



universität
wien

MASTERARBEIT | MASTER'S THESIS

Titel | Title

Thorny-headed worms (Acanthocephala) of freshwater fishes of the Danube:
A comparison of modern and historical distribution

verfasst von | submitted by

Joshua Aditia Rolke B.Sc.

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2026

Studienkennzahl lt. Studienblatt |
Degree programme code as it appears on the
student record sheet:

UA 066 831

Studienrichtung lt. Studienblatt | Degree
programme as it appears on the student
record sheet:

Masterstudium Zoologie

Betreut von | Supervisor:

Dr. Elisabeth Haring Privatdoz.

Table of Contents

Abstract.....	3
Zusammenfassung	4
1. Introduction	5
1.1 General introduction to Acanthocephala	5
1.2. Introduction to <i>Pomphorhynchus</i>	6
1.2.1 Life cycle	7
1.2.2 Adult and Cystacanth morphology of <i>Pomphorhynchus</i>	8
1.3 Taxonomy and species delimitation within the genus <i>Pomphorhynchus</i>	9
1.3.1 Historical view	9
1.3.2 Morphological identification of <i>Pomphorhynchus laevis</i> , <i>P. bosniacus</i> , and <i>P. tereticollis</i>	10
1.3.3 Limitations and misidentification	12
1.3.4 Discovery of cryptic species	12
1.3.5 <i>Pomphorhynchus bosniacus</i> as a potential invasive species	13
1.4 DNA barcoding of <i>Pomphorhynchus</i>	14
1.4.1 What is DNA barcoding?	14
1.4.2 Applications in parasites	14
1.4.3 Other applications of the <i>COI</i> gene	15
1.5 Research goals, questions and hypotheses	16
1.5.1 Current Understanding and Remaining Uncertainties in the European <i>Pomphorhynchus</i> spp.	16
1.5.2 General aim of the study	16
1.5.3 Specific research questions and hypotheses	17
2. Material and Methods	19
2.1 Host and parasite sampling	19
2.2 Identification of species	19
2.2.1 Morphologic identification by light microscopy	20
2.2.2 Genetic identification using DNA barcoding (<i>COI</i>)	21
2.3 DNA sequence analysis	24
3. Results	27
3.1 Overview of isolated parasitic worms and host species	27
3.2 Detected species of <i>Pomphorhynchus</i> (Acanthocephala)	29
3.2.1 Morphological identification through light microscopy	29
3.2.3 Geographic origin of samples	48

3.3 Other parasitic worms.....	53
3.3.1 Nematoda	53
3.3.2 Trematoda.....	55
4. Discussion	58
4.1 General conclusions	58
4.2 Species identification.....	58
4.2.1 Limitations of morphological identification	58
4.2.2 Other parasitic worms	59
4.3 DNA analysis and geographic distribution of the genus <i>Pomphorhynchus</i>	60
4.3.1 Genetic differentiation of <i>Pomphorhynchus</i> species and <i>P. laevis</i> lineages.....	60
4.3.2 Geographic distribution of <i>P. tereticollis</i> and <i>P. laevis</i>	62
4.4 Population genetics and geographic spread of <i>P. bosniacus</i>	63
4.4.1 Population genetics of <i>P. bosniacus</i>	63
4.4.2 Geographic spread of historical and modern samples.....	65
4.5 Invasive status and host-mediated spread of <i>P. bosniacus</i>	66
4.5.1 Recent expansion of <i>P. bosniacus</i>	66
4.5.2 Host associations and dispersal dynamics of <i>P. bosniacus</i>	67
4.6 Evaluation of hypotheses.....	69
4.7 Conclusions and outlook.....	70
4.7.1 Limitations of this study	70
4.7.2 Future directions	70
5. References	72
6. Acknowledgments.....	79
7. Appendix.....	80
Appendix 1	80
Appendix 2	84
Appendix 3	105

Abstract

The acanthocephalan genus *Pomphorhynchus* (Monticelli 1905) comprises widespread intestinal parasites of freshwater fishes in Europe and includes potential cryptic species within the European *Pomphorhynchus* species. In recent years, molecular studies based on *COI* DNA barcoding have demonstrated that *P. laevis*, *P. tereticollis*, and *P. bosniacus* represent genetically distinct species despite their morphological similarity. However, the historical distribution and potential invasion status of *P. bosniacus* remain poorly understood, particularly within the Danube basin. The present study investigated the temporal and spatial distribution of *Pomphorhynchus* species using an integrative morphological and DNA analysis approach. A total of 88 fish specimens belonging to 21 fish taxa from various rivers and lakes in Europe were examined. Out of these, 55 specimens were infested with helminth parasites. Overall, 479 *Pomphorhynchus* were obtained, of which 139 were investigated morphologically and through DNA analysis methods. A fragment of the *cytochrome c oxidase subunit 1* gene (*COI*) was investigated with specific primers designed for the *Pomphorhynchus* genus. Modern material dated from 2014 to 2025, whereas historical material dated from 1826 to 2004, allowing comparison between historical and modern *Pomphorhynchus* populations across Austrian and other European river systems, with an emphasis on the Danube and its tributaries. In addition to species identification, population genetic analyses including haplotype networks, genetic distances, and phylogeographic comparisons between major European river basins were conducted. The results revealed that *P. bosniacus* dominates modern Danube populations, whereas historical museum material contained predominantly *P. laevis* and *P. tereticollis*. The low genetic divergence and high haplotype connectivity observed among modern *P. bosniacus* populations suggest recent expansion and extensive gene flow across European river systems. The distribution of *P. bosniacus* overlaps with regions colonized by invasive Ponto-Caspian gobiids and amphipods, supporting the hypothesis that host-mediated dispersal has contributed to its spread. In contrast, the historical dataset provided limited support for the idea that *P. bosniacus* historically represented a cryptic species within the Danube basin. This study demonstrates the importance of integrative taxonomical approaches and historical museum collections for reconstructing parasite biogeography and invasion history. This conclusion can also be applied to other parasitic worms and invasive species in general. The findings contribute new molecular data on *Pomphorhynchus* species in Austria and provide further insight into the ecological and evolutionary dynamics of parasite dispersal within interconnected European freshwater systems.

Zusammenfassung

Die Acanthocephala-Gattung *Pomphorhynchus* (Monticelli 1905) umfasst weit verbreitete Darmparasiten von Süßwasserfischen in Europa und beinhaltet potenzielle kryptische Arten innerhalb des *Pomphorhynchus laevis*-Komplexes. Molekulargenetische Studien auf Basis des *COI*-DNA-Barcodings zeigten, dass *P. laevis*, *P. tereticollis* und *P. bosniacus* trotz morphologischer Ähnlichkeit genetisch klar getrennte Arten darstellen. Dennoch sind die historische Verbreitung und der potenzielle Invasionsstatus von *P. bosniacus*, insbesondere im Donauraum, bislang unzureichend verstanden. Die vorliegende Arbeit untersuchte die zeitliche und räumliche Verbreitung von *Pomphorhynchus*-Arten mittels eines integrativen morphologischen und molekulargenetischen Ansatzes. Insgesamt wurden 88 Fischproben aus 21 Fisch-Taxa aus verschiedenen europäischen Flüssen und Seen untersucht, von denen 55 mit Parasiten infiziert waren. Insgesamt wurden 479 *Pomphorhynchus*-Exemplare isoliert, von denen 139 morphologisch und molekulargenetisch analysiert wurden. Hierfür wurde ein Fragment des *Cytochrom-c-Oxidase-Untereinheit-1-Gens* (*COI*) mit speziell für die Gattung entwickelten Primern untersucht. Das moderne Material stammte aus den Jahren 2014 bis 2025, das historische Material aus dem Zeitraum 1826 bis 2004, wodurch Vergleiche zwischen historischen und modernen Populationen aus österreichischen und anderen europäischen Flusssystemen ermöglicht wurden. Zusätzlich wurden populationsgenetische Analysen wie Haplotypennetzwerke, genetische Distanzen und phylogeographische Vergleiche durchgeführt. Die Ergebnisse zeigten, dass *P. bosniacus* moderne Donaupopulationen dominiert, während historisches Museumsmaterial überwiegend *P. laevis* und *P. tereticollis* enthielt. Die geringe genetische Divergenz und hohe Haplotyp-Konnektivität moderner *P. bosniacus*-Populationen deuten auf eine rezente Ausbreitung sowie intensiven Genfluss zwischen europäischen Flusssystemen hin. Die Verbreitung von *P. bosniacus* überschneidet sich mit Regionen invasiver ponto-kaspischer Grundeln und Amphipoden, was die Hypothese einer Wirt-vermittelten Ausbreitung unterstützt. Das historische Datenset lieferte dagegen nur begrenzte Hinweise darauf, dass *P. bosniacus* historisch bereits als kryptische Art im Donaubecken vorkam. Diese Arbeit verdeutlicht die Bedeutung integrativer taxonomischer Ansätze und historischer Museumssammlungen für die Rekonstruktion der Biogeographie und Invasionsgeschichte von Parasiten. Die Ergebnisse liefern neue molekulargenetische Daten zu *Pomphorhynchus*-Arten in Österreich und tragen zum besseren Verständnis der ökologischen und evolutionären Dynamiken der Parasitenausbreitung in europäischen Süßwassersystemen bei.

1.Introduction

1.1 General introduction to Acanthocephala

Acanthocephalans, commonly known as thorny-headed worms, are a group of parasitic worms characterized by a retractable, spiny proboscis that they use to anchor themselves to the intestinal walls of their host, usually being a vertebrate (Kennedy 2006). They possess an incomplete digestive system, absorbing nutrients directly through their body surface, due to the lack of an alimentary tract. Their life cycle typically involves arthropods as intermediate hosts and vertebrates such as fish, birds, or mammals, as definitive (end) hosts. However, if arthropods are not part of the definitive host's food chain, a paratenic host can be used as well (Lucius and Loos-Frank 2008). A paratenic host is an organism in which a parasite does not develop further but remains alive and infective. Thus, the paratenic host serves as a temporary carrier that can transmit the parasite to its definitive host (see Fig. 1.1 below). Although rarely harmful to humans, some species can cause disease in livestock, pets, or wildlife, leading to intestinal damage or malnutrition in heavily infected hosts (Buchmann and Karami 2024).

Acanthocephalans as a group have always been difficult to classify, as they are highly specialized parasites and have lost numerous organs and structures over the course of evolution. Currently, classification in the Syndermata is favored, which encompasses only two groups: the Acanthocephalans and the Rotifers. Once thought to be a distinct phylum, acanthocephalans are now considered highly modified rotifers, based on morphological and molecular genetic (Sielaff et al. 2016), suggesting Acanthocephala to be a monophyletic clade inside the paraphyletic Rotifera. Since then, the discovery of a fossil stem-group acanthocephalan has provided further support for the hypothesis that acanthocephalans evolved from rotifer-like ancestors and subsequently underwent morphological reduction associated with their parasitic lifestyle, including the loss of a functional digestive tract (Luo et al. 2025). While acanthocephalans and rotifers differ drastically in appearance (being large, parasitic worms rather than tiny, mostly free-living micro invertebrates), they share features such as a syncytial epidermis (tegument) and similarities in their reproductive and nervous systems. The syncytial tegument is the origin of the name Syndermata. Therefore, acanthocephalans are essentially rotifers that have experienced high evolutionary specialization toward a parasitic lifestyle rather than being a distinct lineage of worms (Hagemann et al. 2024).

1.2. Introduction to *Pomphorhynchus*

The acanthocephalan genus *Pomphorhynchus* (family Pomphorhynchidae), which represents the focus of the present study, comprises intestinal parasites of freshwater fishes that are widely distributed across Europe, particularly within large river systems. Due to their broad distribution, complex life cycles involving multiple hosts, and the presence of cryptic species, *Pomphorhynchus* spp. are of particular interest for studies on parasite taxonomy, biogeography, host-parasite interactions, and the effects of biological invasions in freshwater ecosystems. Of the 30 described species in the genus *Pomphorhynchus*, three are known to exist in Austrian waters:

- *Pomphorhynchus laevis* (Müller 1776)
- *Pomphorhynchus tereticollis* (Rudolphi 1809)
- *Pomphorhynchus bosniacus* Kiškarolj and Čanković 1967

Historically, *P. laevis* was considered one of the most prevalent and widespread acanthocephalan parasites of freshwater fishes in Europe (Kennedy 1984). Regional variability in preferred final and intermediate hosts is linked to its vast geographic dispersion and final host range. In certain intermediate host species, *P. laevis* is known to cause a variety of phenotypic alterations, including a changed response to light as well as altered behavioural patterns (Kennedy et al. 1978). It has been demonstrated that the parasite's behavioural alterations make its intermediate host more susceptible to predation (Kennedy et al. 1978), which is known as parasite-induced trophic transmission (Lafferty 1999).

1.2.1 Life cycle

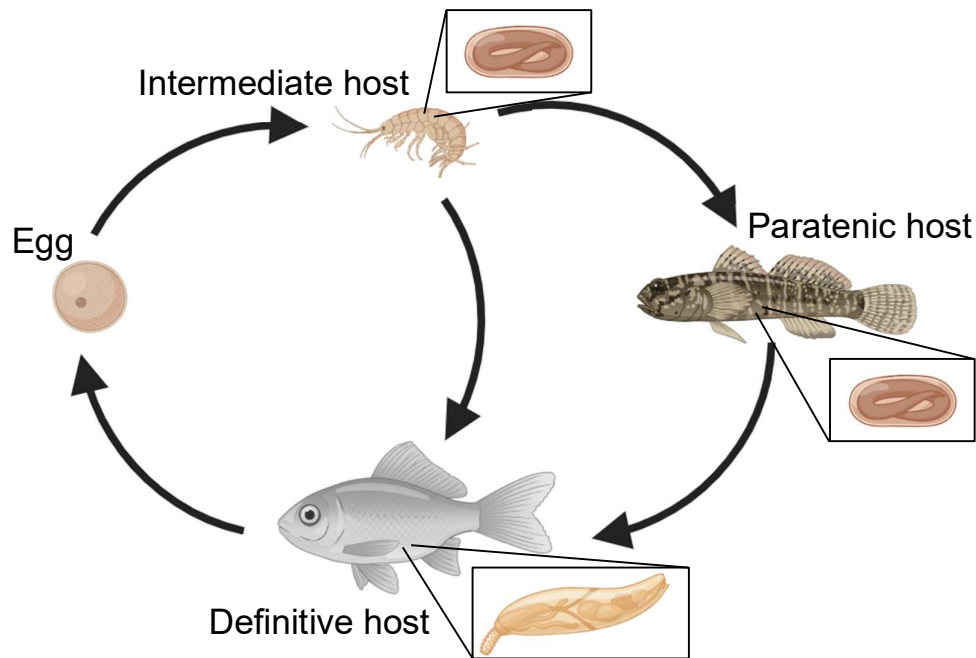


Fig.1.1: Life cycle of *Pomphorhynchus* spp. Showing the transfer of the eggs to an amphipod intermediate host, to fish paratenic and definitive hosts, as well as the progression from the egg to the cystacanth and adult stages. Organisms depicted are not to scale.

The life cycle of *Pomphorhynchus* spp. (as depicted in Fig. 1.1) is indirect and involves aquatic invertebrate and vertebrate hosts. Eggs released by adult worms in the intestines of definitive fish hosts are passed into the environment, where they are ingested by amphipod crustaceans (e.g., *Gammarus* spp. and *Dikerogammarus* spp.), which serve as intermediate hosts (Amin 2013; Kennedy 2006). Within these amphipods, the parasite develops through larval stages to the infective cystacanth. Transmission to the definitive host occurs when infected amphipods are consumed by fish, typically cyprinids such as *Barbus barbus* and *Squalius cephalus*, in which the parasite attaches to the intestinal wall and matures into an adult (Perrot-Minnot 2004; Poulin 2007). In addition, paratenic hosts, often predatory or benthic fish such as gobiids (e.g., *Neogobius melanostomus*, *N. fluviatilis*), can accumulate cystacanths without further development, thereby facilitating transmission to a suitable definitive host through trophic interactions (Hohenadler et al. 2018; Nachev et al. 2024; Ondrackova et al. 2005). This multi-host life cycle, combined with trophic transmission, enables *Pomphorhynchus* spp. to exploit complex food webs and contributes to their widespread distribution in freshwater ecosystems.

1.2.2 Adult and Cystacanth morphology of *Pomphorhynchus*

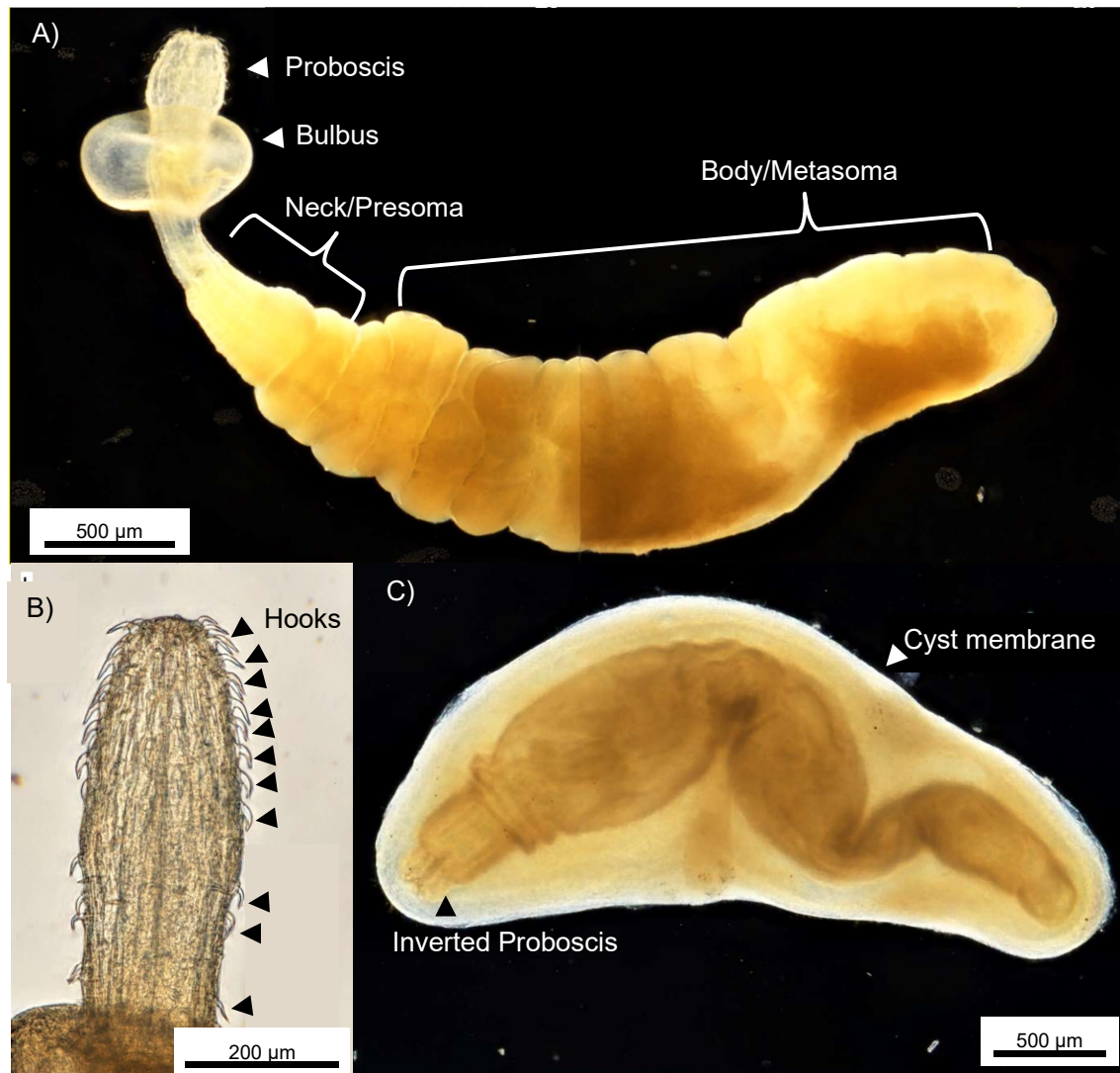


Fig. 1.2: Microscopy of *Pomphorhynchus* spp. showing adult and cystacanth morphology. A) Young adult specimen with everted proboscis, bulbous, neck/prosoma, and trunk/metasma indicated. B) Enlarged view of the adult proboscis showing rows of recurved hooks. C) Cystacanth stage enclosed within a cyst membrane, displaying an inverted proboscis.

Pomphorhynchus are elongate acanthocephalans characterized by a cylindrical neck and a retractable proboscis (Fig. 1.2A) armed with longitudinal rows of recurved hooks (Fig. 1.2B), which are used to anchor the parasite to the intestinal wall of the host (Amin 2013; Kennedy 2006). The neck (also called presoma) and the body (also called metasoma) are generally smooth and unsegmented, although it may appear wrinkled or annulated due to contraction. In the adult stage, individuals are typically found attached to the intestinal lining of fish hosts, where they feed by absorbing nutrients through their tegument (Kennedy 2006; Poulin 2007).

The cystacanth (as depicted in Fig. 1.2C) represents the infective larval stage, developing within intermediate hosts such as amphipods or within paratenic hosts. At this stage, the parasite already possesses a fully formed proboscis with hooks and a developed neck but remains enclosed within a cyst membrane (Amin 2013). Cystacanths are typically more compact and may exhibit a rounded or oval appearance, reflecting their preparatory state prior to transmission to the definitive host.

1.3 Taxonomy and species delimitation within the genus *Pomphorhynchus*

1.3.1 Historical view

The genus *Pomphorhynchus* has long been recognized as a characteristic group of intestinal parasites of freshwater fishes across Europe (Kennedy 2006). The three European *Pomphorhynchus* species are widely distributed across freshwater systems of Central and Eastern Europe, with the Danube basin representing a major core area of occurrence (David et al. 2018; Perrot-Minnot et al. 2018; Reier et al. 2019; Vogel & Taraschewski 2023). Beyond the Danube, *Pomphorhynchus* species are also recorded from other major European river basins, including the Rhine, Weser, Vistula, Rhône, and Loire, reflecting a broad geographic distribution across interconnected freshwater networks (Perrot-Minnot et al. 2018; Reier et al. 2019; Vogel & Taraschewski 2023). For example, *P. laevis* was considered a widespread and ubiquitous species infecting a wide range of hosts across Europe (Kennedy 2006; Hohenadler et al. 2018; David et al. 2018), while *P. tereticollis* is commonly reported from streams and rivers in Western and Central Europe, including the Rhine system (Hohenadler et al. 2018; Perrot-Minnot et al. 2018; Vogel & Taraschewski 2023). More recently, *P. bosniacus*, originally associated with the Ponto-Caspian region, has expanded its range into other basins such as the Rhine and Weser (David et al. 2018; Reier et al. 2019; Vogel & Taraschewski 2023).

Since its early taxonomic description in the 18th and 19th centuries, *P. laevis* has traditionally been regarded as the most widespread and common species within the genus, reported from a broad range of cyprinid hosts and river systems throughout Europe (Kennedy 2006; Perrot-Minnot 2004). Due to its apparent morphological variability and wide geographic distribution, *P. laevis* was historically considered a single, highly adaptable species. However, this view has been increasingly challenged over the past two decades. Detailed morphological studies already suggested inconsistencies within *P. laevis*, particularly regarding variation in hook arrangement and cystacanth morphology (Perrot-Minnot 2004). These observations were later

supported by genetic analyses, which revealed that *P. laevis* represents a complex of cryptic species rather than a single taxon (David et al. 2018; Perrot-Minnot et al. 2018). Among these, *P. bosniacus* was formally recognized as a distinct species within the group, previously masked under the name *P. laevis* due to morphological similarity (Reier et al. 2019).

1.3.2 Morphological identification of *Pomphorhynchus laevis*, *P. bosniacus*, and *P. tereticollis*

To determine *Pomphorhynchus* species morphologically, the proboscis hooks can be examined under a microscope and compared based on several diagnostic characteristics, including the relative length, curvature, and thickness of anterior and posterior hooks, the number of hooks per longitudinal row, and their arrangement along the proboscis (Brown and Thompson 1986; Brown et al. 1986; O'Mahony et al. 2004). In addition, some species differ in the morphology of their hook roots, which anchor the hooks within the proboscis (Špakulová et al. 2011). By measuring these characteristics (as listed in table 1.1 below) and comparing them with published taxonomic keys, the species can be determined.

Table 1.1: Comparison of proboscis and cystacanth morphological characteristics of *P. laevis*, *P. tereticollis* and *P. bosniacus*. Modified from Hohenadler et al. (2018) using (Kiškarolj and Čanković 1969), O'Mahony et al. (2004), Perrot-Minnot et al. (2004), Špakulová et al. (2011), Brown et al. (1986) and Reier et al. (2019).

Morphological characteristics	<i>P. laevis</i>	<i>P. tereticollis</i>	<i>P. bosniacus</i>
Ratio between anterior and posterior proboscis hooks	Anterior hooks significantly larger than posterior hooks	Anterior hooks slightly larger than posterior hooks	Anterior larger than posterior hooks; first three hooks of similar size, posterior hooks shorter and attached near the middle of the hook root
Special feature of basal parts of the proboscis hooks	No proximal projection	Extensions on basal hook roots	Basal hooks attached in hook root middle
Total number of hooks per row	8-13	11	9 (8 in original description)
Total number of longitudinal rows	14-18	18	14
Position of longest anterior hook	4	2 or 3	1-3 (first three hooks equal in size)
Position of shortest anterior hooks	6	4 or 5	6-8 (posterior three hooks shortest)
Cystacanth cyst membrane	Wrinkled	Smooth	Uncertain

Morphological differentiation of cystacanths within the genus *Pomphorhynchus* is limited, although several diagnostic features have been proposed. Traditionally, cystacanths attributed to *P. laevis* have been described as relatively smooth and elongated, whereas those of *P. tereticollis* exhibit a more wrinkled or annulated body surface (Perrot-Minnot 2004). However, these characteristics remain poorly understood and their reliability for species identification is uncertain, as cystacanth morphology may additionally be influenced by contraction state, developmental stage, and preservation condition. Furthermore, the cystacanth morphology of *P. bosniacus* has not yet been clearly characterized, making reliable morphological differentiation of cystacanth stages within the *Pomphorhynchus* species difficult without molecular confirmation.

1.3.3 Limitations and misidentification

Species identification using morphological characteristics alone is still difficult since Pomphorhynchidae generally display a significant intraspecific variability and few differentiating morphological traits (Špakulová et al. 2011; Hohenadler et al. 2018; Li et al. 2017). Furthermore, *P. laevis* appears to possess a significant amount of intraspecific variations between different populations (Perrot-Minnot 2004; Špakulová et al. 2011) making morphological species identification within the *Pomphorhynchus* genus inherently limited and often insufficient for reliable identification. Key diagnostic characters, such as those depicted in Table 1.1, are frequently difficult to observe, particularly in cystacanth stages where the proboscis is commonly invaginated or obscured (Kennedy 2006; Amin 2013). In addition, external traits such as body shape or surface texture (e.g., smooth versus wrinkled cystacanths) show considerable overlap among taxa and can be influenced by specimen condition, host species, or preservation effects (Perrot-Minnot 2004).

Following the recognition of *P. bosniacus* as a distinct but cryptic species, it has become evident that external cystacanth morphology alone is insufficient for reliable species identification, as the specific morphotype of *P. bosniacus* remains poorly defined (Reier et al. 2019). Consequently, reliance on general body shape can lead to misidentification. More robust morphological differentiation is based on proboscis characters, particularly the number of longitudinal hook rows and the structure of basal hook roots, which differ among species (Kennedy 2006; Perrot-Minnot et al. 2018). However, these features are often difficult to assess in cystacanths due to frequent proboscis invagination or limited visibility in intact specimens. As a result, morphological identification at the cystacanth stage is frequently.

1.3.4 Discovery of cryptic species

The understanding of acanthocephalan in Austria is still incomplete. In 1985, *P. laevis* was identified as the sole member of the genus in Austrian waters in the latest survey of Austrian Acanthocephala (Kritscher 1985). The taxonomic position of *P. tereticollis* was previously frequently debated. It was long considered a synonym of *P. laevis* when it was initially described as *Echinorhynchus tereticollis* by Rudolphi in 1908 (Amin et al. 2003). Nevertheless, the species status of *P. tereticollis* was supported by both genetic analyses, and morphological characteristics (Perrot-Minnot 2004; Bombarová et al. 2007) until it was formally resurrected by Špakulová et al. (2011) after a taxonomic revision.

Furthermore, the taxonomic status of *P. bosniacus* was only recently clarified and the species remained unrecognized for a long time due to its close morphological similarity to *P. laevis*.

For decades, specimens now attributed to *P. bosniacus* were routinely identified as *P. laevis*, as no clear morphological characters allowed reliable differentiation between the two species (Perrot-Minnot 2004; Kennedy 2006). However, molecular studies based on mitochondrial DNA sequences revealed the presence of distinct genetic lineages within the *Pomphorhynchus* spp. (David et al. 2018; Perrot-Minnot et al. 2018). Building on this evidence, Reier et al. (2019) further supported the species status of *P. bosniacus* by demonstrating that it forms a well-supported genetic clade despite minimal morphological divergence.

1.3.5 *Pomphorhynchus bosniacus* as a potential invasive species

Pomphorhynchus bosniacus has increasingly been discussed as a potential invasive parasite species associated with the ongoing spread of Ponto-Caspian fauna throughout European freshwater systems. Native to the Ponto-Caspian region, numerous aquatic organisms, including gobiid fishes (*Neogobius* spp.) and amphipods such as *Dikerogammarus villosus*, have expanded westward via the Danube River and connected waterways over recent decades (Bij de Vaate et al. 2002; Bollache et al. 2004; Grabowska et al. 2023). These invasions were further facilitated by anthropogenic modifications such as shipping routes and the Rhine-Main-Danube Canal, which connected previously isolated drainage systems. Reier et al. (2019) proposed that *P. bosniacus* may have spread alongside these invasive hosts, as the species is predominantly associated with the Danube corridor and has increasingly been recorded in western river systems such as the Rhine and Weser (David et al. 2018; Perrot-Minnot et al. 2018; Reier et al. 2019; Vogel & Taraschewski 2023). The apparent predominance of *P. bosniacus* in modern Danube samples, combined with its relatively recent molecular recognition, has therefore raised the possibility that the species represents an expanding invasive parasite rather than a historically overlooked component of the European parasite fauna.

The spread of *P. bosniacus* may furthermore involve ecological processes such as spillover and spillback, which are commonly associated with biological invasions (Torchin et al. 2003; Kelly et al. 2009). Spillover describes the transmission of parasites from invasive hosts to native hosts, whereas spillback occurs when invasive hosts amplify the transmission of parasites already present within the invaded ecosystem (Kelly et al. 2009; Prenter et al. 2004). In the case of *Pomphorhynchus*, invasive gobiids such as *Neogobius melanostomus* can act as highly suitable paratenic hosts, often harbouring large numbers of cystacanths and thereby increasing transmission to definitive fish hosts (Ondračková et al. 2005; Hohenadler et al. 2018). A combined scenario involving both historical presence and recent transmission

amplification through invasive hosts therefore cannot be excluded, potentially reflecting a combination of spillover and spillback processes.

1.4 DNA barcoding of *Pomphorhynchus*

1.4.1 What is DNA barcoding?

Accurate species identification based solely on morphology is often challenging, as many organisms exhibit overlapping or highly variable morphological traits that limit reliable differentiation. This problem is particularly pronounced in groups containing cryptic species, which are genetically distinct but morphologically indistinguishable or very similar (Bickford et al. 2007). In parasitic taxa such as *Pomphorhynchus*, subtle diagnostic characters may be difficult to observe or may vary depending on specimen condition, leading to frequent misidentifications (Kennedy 2006; Perrot-Minnot et al. 2018). As a result, morphology alone is often insufficient for species delimitation, and the presence of cryptic diversity further complicates accurate identification. To overcome the limitations of morphology-based species identification, molecular approaches such as DNA barcoding have been developed.

DNA barcoding is a DNA sequence analysis method for species identification that uses short, standardized gene regions to distinguish taxa based on genetic differences. In animals, the mitochondrial *cytochrome c oxidase subunit 1* (*COI*) gene is most used, as it combines sufficient variability to differentiate closely related species with conserved primer-binding sites that allow for broad amplification across taxa (Hebert et al. 2003). The method involves extracting DNA, amplifying the target gene region via PCR, sequencing it, and comparing the resulting sequence to reference databases such as GenBank or BOLD to determine species identity. DNA barcoding offers several advantages over traditional morphology-based identification, including the ability to detect cryptic species, identify damaged or immature specimens, and provide standardized, reproducible results (Hebert et al. 2003; Ratnasingham and Hebert 2007).

1.4.2 Applications in parasites

In parasitology, DNA barcoding has become an essential tool for accurate species identification, particularly in groups where morphological differentiation is limited or unreliable. By using standardized genetic markers researchers can identify parasites across life stages, including the Cystacanths that lack diagnostic morphological features, and detect cryptic diversity within in *Pomphorhynchus* spp. (Hebert et al. 2003; Poulin 2007). In

acanthocephalans, DNA barcoding has proven especially valuable, as traditional morphological characters are often difficult to assess or overlap among species (Kennedy 2006). Several studies on *Pomphorhynchus* have demonstrated the effectiveness of this approach, revealing previously unrecognized genetic lineages and clarifying species boundaries within the *Pomphorhynchus* spp. (Špakulová et al. 2011; David et al. 2018; Perrot-Minnot et al. 2018). Most notably, DNA sequence analyses based on *COI* sequences led to the identification and formal description of *P. bosniacus* as a distinct species, highlighting the limitations of morphology and the power of genetic tools in resolving cryptic taxa (Reier et al. 2020).

1.4.3 Other applications of the *COI* gene

Beyond species identification, DNA barcoding can also be applied to a wide range of genetic and phylogeographic analyses relevant to ecology and biogeography. Comparisons of *COI* sequences among individuals and populations allow the assessment of genetic relationships, haplotype diversity, nucleotide diversity, and genetic distances, thereby providing insights into population structure and connectivity (Hebert et al. 2003; Avise 2000). Barcode-based haplotype networks can further reveal cryptic diversity, historical dispersal patterns, and geographically structured lineages (Hajibabaei et al. 2007; Avise 2009). In parasitological studies, these approaches are particularly valuable for reconstructing invasion pathways, detecting host-mediated dispersal, and assessing gene flow among populations (Blouin 2002; C. D. Criscione et al. 2005). In the context of *Pomphorhynchus*, *COI* barcoding therefore provides an effective framework not only for distinguishing cryptic species, but also for investigating the spread of *P. bosniacus*, the genetic connectivity between European river basins, and the temporal distribution patterns reconstructed from both modern and museum-derived specimens.

1.5 Research goals, questions and hypotheses

1.5.1 Current Understanding and Remaining Uncertainties in the European *Pomphorhynchus* spp.

Current understanding of European *Pomphorhynchus* recognizes the genus as comprising several closely related but genetically distinct species rather than a single, widely distributed taxon. Molecular studies based on *COI* DNA barcoding have demonstrated clear genetic separation between *P. laevis*, *P. bosniacus*, and *P. tereticollis*, resulting in a substantial revision of their taxonomy and known distribution patterns (David et al. 2018; Perrot-Minnot et al. 2018; Reier et al. 2019; Vogel & Taraschewski 2023). However, significant uncertainties remain regarding the true geographic distribution of these species, as many historical records predate molecular methods and may therefore represent misidentifications. In particular, *P. bosniacus* has attracted increasing attention due to its apparent expansion along major European waterways and its association with invasive Ponto-Caspian hosts (Reier et al. 2019; David et al. 2018; Vogel & Taraschewski 2023). Furthermore, the observed genetic diversity within *Pomphorhynchus* suggests that additional evolutionary lineages may exist, particularly in under-sampled regions of Europe (Perrot-Minnot et al. 2018). These uncertainties highlight the importance of DNA-based species identification and the re-examination of historical museum material for reconstructing the temporal and spatial distribution of *Pomphorhynchus* species.

1.5.2 General aim of the study

The aim of this study is to investigate the historical and contemporary distribution of *Pomphorhynchus* species in Austria by integrating DNA sequence analyses of both recently collected parasites and parasites recovered from ethanol-preserved fish housed in the Natural History Museum Vienna (NHMW). This museum collection possesses some fish dating back to the 19th century and provides a unique opportunity to access historical parasite material and reconstruct temporal patterns of species occurrence. By comparing genetic data from historic and recent samples, this study seeks to assess changes in species composition over time and to evaluate the status of *P. bosniacus* within Austrian river systems. In particular, the study aims to test whether *P. bosniacus* represents a recent invasive species, as previously suggested (Reier et al. 2019, 2020), or whether it has been present historically but remained undetected due to its cryptic morphology.

1.5.3 Specific research questions and hypotheses

To address the unresolved distribution and potential invasive status of *P. bosniacus*, this thesis investigates several research questions concerning the historical occurrence, spread, and ecological dynamics of species within the *Pomphorhynchus* spp. Has *P. bosniacus* historically existed as a cryptic species within the range previously attributed to *P. laevis* and therefore remained undetected due to morphological similarity? Alternatively, is *P. bosniacus* a recently introduced invasive species that has expanded into Austrian river systems and gradually increased in prevalence, potentially replacing *P. laevis* and *P. tereticollis*? If so, how recent is the invasion? Furthermore, a third possibility is that *P. bosniacus* has long represented the dominant *Pomphorhynchus* species within the Danube basin, while the prevalence of *P. laevis* and *P. tereticollis* in this region has been historically overestimated due to misidentifications. Based on these research questions, the following hypotheses were addressed:

H1. *P. bosniacus* has historically existed as a cryptic species within the range of *P. laevis*.

Predictions:

- *P. bosniacus* should be detectable in historical museum fish collected in previous decades or even centuries, alongside *P. laevis* and *P. tereticollis*.
- The geographic distribution of *P. bosniacus* should remain relatively stable across historical and Recently collected parasite samples, at least until invasive Ponto-Caspian hosts arrive.

H2. *P. bosniacus* represents a recently introduced invasive species, that expanded in the Upper Danube in Austria.

Predictions:

- Its occurrence should increase over time in Austrian river systems, particularly in Recently collected samples.
- Recently established populations may show low genetic divergence and high haplotype similarity, consistent with recent expansion.
- *P. laevis* and/or *P. tereticollis* may decline in regions where *P. bosniacus* becomes dominant.

H3. *P. bosniacus* has historically been the dominant species within the Danube basin and was previously misidentified as *P. laevis*.

Predictions:

- Historical museum specimens from the Danube should predominantly be identified as *P. bosniacus*.
- *P. laevis* and *P. tereticollis* is expected to be rare or absent in historical samples originating from the main channel of the Danube. Owing to the limited availability of historical material from tributary systems, however, their historical distribution outside the main river channel cannot be reliably assessed.
- The distribution of *P. bosniacus* should closely follow the main Danube corridor across both historical and recently collected samples.

The specific objective of this thesis is to achieve accurate species identification of *Pomphorhynchus* specimens through an integrative approach combining morphological examination and molecular analysis based on DNA barcoding. Morphological characteristics were assessed where possible, and species identities were confirmed using *COI* sequences to ensure reliable differentiation. Historical museum collections provide a unique opportunity to investigate previously unrecognized distributions of *Pomphorhynchus* species and to reconstruct their temporal spread across European river systems through retrospective molecular analysis of archived specimens. By comparing parasites recovered from historic museum fish with those obtained from Recently collected hosts, this study aims to evaluate temporal changes in species composition and occurrence. Furthermore, the distribution of *Pomphorhynchus* species is analysed across both historical and contemporary datasets to investigate their spatial patterns within Austria and across Europe. In doing so, this work contributes to a better understanding of the prevalence, host associations, and taxonomy of acanthocephalans in Austrian freshwater fish, while also providing new DNA barcode data that may facilitate more accurate species identification in future studies.

2. Material and Methods

2.1 Host and parasite sampling

A total of 45 fish specimens representing various species were collected during 2024 and 2025 from rivers and lakes across the Austrian federal states of Styria, Vienna, Upper Austria, and Lower Austria (Appendix 1). Fish species were identified by in-house ichthyological experts at the Natural History Museum Vienna (NHMW). Some parasite specimens were additionally obtained following host dissections conducted as part of other research projects.

In addition, 40 museum fish specimens were obtained from the ichthyological collection of the NHMW. The collection dates of these fish ranged from 1826 to 2014 (Appendix 1). The original localities of these museum specimens were not limited to Austrian waters but also included the Ponto-Caspian region including Ukraine, Bulgaria, North Macedonia, Greece, Turkey and Croatia. Austrian museum specimens originated from the federal states of Styria, Vienna and Lower Austria.

Throughout this study, the term “recently collected fish” (or RC fish) refers to fish collected in 2024 and 2025, directly from the wild and not previously stored in the NHMW collection. The term “museum collection fish” (or MC fish) refers to historical specimens from and preserved in the NHMW collection, with a collection date prior to 2024. Furthermore, a similar differentiation is made with their corresponding *Pomphorhynchus* parasites: “recently collected parasites” (or RC parasites) refers to parasites recovered from RC fish hosts, whereas “museum collection parasites” (or MC parasites) refers to parasites recovered from MC fish hosts.

Depending on size, fresh material of RC fish was either frozen or kept in 80% ethanol following capture. MC fish were either stored in 80% ethanol or formalin, which is a 10% solution of formaldehyde and water and was commonly used for preservation of biological specimens. Fish intestines were dissected to recover parasites. Parasites were preserved in 75-80% ethanol and temporarily stored at 4°C until further investigations were conducted.

2.2 Identification of species

In addition to *Pomphorhynchus* spp., a variety of other parasitic taxa may be encountered in the intestines and body cavities of the host fish. Among acanthocephalans, genera other than

Pomphorhynchus are commonly reported from European freshwater systems and can co-occur with *Pomphorhynchus* in similar hosts (Amin 2013; Kennedy 2006). Furthermore, nematodes and trematodes are frequently present in fish and may be observed during dissection, often in larval stages that lack clear diagnostic features (Moravec 1994; Niewiadomska 2003). These groups can exhibit morphological similarities with *Pomphorhynchus* at certain life stages, particularly when specimens are degraded or partially digested, which may complicate identification. The presence of parasites other than *Pomphorhynchus* spp. should be considered when examining fish hosts.

Adult *Pomphorhynchus* specimens recovered from the intestinal tract were morphologically assigned to the genus *Pomphorhynchus* based on the long neck and bulbous. Cystacanths were primarily recognized by their round shape and location outside of the intestinal track. Where possible, at least three adult *Pomphorhynchus* and three cystacanths of each host specimen were selected for further investigation. In total, microscopic and genetic analyses were performed on a total of 139 *Pomphorhynchus* ssp. individuals.

2.2.1 Morphologic identification by light microscopy

Clearing in glycerol is required for the morphological identification of preserved specimens using a compound light microscope (Lühe 1911). Specimens were placed in an embryo dish using a 50:50 solution of 75% ethanol and glycerol and were incubated for at least two days at approximately 35°C until the ethanol evaporated (Reier et al. 2020). The samples were then prepared for microscope examination by mounting them in glycerol (Reier et al. 2020).

As glycerol treatment may possibly degrade DNA, MC parasites received special treatment as a precaution. All MC parasites specimens were photographed prior to extraction using a stereoscopic binocular microscope, which, although less suitable for whole-specimen visualization, provided some, albeit limited morphological information. Cystacanths obtained from MC fish were not examined under a compound light microscope at all and were directly prepared for DNA extraction. For adult *Pomphorhynchus* specimens obtained from MC fish, the body was separated from the proboscis and was further prepared for DNA extraction, whereas the proboscis was further examined using a compound light microscope. Consequently, both adult body material and cystacanth specimens were consumed during the extraction process and were not available for subsequent morphological analysis.

Light microscopy photographs were made using a Nikon Eclipse Ni light microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon DS-Ri2 Olympus DP27 microscope camera

(Nikon Corporation, Tokyo, Japan) and the microscopy imaging and analysis software NIS Elements (Nikon Instruments Europe BV, Amsterdam, Netherlands). Figs were assembled using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA).

2.2.2 Genetic identification using DNA barcoding (*COI*)

DNA extraction was performed in the clean room of the Central Research Laboratories of the NHMW, a controlled environment equipped with UV light sterilization, restricted access, and dedicated equipment to minimize the risk of external DNA contamination. The QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used to extract DNA in accordance with the manufacturer's instructions, though instead of Buffer AE, nuclease free water was used. Parasites collected from RC fish were incubated with Proteinase K in a thermoshaker for at least 4 hours, whereas parasites obtained from MC fish were incubated overnight (~16 hours), to ensure complete tissue digestion. Parasites obtained from hosts preserved in formalin were first rinsed to remove residual formalin by keeping them in GTE buffer (Thermo Fisher Scientific, Waltham, MA, USA) for three days.

For DNA barcoding, fragments of the mitochondrial *cytochrome c oxidase subunit 1 gene* (*COI*) were amplified using PCR. Depending on the primer pairs used, the amplicon size was either 111 base pairs (bp) or 710 bp.

Table 2.1: Primer pairs, their sequences and resulting amplicon sizes in base pairs (bp).

Primer pair	Primer	DNA sequence (5' - 3')	Amplicon size (bp)
A	H14AcanCO/Fw1	TTCTACAAATCATAARGATATYGG	710
	H14AcanCO/Rv2	AAAATATAMACTTCAGGATGACCAAA	
B	Acan_short_Fw	CTTTTRATGGAYCGTAATTTGAA	111
	Acan_short_Rv	TGGDCACCCHGAGGTGTATATT	
C	H14AcanCO/Fw1	TTCTACAAATCATAARGATATYGG	710
	Acan_short_Rv	TGGDCACCCHGAGGTGTATATT	
D	Trem-18S-f	GGTTCCTTAGATCGTACATGC	430
	Trem-18S-r	GTACTCATTCGAATTACGGAGC	

The H14AcanCO/* primer pair (primer pair A) was obtained from a previous DNA barcoding study (Reier et al. 2019). It is composed of the forward primer H14AcanCO/Fw1 and the reverse primer H14AcanCO/Rv2. In this study, this primer pair was primarily used for the recent parasites recovered. The resulting PCR had an amplicon size of 710 bp.

The Acan_short_* pair (primer pair B) was created within the present study using AmpliFX Version 2.1.0 (Inst. Neurophysiopathol, Marseille, France) and was designed specifically for the three presumed species of *Pomphorhynchus* known for Central Europe. It is composed of the forward primer Acan_short_Fw and the reverse primer Acan_short_Rv. This primer pair was primarily used for the MC parasites of the NHMW. The resulting PCR had an amplicon size of 111 bp, which is significantly shorter than the other pairs. Even with this drawback, the species can still be identifiable with this shorter *COI* gene section, as there are still enough differences between the three *Pomphorhynchus* species present in Austria, as visible in Fig. 2.1.

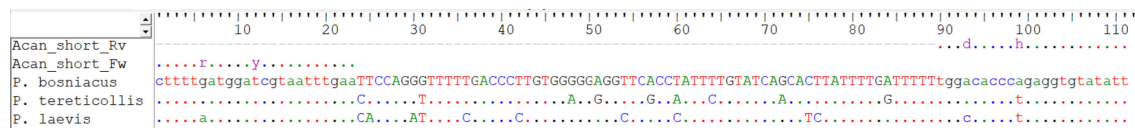


Fig. 2.1: Alignment of COI sequences showing the binding positions of the forward primer (Acan_short_Fw) and reverse primer (Acan_short_Rv) for *Pomphorhynchus bosniacus*, *Pomphorhynchus tereticollis*, and *Pomphorhynchus laevis*. Nucleotide positions identical to the reference sequence are represented as dots, while mismatches are shown as coloured bases.

The forward primer H14AcanCO/Fw1 was used in combination with the reverse primer Acan_short_Rv for recent samples, in samples where PCR failed (primer pair C). As the

reverse primer is more specific to *Pomphorhynchus* species, there is a higher probability of successful amplification. If the PCR failed a second time, these samples were repeated again with the primer pair B, as they replicate a smaller gene section and are therefore more likely to be successful. The latter PCR was only the last resort for the recent samples, as a shorter gene section is less informative when used in population genetic studies as in median joining networks.

Lastly, the Trem-18S-* pair (primer pair D) was used for samples that appeared to be *Pomphorhynchus* at first glance, but the PCR of these samples showed bands of multiple incorrect amplicon lengths in the gel electrophoresis, suggesting a different taxon of parasitic worms. This primer pair is often used for unidentified species belonging to the class Trematoda and was used in this study as such. Unlike the other primer pairs used in this study, which target the mitochondrial *COI* gene, primer pair D amplifies a fragment of the nuclear 18S ribosomal RNA (18S) gene. The 18S gene is a highly conserved genetic marker commonly used for higher-level taxonomic identification and phylogenetic analyses of eukaryotes, including trematodes, particularly when *COI* primers fail or the taxonomic identity of a specimen is uncertain (Olson et al. 2003; Blaxter et al. 1998)

Table 2.2: Contents of performed PCRs. While the Multiplex PCR 5X Master Mix (New England Biolabs) and primer volumes remained the same, the template DNA volume was increased for MC parasites to increase output. The volume of water was adjusted for the total volume to remain at 25 µl.

	Volumes for RC parasite samples (µl)	Volumes for MC parasites samples (µl)
Nuclease Free Water	10	7
Multiplex PCR 5X	12.5	12.5
Reverse Primer	0.25	0.25
Forward Primer	0.25	0.25
Template DNA	2	5
Total Volume	25	25

For the PCRs, the protocol differed depending on the primers used. All amplifications started with an initial denaturation at 95°C for 15 min followed by 35-45 cycles of denaturation, annealing and extension, depending on the primer pair used as visible below in Table 3. To complete elongation, the temperature was held at 72°C for 10 min. After PCR, the samples were assessed using an agarose gel electrophoresis, which was performed in a 1% agarose gel for the larger 710 bp amplicons or, for a better separation of the smaller fragments, a 2% agarose gel for the shorter 111 bp amplicons. When no bands or bands of the wrong size were observed, the PCR of the respective sample was repeated.

Table 2.3: PCR protocols for the different primer pairs.

Primer pair	Denaturation	Annealing	Extension	Cycles
A	95°C, 30 sec.	52°C, 30 sec.	72°C, 30 sec.	40
B	95°C, 30 sec.	50°C, 30 sec.	72°C, 30 sec.	45
C	95°C, 30 sec.	51°C, 30 sec.	72°C, 30 sec.	45
D	95°C, 1 min.	52°C, 2 min.	72°C, 3 min.	35

The PCR products were sequenced by Microsynth (Balgach, Switzerland) using the above-mentioned PCR primers. Samples amplified using the primer pairs A or C were sequenced only from one side (reverse strand). Amplicons obtained with the primer pairs B and D were sequenced in both directions. Single tube sequences were purified using Qiagen Purification Kit (Qiagen, Hilden, Germany), while sequence reactions sent on a plate were sent unpurified, as purification was conducted by Microsynth.

2.3 DNA sequence analysis

Sequences were edited and manually aligned using BioEdit (Hall 1999). To determine species identity, the sequences were compared with reference sequences in NCBI GenBank and BOLD using nucleotide BLAST searches. Afterwards, two datasets were created:

- (i) A long alignment dataset based on the 710 bp amplicons (primer pairs A or C) *Pomphorhynchus* specimens collected from RC fish, (Fig. 2.1). This will henceforth be dataset 1.

- (ii) A short alignment dataset with an amplicon size of 111 bp for *Pomphorhynchus* specimens originating from MC fish, sequenced by using primer pair B (Fig. 2.1). This alignment also includes some sequences of RC parasites, which failed to amplify repeatedly with primer pairs A and C. This will henceforth be dataset 2.

Additional *Pomphorhynchus* sequences retrieved from GenBank were included (Appendix 2). A published sequence of the acanthocephalan *Echinorhynchus truttae* (GenBank accession number DQ089710) was used as an outgroup to root the tree.

After trimming the primer regions, the final aligned datasets comprised 578 sites for dataset 1 and 66 sites for dataset 2. Sequence alignments were saved in FASTA format, and subsequent genetic analyses were performed using MEGA version 12 (Kumar et al. 2024). Phylogenetic relationships were inferred using the Neighbor-Joining (NJ) method, a distance-based clustering approach that generates a single genetic distance tree (Saitou and Nei 1987). NJ trees were constructed using uncorrected *p*-distances, and branch support was assessed by bootstrapping with 1,000 replicates.

Pairwise *p*-distances were calculated in MEGA12. Haplotype diversity and nucleotide diversity of the *COI* dataset was performed in DnaSP v5 (Librado and Rozas 2009). Median-joining networks (MJ) are used in phylogeography and population genetics to visualize haplotype relationships, genetic structure, and historical connectivity among populations (Bandelt et al. 1999). They were generated in PopART v1.7 with the additional *Pomphorhynchus* sequences retrieved from GenBank. Haplotypes were grouped and colour-coded according to their river basin of origin.

Lastly, two geographic maps were generated using QGIS version 3.44 (QGIS Development Team 2025) with *Pomphorhynchus* sequenced in this study as well as with the additional *Pomphorhynchus* sequences retrieved from GenBank (see Fig. 2.2 below), to illustrate the origins of *Pomphorhynchus* specimens analysed in this study, thereby visualizing the spatial and temporal distribution of the species.

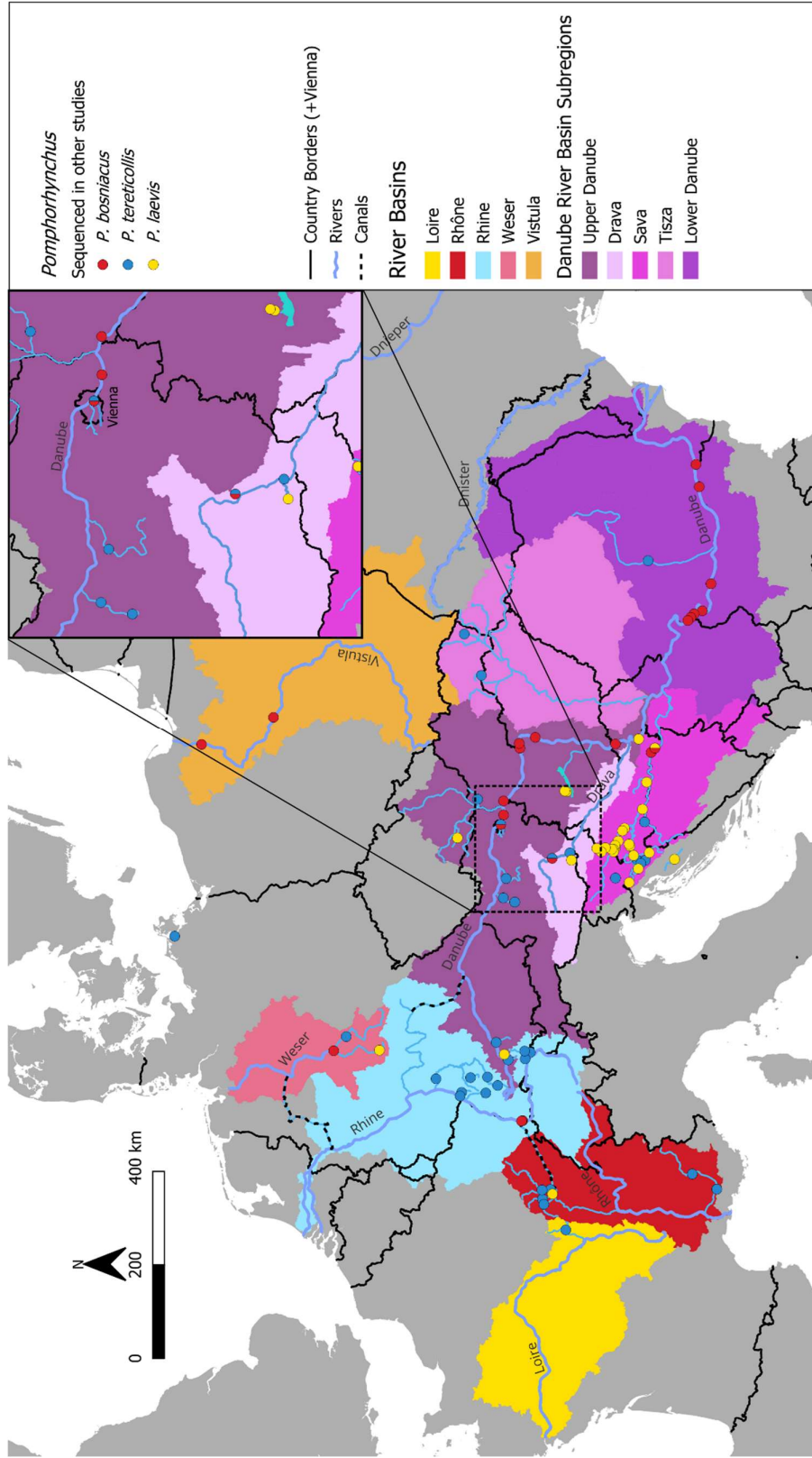


Figure 2.2: Geographic distribution of *Pomphorhynchus* species across major European river basins based exclusively on specimens from previous studies (see Appendix 2 for references) ranging from 1998-2018. The map shows the occurrence of *Pomphorhynchus bosniacus*, *Pomphorhynchus tereticollis*, and *Pomphorhynchus laevis* within the Danube, Rhine, Weser, Vistula, and Dnister systems, including the Upper Danube and tributaries such as the Sava and Drava, with a particular focus on eastern Austria. Sampling locations are represented by circles coloured according to species identity: *Pomphorhynchus bosniacus* = red, *Pomphorhynchus tereticollis* = blue, and *Pomphorhynchus laevis* = yellow. All specimens were identified using COI/DNA barcoding sequences.

3. Results

3.1 Overview of isolated parasitic worms and host species

A total of 85 dissected fish belonging to 21 taxa were recorded, although five taxa could only be identified to genus level. Of these, 55 had a parasite infestation (Table 3.1) and altogether 730 parasitic worms were found (Table 3.2). Of these parasitic worms, 479 belonged to the phylum Acanthocephala, 195 to the phylum Nematoda and 56 to the phylum Platyhelminthes (specifically Trematoda). Twenty instances of distinct parasites coexisting in the same fish were noted: 18 times Acanthocephala plus Nematoda (once even nematodes of different species) and twice an acanthocephalan and a trematode species. No coexistence between two acanthocephalan species was observed.

Table 3.1: Overview of host fish species obtained in this study, including the family they belong to.

Family and species	English common name	German common name	Number of dissected fish
Cottidae <i>Cottus gobio</i>	European bullhead	Groppe, Koppe	3
Cyprinidae <i>Barbus barbus</i> <i>Barbus petenyi</i> <i>Barbus</i> sp.	Common barbel Romanian barbel	Barbe Rumänische Barbe	17 2 3
Gobiidae <i>Babka gymnotrachelus</i> <i>Neogobius fluviatilis</i> <i>Neogobius melanostomus</i> <i>Ponticola kessleri</i> <i>Proterorhinus marmoratus</i> <i>Proterorhinus</i> sp.	Racer-goby Monkey goby Round goby Bighead goby -	Nackthalsgrundel Flussgrundel Schwarzmund-Grundel Kessler-Grundel Marmorierte Grundel	6 2 14 3 3 3
Gobionidae <i>Romanogobio</i> sp.			1
Leuciscidae <i>Alburnus alburnus</i> <i>Alburnus</i> sp. <i>Blicca bjoerkna</i> <i>Chondrostoma nasus</i> <i>Chondrostoma</i> sp. <i>Rutilus rutilus</i> <i>Squalius cephalus</i> <i>Vimba vimba</i>	Common bleak White bream Common nase Rutilus roach European chub Vimba bream	Ukelei, Ablette, Laube Güster Nase Rotauge, Plötze Döbel, Alet, Eitel Zährte, Rußnase	2 1 1 2 4 1 11 2
Percidae <i>Gymnocephalus schraetser</i> <i>Zingel zingel</i>	Schraetzer Zingel	Schrätzer Zingel	2 2
Total			85

Table 3.2: Number of parasitic worms obtained from host fish in this study.

Phylum/taxon	Parasites obtained from RC fish	Parasites obtained from MC fish	Total
Rotifera:			
Acanthocephala	307	172	479
Cystacanth	33	117	150
Adult	274	55	329
Nematoda	16	179	195
Platyhelminthes:			
Trematoda	47	9	56
Total	370	360	730

3.2 Detected species of *Pomphorhynchus* (Acanthocephala)

Based on characteristics unique to the genus, such as the bulbous and long neck, all but two acanthocephalans found could be assigned to the genus *Pomphorhynchus*, including adults and their larval stage, the cystacanth. A total of 479 specimens were recovered in 43 infected fish. Of these specimens, 307 were isolated from 24 RC fish and 172 from 19 MC fish. Furthermore, of the 43 infected fish, only six contained both adult and cystacanth acanthocephalans of the genus *Pomphorhynchus*.

3.2.1 Morphological identification through light microscopy

3.2.1.1. Cystacanths

Approximately 159 larval stages (cystacanths) of *Pomphorhynchus*, were obtained through the dissection of the fish, of which 33 originated from RC fish and 126 from MC fish. Species identification of cystacanths can be difficult, as they lack most morphological characters that are specific to a particular species. Additionally, some cystacanths were discovered with fused cyst membranes. As these fused cyst membranes were difficult to separate mechanically without risking damage to the specimens, each cyst complex was processed as a single individual throughout the study. This approach was maintained consistently during both specimen counting and subsequent DNA extraction, as the fused cystacanths were not separated prior to molecular analyses. Although the number of cystacanths within each complex could be determined morphologically, all complexes yielded unambiguous *COI* sequences without evidence of mixed chromatograms or other anomalies indicative of multiple DNA templates. In total, five fused cyst complexes were observed: two containing two cystacanths, two containing three cystacanths, and one containing four cystacanths (Fig. 3.1). The typical neck and proboscis were not yet developed in most of them, and their bodies were either wrinkled, smooth, or had a membrane around them, known as the cyst membrane.

A total of 70 cystacanths were selected for morphological examination and DNA sequence analysis to test these hypotheses and assess the potential co-occurrence of multiple *Pomphorhynchus* species within individual hosts. As a general rule, at least three cystacanths per fish were analysed further whenever possible. Of the examined specimens, 32 originated from RC fish hosts and 38 from MC specimens.

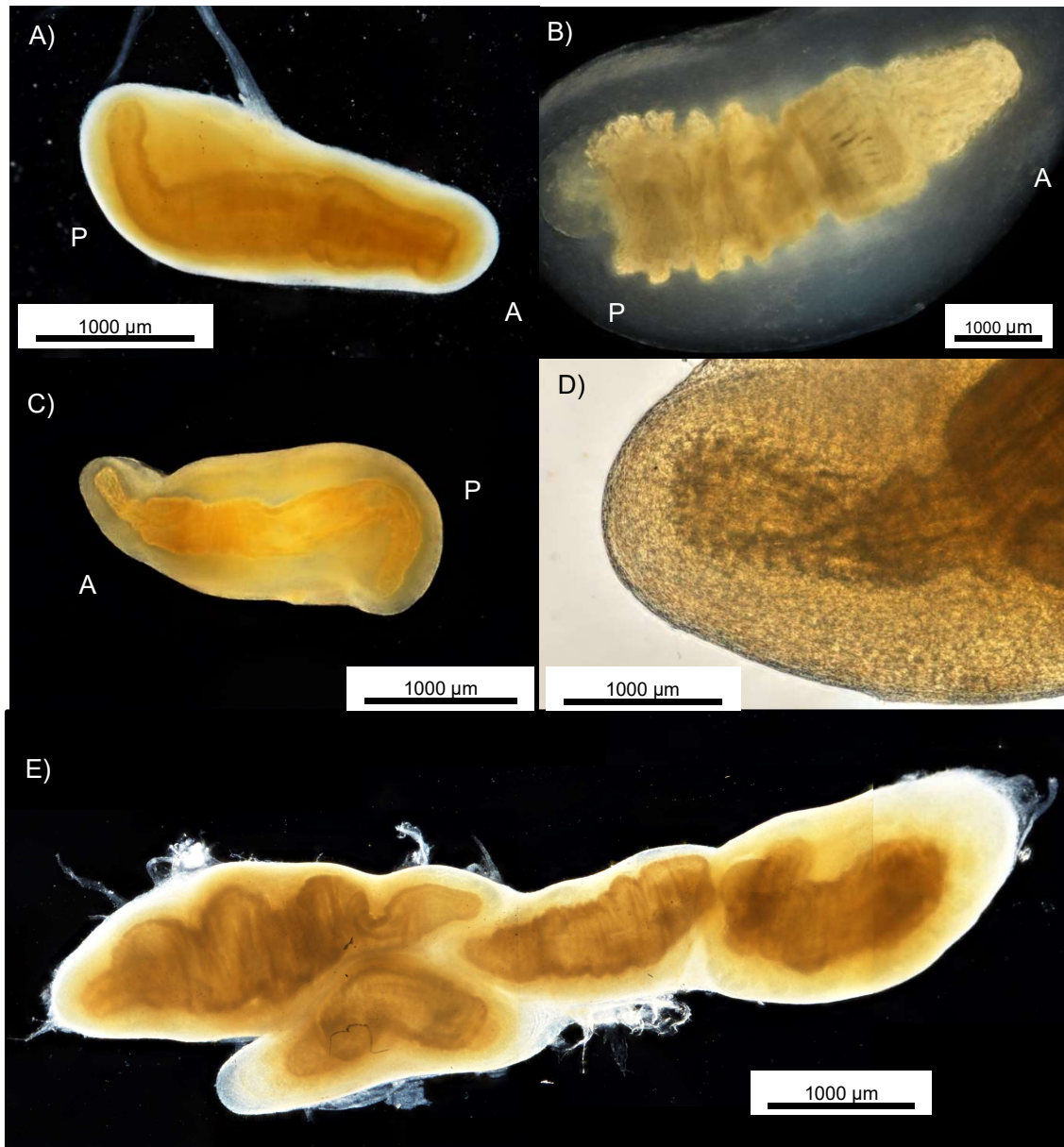


Fig. 3.1: Cystacanths (larval stages) of *Pomphorhynchus* recovered from RC fish. “A” indicates the anterior end and “P” the posterior end. All specimens are enclosed within transparent host-derived cyst membranes, while the enclosed cystacanths appear distinctly wrinkled. DNA barcoding identified all specimens as *Pomphorhynchus bosniacus*. A) BDAF38.2 showing an elongated body with an inverted proboscis. B) BDAF24.1 with an everted proboscis and visible hooks. C-D) Two views of specimen BDAF21.3, including a close-up of the anterior region and proboscis. E) Four cystacanths of specimen BDAF30.3 partially enclosed within adjacent or fused cyst membranes. Images obtained were using phase contrast or transmitted light microscopy.

The composite Fig 3.1 presents multiple developmental appearances of *Pomphorhynchus* cystacanths, illustrating variation in contraction state, cyst integrity, and internal visibility. In all images, the parasites are enclosed within translucent cyst membranes. The cyst walls appear smooth and thin, whereas the enclosed cystacanth bodies exhibit varying degrees of folding and internal differentiation. These folds were possibly already present on the animal before recovery but could also reflect post-mortem contraction or fixation effects.

The cystacanths exhibit an elongated, cylindrical body with smoothly rounded anterior and posterior ends. The interior shows a compact, well-defined structure, consistent with an advanced cystacanth stage. The proboscides are inverted in Fig 3.1A and 3.1E and appear retracted within the anterior region, whereas they appear externally everted in Figs 3.1B-D, with the faint hook structures visible through the cyst wall. However, diagnostic details of the proboscis armature are not sufficiently visible due to invagination and limited optical resolution of the hooks through the cyst membrane, precluding reliable species-level identification. Given the known occurrence of *P. laevis*, *P. bosniacus*, and *P. tereticollis* in the Danube basin, assignment to species based solely on cystacanth morphology is not possible.

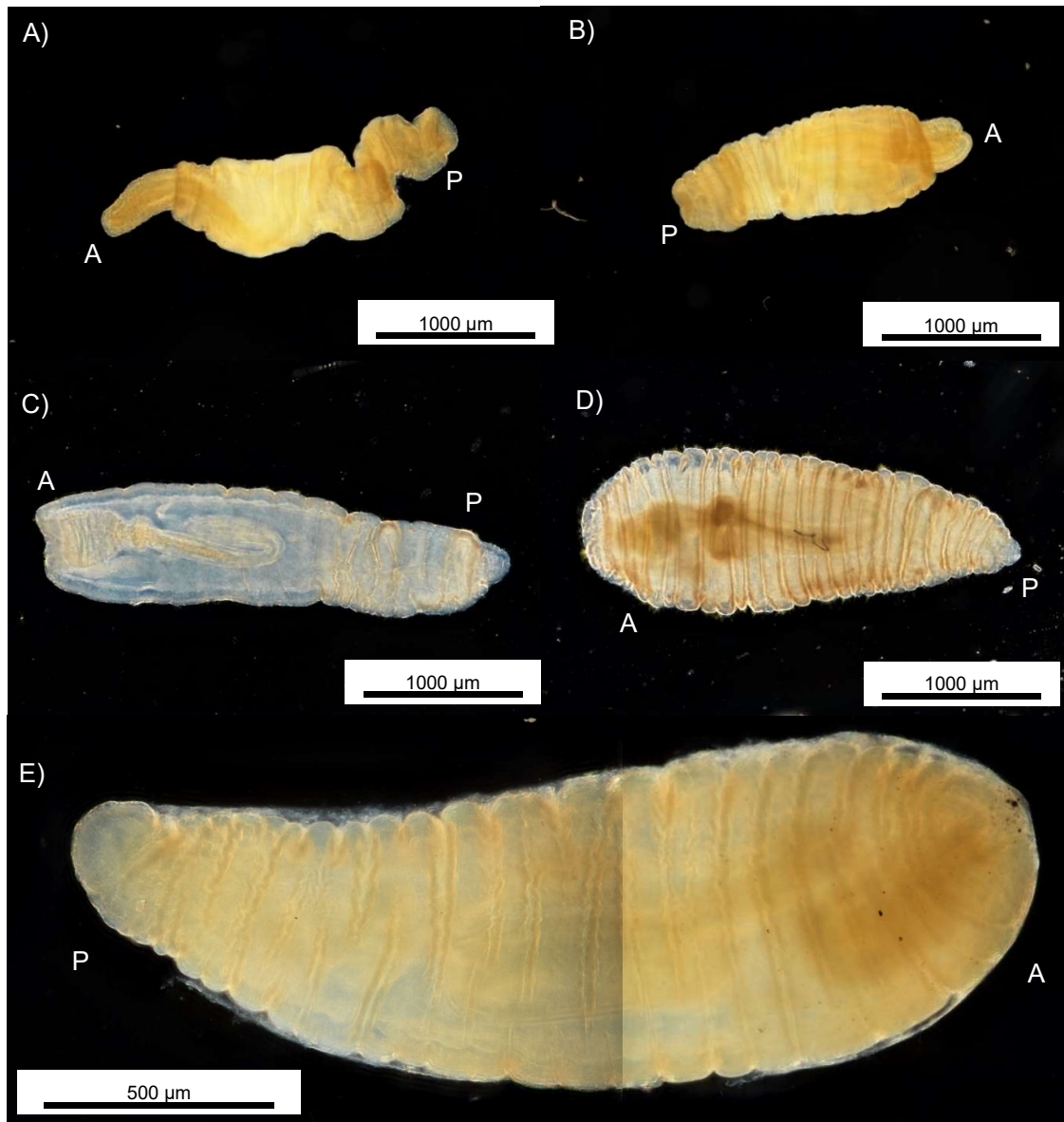


Fig. 3.2: Cystacanths (larval stages) of *Pomphorhynchus* recovered from RC fish with thin surrounding cyst membranes, allowing direct visualization of the larval body. "A" indicates the anterior end and "P" the posterior end. DNA barcoding identified all specimens as *Pomphorhynchus bosniacus*. A-B) Two cystacanths, BDAF21.2 and BDAF21.4 respectively, recovered from *Neogobius melanostomus* showing smooth body surfaces and slightly everted proboscides. C-D) Two cystacanths, BDAF33.3 and BDAF33.4 respectively, recovered from *Gymnocephalus schraetser* with slightly wrinkled body surfaces and invaginated proboscides. E) BDAF25.2, cystacanth recovered from *Neogobius melanostomus* with an invaginated proboscis and a thin translucent cyst membrane remaining close to the tegument. Images were obtained using phase contrast microscopy.

Fig 3.2 depicts images of *Pomphorhynchus* cystacanths showing different degrees of contraction and lacking visible cyst membranes. The specimens depicted in Figs. 3.2A and 3.2B depict compact and contracted cystacanths, with pronounced annulations along the neck and a slightly differentiated anterior region in which the proboscis remains invaginated and no hook armature is externally visible. They exhibit some transverse folds and a somewhat distorted body outline, but the tegument still appears smooth at this magnification. Internal structures are faintly visible through the body. Figs. 3.2C and 3.2D depict cystacanths of differing states of contraction and translucency. Specimen 3.2C appears more relaxed and semi-translucent, allowing clearer visualization of internal anatomy, including elongated internal structures consistent with the proboscis and neck, which is smoother with less pronounced annulation. Specimen 3.2D is moderately contracted, with faintly visible internal structures; the anterior end is rounded, and the proboscis remains invaginated with no externally visible hook armature. Compared to the previous two cystacanths, specimen 3.2E appears broader with less pronounced translucency, while similarly retaining an invaginated proboscis and lacking externally visible hook armature. Furthermore, this specimen apparently retained a very thin residual cyst membrane, unlike the previously mentioned fully exposed cystacanths. This outer layer is distinct from the tegument and likely represents a partially preserved or collapsed cyst membrane that was not completely removed. In contrast, the earlier specimens lacked any visible surrounding membrane and showed the tegument directly exposed to the mounting medium. Similar to Fig 3.1, no diagnostic features of the proboscis armature were visible in all cystacanths in Fig. 3.1, due to the level of invagination and obscured visual of the hooks through the cyst membrane, precluding species-level identification.

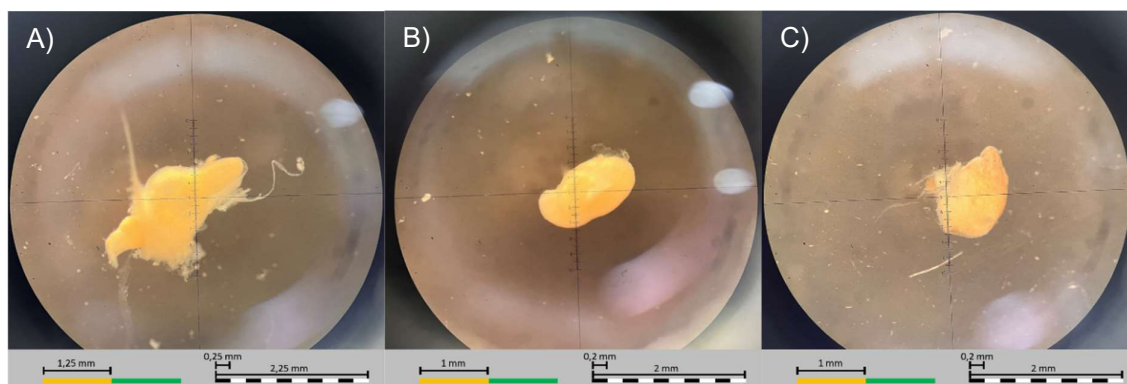


Fig. 3.3: Stereomicroscope images of *Pomphorhynchus* cystacanths recovered from MC fish. DNA barcoding identified specimen A as *Pomphorhynchus laevis*, specimen B as *Pomphorhynchus bosniacus*, and specimen C as *Pomphorhynchus tereticollis*. A) NHMF9.2 recovered from *Squalius cephalus*. B) NHMF10.5 recovered from *Barbus barbus*. C) NHMF13.12 recovered from *Barbus barbus*.

Dissection of cystacanths from MC fish, as depicted in Fig. 3.3, proved more challenging compared to RC material. In these hosts, the cyst membranes appeared thicker and more firmly adherent to the *tunica serosa* of the intestine, the outermost connective tissue layer of the intestinal wall. In several cases, the cyst membrane was closely opposed to, or partially integrated with the *tunica serosa*, requiring more careful mechanical separation during recovery. This increased adherence may be attributable to long-term preservation effects, including gradual dehydration or tissue desiccation over time, which could have altered the physical properties of both host tissue and cyst wall. Consequently, removal of cystacanths from MC fish often required greater force and was associated with a higher likelihood of cyst membrane rupture.

Species-level identification based on morphology was not possible, as diagnostic features such as proboscis hook arrangement and measurements were not observable in the available specimens. Consequently, morphological assessment alone was insufficient for reliable taxonomic resolution.

In host species belonging to the family Gobiidae (including *Neogobius melanostomus*, *Neogobius fluviatilis*, *Babka gymnotrachelus*, *Proterorhinus marmoratus*, and *Ponticola kessleri*), cystacanths were frequently observed distributed throughout the body cavity rather than concentrated in close association with the digestive tract. In these hosts, the parasites were typically only loosely associated with surrounding organs and were occasionally found among the folds of the swim bladder. As a result, removal of the cystacanths from gobiid hosts was generally uncomplicated and required minimal mechanical separation. In contrast, cystacanths recovered from other host species were consistently located in close association

with the intestine. In these cases, the parasites were firmly attached to the outer intestinal surface and were not freely positioned within the body cavity. Several specimens were observed embedded beneath the *tunica serosa*, indicating a closer association with host tissue than that observed in gobiid hosts.

3.2.1.2 Adult *Pomphorhynchus*

From the total of 479 adult *Pomphorhynchus* obtained through dissection, 307 originated from RC fish and 172 from MC fish.

Like the cystacanths, adult parasites from older MC fish specimens were noticeably more difficult to recover than those obtained from recently preserved hosts. In the historical material, the parasites appeared comparatively desiccated and were more firmly adherent to the intestinal lining, often requiring careful and more forceful mechanical separation from the surrounding tissue. The intestinal walls themselves were less pliable and more resistant to tearing, necessitating greater applied force during dissection. In several cases, attempts to remove the worms resulted in separation of the proboscis from the neck region, indicating increased fragility of the anterior structures under mechanical stress. This may be due to the fact that formerly, formalin was often used (especially in the 1980s), which can make the tissue more rigid. In contrast, specimens from RC fish were typically more pliable and less tightly attached, allowing comparatively straightforward removal with minimal structural damage. The increased tissue resistance and structural fragility observed in museum material likely reflect long-term preservation effects, including dehydration and progressive alteration of both host tissues and parasite integument.

Of the 329 adults recovered, 69 were selected for further morphologic as well as DNA sequence analysis: 43 RC and 26 MC specimens.

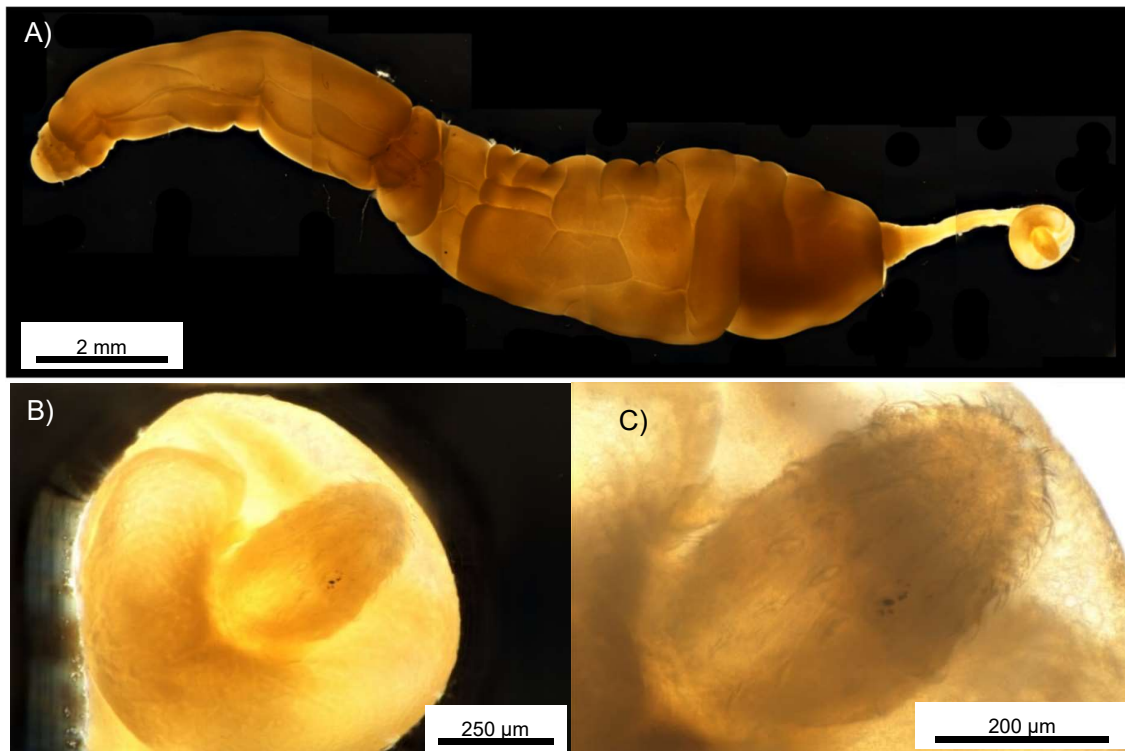


Fig. 3.4: Compound light microscope images of the adult *Pomphorhynchus bosniacus* specimen BDAF4.1 recovered from *Barbus barbus* (Appendix 1). A) Image showing the entire specimen of *Pomphorhynchus* with typical morphology, including an everted hook-bearing proboscis, elongated neck, and characteristic body proportions. B) Anterior region showing the bulbous and everted proboscis. C) Close-up of the proboscis displaying longitudinal rows of recurved hooks characteristic of acanthocephalans. Species identity was confirmed through COI DNA barcoding. Specimen was recovered from RC fish.

Fig. 3.4 shows an exemplary adult *Pomphorhynchus* specimen displaying several diagnostic morphological features characteristic of the genus. In Fig. 3.4A, the specimen exhibits a clear separation between proboscis and neck. The body is elongated and cylindrical, lacking segmentation but showing transverse wrinkles due to muscular contraction or shrinkage due to preparation. A fully everted proboscis is present at the anterior end, armed with hooks (visible as a circular hook arrangement), which are used for attachment to the host's intestinal wall. The neck region is clearly visible as a narrow transition zone between the proboscis and neck. The neck is relatively large and inflated, especially in the mid-body region, giving a characteristic club-shaped appearance often seen in adult *Pomphorhynchus*. The proboscis is relatively small and spherical to oval, and the neck is elongated and slender, distinctly separating the proboscis from the neck, one of the key traits of the genus.

The specimen had a total length of approximately 1.7 cm, which is consistent with the size range reported for adult *Pomphorhynchus* spp. The relatively large body size, together with the well-developed neck and fully everted proboscis, indicates that this individual represents a fully mature adult.

In Fig. 3.4B, the anterior neck is strongly curved, with the bulbus appearing as a rounded, inflated structure from which the proboscis emerges. The transition between neck and proboscis is clearly visible, with the proboscis projecting outward from the anterior end. Fig. 3.4C shows a higher magnification of the proboscis, revealing a densely armed surface with numerous recurved hooks arranged in longitudinal rows. The hooks are directed posteriorly, facilitating anchorage within host tissue. Individual hooks are slender and slightly curved, and their bases appear embedded within the proboscis tegument. The overall shape of the proboscis is elongate-oval, tapering slightly toward the distal end.

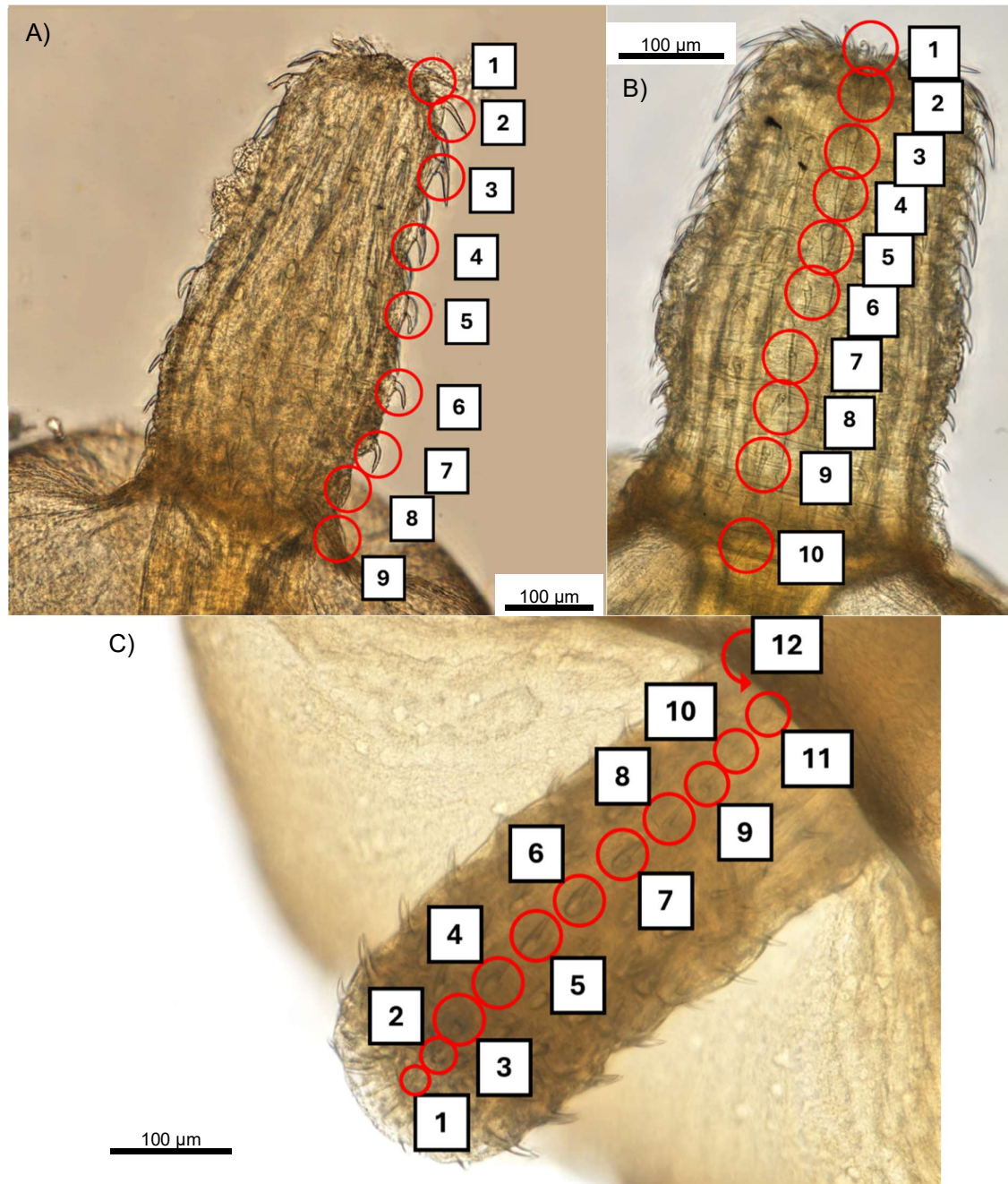


Fig. 3.5: Compound light microscope images of everted proboscides of different *Pomphorhynchus* species recovered from various fish hosts and examined at 200× magnification. The longitudinal rows of hooks represent the primary morphological feature used for species differentiation within the European *Pomphorhynchus* spp. A) *Pomphorhynchus bosniacus* specimen BDAF6.3 recovered from *Barbus barbus*. B) *Pomphorhynchus tereticollis* specimen NHMF2.1 recovered from *Squalius cephalus*. C) *Pomphorhynchus laevis* specimen NHMF1.1 recovered from *Squalius cephalus*.

The number of longitudinal hook rows and the structure of extensions on the basal hook roots is commonly used for species differentiation within the *Pomphorhynchus* spp. However, in the present material, these structures were not consistently visible, limiting the reliability of morphological species identification despite the generally good visibility of the everted proboscis and its armature.

Fig 3.5 shows light microscopic images of everted proboscides of exemplary *Pomphorhynchus* specimens, illustrating the diagnostic arrangement of hooks used for species-level identification within the *Pomphorhynchus* spp. In all three specimens, the proboscis is cylindrical with recurved hooks that are arranged in clearly defined longitudinal rows extending from the distal top toward the base. The hooks are directed posteriorly, an adaptation that would facilitate attachment to a host's intestinal wall. In Fig. 3.5A, nine longitudinal rows of hooks are visible along the proboscis, consistent with the diagnostic morphology of *P. bosniacus*. The rows are evenly spaced, and individual hooks appear slender and slightly curved, with variation in size along the length of the proboscis. Fig. 3.5B displays a specimen with ten longitudinal rows of hooks, corresponding to *P. tereticollis*. The hook rows are clearly delineated and extend uniformly across the proboscis surface, with hooks becoming progressively smaller toward the distal and proximal ends. In Fig. 3.5C, twelve longitudinal rows of hooks can be observed, which is characteristic of *P. laevis*. The increased number of hook rows results in denser armature compared to the other specimens, while maintaining the same general arrangement and posterior orientation of hooks.

Species-level morphological identification was not reliably achievable for most specimens, as the available images did not consistently allow sufficient visualization of diagnostic proboscis structures. In particular, the arrangement of proboscis hooks, which is required for species differentiation, was frequently obscured by low contrast with surrounding tissues, inflation of the bulbus, or partial to complete invagination of the proboscis. Additionally, hook root morphology could not be adequately assessed, as these structures are embedded within the proboscis wall and typically require specialized preparation or exceptionally clear imaging for reliable observation. In the present material, their visibility was further reduced by specimen contraction, preservation artefacts, and limited distinguishability of fine structural details. Consequently, the morphological characters necessary to consistently differentiate between *P. laevis*, *P. bosniacus*, and *P. tereticollis* could not be reliably resolved. Morphological identification based on the number and arrangement of longitudinal hook rows therefore proved unreliable and was consequently discontinued in favour of DNA-based identification.

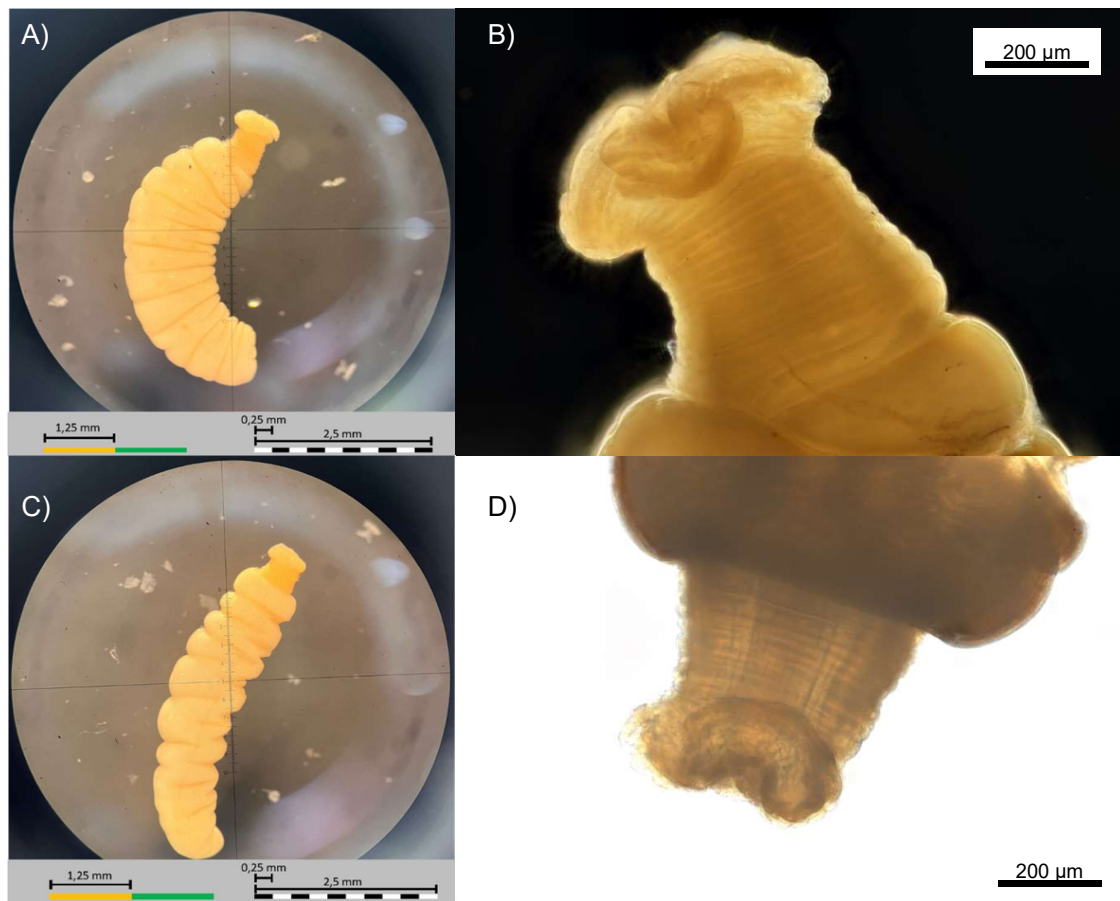


Fig. 3.6: Adult acanthocephalans of unknown species recovered from the intestinal tract of *Squalius cephalus* A-B) Specimen NHMF8.1 examined using stereomicroscopy and compound light microscopy. C-D) Specimen NHMF8.2 examined using stereomicroscopy and compound light microscopy. Both specimens exhibit pronounced transverse annulations and retracted proboscides lacking visible hooks or bulbi, preventing reliable morphological identification. Repeated *COI* DNA barcoding attempts using *Pomphorhynchus*-specific primers failed, leaving the species unknown.

Fig. 3.6 depicts two elongated specimens approximately 3 mm in length, interpreted as a putative acanthocephalan with inverted proboscides. Both specimens exhibit a cylindrical body with pronounced transverse annulations, most likely caused by muscular contraction, or preservation effects associated with long-term shortage in museum collections. The anterior region appears rounded and slightly enlarged, potentially corresponding to the proboscis receptacle region with the proboscis fully retracted and therefore not externally visible. No hook armature can be observed. A distinct neck region is not clearly visible. Overall, morphology is consistent with a strongly contracted or possibly degraded acanthocephalan specimen, although key diagnostic features remain obscured.

Although the specimen was initially presumed to represent a *Pomphorhynchus* individual with an invaginated proboscis, molecular identification through DNA barcoding could not be achieved. PCR amplification of the *COI* fragment failed repeatedly. Consequently, no definitive taxonomic assignment could be made based on DNA data.

3.2.2 DNA analyses of the genus *Pomphorhynchus*

3.2.2.1 Genetic identification using DNA barcoding (*COI*)

For DNA sequence analysis, 138 specimens assigned to the genus *Pomphorhynchus* were selected (Appendix 2). These were separated into dataset 1 and dataset 2, containing long and short sequences respectively (see chapter 2.3 DNA sequence analysis for clarification). Furthermore, as stated previously, PCR amplification failed or produced poor-quality or inconclusive sequences for some *Pomphorhynchus* specimens recovered from RC fish. Inconclusive results likely resulted from low DNA concentration, partial DNA degradation, contamination with non-target DNA, or insufficient sequence quality preventing reliable alignment and species identification. In such cases, PCR amplification was repeated using primer pair B, which targets a shorter amplicon to facilitate species-level identification from degraded or low-quality DNA.

Table 3.3: Numbers of identified *Pomphorhynchus* specimens using DNA barcoding of the *COI* gene as well as *Pomphorhynchus* specimens with inconclusive genetic results. RC parasites were recovered from RC fish (collected 2014-2025, Appendix 1) whereas MC parasites were recovered from MC fish (collected 1826-2004, Appendix 1). Long sequences have an amplicon size of 710 bp, whereas short sequences have an amplicon size of 111 bp (Tab. 2.1)

Species	RC parasites		MC Parasites	Total
	Long sequences	Short sequences	Short sequences	
<i>P. bosniacus</i>	53	10	17	81
<i>P. laevis</i>		6	3	9
<i>P. tereticollis</i>		2	14	16
Inconclusive results	10		22	32

Table 3.3 provides an overview of DNA barcoding success in the *Pomphorhynchus* specimens, separated into recent and museum-derived material, as well as specimens that produced inconclusive genetic results. *Pomphorhynchus bosniacus* represents the dominant species in the dataset, with a total of 81 identified specimens. Of these, 53 RC specimens yielded long sequences, while 10 RC and 17 MC parasites were identified using short sequences. *Pomphorhynchus laevis* was identified in a total of nine specimens, all based on

short sequences, including six RC and three museum-derived individuals. *P. tereticollis* was represented by 16 specimens, comprising two RC and 14 MC parasites identified through short sequences. In addition to the successfully identified specimens, 32 samples produced inconclusive genetic results. This included ten RC and 22 MC parasites for which no reliable species assignment could be achieved, likely due to weak amplification success, fragmented DNA templates, contamination with non-target DNA, or sequences that were too short or error-prone for confident comparison with reference data. In one case, a specimen morphologically identified as *Pomphorhynchus* returned a BLAST match to the host species *Neogobius melanostomus*, most likely due to co-amplification of host DNA or contamination overwhelming the parasite template.

A direct correlation between morphological and genetic species identification could not be established reliably in the present study, as the diagnostic morphological structures required for species-level differentiation, particularly the arrangement of proboscis hooks and hook root morphology, could not be consistently resolved. Consequently, morphological identification based on hook row armature proved unreliable, and molecular identification through *COI* DNA barcoding was ultimately considered the more robust and reproducible approach for species assignment.

Table 3.4: Overview of fish host species infected with the different *Pomphorhynchus* species. Numbers indicate the number of fish individuals in which the respective *Pomphorhynchus* species was detected through DNA barcoding analysis. Fish marked with an asterisk are species considered invasive species of the Ponto-Caspian region.

Host species	<i>P. bosniacus</i>	<i>P. tereticollis</i>	<i>P. laevis</i>	Total
<i>Alburnus alburnus</i>	1			1
<i>Babka gymnotrachelus</i> *	2			2
<i>Barbus barbus</i>	9	2		11
<i>Barbus petenyi</i>	2			2
<i>Gymnocephalus flaviatus</i>	1			1
<i>Gymnocephalus schraetser</i>	1			1
<i>Neogobius melanostomus</i> *	5			5
<i>Ponticola kessleri</i> *	2			2
<i>Squalius cephalus</i>		3	3	6
<i>Zingel zingel</i>	1			1

Table 3.4 demonstrates that *P. bosniacus* was the dominant *Pomphorhynchus* species in the dataset, occurring across the widest host range and infecting both native freshwater fishes and invasive Ponto-Caspian gobiids such as *Neogobius melanostomus*, *Babka gymnotrachelus*, and *Ponticola kessleri* (Bij De Vaate et al. 2002; Grabowska et al. 2023; Cerwenka et al. 2023). In contrast, *P. tereticollis* and *P. laevis* were detected in comparatively few host species, with *P. laevis* occurring only in *Squalius cephalus*.

3.2.2.2 Neighbour joining tree

A Neighbour joining (NJ) tree was constructed using the *COI* dataset 1 comprising 53 RC *P. bosniacus* specimens (dataset 1) from this study and 279 additional sequences retrieved from GenBank, including 103 sequences of *P. bosniacus*, 78 sequences of *P. laevis*, and 97 sequences of *P. tereticollis* from Central and Eastern Europe (Appendix 2).

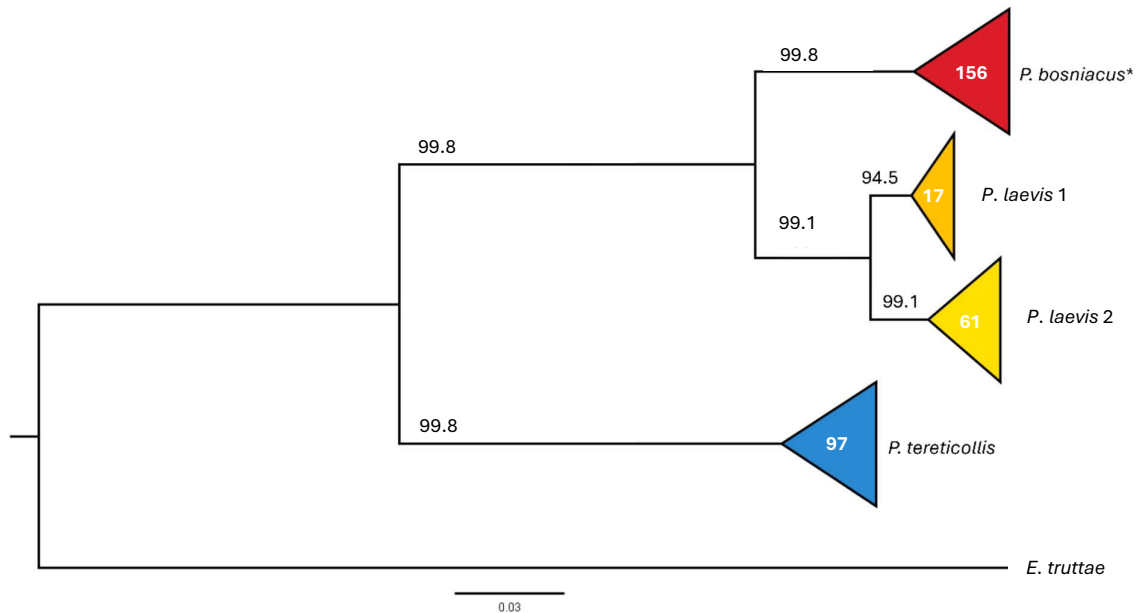


Fig 3.7: Neighbour Joining (NJ) tree showing phylogenetic relationships between species of the genus *Pomphorhynchus* based on a 533 bp *COI* dataset. Clades are collapsed into triangles, with numbers indicating the number of sequences in each clade. Clades containing specimens examined in this study are marked with an asterisk (the 533-bp fragment could be obtained solely from *Pomphorhynchus bosniacus*). Bootstrap values (1000 replicates in %) are shown next to the nodes. Only specimens collected from RC fish are included, as well as sequences obtained from GenBank (Appendix 2) with a total of 332 sequences. The tree was rooted using *Echinorhynchus truttae* as an outgroup.

The four highly supported clades correspond to the previously described groupings of *P. tereticollis*, *P. laevis*, and *P. bosniacus* (Perrot-Minnot et al. 2018; Reier et al. 2019; David et al. 2018). The basal split separating *P. tereticollis* from all other sequences (bootstrap value 99.8) consists exclusively of *P. tereticollis* sequences downloaded from GenBank (n = 97) originating from across Europe (Fig. 3.9). The remaining sequences form two major clades: one corresponding to *P. bosniacus* and the other to *P. laevis* which is further subdivided into two subclades, both supported by high bootstrap values (94.5-99.1). The first (*P. laevis* 1, n = 17) comprises GenBank sequences previously assigned to *P. laevis*, originating from Hungary, France, the Czech Republic, and two locations in Germany. The second (*P. laevis* 2, n = 61) includes sequences from Austria, Bosnia, and Croatia. The largest clade (n = 156) corresponds to *P. bosniacus* and forms a distinct and strongly supported clade (bootstrap value 99.8). It includes both specimens sequenced in this study and published sequences from GenBank, spanning the Ponto-Caspian region, the Danube, the Upper Rhine in France, the Weser in Germany, and the Vistula in Poland (Fig. 3.9). Prior to the taxonomic revision by Reier et al. (2019), this clade would have been interpreted as a subclade of *P. laevis* (David et al. 2018; Perrot-Minnot et al. 2018).

3.2.2.3 Genetic *p*-distances and haplotype network of *P. bosniacus*

Table 3.5: Genetic diversity and pairwise genetic *p*-distances of *P. bosniacus* populations from four European river basins based on the COI dataset 1. *p*-distances (expressed as %) represent the proportion of nucleotide positions differing between sequences. Ø distances indicate the mean intraspecific *p*-distance within each river basin, whereas values between river basins represent the mean interpopulation *p*-distance, with the observed minimum and maximum values shown in parentheses. H = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity.

Basins	no. sequences	H	Hd	π	Ø distances	Rhine	Vistula	Weser
Danube	114	33	0.77	0.00385	0.38	0.49 (0.1-1.1)	0.29 (0.0-0.6)	0.63
Rhine	33	33	1.00	0.00576	0.58		0.39 (0.1-0.9)	0.72 (0.2-1.5)
Vistula	4	3	0.83	0.00188	0.19			0.53
Weser	5	5	1.00	0.00488	0.49			

Pairwise genetic *p*-distances between *P. bosniacus* populations from different European river basins are summarized in Table 3.5. The lowest mean intraspecific divergence was observed in the Vistula population (0.19%), whereas the Rhine population exhibited the highest average divergence (0.58%). The Danube population, which comprised the largest number of sequences, showed a comparatively low mean divergence of 0.38%, while the Weser population had a mean divergence of 0.49%.

Genetic differentiation between river basins was generally low to moderate. The smallest mean interpopulation distance occurred between the Danube and Vistula populations (0.29%; range 0.0-0.6%), whereas the greatest divergence was observed between the Rhine and Weser populations (0.72%; range 0.2-1.5%). The Danube population contained the highest number of haplotypes ($H = 33$), while the Rhine population exhibited the greatest haplotype diversity ($H_d = 1.00$). Despite these differences, nucleotide diversity remained low across all river basins ($\pi = 0.00188$ - 0.00576). Overall, the relatively low p -distances indicate that *P. bosniacus* populations remain genetically closely related despite their broad geographic distribution. However, interpretations regarding the Vistula and Weser populations should be treated cautiously because of their comparatively small sample sizes.

Overall, genetic differentiation between river basins was low to moderate. The lowest mean intraspecific divergence was observed in the Vistula population (0.19%), whereas the Rhine population showed the highest average divergence (0.58%). Even the Danube population, which comprised the largest number of sequences, exhibited comparatively low mean divergence (0.38%). Interpopulation distances were likewise generally low, ranging from 0.29% between the Danube and Vistula populations to 0.72% between the Rhine and Weser populations. Despite this variation, the relatively low p -distances across river basins indicate that *P. bosniacus* populations remain genetically closely related despite their broad geographic distribution. However, interpretations regarding the Vistula and Weser populations should be treated cautiously due to their comparatively small sample sizes.

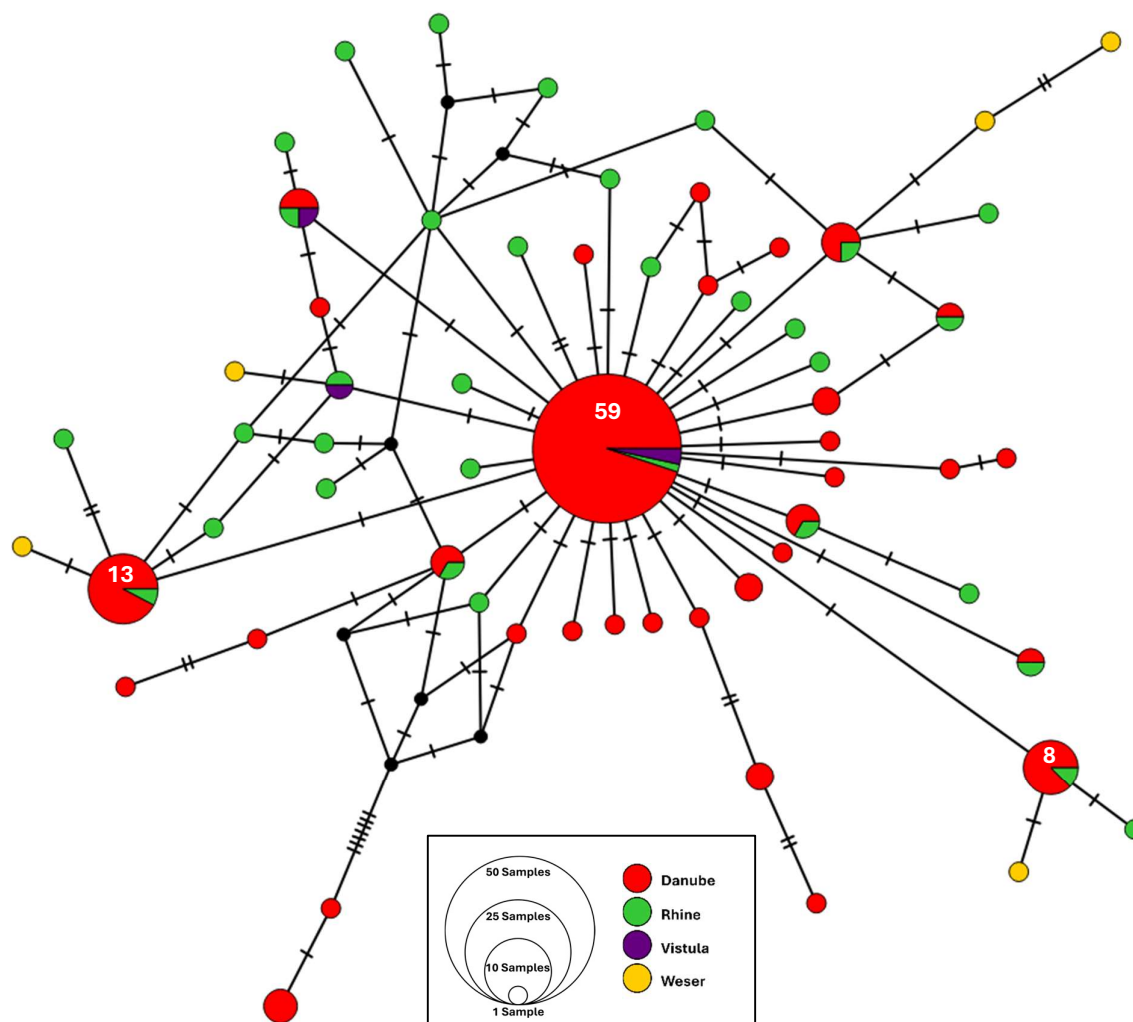


Fig 3.8: Median-joining haplotype network for *P. bosniacus* based on partial *COI* marker sequence found in four major European drainage basins: Danube, Rhine, Vistula and Weser (Colours indicate the drainage basin). The dataset contains 156 samples, originating from this and other studies (Appendix 2). Each circle represents a unique mitochondrial haplotype, with circle size proportional to haplotype frequency (see legend). Colours indicate the drainage basin in which each haplotype occurs (Danube = red, Rhine = green, Vistula = purple, Weser = yellow). Black dots represent median vectors (hypothetical unsampled or ancestral haplotypes). White numbers within the three largest haplotypes indicate the number of specimens of the respective colour. Hatch marks represent how many mutations separate the connected haplotypes. Identical sequences assigned to haplotype nodes are listed in Appendix 3.

Fig. 3.8 shows the median-joining haplotype network of *P. bosniacus* based on partial *COI* sequences from 156 specimens originating from four major European river basins (Danube, Rhine, Vistula, and Weser), while summary statistics of genetic diversity and *p*-distances are presented in Table 3.5. The dataset contained numerous closely related haplotypes connected by relatively few mutational steps, resulting in a predominantly star-like network structure centred around one dominant and widespread haplotype shared by 59 individuals (Haplotype A; Appendix 3). This central haplotype was primarily associated with the Danube basin but also included sequences from other river systems, indicating substantial haplotype sharing across Europe. In general, no pronounced geographic clustering was observed, as haplotypes from different river basins were distributed throughout the network. The high haplotype diversity and comparatively low nucleotide diversity values shown in Table 3.5 are consistent with this network structure, indicating the presence of numerous closely related haplotypes differing by only a small number of mutations. Rhine-associated haplotypes were particularly dispersed across the network, reflecting high haplotype diversity combined with low nucleotide divergence. The Vistula and Weser haplotypes likewise did not form clearly separated clusters, although the Weser-associated haplotypes appeared somewhat more peripheral within the network. However, this pattern may partly reflect the comparatively small sample size from these basins. Overall, the network structure and low *p*-distances summarized in Table 3.5 suggest weak phylogeographic structuring and high genetic connectivity among *P. bosniacus* populations across European freshwater systems.

3.2.3 Geographic origin of samples

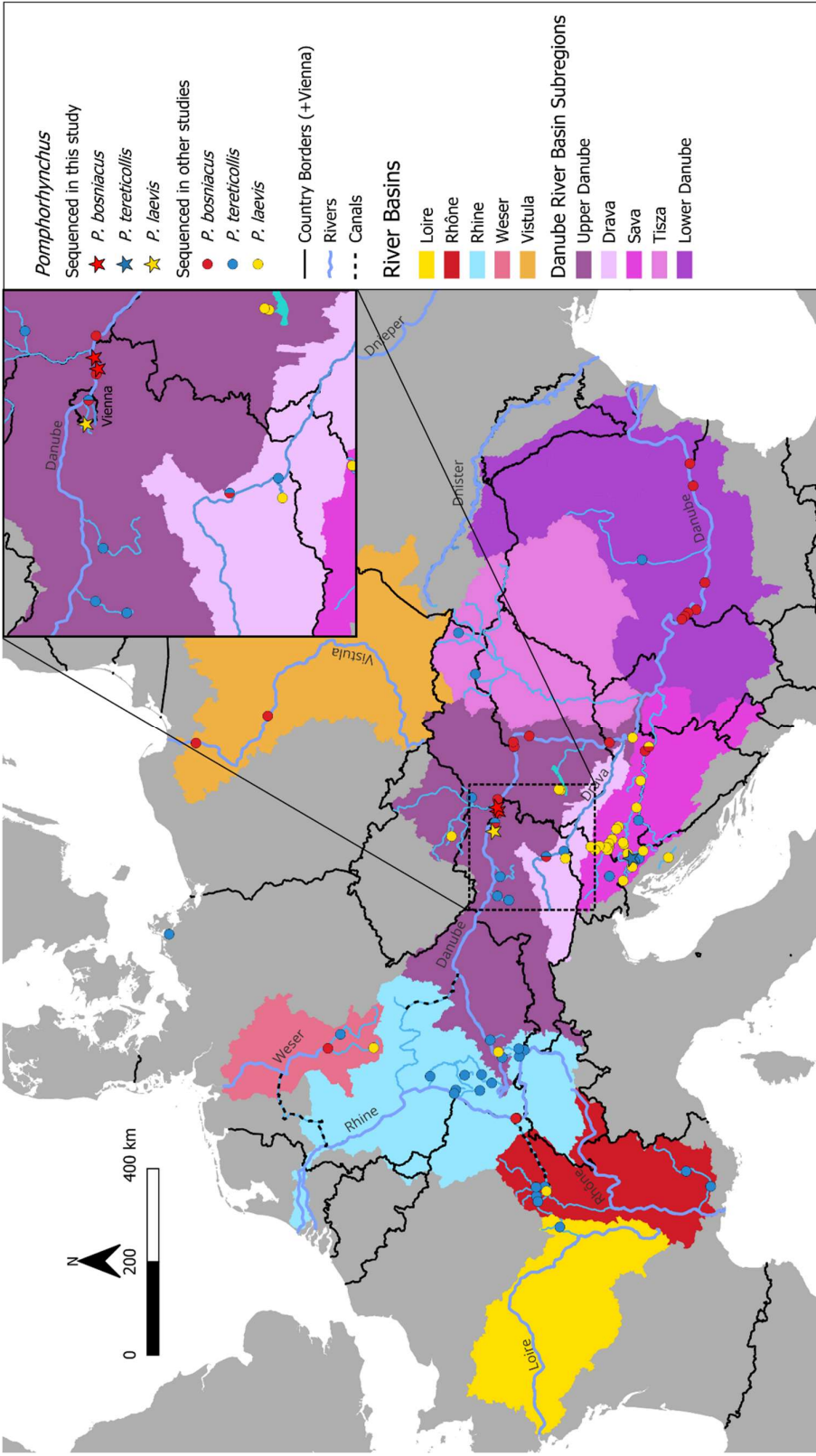


Figure 3.9: Geographic distribution of *Pomphorhynchus* samples from major European river basins analysed in the present study as well as in previous studies. The map shows the occurrence of *Pomphorhynchus bosniacus*, *Pomphorhynchus tereticollis*, and *Pomphorhynchus laevis* in the Danube, Rhine, Weser, Vistula, and Dniester systems, including the Upper Danube and tributaries such as the Sava and Drava. Specimens from other studies (collected between 1998-2018 (Appendix 2)) are shown as circles, while specimens from the present study (collected between 2014-2025) are marked with stars and also appear in Fig. 3.10. Colours indicate species identity: *Pomphorhynchus bosniacus* = red, *Pomphorhynchus tereticollis* = blue, *Pomphorhynchus laevis* = yellow. All species were identified using DNA barcoding sequences of the COI gene.

Fig. 3.9 illustrates the geographic distribution of *Pomphorhynchus* species across major European river basins, with a strong concentration of records within the Danube system. *P. bosniacus* occurs predominantly in the main channel of the Danube, particularly in the eastern Upper Danube downstream of Vienna, but is also present in the Rhine, Weser, and Vistula basins. In contrast, *P. tereticollis* and *P. laevis* are more frequently associated with tributary systems and western regions of the Upper Danube, as well as the Drava and Sava rivers and their tributaries. Similar patterns are visible in the Weser system, where *P. bosniacus* occurs mainly in the main river channel, whereas *P. tereticollis* and *P. laevis* are more common in tributaries such as the Fulda and Werra rivers.

Outside the Danube basin, the Loire and Rhône systems contain comparatively few records and are dominated mainly by *P. tereticollis*, with fewer *P. laevis* records and no confirmed occurrence of *P. bosniacus*. In contrast, the Vistula basin contains confirmed records of *P. bosniacus*, extending the known distribution of the species into northeastern European freshwater systems connected to the Baltic drainage region.

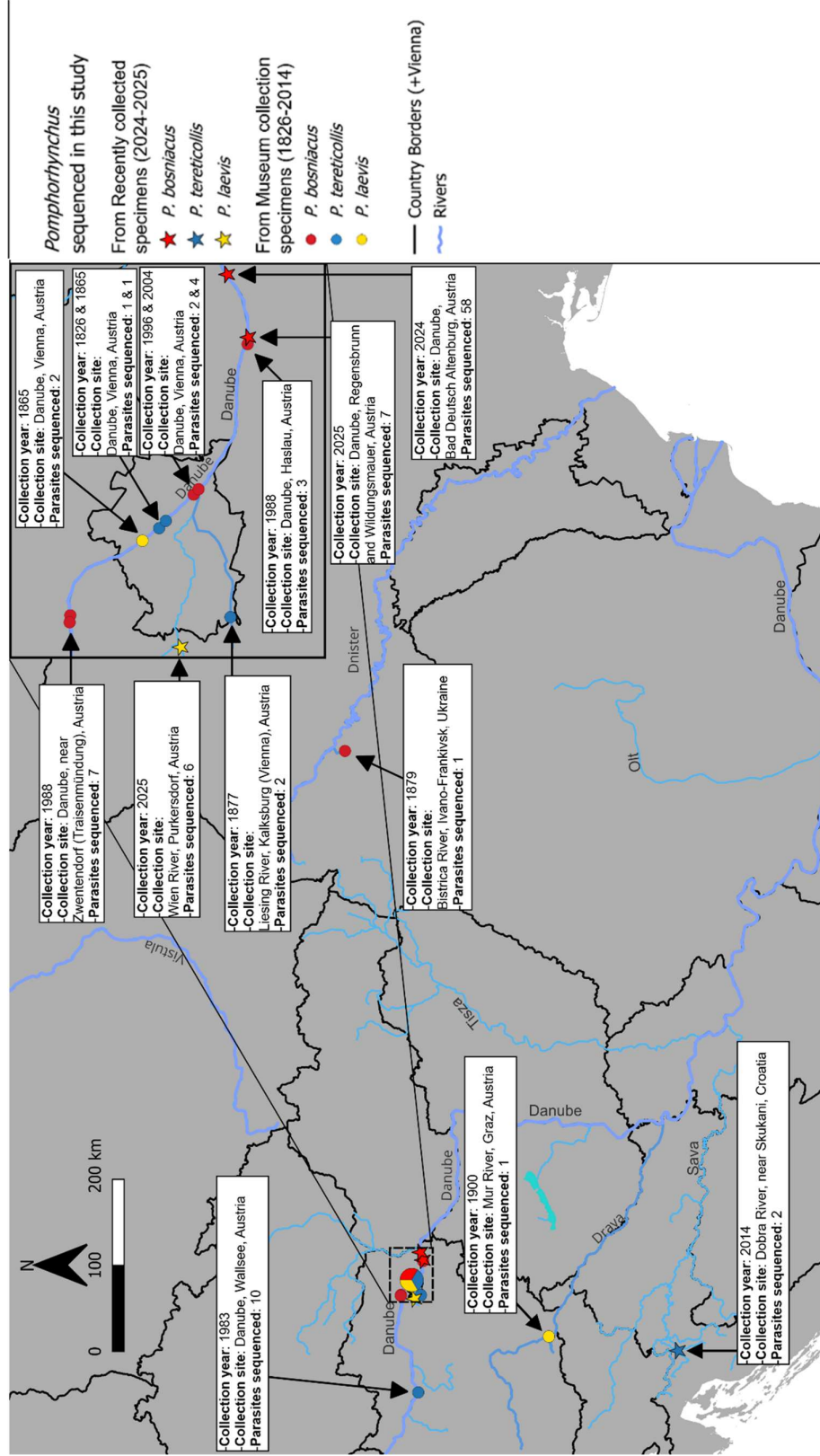


Figure 3.10: Geographic distribution of *Pomphorhynchus* sampling sites across Central and Eastern Europe, with particular focus on the Vienna region. All specimens shown were sequenced in the present study. Sampling sites are represented by coloured symbols indicating species identity: *Pomphorhynchus bosniacus* = red, *Pomphorhynchus tereticollis* = blue, *Pomphorhynchus laevis* = yellow. Circular symbols represent museum collection parasites collected between 1826 and 2014, whereas star-shaped symbols denote recently collected parasites, collected between 2024 and 2025 (and appear in Fig. 3.9). Labels indicate the collection year, sampling locality, and number of sequenced parasites per site. Most records originate from the Danube and its tributaries, including the rivers Drava, Sava, and Wien.

Fig. 3.10 provides a spatial overview of sampling locations and temporal records of *Pomphorhynchus* specimens analysed in the present study, including RC and MC parasites across Central and Eastern Europe, with a focus on Austria and the Danube basin. Most sampling sites are located along the Danube River and its tributaries, including the Drava and Sava River, while the Dnister in Ukraine represents the only major river outside the Danube basin of which samples were investigated.

The Vienna region shows the highest concentration of sampling points (Fig. 3.10, inset). Historical records from Vienna and nearby sites include specimens collected between 1826 and 1865 from the Danube. These MC parasites include individuals of *P. laevis* and *P. tereticollis*. Additional early records include *P. tereticollis* from the Liesing River (1877).

Sampling from 1988-2015 in the Vienna region shows a predominance of *P. bosniacus*. This includes seven museum collection (MC) parasites collected near Zwentendorf in 1988 (this study, Appendix 2), as well as specimens collected in 1996 and 2004 (Appendix 2). Additional records include four individuals collected in 1998 (GenBank accession numbers LN994903, LN994907-LN994909, Appendix 2; Perrot-Minnot & Tougaard 2015) and 27 specimens collected in 2015 (GenBank accession numbers MK612497-MK612523, Appendix 2; Reier et al. 2019). Within the main channel of the Danube, only a single *P. tereticollis* specimen has been recorded in addition to *P. bosniacus* (GenBank accession number MK612533, Appendix 2; Reier et al. 2019). In contrast, *P. laevis* was recovered from the Wien River, a tributary of the Danube, in recently collected material from 2025 (this study, Appendix 2).

In the Danube east of Vienna, three MC parasites of *P. bosniacus* collected in 1988 near Haslau (Appendix 2) were found in close proximity to a sampling location of seven RC *P. bosniacus* specimens collected in 2025 near Regelsbrunn and Wildungsmauer (Appendix 2), as well as additional specimens from Bad Deutsch Altenburg (2024; Appendix 1, Appendix 2). Additional nearby records include *P. bosniacus* collected near Orth an der Donau in 2006 (GenBank accession number LN994918, Appendix 2; Perrot-Minnot & Tougaard 2015) and in Bratislava in 1996 (GenBank accession numbers LN994902, LN994910-LN994911, Appendix 2; Perrot-Minnot & Tougaard 2015).

In contrast, MC parasites from the Danube near Wallsee (Upper Danube) are identified as *P. tereticollis* (Appendix 2). Comparable records from nearby tributaries include *P. tereticollis* collected in the Krems River (GenBank accession numbers MN780978-MN780982, Appendix 2; (Lewisch et al. 2020) and the Ybbs River (GenBank accession numbers MN780983-MN780986, Appendix 2; Lewisch et al. 2020) between 2018 and 2020.

A historical *P. laevis* specimen from the Mur River (1900; Appendix 2) was sampled near RC records of *P. tereticollis* collected in 2014 in the Mur River near Obervogau and Gralla (GenBank accession numbers MK612534-MK612545, Appendix 2; Reier et al. 2019) near RC *P. laevis* specimens collected in 2014 and 2017 in the Sulm River near Gleinstätten (GenBank accession numbers MK612524-MK612532, Appendix 2; Reier et al. 2019).

Two *P. tereticollis* specimens from the Dobra River (collected in 2014; this study, Appendix 2) match with additional *P. tereticollis* records from the same region (GenBank accession numbers OQ569522-OQ569529, Appendix 2; Vardić Smrzlić et al. 2023). These occur alongside *P. laevis* specimens from the same area (GenBank accession numbers OQ569490-OQ569520, Appendix 2; Vardić Smrzlić et al. 2023). All *P. laevis* specimens from the Sava and Drava subregions belong to the *P. laevis* 2 subclade, whereas specimens from other basins and Danube subregions are assigned to the *P. laevis* 1 subclade. The two *P. laevis* subclades are not distinguished in Fig. 3.9; however, the assignment of individual specimens to each subclade is provided in Appendix 2.

Lastly, a specimen of *P. bosniacus* collected in 1879 from the Bistrica River near Ivano-Frankivsk, Ukraine (Appendix 2), represents one of the earliest records included in this dataset. The drainage basin of this river is the Dnister.

3.3 Other parasitic worms

3.3.1 Nematoda

Nematodes constituted the second most abundant parasite group after acanthocephalans. A total of 195 nematode specimens were recovered from the intestinal tracts examined. Of these, 179 individuals (91.79 %) were obtained from MC fish, representing the vast majority of nematodes detected, while only 16 individuals were isolated from RC fish.

Among the 83 dissected fish representing 17 species, nematodes were detected in six host species: *Vimba vimba*, *Barbus barbus*, *Chondrostoma nasus*, *Alburnus alburnus*, *Squalius cephalus*, and *Ponticola kessleri*. In MC fish, nematodes were recovered from all six listed host species. In RC fish, nematodes were detected only in *V. vimba*, *B. barbus*, *C. nasus* and *A. alburnus*.

As nematodes were not the primary focus of the present study, their morphological and genetic identification was conducted only to a limited extent. Nevertheless, all collected specimens were preserved and deposited in the museum collection for potential future investigation. Two specimens were examined microscopically to obtain a general morphological overview and a preliminary assessment of species identity (Fig. 3.11). The specimens appeared morphologically similar based on general external appearance, with the exception of three individuals recovered from a single host (NHMF10, *B. barbus*), which were notably larger than the others (Fig. 3.12).

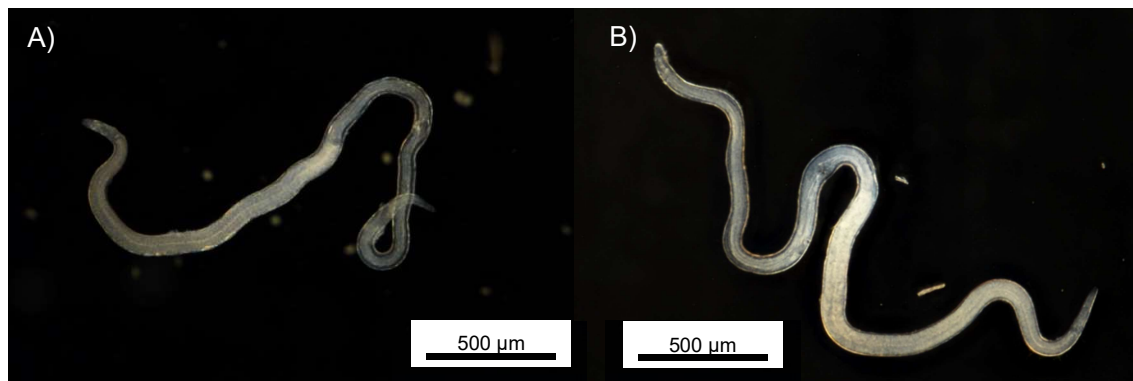


Fig. 3.11: Light microscopy images of nematodes recovered using phase contrast. A) Specimen BDAF10.5. B) Specimen BDAF10.6. Both specimens were recovered from the intestinal tract of *Barbus barbus* specimen BDAF10 (Appendix 1).

In the absence of detailed morphometric analysis or DNA-based confirmation, the examined specimens could only be tentatively assigned to the genus *Contracaecum*. They may belong to the *Contracaecum rudolphii* species complex, which represents the most commonly reported anisakid nematode infecting freshwater fishes of the Danube River basin (Zaharieva and Kirin 2020). This tentative assignment is supported by the overall body morphology, including elongate cylindrical form, smooth cuticle, and general size range typical of *Contracaecum* (Mattiucci et al. 2014). However, without detailed morphological examination or genetic confirmation, this identification remains provisional.

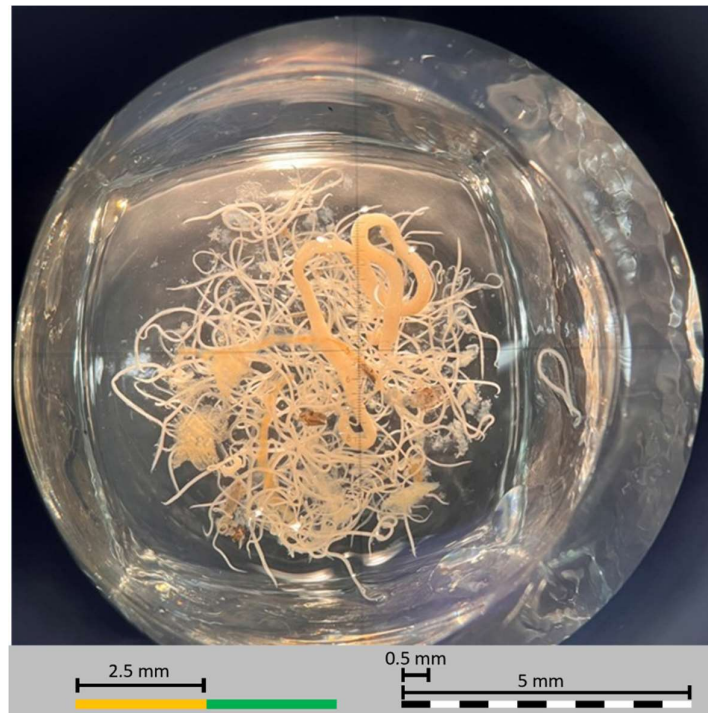


Fig 3.12: Stereomicroscope images of nematodes recovered. All specimens were found in *Barbus barbus* NHMF12 and were located inside of the intestinal tract.

The nematode specimens recovered from MC fish could not be identified beyond a higher taxonomic level. This limitation primarily results from the absence of detailed morphological examination under light microscopy, which is required to assess diagnostic characters.

3.3.2 Trematoda

Trematodes represented the least abundant parasite group in my thesis. In total, 56 trematode specimens were isolated from the examined intestinal tracts. Of these, 40 individuals (71.43 %) were obtained from a single host individual, a *Gymnocephalus schraetser* specimen collected in 2024 (BDAF33, see Appendix 1). Seven additional trematodes were isolated from other RC fish, while nine specimens were obtained from MC fish. As with the nematodes, all trematode specimens were preserved and deposited in the museum collection for potential future investigation.

Among the 83 dissected fish representing 17 species, trematodes were detected in only four species: *Chondrostoma nasus*, *Gymnocephalus schraetser*, *Babka gymnotrachelus*, and *Proterorhinus* spp. The trematodes were recovered in RC fish specimens of *C. nasus*, *G. schraetser*, and *B. gymnotrachelus*, whereas infections in MC parasites were observed only in multiple hosts of *Proterorhinus* spp.

As trematodes were not the primary focus of this study, their morphological and genetic identification was pursued only to a limited extent. Trematodes were originally not meant to be identified to species level using detailed morphological or genetic criteria, with a few exceptions explained below. Consequently, comprehensive morphological characterization or molecular identification was performed for these specimens, and they were treated collectively at a higher taxonomic level.

Based on general external morphology, 41 specimens were identified as adult trematodes, including the 40 individuals recovered from a single host and one additional adult specimen recovered from a different host (Fig. 3.13), while a further 15 specimens were identified as larval stages (metacercariae), and were recovered from multiple fish hosts.



Fig 3.13: Light microscopy image of a trematode (BDAF3.1) using phase contrast. The specimen was recovered from BDAF3 of the species *Chondrostoma nasus*.

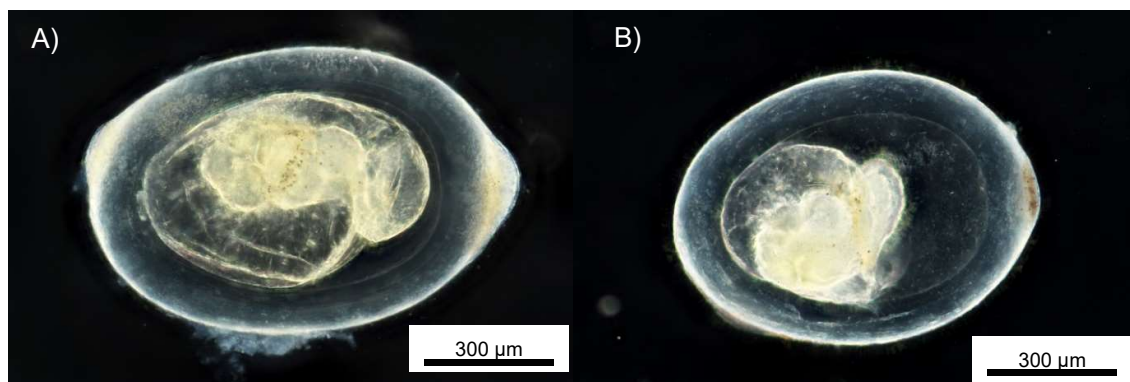


Fig 3.14: Light microscopy images of trematode metacercariae. A) BDAF37.4 and B) BDAF37.5 imaged using phase contrast. Both specimens were recovered from a RC fish (BDAF37; *Babka gymnotrachelus*,) and were located outside of the intestinal tract but inside the body cavity, near the intestines.

The larval specimens of eight trematodes (Figs. 3.14-3.15) were subjected to morphological examination and molecular identification following initial misclassification during gross examination. These specimens were originally presumed to represent larval stages of a *Pomphorhynchus* species due to similarities in size and general appearance and were therefore initially subjected to DNA barcoding using *Pomphorhynchus*-specific primers. Subsequent re-examination and DNA barcoding using trematode-specific primers targeting the 18S rRNA region (Primer pair D; see Table 2.1 in chapter 2.2.2 of Material and Methods) revealed that these individuals were in fact trematodes. DNA sequence analysis therefore enabled correction of the initial morphological assessment and enabled taxonomic reassignment.

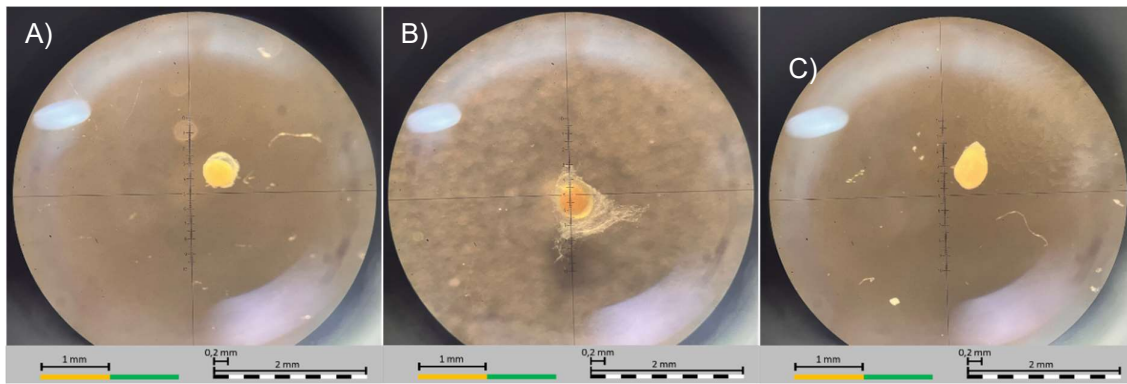


Fig 3.15: Stereomicroscope images of recovered trematodes. A) NHMF21.2 B) NHMF22.3 and C) NHMF23.2. All specimens were recovered from MC fish (NHMF21, NHMF22 and NHMF23 respectively) belonging to the genus *Proterorhinus*. The parasites were located within the body cavity near the intestines.

The microscopy images in Fig. 3.14 and 3.15 depict trematode larvae enclosed within a distinct transparent cyst, giving the specimens an oval to slightly elliptical outline. Internal structures are faintly visible, including differentiated anterior and posterior regions characteristic of strigeid larvae. The cyst walls appear smooth and relatively thick, suggesting a protective encysted stage adapted for persistence within an intermediate or paratenic host. Overall morphology, including the encysted form and body configuration, is consistent with metacercarial stages of strigeid trematodes (Maldonado et al. 2015, Jithila and Prasadnan 2018).

A GenBank BLAST analysis of the sequences obtained from larval trematodes indicated affiliation with the family Strigeidae. However, assignment to species level was not possible. A BOLD BLAST search did not return any matches. This limitation reflects the choice of primer set, which was designed for broad detection of trematodes rather than for resolving finer taxonomic ranks. Consequently, the amplified genetic region provided sufficient resolution for family-level identification but lacked the discriminatory power required for reliable species- or genus-level assignment.

4. Discussion

4.1 General conclusions

The present study confirmed the occurrence of three *Pomphorhynchus* species (*P. bosniacus*, *P. laevis*, and *P. tereticollis*), in historical as well as recently collected fish samples, within Austrian and other European freshwater systems. Species identification based on DNA analysis proved substantially more reliable than morphological examination alone, particularly for cystacanth stages and morphologically similar specimens. RC samples from the Danube were dominated by *P. bosniacus*, whereas historical museum material contained predominantly *P. laevis* and *P. tereticollis*, suggesting patterns consistent with recent expansion of *P. bosniacus*. Population genetic analyses revealed low genetic divergence and high haplotype connectivity among *P. bosniacus* populations, indicating strong genetic connectivity and extensive dispersal across European river systems. In addition, the detection of two geographically structured *P. laevis* lineages supports the pronounced phylogeographic differentiation within this species.

The results further highlight the important role of invasive Ponto-Caspian gobiids and amphipods in the contemporary distribution and transmission dynamics of *Pomphorhynchus* species, particularly within the Danube system. Finally, the integration of morphological observations, DNA analyses, and historical museum material proved valuable for reconstructing distribution patterns and reassessing the historical occurrence of *Pomphorhynchus* species in European freshwater ecosystems.

4.2 Species identification

Understanding species boundaries and distribution patterns within the *Pomphorhynchus* spp. remains a central challenge in freshwater parasitology, particularly due to the prevalence of cryptic diversity and the limitations of morphology-based identification. The present study attempts to address these challenges by combining morphological observations with DNA sequence analysis data to provide new insights into the distribution and phylogeography of *Pomphorhynchus* species in Austria and across Europe.

4.2.1 Limitations of morphological identification

The results demonstrate that reliable species identification within European *Pomphorhynchus* species cannot be generally achieved through morphology alone and strongly benefits from the integration of DNA sequence analysis methods. Although morphological characters such

as the number of longitudinal hook rows are commonly used for species differentiation, these features proved to be variable and often difficult to assess in the examined specimens. In particular, cystacanths and preadult stages (Fig. 3.1-3.2) frequently exhibited invaginated proboscides or insufficient visibility of diagnostic structures, limiting their usefulness for species-level identification. More definitive characters, such as the structure of basal hook roots, could not be consistently observed due to proboscis invagination, visual obstructions, preservation artefacts, or mechanical damage to the specimens. In some cases, such as the acanthocephalans depicted in Fig. 3.6, morphological examination alone did not even allow confident assignment to the genus level, as no hooks, proboscis, bulbus, or neck structures were visible. Although these specimens morphologically resembled contracted *Pomphorhynchus* individuals or members of the family Echinorhynchidae, their exact taxonomic identity remained unresolved (Amin 2013; Kennedy 2006).

Species identification through DNA barcoding likewise failed in a small number of specimens, most likely due to degraded DNA in preserved material or primer-template mismatches caused by the use of primers specifically designed for *Pomphorhynchus* spp. Consequently, failed PCR amplification could not be interpreted as evidence against assignment to *Pomphorhynchus*. Nevertheless, DNA barcoding based on *COI* sequences generally provided clear and reproducible species assignments independent of life stage, highlighting the importance of molecular genetic methods (Reier et al. 2019; Reier et al. 2020). Even historical museum material showed comparatively high identification success, including successful identification of the *P. tereticollis* cystacanth NHMF15.1 (Appendix 2), which was obtained from fish material collected in 1826.

4.2.2 Other parasitic worms

The markedly higher abundance of nematodes in MC fish compared to RC parasites suggests temporal differences in parasite occurrence within the examined fish communities. However, these patterns should be interpreted cautiously, as parasite communities are not static over time and may be influenced by changes in host assemblages, invasive species, and altered food-web dynamics. Since nematodes were not a primary focus of the present study, the available material was not investigated in sufficient detail to allow broader conclusions regarding long-term changes in nematode community composition.

Furthermore, as trematodes were recovered opportunistically during parasite examinations and were also not investigated in detail, no reliable conclusions regarding their diversity, prevalence, or distribution can be drawn from the present dataset.

4.3 DNA analysis and geographic distribution of the genus *Pomphorhynchus*

4.3.1 Genetic differentiation of *Pomphorhynchus* species and *P. laevis* lineages

The NJ tree recovered well-supported clades corresponding to *P. bosniacus*, *P. laevis*, and *P. tereticollis*, consistent with previous molecular genetic studies (Perrot-Minnot et al. 2018; Reier et al. 2019; David et al. 2018). Although these taxa were historically difficult to distinguish morphologically and were therefore frequently misidentified, subsequent detailed morphological investigations revealed consistent diagnostic differences between them (Reier et al. 2019).

Additionally, the subdivision of *P. laevis* into two distinct lineages suggests pronounced phylogeographic structuring within the species rather than unresolved cryptic diversity. Similar patterns were previously described by (Vardić Smrzlić et al. 2024, 2015), Perrot-Minnot et al. (2018), and Reier et al. (2019), who identified a lineage (*P. laevis* 2) associated primarily with the Sava and Drava river system and another lineage (*P. laevis* 1) occurring predominantly in other tributaries of the Upper Danube. The present results are consistent with this pattern, as all *P. laevis* specimens from the Sava and Drava basins? belonged to the same lineage (*P. laevis* 2), including specimens from the Mur River, which hydrologically belongs to the Sava drainage system. In contrast, *P. laevis* specimens recovered from tributaries of the Upper Danube, including the Wien River, as well as specimens collected from the Rhône and Rhine basins, belonged to the *P. laevis* 1 lineage. Although the Wien River specimens were represented by shorter *COI* fragments, these were sufficient to distinguish between the two *P. laevis* lineages and consistently assigned them to *P. laevis* 1.

The observed population genetic patterns could suggest that river basin structure and historical hydrological connectivity play an important role in shaping the distribution and genetic differentiation of *Pomphorhynchus* populations. Populations from hydrologically connected river systems generally exhibited low genetic divergence and high haplotype connectivity, particularly within the Danube and Rhine basins, indicating extensive host-mediated dispersal and ongoing gene flow. In contrast, the distinct subdivision of *P. laevis* into the *P. laevis* 1 and *P. laevis* 2 lineages appears to correspond closely with different drainage systems, especially between the Sava/Drava river systems and the tributaries of the Upper Danube. This pattern suggests that historical isolation of river systems, potentially linked to

palaeohydrological processes and glacial refugia, contributed to lineage divergence and regional differentiation (Bianco 1990; Hewitt 1999). Because *Pomphorhynchus* species rely on fish and amphipod hosts for dispersal, the movement and connectivity of host populations may influence parasite phylogeography. At the same time, modern anthropogenic waterways such as the Rhine-Main-Danube Canal may increasingly reduce historical biogeographic barriers by facilitating recent dispersal between previously isolated river basins (Bij De Vaate et al. 2002). However, as sampling coverage remained limited for several river systems, these interpretations should currently be regarded as preliminary hypotheses requiring further targeted investigation.

An alternative explanation for the observed genetic structuring is that the two *P. laevis* lineages may potentially represent distinct evolutionary entities rather than merely geographically differentiated populations. Similar patterns of genetic divergence within morphologically similar *Pomphorhynchus* taxa previously contributed to the recognition of *P. bosniacus* as distinct from *P. laevis* following combined molecular and detailed morphological investigations (Perrot-Minnot et al. 2018; Reier et al. 2019). Interestingly, variation in the number of longitudinal hook rows has also been observed among *P. laevis* specimens (ranging from 8-13 hook rows), and this characteristic has historically played an important role in species differentiation within the genus. Although hook row number alone is not considered fully reliable due to overlap and variability, consistent differences corresponding to the two genetic lineages could potentially reinforce the idea of distinct taxa. The consistent geographic separation and genetic distinction between *P. laevis* 1 and *P. laevis* 2 may therefore reflect ongoing lineage divergence or cryptic species diversity. Should future studies identify stable morphological differences that align with the observed genetic patterns, a taxonomic re-evaluation similar to the separation of *P. bosniacus* from *P. laevis* may become necessary.

However, the genetic differentiation observed between the two *P. laevis* lineages is considerably lower than that reported between *P. laevis* and *P. bosniacus* and therefore does not, based on the available mitochondrial *COI* data alone, justify their recognition as distinct species. Mitochondrial markers reflect only maternal lineages and are insufficient to resolve species boundaries in isolation. Consequently, comprehensive genomic analyses, ideally combined with detailed morphological comparisons, will be required to determine whether the two *P. laevis* lineages represent geographically structured populations or distinct evolutionary lineages warranting taxonomic recognition.

4.3.2 Geographic distribution of *P. tereticollis* and *P. laevis*

Within the Danube system, multiple *Pomphorhynchus* species co-occur, but distinct spatial patterns emerge at finer scales. *Pomphorhynchus bosniacus* is predominantly associated with the main channel of the Danube, whereas *P. laevis* and *P. tereticollis* occur more frequently in tributary systems. Similar patterns were also observed by (Vogel and Taraschewski 2023) in the Weser system, where *P. bosniacus* occurred mainly in the Weser River itself, while *P. laevis* and *P. tereticollis* were more common in the Fulda and Werra tributaries. These patterns may be influenced by differences in intermediate host communities, as amphipod assemblages differ substantially between large river channels and smaller tributaries. Invasive Ponto-Caspian amphipods such as *Dikerogammarus villosus* frequently dominate large river habitats, whereas native *Gammarus* species are more common in tributaries (Bij de Vaate et al. 2002; Bollache et al. 2004; Mamos et al. 2021). Vogel & Taraschewski (2023) furthermore demonstrated partially overlapping but distinct associations between *Pomphorhynchus* species and amphipod hosts, suggesting preferential host usage rather than strict host specificity.

The geographic distribution patterns observed in this study emphasize the central role of the Danube River as a major dispersal corridor for *Pomphorhynchus* species in Central and Eastern Europe. The strong concentration of records along the Danube and its tributaries highlights the importance of hydrological connectivity and host movement in facilitating parasite dispersal. At the same time, the occurrence of *Pomphorhynchus* species in other river basins, including the Rhine, Weser, and Vistula, indicates broader distribution across European freshwater systems. This wider dispersal likely reflects both natural river connectivity and anthropogenic influences, such as canal construction and international shipping, particularly via systems like the Rhine–Main–Danube Canal, which links previously isolated basins (Bij de Vaate et al. 2002). The genetic patterns observed in the haplotype network further support this interpretation. Most *P. bosniacus* haplotypes from different river basins are separated by only a few mutational steps and cluster closely around a dominant central haplotype largely associated with the Danube population. At the same time, several shared or closely related haplotypes occur across multiple river systems, particularly between the Danube and Rhine, indicating extensive genetic connectivity and recent dispersal between basins. The low genetic divergence and weak phylogeographic structuring therefore suggest ongoing gene flow facilitated by river connectivity and host-mediated dispersal. Similar invasion pathways involving both southern and northern dispersal routes have also been proposed for invasive Ponto-Caspian species such as *Dikerogammarus villosus* and *Neogobius melanostomus* (Wattier et al. 2007; Leuven et al. 2009).

These results provide little support for H3. The first prediction, that historical Danube museum specimens should predominantly represent *P. bosniacus*, was not supported, as the historical material was dominated by *P. laevis* and *P. tereticollis*, with *P. bosniacus* only detected in specimens collected from 1988 onwards. Likewise, the second prediction was not supported, as both *P. laevis* and *P. tereticollis* were present in historical samples from the Danube main channel rather than being rare or absent. Only the third prediction was supported, as *P. bosniacus* showed a strong association with the main Danube corridor in both the available historical and recently collected material. Together, these findings argue against the hypothesis that *P. bosniacus* has historically been the dominant but consistently misidentified *Pomphorhynchus* species within the Danube basin, although the limited historical sample size prevents this possibility from being excluded entirely.

4.4 Population genetics and geographic spread of *P. bosniacus*

4.4.1 Population genetics of *P. bosniacus*

The haplotype network for *P. bosniacus* provides important insights into the population genetic structure. The combination of high haplotype diversity and low nucleotide divergence, particularly in the Rhine and Danube populations, suggests strong genetic connectivity among river systems and may additionally reflect recent population expansion following founder events. This pattern is reflected in the haplotype network by the presence of numerous closely related haplotypes connected by only a few mutational steps. At the same time, the comparatively low genetic differentiation observed between river basins indicates substantial gene flow and ongoing host-mediated dispersal across interconnected freshwater systems (Poulin 2007; Charles D. Criscione and Blouin 2004). Together, these patterns suggest that *P. bosniacus* populations across Europe form a largely interconnected genetic network characterized by weak phylogeographic structuring and potentially recent demographic expansion. Large river systems such as the Rhine and Danube likely function as major dispersal corridors, allowing downstream and upstream transmission via infected definitive, intermediate, and paratenic hosts (Kennedy 2006; Poulin 2007). Anthropogenic waterways such as the Rhine-Main-Danube Canal may further enhance connectivity between previously isolated basins (Bij de Vaate et al. 2002).

Although the smaller sample sizes from the Weser and Vistula limit detailed inference, the similarly low divergence and absence of clear clustering indicate comparable patterns of connectivity and dispersal. At the same time, the occurrence of *P. bosniacus* in the Weser and

especially the Vistula may suggest the existence of multiple invasion routes across Europe rather than a single westward expansion from the Danube system. Similar invasion scenarios involving separate northern and southern dispersal corridors have previously been proposed for invasive Ponto-Caspian species such as *Dikerogammarus villosus* and *Neogobius melanostomus* (Bij de Vaate et al. 2002; Wattier et al. 2007; Leuven et al. 2009). In this context, the observed distribution of *P. bosniacus* is consistent with the established Ponto-Caspian invasion corridors described for numerous aquatic invasive species. The occurrence of closely related haplotypes in the Rhine and Danube systems is compatible with dispersal along the Southern Invasion Corridor, facilitated by the Rhine-Main-Danube Canal, whereas the presence of *P. bosniacus* in the Vistula basin is consistent with the Central Invasion Corridor, which links the Black Sea and Baltic drainage systems. These invasion pathways have been documented for a wide range of Ponto-Caspian fishes and macroinvertebrates and are regarded as the principal routes facilitating their westward expansion across Europe (Bij De Vaate et al. 2002; Leuven et al. 2009; Shiganova et al. 2026; Soto et al. 2023). Together, these findings suggest that host-mediated dispersal along interconnected river systems and established invasion corridors has contributed to shaping the contemporary distribution and genetic structure of *P. bosniacus* across European freshwater systems.

4.4.2 Geographic spread of historical and modern samples

Temporal comparisons between historical and modern samples suggest a shift in *Pomphorhynchus* species composition in the Vienna region, with *P. laevis* and *P. tereticollis* present in earlier records and *P. bosniacus* dominating more recent samples. MC parasites from the NHMW, some dating back to the 19th century, confirm the historical presence of *P. laevis* and *P. tereticollis* in Austrian river systems, whereas *P. bosniacus* was first detected in the examined material from 1988 onwards within and around Vienna. Interestingly, this temporal appearance broadly coincides with the first documented occurrences and subsequent spread of invasive Ponto-Caspian amphipods such as *Dikerogammarus villosus* in the Upper Danube region (Bij De Vaate et al. 2002; Bollache 2004), potentially indicating a connection between host invasions and the establishment or increasing prevalence of *P. bosniacus*.

In several cases, historical and modern records originated from similar geographic locations, suggesting long-term continuity of parasite occurrence within the Danube system. The repeated detection of *P. bosniacus* in the eastern Danube across multiple decades further supports a consistent occurrence of the species in this region, whereas *P. tereticollis* appears to persist mainly in the western Upper Danube and adjacent tributaries. However, given the limited size and geographic coverage of the historical dataset, these patterns should be interpreted cautiously. It cannot be excluded that *P. bosniacus* was already present historically at lower prevalence but remained undetected due to limited sampling effort and the comparatively small number of MC parasites available for molecular analysis. Nevertheless, the inclusion of historical museum material demonstrates its considerable value for reconstructing past parasite distributions and highlights the potential for future studies to expand this approach.

These results provide stronger support for H2 than for H1. Regarding H1, the first prediction, that *P. bosniacus* should be detectable in historical museum material alongside *P. laevis* and *P. tereticollis*, was only partially supported, as *P. bosniacus* was detected exclusively in specimens collected from 1988 onwards and was absent from the oldest examined material. The second prediction, that its geographic distribution should remain relatively stable across historical and recently collected samples, was not supported. In contrast, H2 was supported by the predominance of *P. bosniacus* in recently collected material, consistent with the prediction of increasing occurrence over time, and by the low genetic divergence, high haplotype similarity, and widespread sharing of haplotypes, which are consistent with recent population expansion. The prediction that *P. laevis* and/or *P. tereticollis* may decline in regions

where *P. bosniacus* becomes dominant was only partially supported, as *P. bosniacus* predominated in the modern Danube main channel, while *P. laevis* and *P. tereticollis* were primarily associated with tributary systems. Nevertheless, because the historical dataset remains limited in both sample size and geographic coverage, the possibility that *P. bosniacus* occurred historically at low prevalence but escaped detection cannot be excluded completely.

4.5 Invasive status and host-mediated spread of *P. bosniacus*

4.5.1 Recent expansion of *P. bosniacus*

A key question addressed in this study was whether *P. bosniacus* represents a recently expanding species within Central European river systems (H2) or a historically present but previously overlooked taxon (H1). The widespread occurrence of *P. bosniacus* in RC samples, together with its presence across multiple European river basins, is consistent with previous suggestions of recent range expansion potentially facilitated by invasive Ponto-Caspian hosts such as gobiids and amphipods (Hohenadler et al. 2018; Reier et al. 2019). Although *Pomphorhynchus* specimens were also detected in historical museum collections, these were dominated by *P. laevis* and *P. tereticollis*, whereas *P. bosniacus* occurred predominantly in more recent material (1988 onwards) within the Danube system.

The genetic patterns observed in *P. bosniacus* further support a scenario of recent expansion. The low genetic divergence, high haplotype similarity, and widespread occurrence of shared haplotypes across river systems are consistent with recent population growth and extensive genetic connectivity. In contrast, the available data provide only limited support for the hypothesis that *P. bosniacus* historically represented a widespread but overlooked component of the Austrian parasite fauna. Nevertheless, it cannot be excluded that *P. bosniacus* was already present historically at lower abundance but remained undetected within the comparatively small number of MC parasites available for DNA analyses.

These results provide stronger support for H2 than for H1. The widespread occurrence of *P. bosniacus* in recently collected samples, together with its predominance over *P. laevis* and *P. tereticollis* in the modern Danube system, supports the first and, to some extent, the third prediction of H2. Likewise, the low genetic divergence, high haplotype similarity, and widespread sharing of haplotypes among river systems are consistent with the second prediction of recent population expansion. In contrast, H1 received only limited support: although *P. bosniacus* was detected in museum material, supporting its historical presence, it was absent from the oldest examined specimens and only occurred from 1988 onwards.

Furthermore, the predicted stable historical distribution was not observed. Nevertheless, because the historical dataset remains limited in both sample size and geographic coverage, the possibility that *P. bosniacus* occurred historically at low abundance but remained undetected cannot be excluded.

4.5.2 Host associations and dispersal dynamics of *P. bosniacus*

The spread of parasites in freshwater systems is strongly influenced by host-mediated dispersal and the connectivity of aquatic habitats. Because parasites depend on one or more hosts to complete their life cycles, their geographic distribution is closely linked to the movement and distribution of these hosts, particularly mobile definitive hosts such as fish (Kennedy 2006; Poulin 2007). In European freshwater systems, invasive hosts appear to play an important role in facilitating the spread of *Pomphorhynchus* species. Gobiid fishes, particularly species of the genus *Neogobius* (e.g., *N. melanostomus*, *N. fluviatilis*), as well as *Ponticola kessleri* and *Babka gymnotrachelus*, have expanded their ranges from the Ponto-Caspian region across Europe, primarily via the Danube River and subsequently into river systems such as the Rhine, Weser, Elbe, and Vistula (Bij de Vaate et al. 2002; Grabowska et al. 2023; Cerwenka et al. 2023). These fishes are highly successful invaders due to their ecological flexibility, broad habitat tolerance, and high population densities (Brandner et al. 2013; Dashinov and Uzunova 2020). Importantly, invasive Ponto-Caspian gobies such as the round goby, *N. melanostomus*, and the bighead goby, *P. kessleri*, can function as effective paratenic hosts for *Pomphorhynchus* spp., accumulating cystacanths without further parasite development and thereby facilitating trophic transmission to definitive hosts (Ondračková et al. 2006; Kvach and Skóra 2007; Nachev et al. 2024; Hohenadler et al. 2018). In parallel, Ponto-Caspian amphipods such as *Dikerogammarus villosus* and *D. haemobaphes* have rapidly colonized European river systems and often dominate benthic communities (Bollache 2004; Mamos et al. 2021). As intermediate hosts of acanthocephalans, their expansion may further enhance parasite transmission and dispersal.

Vogel & Taraschewski (2023) demonstrated distinct but partially overlapping associations between *Pomphorhynchus* species and amphipod hosts, suggesting preferential intermediate host usage rather than strict host specificity. In their study, *P. bosniacus* was primarily associated with the invasive amphipod *D. villosus*, whereas *P. laevis* and *P. tereticollis* were more commonly linked to other gammarid species occurring in tributary systems. Similar spatial patterns were observed in the present study, where *P. bosniacus* predominated in the main channel of the Danube, while *P. laevis* and *P. tereticollis* occurred more frequently in tributaries. The predominance of *P. bosniacus* in invasive gobiids within the present dataset may therefore primarily reflect ecological overlap between the parasite, invasive amphipod

intermediate hosts, and gobiid distributions within the main Danube channel rather than strict host specificity. Previous studies demonstrated that invasive gobiids such as *Neogobius melanostomus* can also harbour *P. laevis* and *P. tereticollis* (Emde et al. 2012; Kvach et al. 2017; Kvach and Skóra 2007). Consequently, the predominance of *P. bosniacus* in gobiids may primarily reflect ecological overlap between the parasite, invasive amphipod intermediate hosts, and gobiid distributions within the main Danube channel rather than exclusive host associations.

Invasive species can additionally alter parasite transmission dynamics through processes such as spillover, in which parasites are transmitted from introduced hosts to native species, and spillback, where invasive hosts amplify the transmission of parasites already present within the invaded ecosystem by acting as competent hosts (Torchin et al. 2003; Kelly et al. 2009). However, distinguishing between these processes in natural systems is often difficult, particularly when historical baseline data are limited. The present results are broadly consistent with a spillover-related invasion scenario for *P. bosniacus*, as confirmed records were absent from museum material prior to 1988, while modern populations occur predominantly in river systems colonized by invasive Ponto-Caspian gobiids and amphipods. Furthermore, the low genetic divergence and widespread shared haplotypes observed among modern populations are compatible with recent expansion and high genetic connectivity. Nevertheless, the available data does not allow a definitive distinction between a pure spillover scenario involving a recently introduced parasite and a spillback-like process involving amplification of an already established species. It therefore remains possible that *P. bosniacus* was historically present at low prevalence but increased substantially following the spread of invasive hosts and changes in intermediate host communities.

The occurrence of *P. bosniacus* in several invasive Ponto-Caspian gobiids, including *N. melanostomus* and *P. kessleri*, further supports the importance of these fishes as paratenic hosts facilitating parasite transmission within the Danube system (Bij de Vaate et al. 2002; Grabowska et al. 2023; Cerwenka et al. 2023). Invasive gobiids are known to harbour high parasite loads and facilitate trophic transmission of *Pomphorhynchus* spp. (Ondračková et al. 2005; Hohenadler et al. 2018). Their high abundance, ecological flexibility, and intensive predation on amphipod intermediate hosts likely enhance parasite circulation and transmission efficiency. Together, these findings highlight the importance of invasive host networks and altered intermediate host communities in shaping the contemporary biogeography and transmission dynamics of *Pomphorhynchus* species within interconnected European freshwater ecosystems.

4.6 Evaluation of hypotheses

H1: *P. bosniacus* has historically existed as a cryptic species within the range of *P. laevis*.

This hypothesis is only weakly supported. The first prediction, that *P. bosniacus* should be detectable in historical museum material alongside *P. laevis* and *P. tereticollis*, was partially supported, as *P. bosniacus* was detected in museum specimens but only from 1988 onwards and was absent from the oldest examined material. The second prediction, that its geographic distribution should remain relatively stable across historical and recently collected samples, was not supported. Instead, the observed temporal shift from *P. laevis* and *P. tereticollis* towards *P. bosniacus* predominance is more consistent with a recent expansion. However, because the historical dataset was limited in both sample size and geographic coverage, the possibility that *P. bosniacus* occurred historically at low prevalence but remained undetected cannot be excluded.

H2: *P. bosniacus* represents a recently introduced invasive species, that expanded in the Upper Danube in Austria.

This hypothesis has more support. The first prediction, that *P. bosniacus* should increase in occurrence over time, was supported by its predominance in recently collected material compared with historical museum specimens. The second prediction was also supported, as low genetic divergence, high haplotype similarity, and widespread sharing of haplotypes are consistent with recent population expansion and high connectivity among river systems. The third prediction, that *P. laevis* and/or *P. tereticollis* may decline where *P. bosniacus* becomes dominant, was only partially supported. Although *P. bosniacus* predominated in the modern Danube main channel, the available data do not allow conclusions regarding competitive displacement, and the observed pattern may instead reflect differences in habitat use and intermediate host communities.

H3: *P. bosniacus* has historically been the dominant species within the Danube basin and was previously misidentified as *P. laevis*.

This hypothesis has no support. The prediction that historical Danube museum specimens should predominantly represent *P. bosniacus* was not supported, as the examined historical material was dominated by *P. laevis* and *P. tereticollis*. Likewise, *P. laevis* and *P. tereticollis*

were not rare or absent from historical samples originating from the Danube main channel. Only the prediction that *P. bosniacus* is closely associated with the main Danube corridor was supported. Overall, the available evidence does not support the hypothesis that *P. bosniacus* historically represented the dominant but consistently misidentified *Pomphorhynchus* species within the Danube basin.

4.7 Conclusions and outlook

4.7.1 Limitations of this study

Several limitations of this study should be acknowledged. The number of historical specimens available for analysis was limited, restricting the ability to detect long-term trends with high confidence. Additionally, some specimens failed to yield usable DNA sequences, potentially due to degradation or primer specificity. Moreover, short sequences – despite allowing identification – are not suitable for phylogeographic analyses. Morphological identification was also constrained by specimen condition and the limited visibility of diagnostic features. Despite these limitations, the combined use of morphological and molecular approaches provides a robust framework for species identification and distribution analysis.

4.7.2 Future directions

Future research should expand both the temporal and geographic scope of sampling to further improve our understanding of the historical distribution and spread of *Pomphorhynchus* species across Europe. Although the present results are consistent with a recent expansion of *P. bosniacus*, analysis of additional museum material from other river systems and time periods would allow its invasion history and dispersal pathways to be reconstructed in greater detail. Furthermore, broader population genetic studies using additional molecular markers or genomic approaches may provide deeper insight into phylogeographic structure, gene flow, and the potential presence of additional cryptic lineages within the *Pomphorhynchus* spp.

Although the primary focus of this study was on *Pomphorhynchus* spp., the recovered trematodes and nematodes highlight the potential for broader investigations of freshwater parasite communities. As these specimens were preserved, they provide valuable material for future morphological and molecular analyses, which could improve our understanding of their taxonomy, distribution, and temporal changes in parasite fauna by comparing historical museum collections with recently collected material.

Additional research is also needed to better understand the ecological mechanisms driving the spread of *Pomphorhynchus* species, particularly the role of invasive Ponto-Caspian gobiids and amphipods in parasite transmission. Experimental and DNA analysis studies focusing on intermediate and paratenic host specificity could clarify how host communities influence species distributions and transmission dynamics. Long-term ecological monitoring may further reveal whether the apparent decline of *P. laevis* and *P. tereticollis* in parts of the Danube basin reflects competitive displacement, environmental change, or invasion-driven transmission processes.

Finally, future studies should combine genomic analyses with detailed morphological investigations to further resolve the taxonomic status of the two *P. laevis* lineages identified in this study. In addition, expanding molecular analyses of historical museum specimens from a wider geographic range would improve reconstruction of the temporal and spatial spread of *P. bosniacus* and provide a more comprehensive understanding of long-term changes in European freshwater parasite communities.

5. References

- Amin, Omar M. 2013. "Classification of the Acanthocephala." *Folia Parasitologica* 60 (4): 273–305. <https://doi.org/10.14411/fp.2013.031>.
- Amin, Omar M., Shamall M. A. Abdullah, and Furhan T. Mhaisen. 2003. "Description of Pomphorhynchus Spindletruncatus n. Sp. (Acanthocephala: Pomphorhynchidae) from Freshwater Fishes in Northern Iraq, with the Erection of a New Pomphorhynchid Genus, Pyriproboscis n. g., and Keys to Genera of the Pomphorhynchidae and the Species of Pomphorhynchus Monticelli, 1905." *Systematic Parasitology* 54 (3): 229–35. <https://doi.org/10.1023/A:1022654921523>.
- Avice, John C. 2000. *Phylogeography: The History and Formation of Species*. Harvard University Press. <https://doi.org/10.2307/j.ctv1nzfgj7>.
- Avice, John C. 2009. "Phylogeography: Retrospect and Prospect." *Journal of Biogeography* 36 (1): 3–15. <https://doi.org/10.1111/j.1365-2699.2008.02032.x>.
- Bandelt, H. J., P. Forster, and A. Rohl. 1999. "Median-Joining Networks for Inferring Intraspecific Phylogenies." *Molecular Biology and Evolution* 16 (1): 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>.
- Bianco, P. G. 1990. *Potential Role of the Palaeohistory of the Mediterranean and Paratethys Basins on the Early Dispersal of Euro-Mediterranean Freshwater Fishes*. *Ichthyol. Explor. Freshwat.* 1(2):167-184.
- Bickford, David, David J. Lohman, Navjot S. Sodhi, et al. 2007. "Cryptic Species as a Window on Diversity and Conservation." *Trends in Ecology & Evolution* 22 (3): 148–55. <https://doi.org/10.1016/j.tree.2006.11.004>.
- Bij De Vaate, A., K. Jazdzewski, H. A. M. Ketelaars, S. Gollasch, and G. Van Der Velde. 2002. "Geographical Patterns in Range Extension of Ponto-Caspian Macroinvertebrate Species in Europe." *Canadian Journal of Fisheries and Aquatic Sciences* 59 (7): 1159–74. <https://doi.org/10.1139/f02-098>.
- Blaxter, Mark L., Paul De Ley, James R. Garey, et al. 1998. "A Molecular Evolutionary Framework for the Phylum Nematoda." *Nature* 392 (6671): 71–75. <https://doi.org/10.1038/32160>.
- Blouin, M. S. 2002. *Molecular Prospecting for Cryptic Species of Nematodes: Mitochondrial DNA versus Internal Transcribed Spacer*. *International Journal for Parasitology*, 32(5), 527–531.
- Bollache, L. Devin. 2004. "Rapid Range Extension of the Ponto-Caspian Amphipod Dikerogammarus Villosus in France: Potential Consequences." *Archiv Für Hydrobiologie* 160 (1): 57–66. <https://doi.org/10.1127/0003-9136/2004/0160-0057>.
- Bombarová, Marta, František Marec, Petr Nguyen, and Marta Špakulová. 2007. "Divergent Location of Ribosomal Genes in Chromosomes of Fish Thorny-Headed Worms, Pomphorhynchus Laevis and Pomphorhynchus Tereticollis (Acanthocephala)." *Genetica* 131 (2): 141–49. <https://doi.org/10.1007/s10709-006-9124-3>.
- Brandner, Joerg, Alexander F. Cerwenka, Ulrich K. Schliewen, and Juergen Geist. 2013. "Bigger Is Better: Characteristics of Round Gobies Forming an Invasion Front in the

- Danube River." *PLoS ONE* 8 (9): e73036.
<https://doi.org/10.1371/journal.pone.0073036>.
- Brown, A. F., J. C. Chubb, and C. J. Veltkamp. 1986. "A Key to the Species of Acanthocephala Parasitic in British Freshwater Fishes." *Journal of Fish Biology* 28 (3): 327–34. <https://doi.org/10.1111/j.1095-8649.1986.tb05169.x>.
- Brown, A. F., and D. B. A. Thompson. 1986. "Parasite Manipulation of Host Behaviour: Acanthocephalans and Shrimps in the Laboratory." *Journal of Biological Education* 20 (2): 121–27. <https://doi.org/10.1080/00219266.1986.9654798>.
- Buchmann, Kurt, and A. M. Karami. 2024. "Fish Acanthocephalans as Potential Human Pathogens." *Current Clinical Microbiology Reports* 11 (2): 99–106.
<https://doi.org/10.1007/s40588-024-00226-9>.
- Cerwenka, Alexander F., Joerg Brandner, Dimitriy Dashinov, and Juergen Geist. 2023. "Small but Mighty: The Round Goby (*Neogobius Melanostomus*) as a Model Species of Biological Invasions." *Diversity* 15 (4): 528. <https://doi.org/10.3390/d15040528>.
- Criscione, C. D., R. Poulin, and M. S. Blouin. 2005. *Molecular Ecology of Parasites: Elucidating Ecological and Microevolutionary Processes*. *Molecular Ecology*, 14(8), 2247–2257.
- Criscione, Charles D., and Michael S. Blouin. 2004. "LIFE CYCLES SHAPE PARASITE EVOLUTION: COMPARATIVE POPULATION GENETICS OF SALMON TREMATODES." *Evolution* 58 (1): 198–202. <https://doi.org/10.1111/j.0014-3820.2004.tb01587.x>.
- Dashinov, Dimitriy, and Eliza Uzunova. 2020. "Diet and Feeding Strategies of Round Goby, *Neogobius Melanostomus* (Pallas, 1814) from the Invasion Front in the Danube River Tributaries (Bulgaria): Ontogenetic Shift and Seasonal Variation." *Limnologica* 83 (July): 125796. <https://doi.org/10.1016/j.limno.2020.125796>.
- David, Gwendoline M., Cybill Staentzel, Olivier Schlumberger, Marie-Jeanne Perrot-Minnot, Jean-Nicolas Beisel, and Laurent Hardion. 2018. "A Minimalist Macroparasite Diversity in the Round Goby of the Upper Rhine Reduced to an Exotic Acanthocephalan Lineage." *Parasitology* 145 (8): 1020–26.
<https://doi.org/10.1017/S0031182017002177>.
- Emde, Sebastian, Sonja Rueckert, Harry W. Palm, and Sven Klimpel. 2012. "Invasive Ponto-Caspian Amphipods and Fish Increase the Distribution Range of the Acanthocephalan *Pomphorhynchus tereticollis* in the River Rhine." *PLoS ONE* 7 (12): e53218. <https://doi.org/10.1371/journal.pone.0053218>.
- García-Varela, Martín, and Steven A. Nadler. 2006. "Phylogenetic Relationships among Syndermata Inferred from Nuclear and Mitochondrial Gene Sequences." *Molecular Phylogenetics and Evolution* 40 (1): 61–72.
<https://doi.org/10.1016/j.ympev.2006.02.010>.
- Grabowska, Joanna, Dagmara Błońska, Markéta Ondračková, and Tomasz Kakareko. 2023. "The Functional Ecology of Four Invasive Ponto–Caspian Gobies." *Reviews in Fish Biology and Fisheries* 33 (4): 1329–52. <https://doi.org/10.1007/s11160-023-09801-7>.

- Hagemann, Laura, Katharina M. Mauer, Thomas Hankeln, Hanno Schmidt, and Holger Herlyn. 2024. "Nuclear Genome Annotation of Wheel Animals and Thorny-Headed Worms: Inferences about the Last Common Ancestor of Syndermata (Rotifera s.l.)." *Hydrobiologia* 851 (12–13): 2827–44. <https://doi.org/10.1007/s10750-023-05268-6>.
- Hajibabaei, Mehrdad, Gregory Ac Singer, Elizabeth L. Clare, and Paul Dn Hebert. 2007. "Design and Applicability of DNA Arrays and DNA Barcodes in Biodiversity Monitoring." *BMC Biology* 5 (1): 24. <https://doi.org/10.1186/1741-7007-5-24>.
- Hall, T. A. 1999. *BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT*. *Nucleic Acids Symposium Series*, 41, 95–98. V. Hall, T.A. (1999). Released.
- Hebert, Paul D. N., Alina Cywinska, Shelley L. Ball, and Jeremy R. deWaard. 2003. "Biological Identifications through DNA Barcodes." *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270 (1512): 313–21. <https://doi.org/10.1098/rspb.2002.2218>.
- Hewitt, Godfrey M. 1999. "Post-Glacial Re-Colonization of European Biota." *Biological Journal of the Linnean Society* 68 (1–2): 87–112. <https://doi.org/10.1111/j.1095-8312.1999.tb01160.x>.
- Hohenadler, M. A. A., M. Nachev, F. Thielen, H. Taraschewski, D. Grabner, and B. Sures. 2018. "*Pomphorhynchus laevis*: An Invasive Species in the River Rhine?" *Biological Invasions* 20 (1): 207–17. <https://doi.org/10.1007/s10530-017-1527-9>.
- Kelly, D. W., R. A. Paterson, C. R. Townsend, R. Poulin, and D. M. Tompkins. 2009. "Parasite Spillback: A Neglected Concept in Invasion Ecology?" *Ecology* 90 (8): 2047–56. <https://doi.org/10.1890/08-1085.1>.
- Kennedy, C. R. 2006. *Ecology of the Acanthocephala*. 1st ed. Cambridge University Press. <https://doi.org/10.1017/CBO9780511541902>.
- Kennedy, C. R., P. F. Broughton, and P. M. Hine. 1978. "The Status of Brown and Rainbow Trout, *Salmo Trutta* and *S. Gairdneri* as Hosts of the Acanthocephalan, *Pomphorhynchus laevis*." *Journal of Fish Biology* 13 (2): 265–75. <https://doi.org/10.1111/j.1095-8649.1978.tb03434.x>.
- Kiškarolj and Čanković. 1969. *Kiškarolj, M., & Čanković, M. (1969). Pomphorhynchus Bosniacus Nov. Sp. Aus Barben (Barbus Barbus) Des Savegebietes.*
- Kritscher, E. 1985. *Teil 4, Aschelminthes, Schlauchwürmer; Nemertini, Schnurwürmer: D. Phylum: Acanthocephala. In: Österreichische Akademie Der Wissenschaften: Catalogus Faunae Austriae: Ein Systematisches Verzeichnis Aller Auf Österreichischem Gebiet Festgestellten Tierarten. Wien, Verlag Der Österreichischen Akademie Der Wissenschaften, 15 Pp.*
- Kumar, Sudhir, Glen Stecher, Michael Suleski, Maxwell Sanderford, Sudip Sharma, and Koichiro Tamura. 2024. "MEGA12: Molecular Evolutionary Genetic Analysis Version 12 for Adaptive and Green Computing." *Molecular Biology and Evolution* 41 (12): msae263. <https://doi.org/10.1093/molbev/msae263>.

- Kvach, Yuriy, Markéta Ondračková, Michal Janáč, and Pavel Jurajda. 2017. "The Parasite Community of Round Goby *Neogobius Melanostomus* (Pallas, 1814) (Actinopterygii: Gobiidae) Newly Introduced into the Upper Elbe." *Knowledge & Management of Aquatic Ecosystems*, no. 418: 19. <https://doi.org/10.1051/kmae/2017010>.
- Kvach, Yuriy, and Krzysztof E. Skóra. 2007. "Metazoa Parasites of the Invasive Round Goby *Apollonia Melanostoma* (*Neogobius Melanostomus*) (Pallas) (Gobiidae: Osteichthyes) in the Gulf of Gdańsk, Baltic Sea, Poland: A Comparison with the Black Sea." *Parasitology Research* 100 (4): 767–74. <https://doi.org/10.1007/s00436-006-0311-z>.
- Lafferty, K. D. 1999. "The Evolution of Trophic Transmission." *Parasitology Today* 15 (3): 111–15. [https://doi.org/10.1016/S0169-4758\(99\)01397-6](https://doi.org/10.1016/S0169-4758(99)01397-6).
- Leuven, Rob S. E. W., Gerard Van Der Velde, Iris Baijens, et al. 2009. "The River Rhine: A Global Highway for Dispersal of Aquatic Invasive Species." *Biological Invasions* 11 (9): 1989. <https://doi.org/10.1007/s10530-009-9491-7>.
- Lewisch, E., V. Solymos, K. Waldner, et al. 2020. "Acanthocephalan Parasites Collected from Austrian Fishes: Molecular Barcoding and Pathological Observations." *Diseases of Aquatic Organisms* 139 (April): 103–11. <https://doi.org/10.3354/dao03471>.
- Li, Liang, Hui-Xia Chen, Omar M. Amin, and Yue Yang. 2017. "Morphological Variability and Molecular Characterization of *Pomphorhynchus Zhoushanensis* Sp. Nov. (Acanthocephala: Pomphorhynchidae), with Comments on the Systematic Status of *Pomphorhynchus Monticelli*, 1905." *Parasitology International* 66 (5): 693–98. <https://doi.org/10.1016/j.parint.2017.05.010>.
- Librado, P., and J. Rozas. 2009. "DnaSP v5: A Software for Comprehensive Analysis of DNA Polymorphism Data." *Bioinformatics* 25 (11): 1451–52. <https://doi.org/10.1093/bioinformatics/btp187>.
- Lucius, Richard, and Brigitte Loos-Frank. 2008. *Biologie von Parasiten*. 2. Aufl. 2008. Springer-Lehrbuch. Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-540-37709-2>.
- Lühe, M. 1911. *Acanthocephalen*. In *Die Süßwasserfauna Deutschlands (Vol. 16)*. Gustav Fischer, Jena.
- Luo, Cihang, Luke A. Parry, Brendon E. Boudinot, et al. 2025. "A Jurassic Acanthocephalan Illuminates the Origin of Thorny-Headed Worms." *Nature* 641 (8063): 674–80. <https://doi.org/10.1038/s41586-025-08830-5>.
- Mamos, Tomasz, Michał Grabowski, Tomasz Rewicz, Jamie Bojko, Dominik Strapagiel, and Artur Burzyński. 2021. "Mitochondrial Genomes, Phylogenetic Associations, and SNP Recovery for the Key Invasive Ponto-Caspian Amphipods in Europe." *International Journal of Molecular Sciences* 22 (19): 10300. <https://doi.org/10.3390/ijms221910300>.
- Moravec, F. 1994. *Parasitic Nematodes of Freshwater Fishes of Europe*. Academia, Prague.
- Moret, Yannick, Loïc Bollache, Rémi Wattier, and Thierry Rigaud. 2007. "Is the Host or the Parasite the Most Locally Adapted in an Amphipod–Acanthocephalan Relationship? A Case Study in a Biological Invasion Context." *International Journal for Parasitology* 37 (6): 637–44. <https://doi.org/10.1016/j.ijpara.2006.12.006>.

- Nachev, Milen, Michael Hohenadler, Nicklas Bröckers, Daniel Grabner, and Bernd Sures. 2024. "Suitability of Invasive Gobies as Paratenic Hosts for Acanthocephalans of the Genus *Pomphorhynchus* Sp." *Parasitology* 151 (14): 1522–29. <https://doi.org/10.1017/S0031182024001197>.
- Niewiadomska, K. 2003. *Parasites of Polish Freshwater Fishes (Monogenea, Digenea, Cestoda, Acanthocephala)*. Polish Parasitological Society, Warsaw.
- Olson, P. D., T. H. Cribb, V. V. Tkach, R. A. Bray, and D. T. J. Littlewood. 2003. "Phylogeny and Classification of the Digenea (Platyhelminthes: Trematoda) 11 Nucleotide Sequence Data Reported in This Paper Are Available in the GenBank™, EMBL and DDBJ Databases under the Accession Numbers AY222082–AY222285." *International Journal for Parasitology* 33 (7): 733–55. [https://doi.org/10.1016/S0020-7519\(03\)00049-3](https://doi.org/10.1016/S0020-7519(03)00049-3).
- O'Mahony, E. M., D. G. Bradley, C. R. Kennedy, and C. V. Holland. 2004. "Evidence for the Hypothesis of Strain Formation in *Pomphorhynchus laevis* (Acanthocephala): An Investigation Using Mitochondrial DNA Sequences." *Parasitology* 129 (3): 341–47. <https://doi.org/10.1017/S0031182004005748>.
- Ondrackova, M., M. Davidova, M. Pecinkova, et al. 2005. "Metazoan Parasites of Neogobius Fishes in the Slovak Section of the River Danube." *Journal of Applied Ichthyology* 21 (4): 345–49. <https://doi.org/10.1111/j.1439-0426.2005.00682.x>.
- Ondračková, Markéta, Martina Dávidová, Milan Gelnar, and Pavel Jurajda. 2006. "Susceptibility of Prussian Carp Infected by Metacercariae of *Posthodiplostomum Cuticola* (v. Nordmann, 1832) to Fish Predation." *Ecological Research* 21 (4): 526–29. <https://doi.org/10.1007/s11284-005-0146-6>.
- Perrot-Minnot, Marie-Jeanne. 2004. "Larval Morphology, Genetic Divergence, and Contrasting Levels of Host Manipulation between Forms of *Pomphorhynchus laevis* (Acanthocephala)." *International Journal for Parasitology* 34 (1): 45–54. <https://doi.org/10.1016/j.ijpara.2003.10.005>.
- Perrot-Minnot, Marie-Jeanne, Marta Špakulová, Rémi Wattier, et al. 2018. "Contrasting Phylogeography of Two Western Palaearctic Fish Parasites despite Similar Life Cycles." *Journal of Biogeography* 45 (1): 101–15. <https://doi.org/10.1111/jbi.13118>.
- Poulin, Robert. 2007. *Evolutionary Ecology of Parasites: Second Edition*. Princeton University Press. <https://doi.org/10.1515/9781400840809>.
- Prenter, John, Calum MacNeil, Jaimie T. A. Dick, and Alison M. Dunn. 2004. "Roles of Parasites in Animal Invasions." *Trends in Ecology & Evolution* 19 (7): 385–90. <https://doi.org/10.1016/j.tree.2004.05.002>.
- QGIS Development Team. 2025. *QGIS Geographic Information System (Version 3.40 or Whichever Version You Used)*. Open Source Geospatial Foundation Project. Released.
- Ratnasingham, Sujeevan, and Paul D. N. Hebert. 2007. "BOLD : The Barcode of Life Data System ([Http://Www.Barcodinglife.Org](http://www.barcodinglife.org))." *Molecular Ecology Notes* 7 (3): 355–64. <https://doi.org/10.1111/j.1471-8286.2007.01678.x>.

- Reier, S., H. Sattmann, and E. Haring. 2020. *Optimizing Conflicting Tasks in the Analysis of Parasitic Worms: Morphological Imaging, DNA Yield, Specimen and DNA Preservation*. *Annalen des Naturhistorischen Museums Wien*. 122B: 175–182.
- Reier, Susanne, Helmut Sattmann, Thomas Schwaha, Hans-Peter Fuehrer, and Elisabeth Haring. 2020. “Unravelling the Hidden Biodiversity – the Establishment of DNA Barcodes of Fish-Parasitizing Acanthocephala Koehleuther, 1771 in View of Taxonomic Misidentifications, Intraspecific Variability and Possible Cryptic Species.” *Parasitology* 147 (13): 1499–508. <https://doi.org/10.1017/S0031182020001316>.
- Reier, Susanne, Helmut Sattmann, Thomas Schwaha, Josef Harl, Robert Konecny, and Elisabeth Haring. 2019. “An Integrative Taxonomic Approach to Reveal the Status of the Genus *Pomphorhynchus* Monticelli, 1905 (Acanthocephala: Pomphorhynchidae) in Austria.” *International Journal for Parasitology: Parasites and Wildlife* 8 (April): 145–55. <https://doi.org/10.1016/j.ijppaw.2019.01.009>.
- Saitou, N., and M. Nei. 1987. “The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees.” *Molecular Biology and Evolution* 4 (4): 406–25. <https://doi.org/10.1093/oxfordjournals.molbev.a040454>.
- Shiganova, Tamara A., Henri J. Dumont, and Alexander S. Kazmin. 2026. “Pathways and Vectors of Non-Native Species Introduction in the Ponto-Caspian Seas.” In *Non-Native Species in the Ponto-Caspian Seas*, by Tamara A. Shiganova, Henri J. Dumont, and Alexander S. Kazmin, vol. 96. *Monographiae Biologicae*. Springer Nature Switzerland. https://doi.org/10.1007/978-3-032-18640-9_3.
- Sielaff, Malte, Hanno Schmidt, Torsten H. Struck, et al. 2016. “Phylogeny of Syndermata (Syn. Rotifera): Mitochondrial Gene Order Verifies Epizoic Seisonidea as Sister to Endoparasitic Acanthocephala within Monophyletic Hemirotifera.” *Molecular Phylogenetics and Evolution* 96 (March): 79–92. <https://doi.org/10.1016/j.ympev.2015.11.017>.
- Soto, Ismael, Ross N. Cuthbert, Anthony Ricciardi, et al. 2023. “The Faunal Ponto-Caspianization of Central and Western European Waterways.” *Biological Invasions* 25 (8): 2613–29. <https://doi.org/10.1007/s10530-023-03060-0>.
- Špakulová, M., M. Perrot-Minnot, and B. Neuhaus. 2011. “Resurrection of *Pomphorhynchus tereticollis* (Rudolphi, 1809) (Acanthocephala: Pomphorhynchidae) Based on New Morphological and Molecular Data.” *Helminthologia* 48 (4): 268–77. <https://doi.org/10.2478/s11687-011-0038-y>.
- Torchin, Mark E., Kevin D. Lafferty, Andrew P. Dobson, Valerie J. McKenzie, and Armand M. Kuris. 2003. “Introduced Species and Their Missing Parasites.” *Nature* 421 (6923): 628–30. <https://doi.org/10.1038/nature01346>.
- Vardić Smrzlić, Irena, Barbara Čolić, Damir Kapetanović, Sara Šariri, Tatjana Mijošek, and Vlatka Filipović Marijić. 2024. “Phylogeny and Genetic Variability of Rotifer’s Closest Relatives Acanthocephala: An Example from Croatia.” *Hydrobiologia* 851 (12–13): 2845–60. <https://doi.org/10.1007/s10750-023-05372-7>.
- Vardić Smrzlić, Irena, Damir Valić, Damir Kapetanović, Vlatka Filipović Marijić, Emil Gjurgević, and Emin Teskeredžić. 2015. “*Pomphorhynchus laevis* (Acanthocephala) from the Sava River Basin: New Insights into Strain Formation, mtDNA-like Sequences and Dynamics of Infection.” *Parasitology International* 64 (5): 243–50. <https://doi.org/10.1016/j.parint.2015.02.004>.

- Vogel, Sebastian, and Horst Taraschewski. 2023. "Intermediate Host Patterns of Acanthocephalans in the Weser River System: Co-Invasion vs Host Capture." *Parasitology* 150 (5): 426–33. <https://doi.org/10.1017/S0031182023000124>.
- Wattier, Rémi A., Eleanor R. Haine, Jérémie Beguet, et al. 2007. "No Genetic Bottleneck or Associated Microparasite Loss in Invasive Populations of a Freshwater Amphipod." *Oikos* 116 (11): 1941–53. <https://doi.org/10.1111/j.0030-1299.2007.15921.x>.
- Zaharieva, Petya, and Diana Kirin. 2020. "CIRCULATION OF CADMIUM (CD) IN THE SYSTEM ALBURNUS ALBURNUS (LINNAEUS, 1758), WATER AND SEDIMENTS FROM THE DANUBE RIVER, NORTHWESTERN BULGARIA." *Scientific Papers. Series D. Animal Science, Vol. LXIII, Issue 2, ISSN 2285-5750, 405-412*.

6. Acknowledgments

I would like to express my sincere gratitude to everyone who contributed to the completion of this master's thesis.

First, I would like to thank to Elisabeth Haring, for providing me with the opportunity to work on this project and for sharing his expertise in molecular biology. Your guidance and support throughout the molecular aspects of this study were greatly appreciated.

I am especially grateful to Susanne Reier, whose extensive knowledge of ichthyology and freshwater ecology, together with his continuous guidance, constructive criticism and discussions, were invaluable throughout this project. Your enthusiasm for science and willingness to share his expertise greatly shaped both this thesis and my understanding of the subject.

My sincere thanks also go to the members of the Central Research Laboratories and the 3rd Zoological Department of the Natural History Museum Vienna for their assistance with the molecular laboratory work and for their technical support throughout the experimental phase of this study. In particular, I would like to thank Helmut Sattmann for generously sharing his expertise in parasitology, for the helpful discussions, and for your valuable advice throughout the course of this work. I am also grateful to the in-house experts of the Natural History Museum Vienna who identified the fish specimens used in this study, as well as to everyone who assisted with sample collection, access to the museum collections, and the curation of historical material.

Additionally, I would like to thank everyone at The Nice Guys - Wien. Balancing work and university over the past years would not have been the same without your flexibility, encouragement, and the many enjoyable shifts we shared together. Thank you for making this journey a little easier.

Finally, I would like to express my deepest gratitude to my family and friends for their unwavering encouragement, patience, and support throughout my studies. Their confidence in me has been a constant source of motivation and made the completion of this thesis possible.

7. Appendix

Appendix 1

Appendix 1. List of all dissected host fish, including fish family, fish species, collection site and date, the fish lab ID, Parasites discovered and the number of parasites obtained. Lab ID abbreviations are as follows: BDAF = Bad Deutsch Altenburg Fish, NHMF: Naturhistorisches Museum Fisch (eng.: natural history museum fish), PURK = Purkersdorf, DOB = Dobra.

Host Family	Host Species	Collection site (Country, Administrative division, City/Village, River)	Collection date	Lab ID	Parasite	Number of Parasites
Cottidae	<i>Cottus gobio</i>					
			15.10.2024	BDAF22	No	-
				BDAF23	No	-
				BDAF29	No	-
Cyprinidae	<i>Barbus barbus</i>					
		Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
			15.10.2024	BDAF1	<i>Pomphorhynchus</i> Adult	31
				BDAF4	<i>Pomphorhynchus</i> Adult	20
				BDAF5	<i>Pomphorhynchus</i> Adult	40
					<i>Pomphorhynchus</i>	
					Cystacanth	16
				BDAF6	<i>Pomphorhynchus</i> Adult	34
				BDAF10	<i>Pomphorhynchus</i> Adult	18
					<i>Pomphorhynchus</i>	
					Cystacanth	2
					Nematoda	10
				BDAF32	<i>Pomphorhynchus</i> Adult	1
					Nematoda	1
		Austria, Lower Austria, near Greifenstein, Danube				
			5.5.1988	NHMF10	<i>Pomphorhynchus</i> Adult	12
					<i>Pomphorhynchus</i>	
					Cystacanth	3
					Nematoda	30
				NHMF11	<i>Pomphorhynchus</i> Adult	10
					Nematoda	10
		Austria, Lower Austria, near Wallsee, Danube				
			1983	NHMF12	<i>Pomphorhynchus</i> Adult	1
					Nematoda	30
				NHMF13	<i>Pomphorhynchus</i> Adult	12
					<i>Pomphorhynchus</i>	
					Cystacanth	12
					Nematoda	30
		Austria, Lower Austria, between Haslau and Wildungsmauer, Danube				
			6.9.1988	NHMF14	<i>Pomphorhynchus</i> Adult	7
					Nematoda	10
		Austria, Vienna, Danube				

			<i>Pomphorhynchus</i>	
1826	NHMF15		Cystacanth	25
	NHMF16		Nematoda	4
	NHMF17		<i>Pomphorhynchus</i> Adult	1
			Nematoda	10
1876	NHMF18		Nematoda	30
			<i>Pomphorhynchus</i>	
1874	NHMF19		Cystacanth	1
			<i>Pomphorhynchus</i>	
1856	NHMF20		Cystacanth	5
<i>Barbus petenyi</i>				
Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
15.10.2024	BDAF8		<i>Pomphorhynchus</i> Adult	8
	BDAF12		<i>Pomphorhynchus</i> Adult	20
			<i>Pomphorhynchus</i>	
			Cystacanth	3
<i>Barbus</i> sp.				
Greece, Thessaly, Larissa, Pineios				
1894	NHMF38	No		-
	NHMF39	No		-
	NHMF40	No		-
Gobiidae				
<i>Babka gymnotrachelus</i>				
Austria, Lower Austria, near Wildungsmauer, Danube				
			<i>Pomphorhynchus</i>	
7.3.2025	BDAF37		Cystacanth	7
			Trematoda	5
	BDAF43	No		-
	BDAF44		Trematoda	1
Austria, Lower Austria, near Regelsbrunn, Danube				
27.3.2025	BDAF39	No		-
			<i>Pomphorhynchus</i>	
	BDAF40		Cystacanth	1
	BDAF41	No		
<i>Neogobius fluviatilis</i>				
Ukraine, Ivano-Frankivsk, Ivano-Frankivsk, Bistrica River				
			<i>Pomphorhynchus</i>	
1879	NHMF26		Cystacanth	1
	NHMF27	No		-
<i>Neogobius melanostomus</i>				
Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
15.10.2024	BDAF21		<i>Pomphorhynchus</i> Adult	10
			<i>Pomphorhynchus</i>	
	BDAF24		Cystacanth	4
	BDAF25		<i>Pomphorhynchus</i> Adult	2
	BDAF30		<i>Pomphorhynchus</i> Adult	40
	BDAF35		<i>Pomphorhynchus</i> Adult	10
Austria, Lower Austria, near Wildungsmauer, Danube				
27.3.2025	BDAF42	No		
			<i>Pomphorhynchus</i>	
7.3.2025	BDAF38		Cystacanth	2
Ukraine, Odessa, Odessa, Black Sea				
1862	NHMF28	No		-
Turkey, Istanbul, Istanbul, Black Sea				
			<i>Pomphorhynchus</i>	
1894	NHMF29		Cystacanth	2
	NHMF30	No		-

		NHMF31	No	-
	Bulgaria, Plovdiv, Plovdiv, Maritsa			
	1894	NHMF32	No	-
		NHMF33	No	-
		NHMF34	No	-
<i>Ponticola kessleri</i>				
	Austria, Lower Austria, near Regelsbrunn, Danube			
	7.3.2025	BDAF36	<i>Pomphorhynchus</i> Cystacanth	2
	1996	NHMF24	<i>Pomphorhynchus</i> Cystacanth	23
			Nematoda	1
	2004	NHMF25	<i>Pomphorhynchus</i> Cystacanth	24
<i>Proterorhinus marmoratus</i>				
	Bulgaria, Plovdiv, Plovdiv, Maritsa			
	1879	NHMF35	No	-
		NHMF36	No	-
		NHMF37	No	-
<i>Proterorhinus</i> sp.				
	Austria, Lower Austria			
	1969	NHMF21	Trematoda	2
		NHMF22	Trematoda	5
		NHMF23	Trematoda	2
Gobionidae				
<i>Romanogobio</i> sp.				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF17	No	-
Leuciscidae				
<i>Alburnus alburnus</i>				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF27	<i>Pomphorhynchus</i> Adult	1
			Nematoda	3
		BDAF34	<i>Pomphorhynchus</i> Adult	1
<i>Alburnus</i> sp.				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF14	<i>Pomphorhynchus</i> Adult	2
<i>Blicca bjoerkna</i>				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF7	No	-
<i>Chondrostoma nasus</i>				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF2	Trematoda	1
		BDAF3	No	
<i>Chondrostoma</i> sp.				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF20	Nematoda	1
		BDAF26	No	-
		BDAF28	No	-
		BDAF31	No	-
<i>Rutilus rutilus</i>				
	Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
	15.10.2024	BDAF15	No	-
<i>Squalius cephalus</i>				
	Austria, Vienna, Purkersdorf, Wienfluss			
	05.06.2025	PURK1	<i>Pomphorhynchus</i> Adult	2

Austria, Vienna, Danube				
1865	NHMF1	<i>Pomphorhynchus</i> Adult	3	
		<i>Pomphorhynchus</i>		
		Cystacanth	2	
	NHMF2	<i>Pomphorhynchus</i> Adult	1	
		Nematoda	6	
Austria, Vienna, Liesing				
1877	NHMF3	No		
		<i>Pomphorhynchus</i>		
	NHMF4	Cystacanth	10	
		Nematoda	1	
	NHMF5	<i>Pomphorhynchus</i> Adult	2	
		Nematoda	2	
Austria, Lower Austria				
24.4.1890	NHMF6	Nematoda	3	
	NHMF7	Nematoda	4	
Austria, Styria, Graz, Mur				
1900	NHMF8	Acanthocephala	2	
		Nematoda	4	
		<i>Pomphorhynchus</i>		
	NHMF9	Cystacanth	3	
		Nematoda	4	
<i>Squalius cephalus</i>				
Croatia, Dobra				
2014	DOB1	<i>Pomphorhynchus</i> Adult	6	
<i>Vimba vimba</i>				
Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
15.10.2024	BDAF9	Nematoda	1	
	BDAF16	No	-	
Percidae				
<i>Gymnocephalus schraetser</i>				
Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
15.10.2024	BDAF18	<i>Pomphorhynchus</i> Adult	4	
	BDAF33	<i>Pomphorhynchus</i> Adult	20	
		Trematoda	40	
<i>Zingel zingel</i>				
Austria, Lower Austria, Bad Deutsch Altenburg, Danube				
15.10.2024	BDAF11	No	-	
	BDAF19	<i>Pomphorhynchus</i> Adult	4	

Appendix 2

Appendix 2. List of all parasite specimens which were used for further investigations, Parasite species (and assigned lineage for *P. laevis*), collection site and date, the host species, fish lab ID, life stage of the parasite as well as the reference for parasites obtained from other studies. Abbreviations are as follows: BDAF= Bad Deutsch Altenburg Fish, NHMF = Naturhistorisches Museum Fisch (eng.: natural history museum fish), PURK = Purkersdorf, DOB = Dobra, BOKU = Universität für Bodenkultur Wien (eng.: University of Natural Resources and Life Sciences, Vienna). Bracketed numbers after the Cystacanth represents the amount of individuals attached to each other.

Parasite species (and lineage)

Collection site (Country, Administrative division, City/Village, River)

Collection date

Collector

Latitude and Longitude

Host species

	Lab ID	GenBank	Stage
<i>P. bosniacus</i>			
Austria, Lower Austria, Bad Deutsch Altenburg, Danube			
15.10.2024			
BOKU			
48°08'29.4"N 16°54'09.2"E			
<i>A. alburnus</i>			
BDAF34.1	-		Pre-Adult
<i>Alburnus sp.</i>			
BDAF14.1	-		Adult
BDAF14.2	-		Adult
<i>B. barbus</i>			
BDAF1.1	-		Adult
BDAF1.2	-		Adult
BDAF4.1	-		Adult
BDAF4.2	-		Adult
BDAF4.3	-		Adult
BDAF4.4	-		Adult
BDAF4.5	-		Adult
BDAF4.6	-		Adult
BDAF5.3	-		Cystacanth
BDAF5.4	-		Cystacanth
BDAF6.1	-		Adult
BDAF6.3	-		Adult
BDAF6.4	-		Adult
BDAF10.1	-		Adult
BDAF10.2	-		Adult
BDAF10.3	-		Adult
BDAF10.4	-		Adult
BDAF10.8	-		Cystacanth
BDAF10.9	-		Cystacanth (2)

		BDAF32.1	-	Adult
		<i>B. petenyi</i>		
		BDAF8.1	-	Adult
		BDAF8.2	-	Adult
		BDAF8.3	-	Adult
		BDAF12.1	-	Adult
		BDAF12.2	-	Adult
		BDAF12.3	-	Adult
		<i>G. schraetser</i>		
		BDAF18.1	-	Adult
		BDAF33.1	-	Adult
		BDAF33.2	-	Adult
		BDAF33.3	-	Cystacanth
		BDAF33.4	-	Cystacanth
		BDAF33.5	-	Cystacanth
		<i>N. melanostomus</i>		
		BDAF21.1	-	Pre-Adult
		BDAF21.2	-	Pre-Adult
		BDAF21.3	-	Cystacanth
		BDAF21.4	-	Pre-Adult
		BDAF24.1	-	Cystacanth
		BDAF24.2	-	Cystacanth (3)
		BDAF25.1	-	Pre-Adult
		BDAF30.1	-	Pre-Adult
		BDAF30.2	-	Pre-Adult
		BDAF30.3	-	Cystacanth (4)
		BDAF30.4	-	Cystacanth (3)
		BDAF30.5	-	Cystacanth
		BDAF30.6	-	Pre-Adult
		BDAF30.8	-	Cystacanth (2)
		BDAF35.1	-	Pre-Adult
		BDAF35.2	-	Pre-Adult
		BDAF35.3	-	Cystacanth
		BDAF35.4	-	Cystacanth
		BDAF35.5	-	Cystacanth
		<i>Z. zingel</i>		
		BDAF19.1	-	Adult
		BDAF19.2	-	Adult
		BDAF19.3	-	
Austria, Lower Austria, between Haslau and Wildungsmauer, Danube				
1988				
B. Herzig				
48°06'54.7"N 16°45'57.6"E				
		<i>B. barbatus</i>		
		NHMF14.1	-	Adult
		NHMF14.2	-	Adult
		NHMF14.4	-	Adult
Austria, Lower Austria, near Regelsbrunn, Danube				
27.3.2025				
T. Leister				
48°06'50.1"N 16°46'46.4"E				

		<i>B. gymnotrachelus</i>			
		BDAF40.1	-		Cystacanth
Austria, Lower Austria, near Wildungsmauer, Danube					
7.3.2025					
T. Leister					
48°06'52.1"N		16°47'43.3"E			
		<i>B. gymnotrachelus</i>			
		BDAF37.1	-		Cystacanth
		BDAF37.2	-		Cystacanth
		BDAF37.3	-		Cystacanth
		<i>N. melanostomus</i>			
		BDAF38.1	-		Cystacanth
		BDAF38.2	-		Cystacanth
Austria, Lower Austria, near Zwentendorf (Traisenmündung), Danube					
1988					
B. Herzig					
48°21'50.6"N		15°51'43.5"E			
		<i>B. barbatus</i>			
		NHMF10.1	-		Adult
		NHMF10.3	-		Adult
		NHMF10.4	-		Cystacanth
		NHMF10.5	-		Cystacanth
		NHMF11.1	-		Adult
		NHMF11.2	-		Adult
		NHMF11.3	-		Adult
Austria, Orth a.d. Donau, Danube					
2006					
M.-J. Perrot-Minnot					
48°07'37.7"N		16°43'23.2"E			
		Unkown			
			-	LN994918	-
Austria, Styria, near Andritzbach, Stattegg (fishpond)					
2017					
S. Koblmüller					
47°08'37.4"N		15°25'08.2"E			
		Unkown			
			-	MT682952	-
			-	MT682953	-
Austria, Vienna, Danube					
1996					
H. Ahnelt					
48°11'08.5"N		16°28'16.5"E			
		<i>P. kessleri</i>			
		NHMF24.1	-		Cystacanth
		NHMF24.3	-		Cystacanth
1998					
M.-J. Perrot-Minnot					
48°11'08.5"N		16°28'16.5"E			
		Unkown			
			-	LN994903	-
			-	LN994907	-
			-	LN994908	-
			-	LN994909	-
2004					

H. Ahnelt					
48°11'08.5"N			16°28'16.5"E		
			<i>P. kessleri</i>		
			NHMF25.1	-	Cystacanth
			NHMF25.2	-	Cystacanth
			NHMF25.3	-	Cystacanth
			NHMF25.4	-	Cystacanth
Austria, Vienna, Freudenau, Danube					
2015					
H. Sattmann & R. Konecny					
48°11'05.7"N			16°26'12.6"E		
			Unkown		
			-	MK612497	-
			-	MK612498	-
			-	MK612499	-
			-	MK612500	-
			-	MK612501	-
			-	MK612502	-
			-	MK612503	-
			-	MK612504	-
			-	MK612505	-
			-	MK612506	-
			-	MK612507	-
			-	MK612508	-
			-	MK612509	-
			-	MK612510	-
			-	MK612511	-
			-	MK612512	-
			-	MK612513	-
			-	MK612514	-
			-	MK612515	-
			-	MK612516	-
			-	MK612517	-
			-	MK612518	-
			-	MK612519	-
			-	MK612520	-
			-	MK612521	-
			-	MK612522	-
			-	MK612523	-
Bosnia and Herzegovina, Sava River					
2017					
Z. Nedić					
45°00'00.32"N			18°43'45.44"E		
			Unkown		
			-	MK133340	-
			-	MH282838	-
			-	MH282839	-
Bulgaria, Kozloduy, Danube					
2006					
M.-J. Perrot-Minnot					
43°47'49.7"N			23°41'35.2"E		
			Unkown		
			-	LN994900	-
			-	LN994901	-

Bulgaria, Novo Selo, Danube					
1998					
M.-J. Perrot-Minnot					
44°09'43.7"N	22°47'25.8"E				
Unkown					
-		LN994899	-		
-		LN994905	-		
Bulgaria, Silistra, Danube					
1998					
M.-J. Perrot-Minnot					
44°07'16.3"N	27°16'47.8"E				
Unkown					
-		LN994904	-		
Bulgaria, Szob, Danube					
1998					
M.-J. Perrot-Minnot					
47°48'49.7"N	18°52'18.7"E				
Unkown					
-		LN994912	-		
-		LN994913	-		
Bulgaria, Tutrakan, Danube					
1999					
M.-J. Perrot-Minnot					
44°03'03.2"N	26°36'24.5"E				
Unkown					
-		LN994894	-		
-		LN994895	-		
Bulgaria, Vidin, Danube					
2006					
M.-J. Perrot-Minnot					
43°59'11.1"N	22°52'53.5"E				
Unkown					
-		LN994896	-		
-		LN994897	-		
-		LN994898	-		
Croatia, Batina, Danube					
2005					
M.-J. Perrot-Minnot					
45°49'59.5"N	18°51'06.9"E				
Unkown					
-		LN994919	-		
France, Grand Est, near Ottmarsheim, Rhine					
2016					
G.M. David					
47°44'43.95"N	7°32'38.72"E				
Unkown					
-		MF563507	-		
-		MF563505	-		
-		MF563509	-		
-		MF563502	-		
-		MF563495	-		
-		MF563496	-		
-		MF563497	-		
-		MF563498	-		

		-	MF563499	-
		-	MF563500	-
		-	MF563501	-
	47°44'51.87"N	7°32'38.72"E		
		Unkown		
		-	MF563503	-
		-	MF563504	-
		-	MF563506	-
		-	MF563508	-
		-	MF563510	-
		-	MF563511	-
		-	MF563512	-
		-	MF563513	-
		-	MF563514	-
		-	MF563515	-
		-	MF563516	-
	47°46'03.15"N	7°31'54.84"E		
		Unkown		
		-	MF563517	-
		-	MF563518	-
		-	MF563519	-
		-	MF563520	-
		-	MF563521	-
		-	MF563522	-
		-	MF563523	-
		-	MF563524	-
		-	MF563525	-
		-	MF563526	-
		-	MF563527	-
Germany, Hann. Münden, Weser				
2018				
S. Vogel				
	51°25'29.9"N	9°38'34.6"E		
		Unkown		
		-	ON649693	-
		-	ON649694	-
		-	ON649695	-
		-	ON649696	-
		-	ON649697	-
Hungary, Budapest, Danube				
1998				
M.-J. Perrot-Minnot				
	47°29'19.5"N	19°03'04.9"E		
		Unkown		
		-	LN994906	-
		-	LN994914	-
Poland, Gniew, Vistula				
2008				
M.-J. Perrot-Minnot				
	53°50'09.8"N	18°50'18.8"E		
		Unkown		
		-	LN994923	-
Poland, Plock, Vistula				
2008				

M.-J. Perrot-Minnot		52°32'25.7"N	19°40'43.8"E			
		Unkown				
				-	LN994920	-
				-	LN994921	-
				-	LN994922	-
Romania, Pristol, Danube						
1998						
M.-J. Perrot-Minnot		44°13'56.1"N	22°40'47.0"E			
		Unkown				
				-	LN994915	-
Serbia, Prahovo, Danube						
1998						
M.-J. Perrot-Minnot		44°17'46.4"N	22°35'27.3"E			
		Unkown				
				-	LN994890	-
				-	LN994891	-
				-	LN994892	-
				-	LN994893	-
Slovakia, Bratislava, Danube						
1998						
M.-J. Perrot-Minnot		48°07'44.4"N	17°08'29.1"E			
		Unkown				
				-	LN994902	-
				-	LN994910	-
				-	LN994911	-
Slovakia, Štúrovo, Danube						
2006						
M.-J. Perrot-Minnot		47°47'52.6"N	18°43'39.3"E			
		Unkown				
				-	LN994916	-
				-	LN994917	-
Ukraine, Ivano-Frankivsk, Ivano-Frankivsk, Bistrice River						
1879						
		Unknown				
		48°55'36.9"N	24°45'21.3"E			
		<i>N. fluviatilis</i>				
				NHMF26.1	-	Cystacanth
<i>P. laevis</i>						
Austria, Lower Austria, Purkersdorf, Wienfluss						
05.06.2025						
S. Reier		48°12'08"N	16°10'21"E			
		<i>S. cephalus</i>				
				PURK1.1	-	Adult
				PURK1.2	-	Adult
				PURK1.3	-	Adult
				PURK1.4	-	Adult
				PURK1.5	-	Adult

Austria, Styria, Graz, Mur	1900	Joanneum	46°46'48.9"N	15°34'58.3"E	PURK1.6	-	Adult
					NHMF9.1	-	Cystacanth
Austria, Vienna, Danube	1865	Unknown	48°11'08.5"N	16°28'16.5"E	NHMF1.1	-	Adult
<i>P. laevis</i> 1	Czechia, Rokytná River	2004	Y. Moret	49°02'48.7"N	16°02'02.8"E	Unkown	-
France, Dijon, Ouche	2003	M.-J. Perrot-Minnot	47°18'50.9"N	5°01'46.2"E	Unkown	-	-
France, Dijon, Ouche	2004	Y. Moret	47°18'50.9"N	5°01'46.2"E	Unkown	-	-
France, Les Maillys, Tille	2004	Y. Moret	47°08'12.9"N	5°20'37.3"E	Unkown	-	-
Germany, Durmentingen, Kanzach	2018	A. Ros	48°6'51"N	9°32'6"E	Unkown	-	-
Germany, Fulda, Fulda	2018	S. Vogel	50°33'35.9"N	9°39'36.6"E	Unkown	-	-

			-	ON649687	-
Hungary, Lake Balaton, Tapolca river					
2004					
Y. Moret					
46°50'55.3"N	17°25'31.7"E				
	Unkown				
			-	EF051067	-
46°52'58"N	17°26'29"E				
	Unkown				
			-	EF051066	-
			-	EF051068	-
			-	EF051069	-
Hungary, near lake Balaton, Tapolca					
2003					
M.-J. Perrot-Minnot					
46°52'58"N	17°26'29"E				
	Unkown				
			-	AY423349	-
			-	AY423350	-
<i>P. laevis</i> 2					
Austria, Styria, Gleinstaetten, Sulm					
2014					
S. Reier					
46°44'56.0"N	15°21'54.5"E				
	Unkown				
			-	MK612524	-
			-	MK612532	-
			-	MK612525	-
			-	MK612526	-
			-	MK612527	-
			-	MK612528	-
			-	MK612529	-
			-	MK612530	-
			-	MK612531	-
Bosnia and Herzegovina, Hum, Sutla					
2008-2010					
D. Valic					
46°13'20.34"N	15°43'11.22"E				
	Unkown				
			-	KF559284	-
			-	KF559285	-
			-	KF559286	-
Bosnia and Herzegovina, Jarun, Sava					
2005-2007					
D. Valic					
45°46'34.32"N	15°56'31.44"E				
	Unkown				
			-	KF559294	-
			-	KF559295	-
Bosnia and Herzegovina, Klanjec, Sutla					
2008-2010					
D. Valic					
46°02'43.68"N	15°44'14.22"E				
	Unkown				

	-	KF559287	-
	-	KF559288	-
Bosnia and Herzegovina, Ključ Brdovečki, Sutla			
2008-2010			
D. Valić			
45°52'25.98"N	15°37'01.20"E		
	Unkown		
	-	KF559292	-
	-	KF559293	-
Bosnia and Herzegovina, Kraj, Sutla			
2008-2010			
D. Valić			
45°54'36.12"N	15°41'31.02"E		
	Unkown		
	-	KF559289	-
Bosnia and Herzegovina, Kumrovec, Sutla			
2008-2010			
D. Valić			
46°07'49.80"N	15°37'01.20"E		
	Unkown		
	-	KF559290	-
	-	KF559291	-
Bosnia and Herzegovina, Oborovo, Sava			
2005-2007			
D. Valić			
45°41'17.16"N	16°14'52.50"E		
	Unkown		
	-	KF559298	-
Bosnia and Herzegovina, Otok Samoborski, Sava			
2005-2007			
D. Valić			
45°50'32.58"N	15°43'29.82"E		
	Unkown		
	-	KF559297	-
Bosnia and Herzegovina, Sava			
2017			
Z. Nedić			
45°00'00.32"N	18°43'45.44"E		
	Unkown		
	-	MK133342	-
	-	MK133343	-
	-	MK133344	-
	45°04'54.58"N	18°35'58.49"E	
	Unkown		
	-	MH282840	-
2005-2007			
D. Valić			
45°46'34.32"N	15°56'31.44"E		
	Unkown		
	-	KF559299	-
Bosnia and Herzegovina, Sava River, Jasenovac, Sava			
2005-2007			
D. Valić			
45°15'49.50" N	16°53'39.48"E		

		Unkown			
		-		KF559296	-
Croatia, Čabranka river					
	2005-2016				
	I. Vardić Smrzlić				
	45°32'43.4"N	14°41'30.8"E			
		Unkown			
		-		OQ569519	-
Croatia, Dobra river					
	2005-2016				
	I. Vardić Smrzlić				
	45°17'59.6"N	15°16'12.7"E			
		Unkown			
		-		OQ569495	-
		-		OQ569504	-
	45°21'15.1"N	15°06'25.2"E			
		Unkown			
		-		OQ569520	-
Croatia, Korna river					
	2005-2016				
	I. Vardić Smrzlić				
	45°07'16.7"N	15°35'22.2"E			
		Unkown			
		-		OQ569490	-
		-		OQ569491	-
Croatia, Krapina river					
	2005-2016				
	I. Vardić Smrzlić				
	45°49'59.9"N	15°49'34.3"E			
		Unkown			
		-		OQ569497	-
		-		OQ569505	-
		-		OQ569512	-
Croatia, Kupa river					
	2005-2016				
	I. Vardić Smrzlić				
	45°31'53.4"N	15°47'21.8"E			
		Unkown			
		-		OQ569510	-
	45°32'56.4"N	15°50'43.1"E			
		Unkown			
		-		OQ569507	-
	45°36'53.3"N	15°28'29.6"E			
		Unkown			
		-		OQ569493	-
		-		OQ569499	-
		-		OQ569502	-
		-		OQ569516	-
	45°49'59.9"N	14°49'34.3"E			
		Unkown			
		-		OQ569508	-
Croatia, Lika river					
	2005-2016				
	I. Vardić Smrzlić				

	44°34'56.6"N	15°23'30.1"E			
		Unkown	-	OQ569496	-
Croatia, Mrežnica river	2005-2016				
	I. Vardić Smrzlić				
	45°27'10.8"N	15°30'23.4"E			
		Unkown	-	OQ569501	-
Croatia, near Vukovar city, Danube	2005-2016				
	I. Vardić Smrzlić				
	45°20'40"N	19°00'09"E			
		Unkown	-	OQ569494	-
			-	OQ569514	-
Croatia, Orłjava river	2005-2016				
	I. Vardić Smrzlić				
	45°10'31.8"N	17°42'19.8"E			
		Unkown	-	OQ569500	-
			-	OQ569515	-
			-	OQ569518	-
Croatia, Ribnjak	2005-2016				
	I. Vardić Smrzlić				
	45°12'23.4"N	15°21'38.9"E			
		Unkown	-	OQ569509	-
Croatia, Sava river	2005-2016				
	I. Vardić Smrzlić				
	45°38'37.7"N	16°19'23.2"E			
		Unkown	-	OQ569517	-
	45°50'32.3"N	15°43'29.6"E			
		Unkown	-	OQ569503	-
	45°50'37.3"N	15°48'34.9"E			
		Unkown	-	OQ569511	-
	45°52'25.7"N	15°41'25.4"E			
		Unkown	-	OQ569492	-
	46°02'43.4"N	15°44'13.9"E			
		Unkown	-	OQ569506	-
			-	OQ569513	-
	46°13'20.3"N	15°43'10.9"E			
		Unkown	-	OQ569498	-
<i>P. tereticollis</i>					
Austria, Lower Asutria, Hausmening, Ybbs					

2018-2020	E. Lewisch	48°04'39.6"N	14°48'54.7"E	Unkown	-	MN780983	-
					-	MN780984	-
					-	MN780985	-
					-	MN780986	-
Austria, Lower Austria, near Wallsee, Danube							
1983	Janisch	48°09'57.3"N	14°42'27.9"E	<i>B. barbus</i>			
				NHMF13.1	-	Adult	
				NHMF13.1			
				0	-	Cystacanth	
				NHMF13.1			
				1	-	Cystacanth	
				NHMF13.1			
				2	-	Cystacanth	
				NHMF13.2	-	Adult	
				NHMF13.3	-	Adult	
				NHMF13.4	-	Cystacanth	
				NHMF13.5	-	Cystacanth	
				NHMF13.7	-	Adult	
				NHMF13.8	-	Adult	
Austria, Styria, near Andritzbach, Stattegg (fish pond)							
2018	C. Gumpinger	47°08'37.4"N	15°25'08.2"E	Unkown			
					-	MT682956	-
					-	MT682954	-
					-	MT682955	-
Austria, Styria, near Obervogau and Gralla, Mur							
2014	S. Reier	46°46'48.9"N	15°34'58.3"E	Unkown			
					-	MK612534	-
					-	MK612535	-
					-	MK612536	-
					-	MK612537	-
					-	MK612538	-
					-	MK612539	-
					-	MK612540	-
					-	MK612541	-
					-	MK612542	-
					-	MK612543	-
					-	MK612544	-
					-	MK612545	-
Austria, Upper Austria, Neuhofer an der Krems, Krems							
2018-2020	E. Lewisch	47°54'14.6"N	14°06'24.9"E				

		Unkown			
		-	MN780980	-	
		-	MN780981	-	
		-	MN780982	-	
	48°8'1"N	14°14'0"E			
		Unkown			
		-	MN780978	-	
		-	MN780979	-	
Austria, Vienna, Danube					
1826					
"Laboratorium"					
48°11'08.5"N	16°28'16.5"E				
	<i>B. barbatus</i>				
	NHMF15.1	-		Cystacanth	
1865					
Unknown					
48°11'08.5"N	16°28'16.5"E				
	<i>S. cephalus</i>				
	NHMF2.1	-		Adult	
Austria, Vienna, Freudenau, Danube					
2015					
S. Reier					
48°11'05.7"N	16°26'12.6"E				
	Unkown				
	-	MK612533	-		
Austria, Vienna, Liesing river					
1877					
Wiesbauer					
48°08'15.5"N	16°13'52.9"E				
	<i>S. cephalus</i>				
	NHMF4.2	-		Cystacanth	
	NHMF4.3	-		Cystacanth	
Croatia, Dobra river					
2005-2016					
I. Vardić Smrzlić					
45°17'59.64"N	15°16'12.72"E				
	Unkown				
	-	OQ569525	-		
45°20'53.5194'					
'N	15°20'52.8"E				
	Unkown				
	-	OQ569522	-		
	-	OQ569529	-		
45°22'23.5194'					
'N	15°21'38.8794"E				
	Unkown				
	-	OQ569523	-		
	-	OQ569524	-		
Croatia, Karlovac, near Skukani, Dobra					
2014					
I. Vardić Smrzlić					
45°22'24"N	15°21'39"E				
	<i>S. cephalus</i>				
	DOB1.1	-		Adult	
	DOB1.2	-		Adult	

Croatia, Kupa river	2005-2016	I. Vardić Smrzlić	45°49'59.88"N	14°49'34.32"E	Unkown	-	OQ569528	-
Croatia, Ribnjak	2005-2016	I. Vardić Smrzlić	45°12'23.3994'	15°21'38.8794"E	Unkown	-	OQ569526	-
Croatia, Una river	2005-2016	I. Vardić Smrzlić	45°13'17.04"N	16°30'43.9194"E	Unkown	-	OQ569527	-
France, Chevigny, Ruisseau de Borne	2003	M.-J. Perrot-Minnot	47°10'39"N	5°28'34"E	Unkown	-	AY423353	-
France, Dijon, Ouche	2005	M.-J. Perrot-Minnot	47°18'50.9"N	5°01'46.2"E	Unkown	-	LN994951	-
						-	LN994952	-
						-	LN994953	-
						-	LN994954	-
						-	LN994955	-
						-	LN994956	-
						-	LN994957	-
						-	LN994958	-
France, Durance, Le Buëch	2008	M.-J. Perrot-Minnot	44°12'07.3"N	5°56'34.3"E	Unkown			
	2003	M.-J. Perrot-Minnot	44°12'07.3"N	5°56'34.3"E	Unkown	-	LN994965	-
						-	LN994966	-
						-	AY423351	-
France, Le Mousseau, Mesvrin	2009	M.-J. Perrot-Minnot	46°51'52.8"N	4°15'44.8"E	Unkown			

			-	LN994969	-
France, Maillys, Tille	2003	M.-J. Perrot-Minnot			
		47°08'12.9"N	5°20'37.3"E		
		Unkown			
			-	AY423352	-
			-	LN994963	-
			-	LN994964	-
France, Pertuis, Durance	2008	M.-J. Perrot-Minnot			
		43°40'11.6"N	5°29'11.2"E		
		Unkown			
			-	LN994967	-
			-	LN994968	-
France, Talmay, Vingeanne	2005	M.-J. Perrot-Minnot			
		47°21'05.2"N	5°27'45.9"E		
		Unkown			
			-	LN994959	-
			-	LN994960	-
			-	LN994961	-
			-	LN994962	-
Germany, Altneudorf, Steinach	2018	A. Ros			
		49°28'01.2"N	8°48'07.2"E		
		Unkown			
			-	MT216161	-
Germany, Beiertheim, Alb	2018	A. Ros			
		48°57'46.8"N	8°23'34.8"E		
		Unkown			
			-	MT216170	-
Germany, Beizkofen, Ostrach	2018	A. Ros			
		48°02'09.6"N	9°22'08.4"E		
		Unkown			
			-	MT216162	-
			-	MT216166	-
Germany, between Bieringen und Obernau, Neckar	2018	A. Ros			
		48°27'00.0"N	8°51'18.0"E		
		Unkown			
			-	MT216168	-
Germany, Epfendorf, Neckar	2018	A. Ros			
		48°14'49.2"N	8°36'03.6"E		

	Unkown			
	-		MT216169	-
Germany, Eschwege, Werra Fluss				
2018				
S. Vogel				
51°11'21.4"N	10°04'01.7"E			
	Unkown			
	-		ON649688	-
	-		ON649689	-
	-		ON649690	-
	-		ON649691	-
	-		ON649692	-
Germany, Friedrichstal	Unkown	Huttenwerk, Forbach		
2018				
A. Ros				
48°28'58.8"N	8°22'33.6"E			
	Unkown			
	-		MT216163	-
Germany, Hausen alte Bogenbruecke, Wuerm				
2018				
A. Ros				
48°47'13.2"N	8°50'09.6"E			
	Unkown			
	-		MT216164	-
Germany, Kluftern, Lipbach				
2018				
A. Ros				
47°41'38.4"N	9°23'52.8"E			
	Unkown			
	-		MT216171	-
Germany, Muehlenholz, Schwarzach				
2018				
A. Ros				
47°42'17.3"N	9°38'02.4"E			
	Unkown			
	-		MT216160	-
Germany, near Karlsruhe, Rhine				
2005				
M.-J. Perrot-Minnot				
48°59'35.7"N	8°17'02.5"E			
	Unkown			
	-		LN994970	-
	-		LN994971	-
Germany, Nonnenbach				
2018				
A. Ros				
47°35'24.0"N	9°35'31.2"E			
	Unkown			
	-		MT216159	-
	-		MT216165	-
	-		MT216172	-
Germany, Stetten, Rot				
2018				
A. Ros				

	48°16'37.2"N	9°53'42.0"E			
		Unkown	-	MT216167	-
Germany, Stralsund					
2006					
M. Spakulova					
54°18'33"N	13°04'55"E				
	Unkown	-	JF706706	-	
		-	JN695505	-	
		-	JN695506	-	
		-	JN695507	-	
		-	JN695508	-	
M.-J. Perrot-Minnot					
54°18'33"N	13°04'55"E				
	Unkown	-	LN994972	-	
		-	LN994973	-	
		-	LN994974	-	
		-	LN994975	-	
		-	LN994976	-	
		-	LN994977	-	
		-	LN994978	-	
Romania, Olteni, Olt					
Unkown					
M.-J. Perrot-Minnot					
45°08'46.6"N	24°23'13.2"E				
	Unkown	-	LN994987	-	
Slovakia, Buzica, Ida					
2006					
M.-J. Perrot-Minnot					
48°34'16.0"N	20°56'27.6"E				
	Unkown	-	LN994979	-	
		-	LN994980	-	
Slovakia, Mylavsky, Myjava					
2004					
M.-J. Perrot-Minnot					
48°38'48.2"N	17°11'42.8"E				
	Unkown	-	LN994981	-	
Slovakia, Vihorlat, Male Morske Okna					
2004					
M.-J. Perrot-Minnot					
48°54'41.2"N	22°11'01.0"E				
	Unkown	-	LN994982	-	
		-	LN994983	-	
		-	LN994984	-	
		-	LN994985	-	
		-	LN994986	-	

Pomphorhynchus sp.

Austria, Lower Austria					
1874					
Unknown					
Unknown	Unknown				
	<i>B. barbus</i>				
	NHMF19.1	-			Cystacanth
Austria, Lower Austria, Bad Deutsch Altenburg, Danube					
15.10.2024					
BOKU					
48°08'29.4"N	16°54'09.2"E				
	<i>A. alburnus</i>				
	BDAF27.1	-			Adult
	<i>B. barbus</i>				
	BDAF1.3	-			Adult
	BDAF5.1	-			Pre-Adult (?)
	BDAF5.2	-			Adult
	BDAF6.2	-			Adult
	<i>G. schraetser</i>				
	BDAF18.2	-			Adult
	<i>N. melanostomus</i>				
	BDAF25.2	-			Cystacanth
	BDAF30.7	-			Cystacanth
Austria, Lower Austria, between Haslau and Wildungsmauer, Danube					
1988					
B. Herzig					
48°06'54.7"N	16°45'57.6"E				
	<i>B. barbus</i>				
	NHMF14.3	-			Adult
Austria, Lower Austria, near Regelsbrunn, Danube					
7.3.2025					
T. Leister					
48°06'50.1"N	16°46'46.4"E				
	<i>P. kessleri</i>				
	BDAF36.1	-			Cystacanth
	BDAF36.2	-			Cystacanth
Austria, Lower Austria, near Wallsee, Danube					
1983					
Janisch					
48°09'57.3"N	14°42'27.9"E				
	<i>B. barbus</i>				
	NHMF12.1	-			Adult
	NHMF13.6	-			Cystacanth
	NHMF13.9	-			Adult
Austria, Lower Austria, near Zwentendorf (Traisenmündung), Danube					
1988					
B. Herzig					
48°21'50.6"N	15°51'43.5"E				
	<i>B. barbus</i>				
	NHMF10.2	-			Adult
	NHMF10.6	-			Cystacanth
Austria, Styria, Graz, Mur					
1900					
Joanneum					
46°46'48.9"N	15°34'58.3"E				

		<i>S. cephalus</i>		
		NHMF9.2	-	Cystacanth
		NHMF9.3	-	Cystacanth
Austria, Vienna, Danube				
1826				
"Laboratorium"				
48°11'08.5"N	16°28'16.5"E			
		<i>B. barbus</i>		
		NHMF15.2	-	Cystacanth
		NHMF15.3	-	Cystacanth
1856				
Unknown				
48°11'08.5"N	16°28'16.5"E			
		<i>B. barbus</i>		
		NHMF20.1	-	Cystacanth
		NHMF20.2	-	Cystacanth
		NHMF20.3	-	Cystacanth
1865				
Unknown				
48°11'08.5"N	16°28'16.5"E			
		<i>S. cephalus</i>		
		NHMF1.3	-	Adult
1876				
Unknown				
48°11'08.5"N	16°28'16.5"E			
		<i>B. barbus</i>		
		NHMF17.1	-	Adult
1996				
H. Ahnelt				
48°11'08.5"N	16°28'16.5"E			
		<i>P. kessleri</i>		
		NHMF24.2	-	Cystacanth
Austria, Vienna, Liesing river				
1877				
Wiesbauer				
48°08'15.5"N	16°13'52.9"E			
		<i>S. cephalus</i>		
		NHMF4.1	-	Cystacanth
		NHMF5.1	-	Adult
		NHMF5.2	-	Adult
Turkey, Istanbul, Istanbul, Black sea				
1894				
Steindachner				
Unknown		Unknown		
		<i>N. melanostomus</i>		
		NHMF29.1	-	Cystacanth
		NHMF29.2	-	Cystacanth
Acanthocephala				
Austria, Styria, Graz, Mur				
1900				
Joanneum				
46°46'48.9"N	15°34'58.3"E			
		<i>S. cephalus</i>		
		NHMF8.1	-	Adult

Echinorhynchus truttae				NHMF8.2	-	Adult
Unkown						
	Unkown					
	Unkown					
		Unkown				
			Unkown			
				-	DQ089710	-
Nematoda						
Austria, Lower Austria, Bad Deutsch Altenburg, Danube						
15.10.2024						
BOKU						
48°08'29.4"N		16°54'09.2"E				
		<i>B. barbus</i>				
			BDAF10.5	-		-
			BDAF10.6	-		-
Trematoda						
Austria, Lower Austria						
1969						
Steiner						
	Unknown	Unknown				
		<i>Proterorhinus sp.</i>				
			NHMF21.1	-		Metacercaria
			NHMF21.2	-		Metacercaria
			NHMF22.1	-		Metacercaria
			NHMF22.2	-		Metacercaria
			NHMF22.3	-		Metacercaria
			NHMF23.1	-		Metacercaria
			NHMF23.2	-		Metacercaria
Austria, Lower Austria, Bad Deutsch Altenburg, Danube						
15.10.2024						
BOKU						
48°08'29.4"N		16°54'09.2"E				
		<i>C. nasus</i>				
			BDAF2.1	-		Adult
27.3.2025						
T. Leister						
48°06'52.1"N		16°47'43.3"E				
		<i>B. gymnotrachelus</i>				
			BDAF44.1	-		Metacercaria
7.3.2025						
T. Leister						
48°06'52.1"N		16°47'43.3"E				
		<i>B. gymnotrachelus</i>				
			BDAF37.4	-		Metacercaria
			BDAF37.5	-		Metacercaria

Appendix 3

Appendix 3: Overview of sequences assigned to a haplotype in the *P. bosniacus* COI haplotype network (Fig. 3.8). The table lists all specimens sharing the same haplotype, including the number of matching sequences, list of matching sequences, data source, and associated river basin.

Basin	Reference	List of matching sequences
Haplotype A (59 sequences)		
Danube	This study	BDAF1.1
Danube	This study	BDAF1.2
Danube	This study	BDAF4.2
Danube	This study	BDAF4.3
Danube	This study	BDAF4.4
Danube	This study	BDAF4.6
Danube	This study	BDAF5.3
Danube	This study	BDAF5.4
Danube	This study	BDAF8.2
Danube	This study	BDAF10.3
Danube	This study	BDAF14.1
Danube	This study	BDAF14.2
Danube	This study	BDAF19.2
Danube	This study	BDAF21.1
Danube	This study	BDAF21.2
Danube	This study	BDAF21.3
Danube	This study	BDAF30.3
Danube	This study	BDAF30.4
Danube	This study	BDAF30.5
Danube	This study	BDAF30.8
Danube	This study	BDAF33.2
Danube	This study	BDAF35.3
Danube	This study	BDAF38.1
Danube	This study	BDAF40.1
Danube	Perrot-Minnot and Tougard, 2015	LN994891
Danube	Perrot-Minnot and Tougard, 2015	LN994896
Danube	Perrot-Minnot and Tougard, 2015	LN994897
Danube	Perrot-Minnot and Tougard, 2015	LN994900
Danube	Perrot-Minnot and Tougard, 2015	LN994909
Danube	Perrot-Minnot and Tougard, 2015	LN994910

Danube	Perrot-Minnot and Tougard, 2015	LN994911
Danube	Perrot-Minnot and Tougard, 2015	LN994912
Danube	Perrot-Minnot and Tougard, 2015	LN994913
Danube	Perrot-Minnot and Tougard, 2015	LN994914
Danube	Perrot-Minnot and Tougard, 2015	LN994915
Danube	Perrot-Minnot and Tougard, 2015	LN994916
Danube	Perrot-Minnot and Tougard, 2015	LN994917
Danube	Perrot-Minnot and Tougard, 2015	LN994918
Vistula	Perrot-Minnot and Tougard, 2015	LN994922
Vistula	Perrot-Minnot and Tougard, 2015	LN994923
Rhine	David et al., 2018	MF563497
Danube	Reier et al., 2019	MK612498
Danube	Reier et al., 2019	MK612499
Danube	Reier et al., 2019	MK612500
Danube	Reier et al., 2019	MK612501
Danube	Reier et al., 2019	MK612503
Danube	Reier et al., 2019	MK612505
Danube	Reier et al., 2019	MK612508
Danube	Reier et al., 2019	MK612509
Danube	Reier et al., 2019	MK612510
Danube	Reier et al., 2019	MK612513
Danube	Reier et al., 2019	MK612516
Danube	Reier et al., 2019	MK612517
Danube	Reier et al., 2019	MK612518
Danube	Reier et al., 2019	MK612520
Danube	Reier et al., 2019	MK612521
Danube	Reier et al., 2019	MK612523
Danube	Reier et al., 2020	MT682952
Danube	Reier et al., 2020	MT682953
Haplotype B (13 sequences)		
Danube	This study	BDAF4.5
Danube	This study	BDAF8.3
Danube	This study	BDAF24.1
Danube	This study	BDAF24.2
Danube	This study	BDAF35.5
Danube	This study	BDAF37.2
Danube	This study	BDAF38.2

Danube	Perrot-Minnot and Tougard, 2015	LN994899
Rhine	David et al., 2018	MF563498
Danube	Nedić et al., 2019	MH282839
Danube	Nedić et al., 2020	MK133340
Danube	Reier et al., 2019	MK612502
Danube	Reier et al., 2019	MK612511
Haplotype C (28 sequences)		
Danube	This study	BDAF8.1
Danube	This study	BDAF12.3
Danube	This study	BDAF19.3
Danube	This study	BDAF21.4
Danube	This study	BDAF33.5
Danube	Perrot-Minnot and Tougard, 2015	LN994890
Danube	Perrot-Minnot and Tougard, 2015	LN994919
Rhine	David et al., 2018	MF563501
Haplotype D (5 sequences)		
Danube	This study	BDAF37.3
Vistula	Perrot-Minnot and Tougard, 2015	LN994921
Rhine	David et al., 2018	MF563500
Danube	Nedić et al., 2018	MH282838
Danube	Reier et al., 2019	MK612519
Haplotype E (4 sequences)		
Rhine	David et al., 2018	MF563495
Danube	Reier et al., 2019	MK612512
Danube	Reier et al., 2019	MK612514
Danube	Reier et al., 2019	MK612515
Haplotype F (3 sequences)		
Danube	Perrot-Minnot and Tougard, 2015	LN994903
Danube	Perrot-Minnot and Tougard, 2015	LN994904
Danube	Perrot-Minnot and Tougard, 2015	LN994905
Haplotype G (3 sequences)		
Danube	This study	BDAF37.1
Rhine	David et al., 2018	MF563496
Danube	Reier et al., 2019	MK612497
Haplotype H (3 sequences)		
Danube	Perrot-Minnot and Tougard, 2015	LN994901
Danube	Perrot-Minnot and Tougard, 2015	LN994902

Rhine	David et al., 2018	MF563505
Haplotype I (3 sequences)		
Danube	This study	BDAF4.1
Rhine	David et al., 2018	MF563502
Haplotype J (3 sequences)		
Danube	Perrot-Minnot and Tougard, 2015	LN994907
Danube	Perrot-Minnot and Tougard, 2015	LN994908
Haplotype K (3 sequences)		
Vistula	Perrot-Minnot and Tougard, 2015	LN994920
Rhine	David et al., 2018	MF563511
Haplotype L (3 sequences)		
Danube	Perrot-Minnot and Tougard, 2015	LN994894
Rhine	David et al., 2018	MF563521
Haplotype M (3 sequences)		
Danube	Perrot-Minnot and Tougard, 2015	LN994898
Danube	Reier et al., 2019	MK612507
Haplotype N (3 sequences)		
Danube	This study	BDAF35.1
Danube	This study	BDAF35.4