genera: *Pomphorhynchus*, *Dentitruncus* and *Acanthocephalus* in Croatia using molecular markers: 18S rRNA, COI and ITS; 2) determine the genetic position of Croatian Acanthocephala haplotypes in the context of phylogroups of the European population; 3) discuss the phylogeographic patterns of their distribution in relation to hosts.

**Materials and methods**

*Specimen sampling and identification*

The Acanthocephala specimens used for analysis in this study were obtained from freshwater fish collected in various rivers and streams in Croatia between 2005 and 2015 (Figure 1, Table 1). The fish were collected by electrofishing according to the Croatian standard protocol HRN EN 14011 (2005). All fish were anaesthetized with clove oil (Sigma Aldrich) and sacrificed. Acanthocephala from the gut were collected and fixed in 75% ethanol for morphological identification and/or stored at -80◦C for molecular analysis. Specimens used for morphological identification (3-5 specimens of different species per site) were cleared with methyl salicylate and examined under a magnifying glass (Olympus) or a BH-2 microscope (Olympus). Identification was done at the genus level for *Pomphorhynchus* spp. due to the damaged proboscis in several specimens or at the species level for *Acanthocephalus* spp. and *D. truttae* according to the keys for Acanthocephala (Petroschenko, 1956; Moravec, 2004; Amin et al., 2008; Dezfuli et al., 2008). Molecular methods were also used to confirm species-level identification and to determine genetic variability among specimens.

**Figure 1.** Map of all sites with records of different Acanthocephala species in freshwaters of Croatia. Brown dashed line represents the divide between the Black Sea (Danube) and the Adriatic Sea basins (Figure adapted from Ćaleta et al., 2019).

Table 1. Data of the samples: name of the isolates, fish host, river, location and haplotype from the Median Joining network (from Fig. 2B)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***Isolate*** | ***Fish host*** | ***River*** | ***Location Φ (kN) Λ (E)*** | ***Haplotype (MJ network, Figure 1B)*** |
| ***P. laevis*** |
| C 10  | *Salmo trutta* | Cabranka | 45,5454 14,6919 | Hap\_20 |
| GOJ4 | *Squalius cephalus* | Dobra | 45,2999 15,2702  | Hap\_11 |
| GOJ19 | *Barbus barbus* | Dobra | 45,2999 15,2702 | Hap\_9 |
| DOBP | *Salmo trutta* | Dobra | 45,3542 15,1070 | Hap\_22 |
| R1 | *Barbus barbus* | Ribnjak | 45,2065 15,3608 | Hap\_14 |
| DUN 3 | *Barbus barbus*  | Danube | near Vukovar city | Hap\_17 |
| DUN 11 | *Leuciscus idus* | Danube | near Vukovar city | Hap\_4 |
| KOK | *Squalius cephalus* | Korana | 45,1213 15,5895 | Hap\_1 |
| KOU | *Squalius cephalus* | Korana | 45,1213 15,5895 | Hap\_1 |
| KR 1 | *Squalius cephalus* | Krapina | 45,8333 15,8262 | Hap\_13 |
| KR 2 | *Squalius cephalus* | Krapina | 45,8333 15,8262 | Hap\_4 |
| KR 3 | *Squalius cephalus* | Krapina | 45,8333 15,8262 | Hap\_4 |
| KK5 | *Thymallus thymallus* | Kupa | 45,5315 15,7894 | Hap\_12 |
| KU2 | *Squalius cephalus* | Kupa | 45,6148 15,4749 | Hap\_4 |
| KU3 | *Squalius cephalus* | Kupa | 45,5490 15,8453 | Hap\_16 |
| KU4 | *Squalius cephalus* | Kupa | 45,6148 15,4749 | Hap\_4 |
| KU10 | *Squalius cephalus* | Kupa | 45,6148 15,4749 | Hap\_7 |
| KU33 | *Squalius cephalus* | Kupa | 45,6148 15,4749 | Hap\_15 |
| KT2 | *Squalius cephalus* | Kupa | 45,8333 14,8262 | Hap\_16 |
| L 4 | *Esox lucius* | Lika | 44,5824 15,3917 | Hap\_4 |
| M1 | *Squalius cephalus* | Mrežnica | 45,4530 15,5065 | Hap\_21 |
| OR 7 | *Squalius cephalus* | Orljava | 45,1755 17,7055 | Hap\_4 |
| OR 11 | *Squalius cephalus* | Orljava | 45,1755 17,7055 | Hap\_4 |
| OR 14 | *Squalius cephalus* | Orljava | 45,1755 17,7055 | Hap\_8 |
| IG1 | *Barbus barbus* | Sava | 45,6438 16,3231 | Hap\_4 |
| S36 | *Squalius cephalus* | Sava | 45,8423 15,7249 | Hap\_6 |
| S2 | *Squalius cephalus* | Sava | 45,8437 15,8097 | Hap\_2 |
| SUT1 | *Squalius cephalus* | Sutla | 46,2223 15,7197 | Hap\_10 |
| SUT6 | *Squalius cephalus* | Sutla | 46,0454 15,7372 | Hap\_18 |
| SUT9 | *Squalius cephalus* | Sutla | 46,0454 15,7372 | Hap\_19 |
| SUT21 | *Squalius cephalus* | Sutla | 45,8738 15,6904 | Hap\_5 |
| ***P. tereticollis*** |
| A7 | *Squalius cephalus* | Dobra | 45,3482 15,3480 | Hap\_6 |
| A8 | *Squalius cephalus* | Dobra | 45,3482 15,3480 | Hap\_7 |
| HL6 | *Squalius cephalus* | Dobra | 45,3732 15,3608 | Hap\_1 |
| HL19 | *Squalius cephalus* | Dobra | 45,3732 15,3608 | Hap\_1 |
| GOJP | *Salmo trutta* | Dobra | 45,2999 15,2702 | Hap\_2 |
| R7K | *Squalius cephalus* | Ribnjak | 45,2065 15,3608 | Hap\_3 |
| KT4 | *Barbus barbus* | Kupa | 45,8333 14,8262 | Hap\_5 |
| U4 | *Squalius cephalus* | Una | 45,2214 16,5122 | Hap\_4 |
| ***D. truttae*** |
| U2 | *Salmo trutta* | Una | 44,2499 16,6319 | Hap\_4 |
| U3 | *Salmo trutta* | Una | 44,2499 16,6319 | Hap\_5 |
| U6 | *Salmo trutta* | Una | 45,6584 16,2357 | Hap\_6 |
| U8 | *Salmo trutta* | Una | 45,6584 16,2357 | Hap\_7 |
| BRLJ2 | *Salmo trutta* | Krka | 44,0034 16,0244 | Hap\_1 |
| BRLJ5 | *Salmo trutta* | Krka | 44,0034 16,0244 | Hap\_1 |
| BUT8 | *Salmo trutta* | Krka | 44,0229 16,1034 | Hap\_2 |
| KRC2 | *Salmo trutta* | Krka | 44,0256 16,1441 | Hap\_3 |
| ***A. anguillae*** |
| S3\_1 | *Cyprinus carpio* | Kupa | 45,6148 15,4749 | Hap\_1 |
| S3\_2 | *Cyprinus carpio* | Kupa | 45,6148 15,4749 | Hap\_1 |
| B1 | *Carassius gibelio*  | Kupa | 45,6148 15,4749 | Hap\_4 |
| KLENT | *Squalius cephalus* | Dobra | 45,2999 15,2702  | Hap\_2 |
| KLEN22 | *Squalius cephalus* | Dobra | 45,2999 15,2702  | Hap\_3 |
| TROS1 | *Squalius cephalus* | Dobra | 45,2065 15,3608 | Hap\_5 |
| V1 | *Squalius cephalus* | Dobra | 45,3732 15,3608 | Hap\_6 |

*DNA extraction, amplification and sequencing*

A total of 58 specimens (41 *Pomphorhynchus* spp., 9 *Acanthocephalus* spp. and 8 *D. truttae*), which were not used for the morphological analyses, were prepared for DNA extraction. DNA extraction was performed using the DNeasy® Blood & Tissue (Qiagen) kit from each individual specimen according to the manufacturer's instructions. PCR amplifications (50 µl) were performed using 1x PCR Master Mix (Jump Start Mix or AccuTaq Mix buffer), 20 pmol of each primer, 3 µl (~10 ng) total DNA and molecular biology grade water (Sigma). The primers used for amplification of the 18S rRNA, ITS and COI regions have been described previously (Perrot-Minnot, 2004). The reaction conditions were as follows: 10 min at 94°C (initial denaturation), 35 cycles of 30 s at 94°C (denaturation), 45 s at 46°C or (annealing) and 1 min at 72°C (extension), with a final elongation step of 10 min at 72°C for the COI marker, while the conditions for amplification of 18S and ITS PCR were described by Perrot-Minnot (2004). The amplicons were purified with the Gel Extraction Kit (Qiagen) and commercially sequenced (Macrogen, The Netherlands). The sequences were deposited in NCBI GenBank under the accession numbers: OQ569490-OQ569520, OQ569522-OQ569529, OQ569535-OQ569536 and OQ569921-OQ569928 (COI marker); OQ572460-OQ572482, OQ572448-OQ572454, OQ572455-OQ572459 and OQ572444-OQ572447 (ITS region); OQ581714, OQ581740, OQ581743, OQ581789 and OQ592254 (18S rRNA).

*Phylogenetic analyses and haplotypes networks*

The DNA sequences were intact and there was no evidence of NUMTs (nuclear pseudogenes of mt sequences) in COI sequences (Benesh et al., 2006; Vardić Smrzlić et al., 2015). Sequences of three markers evolving at different rates (12 sequences of 18S rDNA, 54 sequences of COI, and 42 sequences of ITS) were edited and aligned using the program BioEdit (<http://www.mbio.nscu.edu/BioEdit/BioEdit.html>; Hall, 1999). NCBI Blast was used to check the percentage identity with the closest sequences. The final alignment of the 18S rRNA marker contained 50 sequences from Croatia and European isolates and was trimmed to the length of 1720 bp. The COI marker of Croatian Acanthocephala contained 54 sequences and was trimmed to the same length (565 bp), while the alignment of the ITS marker contained 42 sequences which were also trimmed to a length of 614 bp. To compare our sequences with the dataset from the NCBI GenBank we used sequences under the following accession numbers: AY218096, AY423348-AY423353, EF051062-EF051071, KF559284-KF559300, LN994875-LN995000, LN994844-LN994873, LN994840-LN994842, JF06706, JN695504-JN695508, MF563497-MF563527, MK612497-MK612545 (COI marker for *Pomphorhynchus* spp.) and MN416028-MN416031; MT682931, MT682932, MT682934; MN780911-MN780919 (COI marker for *Acanthocephalus anguillae*); AY424669-AY424670, AY135413-AY135418, JF706705, KF559305-KF559308, KJ756498-KJ756500, KY075791-KY075793, LN995001- LN995017, LN995038-LN995058, MH319898-MH319899, MK157041, MT216138-MT216148, OP681185 (ITS marker for *Pomphorhynchus* spp.), MN394422- MN394423, MN394424 (ITS for *Acanthocephalus* *anguillae*). The final alignment contained 243 sequences for the COI dataset of *P. laevis* and was trimmed to 559 bp, while the final alignment contained 84 sequences for the COI dataset of *P. tereticollis* and was trimmed to 604 bp. ITS regions of the European isolates of *P. laevis* and *P. tereticollis* have mostly incomplete sequences in the database. Therefore, a significantly shorter segment of the ITS regions was used for the analyses, namely 149 bp for *P. laevis* and 277 bp for *P. tereticollis*. The sequences of the COI marker of *A. anguillae* were trimmed to 614 bp. Phylogenetic trees were constructed using MEGA 11 (Tamura et al., 2021) and the evolutionary history was inferred by using the Maximum Likelihood methods and the following models: GTR model (for 18S rRNA, for the Croatian + European COI dataset) and HKY (for the Croatian COI dataset, for Croatian ITS dataset and for the Croatian+European ITS dataset). Haplotype diversity (Hd) and nucleotide diversity (π) of the COI and ITS datasets were performed in DnaSP v5 (http://www.ub.edu/dnasp; Librado and Rozas, 2009).

Median-Joining (MJ) Haplotype Networks (Bandelt et al., 1999) were calculated using PopART 1.7 software (<http://www.popart.otago.ac.nz>, Leigh and Bryant, 2015).

**Results**

*Morphological determination*

The results of the morphological identification of Acanthocephala from the Croatian rivers showed the presence of: *Pomphorhynchus* spp., *Acanthocephalus anguillae* (Mueller, 1780), *Acanthocephalus lucii* (Mueller, 1776) and *D. truttae*. Acantocephala from the rivers: Dobra, Kupa Sava and Sutla (five individuals per river), Danube, Orljava and Krapina (four individuals per river), Čabranka, Ribnjak, Korana, Lika, Mrežnica and Una (three individuals per river) had a long neck with a spherical bulb at the anterior end, which allowed us to classify them in the genus *Pomphorhynchus*. Their body length was 6.07-21.8 mm, while the width was 1.68-2.8 mm. Due to the damaged proboscis, the number and shape of the hooks could not be determined, so that an identification on the species level was not possible. Three specimens from the Kupa River and four from the Dobra River had bodies 5.56-14.43 mm long and 1.28-1.83 mm wide. Six hooks were arranged in a row on the 0.57-0.78 mm long proboscis. The hook roots were well developed and contained two distinct lateral outgrowths, while the posterior hooks were without lateral extensions. These morphological features allowed us to classify them as *A. anguillae*. Three specimens of Acanthocephala from the Lika River had bodies 6.20-11.43 mm long and 0.98-1.52 mm wide. The 0.53-0.82 mm long proboscis was armed with seven wing-shaped hooks in a row, without lateral outgrowths on the distended roots. These characteristics allowed us to identify the individuals as *A. lucii*. Five specimens from the Krka River and three from the Una River had bodies 6.2-11.4 mm long and 0.98-1.52 mm wide. There were 17-18 hooks in longitudinal rows on the 0.60-0.99 mm long proboscis. The trunk was characteristically spined anteriorly, so that these individuals could be assigned to the species *D. truttae*. The results of the morphological analysis agree with the molecular data. In the case of the *Pomphorhynchus* sister species, the identification of *P. laevis* and *P. tereticollis* was possible by ITS and COI sequence analysis.

*DNA sequence analysis*

Variability in 18S rRNA sequences was absent or very low in the species studied (Table 1, Supplementary), whereas ITS and COI were more informative (Table 2). Phylogenetic analysis of 18S rRNA showed clear separation in well supported clades (Fig. 1 supplementary). Because *A. lucii* was represented by only two specimens, we did not use it for genetic diversity analyses (Figure 2, Supplementary).

The lowest nucleotide and haplotype diversity of the ITS region was found for *P. tereticollis* and *P. laevis* and the highest for *D. truttae* (Table 2). The lowest nucleotide diversity of the partial COI marker was found in *P. laevis* and the highest in *P. tereticollis*. *A. anguillae* and *D. truttae* had similar values for COI nucleotide and haplotype diversity.

Table 2. Nucleotide diversity of ITS and COI region in analysed acanthocephalan species. Number of haplotypes (H), number of polymorphic sites (S), nucleotide diversity (% π) and haplotype diversity (Hd) are given.

|  |  |  |
| --- | --- | --- |
| **Species** | **ITS** | **COI** |
| N | H | S | % π | % Hd | N | H | S | % π | % Hd |
| *P. laevis* | 25 | 8 | 9 | 0.230 | 63.2 | 31 | 22 | 28 | 0.446 | 92.0 |
| *P. tereticollis* | 10 | 3 | 2 | 0.111 | 60.0 | 8 | 7 | 22 | 1.156 | 96.4 |
| *D. truttae* | 5 | 4 | 7 | 0.654 | 90.0 | 8 | 7 | 14 | 0.732 | 96.4 |
| *A. anguillae* | 4 | 3 | 3 | 0.250 | 83.3 | 7 | 6 | 14 | 0.892 | 95.2 |

Phylogenetic analysis based on partial COI and ITS sequences confirmed the separate grouping of Acanthocephala species (Figure 2 and 3).

*Pomphorhynchus laevis*

COI dataset

MJ network analysis of *P. laevis* based on COI sequence analyses consists of 22 haplotypes (out of 31 specimens) and is roughly divided into five clusters that do not reflect a clear geographic structuring (Figure 2). The low nucleotide diversity (π=0.00446) is partly due to the high frequency of a very common haplotype. This haplotype originated from several fish species (6 *Squalius cephalus*, 1 *Esox lucius*, 1 *Barbus barbus*, and 1 *Leuciscus idus*) and rivers (Krapina, Kupa, Orljava, Sava, Danube and Lika). The high haplotype diversity (HD =0.920) is due to the large number of haplotypes shared by only one or two individuals.

When we compared our *P. laevis* COI dataset with 212 other *P. laevis* and *P. bosniacus* COI sequences from NCBI GenBank, inferred Maximum Likelihood bootstrap tree showed similar clustering into different clades as it was shown by Perrot-Minnot (2018) and Reier (2019) (Figure 3A and B, Supplement). All Croatian sequences of *P. laevis* clustered within Ponto-Caspian – Balkan clade, except for two haplotypes from the Danube (Figure 3A and B, Supplement). However, it is it is necessary to emphasize that two other Croatian strains from the Danube fell under the Ponto-Caspian – Balkans clade. This clade, apart from Croatian haplotypes also contains most haplotypes from Serbia, Slovenia, Romania and Austria (Figure 3A and B, Supplement).

 ITS dataset

MJ network analysis of *P. laevis* based on ITS sequence analyses consists of 8 haplotypes (out of 25 specimens) and is divided into six clusters that do not reflect a clear geographic structuring (Figure 3). The main haplotype consists of 13 specimens from different rivers. Two haplotypes of Dobra River are clustered 26 and 27 mutational steps away from the main haplotypes, while two Danube haplotypes were 4 mutational steps distant from the main haplotype.

MJ haplotype network which was constructing to show assignation of Croatian *P. laevis* haplotypes to the other European haplotypes based on 82 ITS sequence analyses showed 2 clear groups of sequences divided by 41 mutation steps (Figure 4, Supplementary). Largest cluster included 33 specimens from: Croatia (all sequences from this study), all German haplotypes, partly Czech, Italian, French and Slovakian haplotypes, and also haplotypes from Bosnia and Herzegovina and Uzbekistan. Near to the main haplotype was another one included *P. bosniacus* from Bosnia and Herzegovina (Figure 4, Supplementary). Other large cluster (23 specimens) included French, Serbian, Romanian, Russian, Bulgarian, Slovenian and Turkey specimens. Close to it, haplotypes including specimens from Italy and Turkey, and also Poland, Bulgaria and Austria together with one Croatian specimen done by another authors (Perrot-Minot, 2018) were obtained. This haplotype network could not clearly differentiate Ponto-Caspian – Balkan clade, as some specimens from Balkan countries were divided into two separated clades. However, it is necessary to emphasize that we used short part of ITS sequences (143 bp) for the haplotype network construction, due to incomplete DNA sequences data in NCBI GenBank.

*Pomphorhynchus tereticollis*

COI dataset

Haplotype network analysis of *P. tereticollis* revealed the presence of 7 haplotypes (out of 8 specimens) and the clusters obtained reflect a geographical structuring (Figure 2). Specimens from the Dobra River and its tributary Ribnjak are clustered separately from specimens from the Kupa and Una Rivers. Only one haplotype from the Dobra River was separated from the nearest haplotype by ten mutational steps. When we compared Croatian *P. tereticollis* COI dataset with other 76 *P. tereticollis* COI sequences from NCBI GenBank, the haplotype network showed 46 haplotypes divided into four clusters (Figure 5, Supplement). The largest cluster includes Croatian haplotypes which partly formed sub-cluster itself and partly clustered with Austrian of French isolates (Figure 5, Supplement). Other three clusters included NCBI GenBank datasets from: Austria, France and from Central and Northern Europe (mostly from marine fish species (Figure 5, Supplement). This „marine“ cluster was divided from the other European haplotypes by 7 mutation steps (Figure 5, Supplement).

ITS dataset

MJ network analysis of *P. tereticollis* based on ITS sequence analyses consists of 3 haplotypes (out of 10 specimens) and is divided into three clusters that do not reflect a clear geographic structuring (Figure 3). The main haplotype consists of 5 specimens from all studied rivers (Figure 3).

MJ haplotype network which was constructing to show assignation of Croatian *P. tereticollis* haplotypes to the other European haplotypes based on ITS sequence analyses showed four different clusters not clearly geographically distributed (Figure 6, Supplement). All Croatian haplotypes were clustering together with haplotypes from Germany, France and Slovakia. Another large cluster included Baltic Sea and Western Europe haplotypes, but also some Balkan haplotypes (Figure 6, Supplement). In these analyses we also used short part of ITS sequences (277 bp) for the haplotype network construction, due to incomplete DNA sequences data in NCBI GenBank.

*Dentitruncus truttae*

COI dataset

The MJ network analysis of *D. truttae* based on COI sequence analyses consists of 7 haplotypes (out of 8 specimens) and do not reflect clear geographic structuring of Krka River and Una River specimens (Figure 2). The large haplotype diversity (HD=0.964) is due to the large number of haplotypes shared by only one or two individuals. Due to missing NCBI GenBank data for this species from other geographical regions, we could not compare our data to the other European specimens.

ITS dataset

The MJ network analysis of *D. truttae* based on ITS sequence analyses consists of 4 haplotypes just from the Krka River (Una River specimens were not available for the analysis) (Figure 3). Obtained haplotypes showed diversification according to different sampling sites in the Krka River. However, only 5 samples were included into analysis and we could not compare our data to the other European specimens due to missing NCBI GenBank data for this species from other geographical regions.

*Acanthocephalus anguillae*

COI dataset

The haplotype network of *A. anguillae* based on COI dataset showed low nucleotide diversity and high haplotype diversity, similar to the other species studied. A value of nucleotide diversity π=0.00892 is due to missing haplotypes connecting clusters, but in general a geographical separation of specimens from the Kupa and Dobra rivers could be detected. However, these haplotypes also showed differences among themselves (more mutation steps) (Figure 2). When we compared Croatian *A. anguillae* COI dataset to the other 16 *A. anguillae* COI sequences from the NCBI GenBank, the haplotype network showed clustering of 2 main clusters with more subclusters. Croatian haplotypes were clustered together with *Acanthocephalus* sp. from Austria (Figure 7, Supplement), while German and Austrian *A. anguillae* formed separated cluster. Two sequences of *A. anguillae balcanicus* from Slovenia were divided by 7 mutation steps from *A. anguillae* from Austria (Figure 7, Supplement).

ITS dataset

The MJ network of *A. anguillae* specimens based on ITS sequence analyses consists of 3 haplotypes (out of 4 specimens) and one haplotype consisted both from Kupa and Dobra River specimens, indicating no clear geographical distribution (Figure 3). When we compared Croatian *A. anguillae* haplotypes to the other European haplotypes based on ITS sequence analyses (also just three specimens from the NCBI GenBank, including two *A. anguillae* balcanicus) due to different sequences length only 344 bp sequences could be compared and ITS alignment showed 100% of identity.



**Figure 2.** Phylogenetic analyses of members of three Acanthocephala genera from Croatia based on partial COI marker sequence (565 bp). Evolutionary history was inferred using the maximum likelihood method and the Hasegawa-Kishino-Yano model in MEGA11. The percentage of trees in which the associated taxa clustered is shown next to the branches. Median linkage networks of four Acanthocephalan species: *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis*, *Dentitruncus truttae*, and *Acanthocephalus anguillae*, performed in PopART 1.7. Mutation steps are indicated by vertical lines. Black dots represent haplotypes missing from the study sample. Coloured dots represent haplotypes from different sites, while the size of the dot indicates the number of haplotypes.



**Figure 3.** Phylogenetic analyses of members of three Acanthocephala genera from Croatia based on partial ITS sequence (614 bp). Evolutionary history was inferred using the maximum likelihood method and the Hasegawa-Kishino-Yano model in MEGA11. The percentage of trees in which the associated taxa clustered is shown next to the branches. Median linkage networks of four Acanthocephalan species: *Pomphorhynchus laevis*, *Pomphorhynchus tereticollis*, *Dentitruncus truttae*, and *Acanthocephalus anguillae*, performed in PopART 1.7. Mutation steps are indicated by vertical lines. Black dots represent haplotypes missing from the study sample. Coloured dots represent haplotypes from different sites, while the size of the dot indicates the number of haplotypes.

**Discussion**

*Distribution and phylogeographic patterns of Acanthocephala in Croatia*

The high level of endemism of Balkan freshwater fauna, including the intermediate and final hosts of Acanthocephala, is well known (Žganec et al., 2016). Members of the genera *Gammarus* and *Echinogammarus*, two largest European genera of the family Gammaridae, which are known intermediate hosts of Acanthocephala, have restricted distribution with centers of endemism at Balkan Peninsula (Žganec et al., 2010). As far as fish hosts are concerned, Croatia is mainly located in the Central Peri-Mediterranean biogeographical region identified by Reyjol et al. (2007) and has at least 137 freshwater species (Ćaleta et al., 2019). Faunistic peculiarity of the Balkan Peninsula is due to both geotectonic events that separated the Mediterranean and Pontian river basins during the Tertiary and climatic events that occurred at the beginning of the Quaternary (Hewitt et al., 2017). However, range expansions of a high number of alien and invasive species across Croatian freshwaters, including species that may serve as intermediate (Kralj et al., 2022) and final (Ćaleta et al., 2019) hosts of Acanthocephala may cause changes in their biodiversity (Vogel and Taraschewski, 2023). The possible influence of hosts on the genetic structure of Acanthocephala is important to determine their phylogeographic patterns. In the present study, we analysed the genetic variability of different members of four Acanthocephala species: *P. laevis*, *P. tereticollis*, *A. anguillae* and *D. truttae* from 28 different sites, including rivers of the Black Sea basin (Cabranka, Dobra, Danube, Korana, Krapina, Kupa, Mreznica, Orljava, Sava, Sutla, Ribnjak, Una) and the Adriatic basin (Lika, Krka) in Croatia (Figure 1).

*P. laevis* was found in all mentioned rivers of the Black Sea basin except for Una River, but only two sampling sites of Una River were included in the investigation. As expected, *S. cephalus*, a native fish species in the rivers of Danube drainage area in Croatia was dominant final host (71% of all fish species) for *P. laevis*. It seems that *P. laevis* together with its final host have a long history in mentioned rivers systems. Regarding possible intermediate hosts, in all rivers where *P. laevis* was found, characteristic native gammarids are also distributed, e.g. *Gammarus fossarum* (Sava – upper flow, Una, Kupa, Dobra and tributaries, Orljava, Sutla) although down streams of Sava and Croatian part of Danube are almost completely inhabited by invasive species: *Chelicorophium sowinskyi* (Martynov, 1924), *C. curvispinum* (G.O. Sars, 1895), *Dikerogammarus haemobaphes* (Eichwald, 1841) and *D. villosus* (Sovinsky, 1894) (Žganec et al., 2009; Kralj et al., 2020). As *P. laevis* was common in Danube, Sava and its tributaries such as Sutla, Krapina and Orljava River, we can create hypothesis of expansion of this species from the largest Black Sea drainage rivers to their tributaries. Such pattern could be confirmed by spreading of invasive gammarid species as intermediate hosts throughout large rivers of Danube drainage, e.g., *D. villosus*, *D. haemobaphes* or *C. curvispinum*. *D. villosus* is a P.-C. invasive gammarid described as a very successful intermediate host that promotes expansion of *Pomphorhynchus* sp. (Reier et al., 2019; Emde et al., 2012; Hohenadler et al., 2018). Invasive *Neogobius* specimens spread within Danube and Sava River could promote expansion of *P. bosniacus* in Austria (Reier et al., 2019).

*P. tereticollis* was detected at the three sites at Dobra River, one site at Kupa River and one downstream site at Una River. Final host also predominately was *S. cephalus* (75%), while native gammarid *G. fossarum* is presented in all rivers. We need to emphasise the fact that on the localities where *P. tereticollis* was found at Dobra River, endemic species *Echinogammarus cari* (Karaman 1931), the only native species of this genus present in the Black Sea drainage basin of Croatia, was presented (Žganec et al., 2009). It is possible that this species is the original intermediate host for *P. tereticollis* in Dobra River. Due to the construction of the dam, its population is decreasing which can also affect *P. tereticollis* population (Žganec et al., 2009). At Una River locality of *P. tereticollis* was also inhabited by *G. roeseli* (Žganec et al., 2010), which is a well-known final host for *Pomphorhynchus* species. We need to emphasize that in Dobra River with common *P. tereticollis* species no invasive gammarid species were detected (Žganec et al., 2009; Žganec et al. 2020).

*A. anguillae* was found in one Kupa and two Dobra River localities, in co-occurence with *Pomphorhynchus* species (Figure 1). It is also found only in cyprinid species (*S. cephalus*, *C. carpio* and *C. gibelio*, Table 1). Bates and Kennedy (1990) published that in vitro co-infection of salmond fish with *A. anguillae* and *P. laevis* affected and reduced range of intestine occupation of *A. anguillae*, but not vice versa. It seems that such interference competition also exists in the nature, as we found *P. laevis* to be predominant species at the sampling sites with coinfection.

*D. truttae* is an endemic Acanthocephala species and its occurrence was confirmed in the Krka River (Croatia) and Una River (Croatia, Bosnia and Herzegovina) (Šinžar, 1956). The Krka River belongs to the Adriatic Basin, while the Una River belongs to the Black Sea Basin, and in both rivers *Salmo trutta* specimens were caught as fish hosts for this parasite. However, both rivers contain the same endemic gammarid species *Echinogammarus acarinatus* (Karaman, 1931) and *Fontogammarus dalmatinus* (Karaman, 1931) (Gottstein et al., 2007; Žganec et al., 2010), which could serve as an intermediate host for *D. truttae*. Close proximity of the upper Una and Butiznica (Krka tributary) indicates that river capture events could explain the presence of the same species in different drainage basins. River capture events probably happened during the Pliocene period when Dalmatian plateau uplift and active incision of rivers occurred (Žganec et al., 2016).

*Genetic variability and haplotype networks*

Analysis of 18S rDNA sequences confirmed conservation of this nuclear marker within studied acanthocephalan specimens (Table 1, Supplement), as all sequences in our study showed no variability. Nevertheless, this marker was suitable for elucidating evolutionary relationships among acanthocephalan taxa (Garciá-Varela and Nadler, 2005; Vardić Smrzlić et al., 2013), as well as for differentiating close specimens e.g., *P. laevis* sensu stricto and *P. tereticollis* (Perrot-Minnot, 2004). In our study, we could differentiate species within *Pomphorhynchus* and *Acanthocephalus* genera, based on 18S rDNA phylogenetic tree clustering (Figure 1, Supplement).

Analysis of ITS region showed low nucleotide and haplotype diversity and was less informative about studied acanthocephalan phylogroups. This marker is conserved within the species but could have a high degree of variation even between closely related species. In our study, we used this marker for *P. laevis* and *P. tereticollis* diversification (Figure 3). The values of nucleotide and haplotype diversity of ITS are similar to those that we published earlier (Vardić Smrzlić et al., 2013; Vardić Smrzlić et al., 2015). Haplotype network analysis of *A. anguillae* was not informative as one large haplotype contained sequences from all sampling sites where this species was found (Figure 2). When we compared our ITS sequences to the other European available from the NCBI GenBank, we used trimmed ITS sequences of *P. laevis* (149 bp) and *P. tereticollis* (277 bp) for haplotype network calculation and such short sequences could be the reason for weak geographical structuring.

In general, the COI marker proved to be more variable (π=0.00446-0.01156) and was most informative for phylogeographic and haplotype network analysis. The MJ network shows high haplotype diversity but significantly low nucleotide diversity, which is consistent with the results of Reier et al. (2019). They found higher nucleotide diversity in *P. laevis* specimens belonging to the more distant geographic regions in Austria (Reier et al., 2019). The fact that the Lika from the Adriatic Basin and the Krapina, Kupa, Orljava, Sava, and Danube rivers from the Black Sea Basin share the same *P. laevis* haplotype suggests that haplotypes are not strictly separated by geographic location. Lika is the underground and isolated river and we cannot explain presenceof such common haplotype of *P. laevis* by expansion from another watershed. It is possible that due to the frequent practice of stocking rivers in Croatia in the past, where individual adult fish are transferred from one river to another, *Acanthocephala* are also transferred.

Recent studies have documented phylogeographical patterns of widely distributed *Pomphorhynchus* species, identifying 5 genetic lineages of *P. laevis* in Europe (Perrot-Minnot et al., 2018; Reier et al., 2019) and showed misidentifications, intraspecific variability and possible cryptic speciation within different acanthocephalan species from Austria (Reier et al., 2020). Croatian haplotypes as expected were clustered within Ponto-Caspian Balkans clade, that includes specimens from the tributaries of the Danube River (Figure 3A and B, Supplement) (Perrot-Minnot, 2018). However, two haplotypes from the Danube River were clustered within group that contained *P. bosniacus* from Austria (Reier et al., 2019). Due to damaged proboscis we could not morphologically exanimate these specimens, but it is possible that it was misidentified, and that these haplotypes are *P. bosniacus* specimens. ITS region analyses also can confirm this finding, as Croatian Danube ITS haplotypes were clustered with Bosnian *P. laevis* sequences from the NCBI GenBank. More in depth morphological examination of the species from the Danube should be done.

Of the species studied, *P. tereticollis* showed the highest nucleotide diversity (π=0.01556). However, this value is generally low, and such a result together with high haplotype diversity was also published for *P. tereticollis* studied by Reier et al. (2019). The clustering of haplotypes of *P. tereticollis* might be related to the different sampling sites on the Dobra River. Two haplotypes separated from the main haplotype of *P. tereticollis* by four and fourteen mutation steps originated from the same sites (Table 1) and other haplotypes from the different sites.

Vardić Smrzlić et al. (2013) showed low genetic variability within *D. truttae* specimens from the Krka River and our results did not indicate a clear separation of *D. truttae* haplotypes between Krka and Una (Figure 2). *D. truttae* is an endemic Acanthocephala species and its occurrence was also confirmed in the Una River from neighbouring Bosnia and Herzegovina (Šinžar, 1956). As expected, the nucleotide diversity of the studied *D. truttae* specimens was generally low (π=0.00732), but higher than the diversity within specimens from the Krka River (π= 0.00076 - 0.00490, at different sites) (Vardić Smrzlić et al., 2013).

The haplotype network of *A. anguillae* showed low nucleotide diversity and high haplotype diversity, similar to the other species studied. Amin et al. (2019) found that the average uncorrected genetic distance within *A. anguillae* and *A. anguillae* subsp. *balcanicus* was 2.1%, which is also a low value. The geographical differentiation of the haplotypes was not clear, probably because the haplotypes from the Dobra River were collected from different locations, although they came from the same final host, *S. cephalus*. Two specimens of *A. anguillae* from *Cyprinus carpio* from the Kupa River formed the same haplotype, separate from the haplotype of *A. anguillae* from *Carassius gibelio* from the same location. Phylogenetic analysis showed that our sequences were most closely related to the other *Acanthocephalus* sp*.* (Lewisch et al. et al., 2020) with 98.4 - 99.4% identity and to the haplotype of *A. anguillae* (Reier et al., 2020) with 98.3%, all from Austria (Figure 7 Supplementary). Both morphological analysis and the 18S rRNA sequence analysis of our 7 specimens identified them as *A. anguillae*. During their study, Reier et al. (2020) found that a COI sequence of a specimen of the genus *Acanthocephalus* differed from *A. anguillae* and *A. lucii* by a high genetic distance with p-values of 27.8% and 24.1%, respectively. Based on these results, they point to the possibility of the existence of unknown *Acanthocephalus* species. *Acanthocephalus* taxonomy is further complicated by the fact that Benesh et al. (2006) found the presence of nuclear pseudogenes (NUMTs) of the mitochondrial COI gene in *A. lucii*. Furthermore, it appears that the COI sequence from the NCBI GeneBank used to construct the phylogenetic tree in our study (Figure 2, Supplementary) was likely misidentified, indicating specimens of *A. dirus* (Amin et al., 2019; Reier et al., 2020). All these problems make the taxonomy of the genus *Acanthocephalus* a challenge and underline the need for more DNA sequence data and morphological data of the extant *Acanthocephalus* species.

The nucleotide divergence of the COI and the ITS markers of Acanthocephala is variable at intra- and interspecific levels (Garcia-Varela and Pérez-Ponce de León 2008, Špakulová et al. 2011) and they have been used to distinguish five genetic lineages of *P. laevis* in Europe with genetic distances ranging from 10.5% to 20.3% (Perrot-Minnot et al. (2018)). Although ITS is a valuable marker for distinguishing *P. laevis* from *P. tereticollis* (Špakulová et al. 2011), the COI marker exhibited higher nucleotide and haplotype diversity within a species and was therefore more suitable for studying interpopulation diversity of Acanthocephala. In addition to phylogeographic studies, clear taxonomic status and even haplotype profiling of the Acanthocephala species studied could be important to accurately define specimens used as bioindicators in freshwater ecosystem contamination studies.

**Conflict of interest**

The authors have no financial or non-financial interests to declare that are relevant to the content of this article.

**Data Availability Statement**

Generated DNA sequences files are available in NCBI Gen Bank.

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