Trematode diversity in freshwater ecosystems: from individuals to communities

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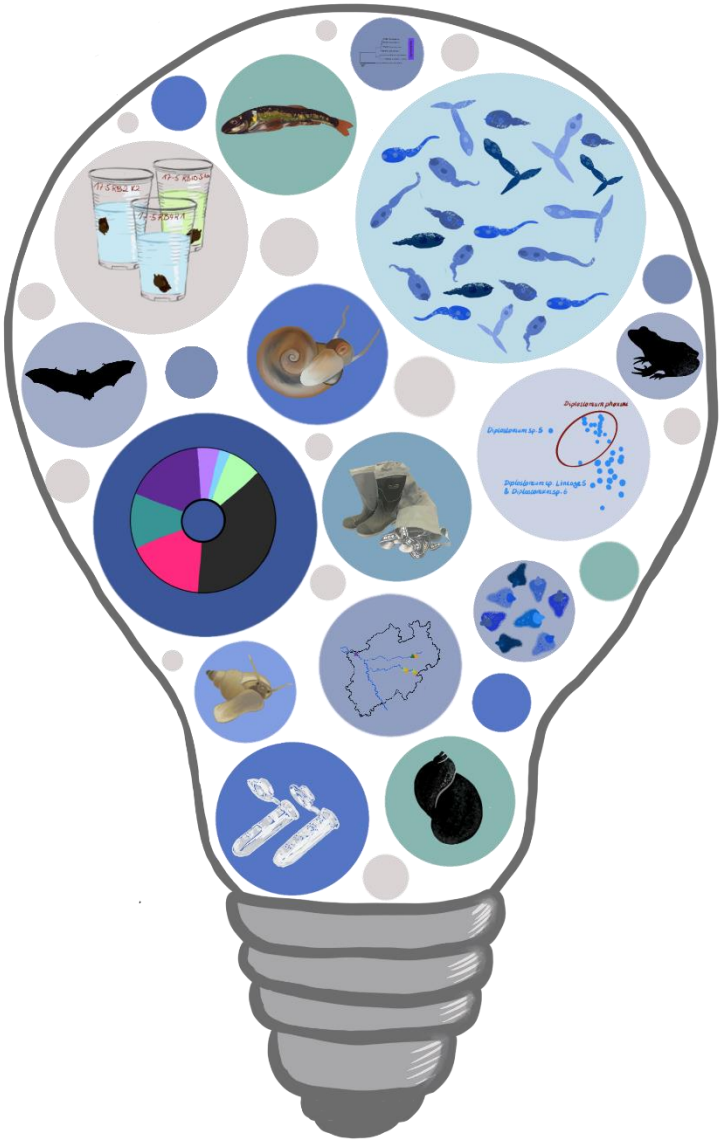


1.1. Summary

Biodiversity is declining worldwide at a rapid pace. Despite the alarming rate of biodiversity loss, the predictions made to date are probably still rather conservative, as a large proportion of the species on our planet have not yet been officially described (Singh 2002; Poulin and Morand 2004; Barnosky et al. 2011; Mora et al. 2011; Engel et al. 2021). Despite conservative estimates, a multitude of studies predict that the rate of biodiversity loss will accelerate in the future, exacerbated by anthropogenic impact (e.g., De Vos et al. 2017; Diaz et al. 2019; Andermann 2020; Bradshaw et al. 2021). Human activities have altered large areas of our planet (Sanderson et al. 2002; Halpern et al. 2008; Birk et al. 2020), with freshwater habitats being particularly affected by degradation and species loss (Birk et al. 2020; Tickner et al. 2020). Changes in water usage, distribution, and flow, combined with changes in climate and land-use, and a rapid human population increase, have detrimental consequences for biodiversity and ecosystem services (Elmhagen et al. 2015; Albert, 2021). Although the threat to our planet from the loss of biodiversity and intact ecosystems rivals that from climate change, it remains difficult to find a common approach to the protection of ecosystems, plants and animals that will effectively address the biodiversity crisis in the long term.

Besides our moral duty to care for the well-being of all organisms living on this planet, there are several reason why biodiversity conservation, including that of parasites, is critical (Poulin and Morand 2004). Parasites, like other organisms, are part of the ecosystem and fulfill important roles, for instance, modulating ecological speciation (Karvonen and Seehausen 2012; Betts et al. 2018) acting as ecosystem engineers (Thomas et al. 1999), regulating host populations (Hudson et al. 2006; Lefèvre et al. 2009; Frainer et al. 2018), and altering predator-prey dynamics (Lafferty and Morris 1996; Mouritsen and Poulin 2002). Although, parasitism is regarded as one of the most successful and ubiquitous life forms (Dobson et al. 2008; Poulin 2014; Carlson et al. 2019), our understanding of parasites and their critical roles in ecosystems is still very limited, particularly for those having complex life cycle, such as digenean trematodes. Digenean trematodes belong to one of the most diverse and widespread groups of metazoan parasites on the planet (Esch et al. 2002). Due to their complex life cycles, involving mostly two or three hosts, including snails, digenean trematodes may be particularly

suitable as bioindicators to assess changes in environmental conditions and ecosystem functioning (Vidal-Martnez et al. 2010; Shea et al. 2012; Sures et al. 2017b). However, knowledge about their distribution, life cycle, and taxonomic relationships is relatively incomplete (Poulinand Morand 2004; Poulin 2014; Sures et al. 2017a; Jorge and Poulin 2018). Given the significance of parasites in ecosystems and the current gap of knowledge relative to their diversity and ecology, we are unable to fully understand and consequently preserve ecosystems effectively. Moreover, in view of enhanced biodiversity loss, a lack of these fundamental information may also imply that many parasite species may become extinct before even being detected. Thus, the knowledge of their role in the ecosystem may be lost forever.

The aim of this thesis is to explore the yet unknown trematode diversity in European freshwater ecosystems at the species, host, and ecosystem levels. Hence aquatic molluscs, the first intermediate host of trematodes, were collected in 2016, 2017, and 2019 at several sampling sites in three German rivers: the Ruhr, the Lippe, and the Rhine. Among the snails collected were representatives of 20 different species from eight families, of which members of the families Lymnaeidae and Planorbidae were the most abundant. Additionally, 15 European minnows (*Phoxinus phoxinus*) from the river Ruhr were examined for infections with metacercariae. Trematode stages were identified by morphological characteristics and, when necessary and appropriate, by molecular methods.

The individual studies of this thesis reveal exceptional and novel insights into parasite diversity at different levels. At the species level *Diplostomum phoxini* was characterised morphologically and molecularly using cercariae isolates from the gastropod *Ampullaceana balthica* and metacercariae from *P. phoxinus*. Phylogenetic analyses targeting the cytochrome c oxidase subunit 1 (*cox*1) gene yielded 44 molecularly characterised species and genetically distinct lineages of *Diplostomum* and resulted in a re-identification/re-classification of 98 isolates, a redefinition of the composition of the *D. baeri* and *D. mergi* species complexes, and an actualised nomenclature for the molecularly characterised species/lineages of *Diplostomum* (Schwelm et al. 2021a; Chapter I).

In Chapter II, the trematode diversity at the level of the first intermediate host was investigated. For this purpose, *Bithynia tentaculata*, a member of the family Bithynidae, for which little information is available on its trematode communities despite its wide distribution in European freshwater systems, was specifically studied. Overall, *B. tentaculata* showed a

high trematode prevalence and molecular and morphological data analysis revealed a species-rich trematode fauna comprising 20 species belonging to 10 families demonstrating a unique species composition when compared to well-studied snail host families, such as Lymnaeidae and Planorbidae (Schwelm et al. 2020; Chapter II).

In Chapter III, a protected natural freshwater system was investigated to assess the trematode diversity on the ecosystem level. In view of the ongoing destruction of habitats and the biodiversity crisis, protected areas are important refuges for wildlife and thus also for parasites that use those organisms as hosts. In total, fifteen snail species were studied, which revealed a high trematode species richness of 40 species, with a substantial proportion of species with complex life cycles (Schwelm et al. 2021b; Chapter III).

In this work, essential aspects, such as the importance of taxonomic resolution, reconstruction of life-cycles and the important role of parasites in food webs and ecosystem health were discussed. Taxonomic resolution is a cornerstone for reliable estimates of parasite diversity, extinction rates, life cycle reconstruction and food web assessment. Within this thesis, it could be demonstrated that the application of molecular methods can contribute significantly to the clarification of species relationships. Although, no cryptic diversity could be detected within this study, two species complexes could be redefined, numerous isolates could be reclassified, and isolates of the cercariae of *A. balthica* and metacercariae of *P. phoxinus* were matched, thus underlining the host specificity of the parasite for its second intermediate fish host. The study of the previously neglected host *B. tentaculata* revealed a unique trematode community compared to well studied snail host families. In total, 20 species could be identified, some of which could only be determined at genus or family level due to a lack of identification literature and comparative isolates from GenBank. This highlights the importance of collecting molecular reference material anchored in reliably determined and detailed morphological descriptions. The reconstruction of life cycles, which can provide valuable insights into trophic interactions and local free-living diversity (Hechinger et al. 2007; Byers et al. 2010; Shea et al. 2012), also relies on reliable identified species. Based on the identified parasites, statements could be made about trophic relationships and the presence of free-living organisms in the habitat studied, e.g., bats and herons. On the other hand, parasites have also been detected whose life cycle we still know too little about which shows the patchy and heterogeneous data situation on parasites and their life cycles (Poulin and Morand 2004; Kudlai et al. 2015; Sures

et al. 2017a). Parasites play crucial roles in the ecosystem equilibrium, for example by contributing to diversity, influencing host population density and predator prey interactions. In this thesis, the brain dwelling metacercariae *D. phoxini* was studied, which is known for altering the flight behaviour of its host and therefore, most likely influencing interaction strength between its intermediate fish host and its potential final host. Whilst the characteristics of digenean trematodes discussed in this dissertation allow for a detailed and comprehensive analysis of these parasites at all levels of diversity, hurdles and knowledge gaps have also been identified that impede the use of parasites in biodiversity assessments and prevent their great potential as bioindicators from being fully realised. To be able to benefit from this opportunity in the future, it would be advisable to focus on collecting life cycle data based on reliably molecularly and morphologically determined specimens, which are collected and made available in large-scale databases.

In conclusion, this work has shown that trematodes contribute in multiple ways to biodiversity, energy flow and the structure of the ecosystems in which they occur. The impending loss of biodiversity will not only result in a significant decline of charismatic megafauna diversity, but it will also eradicate their parasites, impacting undiscovered essential and functional components of the ecosystem. Although our findings are merely glimpses, they open the door to further developments in parasite taxonomy and ecological parasitology, as well as prompting a rethinking of methodological approaches and conservation regimes. Despite the fact that we still have a long way to go before we obtain a more holistic view of their great diversity and functional roles in ecosystems, we should aim to include parasites in our conception of biodiversity. Consequently, the current thesis provides the base for an important step towards including parasites in ecosystem studies and biodiversity conservation efforts. We will only be able to successfully and sustainably protect an ecosystem if we fully comprehend the underlying functions of all its members.

1.2 Zusammenfassung

Die biologische Vielfalt nimmt weltweit in rasantem Tempo ab. Trotz der alarmierenden Verlustrate sind die bisherigen Prognosen wahrscheinlich noch recht konservativ, da ein großer Teil der Arten auf unserem Planeten noch nicht offiziell beschrieben wurde (Singh 2002; Poulin und Morand 2004; Barnosky et al. 2011; Mora et al. 2011; Engel et al. 2021). Trotz vorsichtiger Schätzungen sagen zahlreiche Studien voraus, dass sich der Verlust der biologischen Vielfalt in Zukunft beschleunigen wird, verstärkt durch anthropogene Aktivitäten (z. B. De Vos et al. 2017; Diaz et al. 2019; Andermann 2020; Bradshaw et al. 2021). Menschliche Aktivitäten haben große Gebiete unseres Planeten verändert (Sanderson et al. 2002, Halpern et al. 2008, Birk et al. 2020), wobei Süßwasserlebensräume besonders von Degradation und Artenverlust betroffen sind (Birk et al. 2020; Tickner et al. 2020). Veränderungen in der Wassernutzung, -verteilung und -strömung haben in Verbindung mit Klima- und Landnutzungsänderungen und einem raschen Anstieg der menschlichen Bevölkerung nachteilige Folgen für die biologische Vielfalt und die Ökosystemleistungen (Elmhagen et al. 2015; Albert, 2021). Obwohl die Bedrohung unseres Planeten durch den Verlust der biologischen Vielfalt und intakter Ökosysteme derjenigen durch den Klimawandel in nichts nachsteht, ist es nach wie vor schwierig, einen gemeinsamen Ansatz für den Schutz von Ökosystemen, Pflanzen und Tieren zu finden, mit dem die Biodiversitätskrise auf lange Sicht wirksam angegangen werden kann.

Neben unserer moralischen Pflicht, für das Wohlergehen aller auf diesem Planeten lebenden Organismen zu sorgen, gibt es mehrere Gründe, warum die Erhaltung der biologischen Vielfalt, einschließlich der von Parasiten, entscheidend ist (Poulin und Morand 2004). Parasiten sind, wie andere Organismen auch, Teil des Ökosystems und erfüllen wichtige Aufgaben, wie z. B. die Einflussnahme auf die ökologische Artbildung (Karvonen und Seehausen 2012; Betts et al. 2018), die Funktion eines Ökosystemingenieurs (Thomas et al. 1999), die Regulierung von Wirtspopulationen (Hudson et al. 2006; Lefèvre et al. 2009; Frainer et al. 2018) und die Veränderung von Räuber-Beute-Dynamiken (Lafferty und Morris 1996; Mouritsen und Poulin 2002). Obwohl Parasitismus als eine der erfolgreichsten und

allgegenwärtigsten Lebensformen gilt (Dobson et al. 2008; Poulin 2014; Carlson et al. 2019), ist unser Verständnis von Parasiten und ihrer entscheidenden Rolle in Ökosystemen immer noch sehr begrenzt, insbesondere bei Parasiten mit komplexem Lebenszyklus, wie z. B. digenenen Trematoden. Digene Trematoden gehören zu einer der vielfältigsten und am weitesten verbreiteten Gruppen metazoischer Parasiten auf dem Planeten (Esch et al. 2002). Aufgrund ihrer komplexen Lebenszyklen, an denen meist zwei oder drei Wirte, einschließlich Schnecken, beteiligt sind, eignen sich digene Trematoden besonders gut als Bioindikatoren zur Bewertung von Veränderungen der Umweltbedingungen und der Funktionsweise von Ökosystemen (Vidal-Martnez et al. 2010; Shea et al. 2012; Sures et al. 2017b). Das Wissen über ihre Verbreitung, ihren Lebenszyklus und ihre taxonomischen Beziehungen ist jedoch relativ unvollständig (Poulin und Morand 2004; Poulin 2014; Sures et al. 2017a; Jorge und Poulin 2018). Angesichts der Bedeutung von Parasiten in Ökosystemen und der derzeitigen Wissenslücke in Bezug auf ihre Vielfalt und Ökologie sind wir nicht in der Lage, Ökosysteme vollständig zu verstehen und folglich langfristig effektiv zu erhalten. In Anbetracht des zunehmenden Verlusts an biologischer Vielfalt kann ein Mangel an diesen grundlegenden Informationen auch bedeuten, dass viele Parasitenarten aussterben, bevor sie überhaupt entdeckt werden. Damit könnte das Wissen über ihre Rolle im Ökosystem für immer verloren gehen.

Ziel dieser Arbeit ist es, die bisher unbekannte Trematodenvielfalt in europäischen Süßwasserökosystemen auf der Ebene der Arten, der Wirte und des Ökosystems zu erforschen. Dazu wurden in den Jahren 2016, 2017 und 2019 aquatische Schnecken, die Zwischenwirte der Trematoden, an mehreren Probenahmestellen in den Flüssen Ruhr, Lippe und Rhein gesammelt. Bei den gesammelten Schnecken handelte es sich um Vertreter von 20 verschiedenen Arten aus acht Familien, von denen Mitglieder der Familien Lymnaeidae und Planorbidae am häufigsten vorkamen. Zusätzlich wurden 15 Elritzen (*Phoxinus phoxinus*) aus der Ruhr auf Infektionen mit Metazerkarien untersucht. Die Trematodenstadien wurden anhand morphologischer Merkmale und, wenn nötig und sinnvoll, mit molekularen Methoden identifiziert.

Die einzelnen Studien dieser Arbeit zeigen außergewöhnliche und neue Einblicke in die Parasitendiversität auf verschiedenen Ebenen. Auf Artniveau wurde *Diplostomum phoxini* morphologisch und molekularbiologisch charakterisiert, indem Zerkarienisolate aus der

Schnecke *Ampullaceana balthica* und Metazerkarien aus der Elritze *P. phoxinus* verwendet wurden. Phylogenetische Analysen, die auf das Cytochrom c-Oxidase Untereinheit 1 (*cox*1) Gen abzielten, ergaben 44 molekular charakterisierte Arten und genetisch unterschiedliche Linien von *Diplostomum* und führten zu einer Re-Identifizierung/Reklassifizierung von 98 Isolaten, einer Neudefinition der Zusammensetzung der *D. baeri* und *D. mergi* Artenkomplexe und einer aktualisierten Nomenklatur für die molekular charakterisierten Arten/Linien von *Diplostomum* (Schwelm et al. 2021a; Kapitel I).

In Kapitel II wurde die Trematodenvielfalt auf der Ebene des ersten Zwischenwirts untersucht. Zu diesem Zweck wurde gezielt *Bithynia tentaculata*, ein Vertreter der Familie Bithynidae, untersucht, über den trotz seiner weiten Verbreitung in europäischen Süßwassersystemen nur wenige Informationen über seine Trematodengemeinschaften vorliegen. Insgesamt zeigte *B. tentaculata* eine hohe Trematodenprävalenz, und die molekulare und morphologische Datenanalyse ergab eine artenreiche Trematodenfauna bestehend aus 20 Arten (10 Familien), welche eine einzigartige Artenzusammensetzung im Vergleich zu gut untersuchten Schneckenwirtsfamilien, wie Lymnaeidae und Planorbidae, aufweisen (Schwelm et al. 2020; Kapitel II).

In Kapitel III wurde ein geschütztes natürliches Süßwassersystem untersucht, um die Trematodenvielfalt auf der Ebene des Ökosystems zu bewerten. Angesichts der fortschreitenden Zerstörung von Lebensräumen und der Krise der biologischen Vielfalt sind Schutzgebiete wichtige Rückzugsgebiete für Wildtiere und damit auch für Parasiten, die diese Organismen als Wirte nutzen. Insgesamt wurden fünfzehn Schneckenarten untersucht und ein hoher Trematoden-Artenreichtum von 40 Arten festgestellt, mit einem hohen Anteil an Arten mit komplexen Lebenszyklen (Schwelm et al. 2021b; Kapitel III).

In dieser Arbeit werden wesentliche Aspekte, wie die Bedeutung der taxonomischen Auflösung, die Rekonstruktion von Lebenszyklen und die wichtige Rolle von Parasiten in Nahrungsnetzen und der Gesundheit von Ökosystemen erörtert. Die taxonomische Auflösung ist ein Grudstein für zuverlässige Schätzungen der Parasitenvielfalt, der Aussterberaten, der Rekonstruktion von Lebenszyklen und der Bewertung von Nahrungsnetzen. Im Rahmen dieser Arbeit konnten gezeigt werden, dass die Anwendung molekularer Methoden wesentlich zur Klärung von Artbeziehungen beitragen kann. Obwohl wir im Rahmen unserer Studie keine kryptische Diversität nachweisen konnten, gelang es, auf der Grundlage der durchgeführten

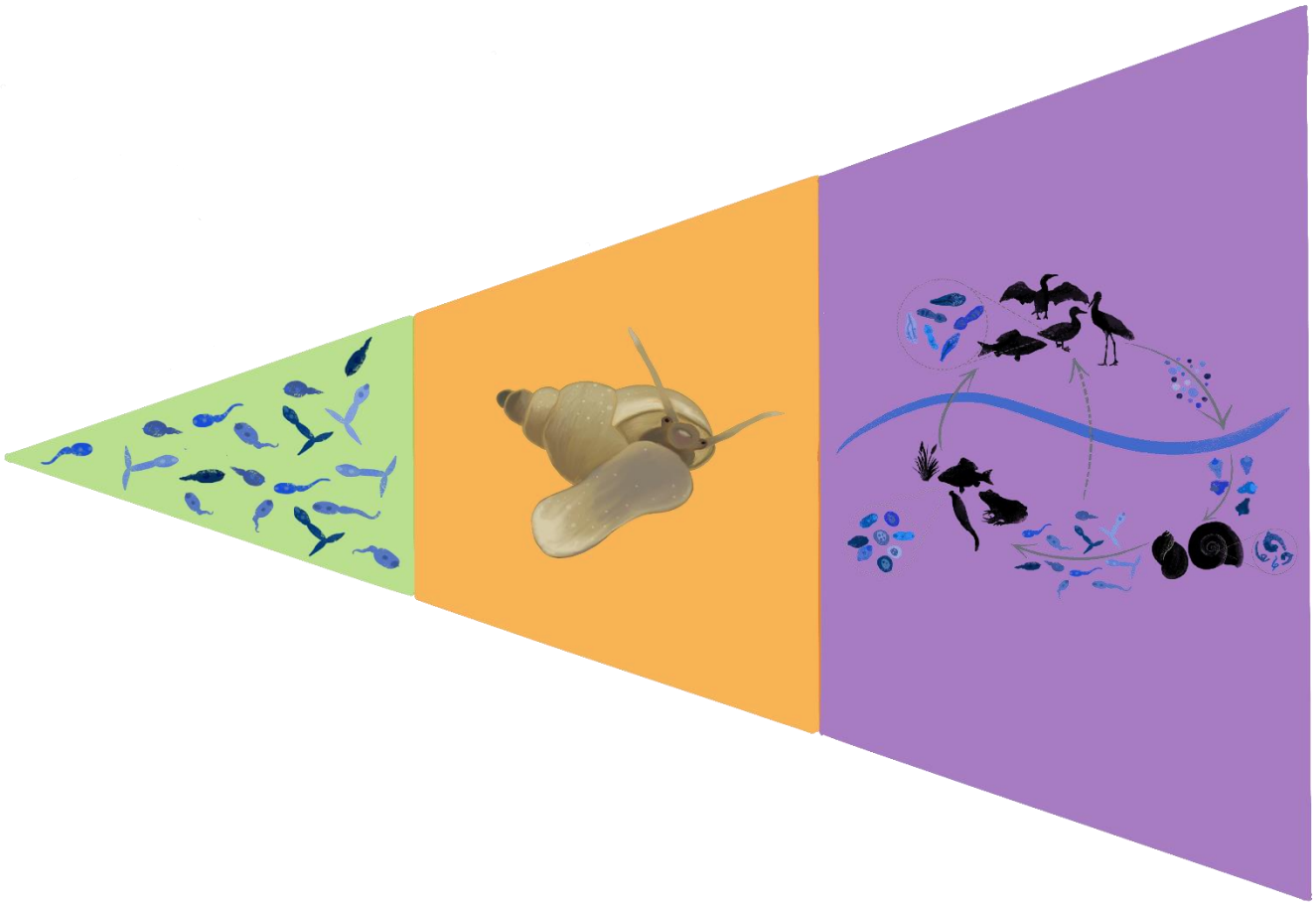
Analysen zwei Artenkomplexe neu zu definieren, zahlreiche Isolate neu zu klassifizieren und Isolate der Zerkarien von *A. balthica* und Metacercarien von *P. phoxinus* einander zu zuordnen, was die Wirtsspezifität des Parasiten für seinen zweiten Fischzwischenwirt unterstreicht. Die Untersuchung des bisher vernachlässigten Wirts *B. tentaculata* ergab eine einzigartige Trematodengemeinschaft im Vergleich zu gut untersuchten Wirtsfamilien, wie den Lymnaeidae und Planorbidae. Insgesamt wurden 20 Arten, von denen einige aufgrund fehlender Bestimmungsliteratur und vergleichbarer Isolate aus GenBank nur auf Gattungs- oder Familienebene bestimmt werden konnten, identifiziert. Dies verdeutlicht, wie wichtig es ist, molekulares Referenzmaterial zu sammeln, das sich auf verlässliche und detaillierte morphologische Beschreibungen stützt. Die Rekonstruktion von Lebenszyklen, die wertvolle Einblicke in trophische Interaktionen und die lokale freilebende Vielfalt liefern kann (Hechinger et al. 2007; Byers et al. 2010; Shea et al. 2012), hängt ebenfalls von zuverlässig identifizierten Arten ab. Anhand der identifizierten Parasiten konnten Aussagen über trophische Beziehungen und das Vorhandensein freilebender Organismen im untersuchten Habitat, z. B. Fledermäuse und Reiher, getroffen werden. Andererseits wurden auch Parasiten gefunden, über deren Lebenszyklus man noch zu wenig weiß, was die lückenhafte und heterogene Datenlage zu Parasiten und ihren Lebenszyklen unterstreicht (Poulin und Morand 2004; Kudlai et al. 2015; Sures et al. 2017a). Parasiten spielen eine entscheidende Rolle im Gleichgewicht des Ökosystems, indem sie beispielsweise zur Diversität beitragen, die Wirtspopulationsdichte und die Räuber-Beute-Interaktionen beeinflussen. In dieser Arbeit untersuchten wir die hirnparasitierenden Metazerkarien von *D. phoxini*, über die man weiß, dass sie das Fluchtverhalten ihres Wirts verändern und damit höchstwahrscheinlich die Interaktionsstärke zwischen ihrem Fischzwischenwirt und ihrem potenziellen Endwirt beeinflussen.

Obwohl die in dieser Dissertation erörterten Charakteristika von digenen Trematoden eine detaillierte und umfassende Analyse dieser auf allen Diversitätsebenen ermöglichen, wurden auch Hürden und Wissenslücken festgestellt, die den Einsatz von Parasiten bei der Bewertung der biologischen Vielfalt erschweren und verhindern, dass ihr großes Potenzial als Bioindikatoren voll ausgeschöpft werden kann. Um diese Möglichkeit in Zukunft besser nutzen zu können, wäre es ratsam, sich auf die Erhebung von Lebenszyklusdaten zu konzentrieren,

die auf zuverlässig molekular und morphologisch bestimmten Exemplaren basieren, die gesammelt und in groß angelegten Datenbanken verfügbar gemacht werden.

Insgesamt konnten in dieser Doktorarbeit gezeigt werden, dass Trematoden in mehrfacher Hinsicht zur Artenvielfalt, zum Energiefluss und zur Struktur der Ökosysteme, in denen sie vorkommen, beitragen. Der drohende Verlust der Artenvielfalt wird nicht nur zu einem erheblichen Rückgang der Vielfalt charismatischer Megafauna führen, sondern auch ihre Parasiten ausrotten, was sich auf bisher unzureichend untersuchte, aber wichtige und funktionelle Komponenten des Ökosystems auswirken wird. Auch wenn die Erkenntnisse dieser Arbeit nur einen kleinen Einblick gewähren, öffnen sie die Tür für weitere Entwicklungen in der Taxonomie der Parasiten und der ökologischen Parasitologie und regen dazu an, methodische Ansätze und Schutzmaßnahmen zu überdenken. Trotz der Tatsache, dass wir noch einen weiten Weg vor uns haben, bis wir einen ganzheitlicheren Blick auf ihre große Vielfalt und ihre funktionelle Rolle in Ökosystemen erhalten werden, sollten wir anstreben, Parasiten in unser Konzept der biologischen Vielfalt einzubeziehen. Die vorliegende Arbeit bildet daher die Grundlage für einen wichtigen Schritt zur Einbeziehung von Parasiten in die Erforschung von Ökosystemen und die Bemühungen zur Erhaltung der biologischen Vielfalt. Wir werden nur dann in der Lage sein, ein Ökosystem erfolgreich und nachhaltig zu schützen, wenn wir die zugrundeliegenden Funktionen all seiner Mitglieder vollständig verstehen.

2. Introduction



2. Introduction

Biodiversity is declining worldwide at a rapid pace. Despite the alarming rate of biodiversity loss, the predictions made to date are probably still rather conservative, as a large proportion of the species on our planet have not been officially described yet (Singh 2002; Poulin and Morand 2004; Barnosky et al. 2011; Mora et al. 2011; Engel et al. 2021). The biodiversity crisis is no longer just a topic of concern to biologists, but due to its extensive negative consequences, a global political and social issue that is becoming increasingly important in our everyday lives. Along with the repercussions of the ongoing climate change, the biodiversity crisis is one of the most urgent problems of the Anthropocene (Albert et al. 2021).

To begin discussing the biodiversity crisis, we must first clarify what the term "biodiversity" implies. There are several definitions, as is typically for such a complex term (see also Hamilton 2005; Hossain et al. 2020). However, one of the most thorough definitions is the following:

> ***Biodiversity:***
>
> *The variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are a part. This includes variation in genetic, phenotypic, phylogenetic, and functional attributes, as well as changes in abundance and distribution over time and space within and among species, biological communities and ecosystems* (IPEBS 2019a).

Accordingly, the term *biodiversity crisis* refers to the massive decline in the number and abundance of species and other biological entities worldwide. The latest Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services report (IPBES 2019b) portrays a bleak picture of biodiversity: 25 % of known plant and animal species are considered to be on the verge of extinction, the abundance of naturally occurring species has decreased by an average of 23 % in terrestrial communities, wildlife biomass has decreased by 82 % since prehistory, and the area of natural ecosystems has shrunk by 47 %. Moreover, there is broad consensus that the biodiversity crisis is directly caused and amplified by anthropogenic activities (e.g., De Vos et al. 2017; Diaz et al. 2019; Andermann et al. 2020; Bradshaw et al. 2021). The causes of the massive species extinctions and ecosystem degradation are

numerous, but land/sea use change, direct exploitation, pollution, invasive alien species, and unsustainable use of natural resources have been recognized as the primary direct drivers (IPEBS 2019b). A rapidly increasing trend of mammalian species extinctions in the recent past has been mirrored by comparable patterns in other animal groups, including birds, reptiles, amphibians, and ray-finned fishes, prompting scientists to declare the present biodiversity crisis (Barnosky et al. 2011; Andermann et al. 2020; Bradshaw et al. 2021). In the next 50 years, up to half of all species are predicted to become extinct (Pimm et al. 2000; Thomas et al. 2004). Some studies suggest that current extinction rates are 1,000 times higher than natural background extinction rates and future rates being likely to be 10,000 times higher (De Vos et al. 2014). Based on current extinction rates for mammals, projected estimates imply a significant rise in extinction rates by 2100 (Andermann et al. 2020). For some time now, experts have been contemplating a sixth mass extinction due to its alarming speed and severity of these dynamics (Barnosky et al. 2011; Ceballos et al. 2015, 2017; Andermann et al. 2020).

Even on small geographical and temporal dimensions, biodiversity has the ability to impact ecosystem functioning on a measurable scale (Loreau 2010). Loss of species has serious consequences for ecosystem resilience, recovery capacity, and adaptation to climate change (Arighton 2021). Humanity is strongly dependent on nature and its countless contributions. Nature's capacity to provide essential benefits, including environmental processes underpinning human health and nonmaterial contributions to human quality of life, has declined as a result of the decrease in the number and population size of wild species, the distinctness of ecological communities, and the extent and integrity of many terrestrial and aquatic ecosystems. (Diaz et al. 2019; Bradshaw et al. 2021). As reviewed in Bradshaw et al. (2021), a decrease of ecosystem services results in reduced carbon sequestration (Heath et al. 2005; Lal 2008), decreased pollination (Potts et al. 2016), degradation of soil (Lal 2015), deterioration of air and water quality (Smith et al. 2013), accumulation and intensification of flooding events and fires (Bradshaw et al. 2007; Hinkel et al. 2014; Boer et al. 2020; Bowman et al. 2020), and adverse effects on human health (Díaz et al. 2006; Bradshaw et al. 2019).

As described above, human activities have a major impact on the current state of our planet. Globally, large fractions of terrestrial areas (Sanderson et al. 2002), oceans (Halpern et al.

2008), and freshwater habitats (Birk et al. 2020) are anthropogenically altered. The latter are among the heavily affected and threatened ecosystems suffering from multiple stressors (Birk et al. 2020; Tickner et al. 2020). Since 1970, about 30 % of natural freshwater habitats have disappeared, and 87 % of inland wetlands have perished since 1700 (Davidson 2014; Dixon et al. 2016; Tickner et al. 2020). Numerous human activities jeopardize freshwater species and ecosystems, including habitat modification, water contamination, overfishing, exotic species introduction, river diversions, fragmentation and flow regulation, agricultural and urban landscape expansion, climate change, rising sea levels, and altered precipitation regimes (Dudgeon 2019; Grill et al. 2019; IPBES 2019b; Albert et al. 2021). However, freshwater ecosystems are also among the most diverse per habitat unit on Earth (Albert et al. 2021). Although they account for only about 1-2 % of the world's surface area and 0.007 % of the total planetary water supply, they host over 140 000 species (fungi, plants, invertebrates and vertebrates), representing over a thenth of all described species (Reid et al. 2019; Tickner et al. 2020; Albert et al. 2021). An evaluation of historical trends reveals that freshwaters are dwindling and ecosystems deteriorating at a faster rate than their terrestrial equivalents (Albert et al. 2021). Consequently, freshwater environments have higher rates of biodiversity loss than terrestrial ecosystems (Turak et al. 2017; Tickner et al. 2020), with freshwater vertebrates (fishes and amphibians) being the most endangered category of vertebrates (Reid et al. 2019; Albert et al. 2021). The massive decline of both freshwater and terrestrial groups is also closely associated with the extent of wetland loss (Albert et al. 2021).

Changes in water usage, distribution, and flow changes, in combination with climate change, land use change, and fast human population increase, have consequences for biodiversity and ecosystem services (Elmhagen et al. 2015; Albert et al. 2021). Freshwater withdrawal and water quality deterioration are currently well above levels that can maintain existing biodiversity, critical ecological processes, or good ecological status, as required by the EU Water Framework Directive (Destouni et al. 2017; Albert et al. 2021). The fast growth in human population and accompanying food production (e.g., agriculture and livestock) is putting additional strain on freshwater resources in many parts of the world (Albert et al. 2021).

Although, the dangers of climate change on people are considerably more obvious than those of biodiversity loss (Legagneux et al. 2018), society still struggles to cope with them effectively (Bradshaw et al. 2021). As a result, it is not surprising that, despite the fact that global civilisation is rapidly approaching the limits of what the Earth's biosphere can support (Steffen et al. 2015; Röckström et al. 2009; Bradshaw et al. 2021), and thus the basis of human life on Earth is severely threatened, it remains difficult to enthuse people to protect ecosystems, plants and animals. One exception might be large mammals and colourful birds, the so-called charismatic megafauna (Poulin and Morand 2004). Besides our moral duty to care for the well-being of all organisms living on this planet, there are several reasons why it is critical to conserve not only the visually appealing creatures, but all organisms (Poulin and Morand 2004). At first glance, the conservation of parsites might seen controversial, since parasites, by definition, harm and weaken other organisms. However, as described above, all organisms are part of an ecosystem and fulfil important functional roles in its complex interplay. This is also true for parasites and even though our knowledge on parasites is still very limited, there is overwhelming evidence that they are drivers of ecological speciation (Karvonen and Seehausen 2012; Betts et al. 2018) and affect ecosystems processes in various ways (Sures et al. 2017a).

As previously mentioned, our understanding of parasites is quite restricted. Parasites of medical significance for humans or livestock, on the other hand, are an exception. Species, such as *Plasmodium* spp., *Trypanosoma* spp., *Schistosoma* spp. or *Fasciola* spp., are usually examined in great detail and actively addressed (e.g., Hoffmann et al. 2014; Mishra et al. 2019; WHO 2020). However, due to their generalized poor reputation as pathogens, as well as their small size and hidden way of life, parasites are frequently disregarded or neglected in conservation approaches and ecological assessments of biodiversity (Poulin and Morand 2004; Selbach et al. 2010). Nonetheless, most free-living animals are assumed to host at least one parasite species (Marcogliese et al. 2003; Poulin and Morand 2004), and a correspondingly high diversity of parasites must be expected. Parasitism is regarded as one of the most successful and ubiquitous life forms, with estimates ranging from 30 to 50 percent of parasite contribution to global species diversity (Price 1980; Dobson et al. 2008; Poulin 2014; Carlson et al. 2019).

Despite the fact that parasites are frequently disregarded (or ignored) in environmental conservation approaches and debates, they play critical roles in every ecosystem, affecting and altering it via a variety of mechanisms. For instance, it has been demonstrated that they act as ecosystem engineers (Thomas et al. 1999) and shape and regulate host population dynamics (Hudson et al. 2006; Lefèvre et al. 2009; Frainer et al. 2018). They are known to affect intraspecific (Ruehle and Poulin 2021) as well as interspecific relations, e.g., predator-prey interactions (Lafferty and Morris 1996; Mouritsen and Poulin 2002) by influencing host growth, mortality, fitness, and behaviour (Macrogliese 2004; Lagrue and Poulin 2008; Rosenkranz et al. 2018). As a result, parasites should be regarded as major structural factors in food webs and ecosystems (Lafferty et al. 2008; Thieltges et al 2013; Dunn et al. 2013; Sánchez Barranco et al. 2020; Morton et al. 2021). Besides that, they contribute considerably to the flow of energy within and between ecosystems and account for a substantial part of the biomass in many habitats (Kuris et al. 2008; Thieltges et al. 2008; Preston et al. 2013; Lagrue and Poulin 2015; Soldánová et al. 2016). Due to their complex life cycles, parasites may also serve as bioindicators to assess environmental conditions and changes (Vidal-Martínez et al. 2010; Shea et al. 2012; Sures et al. 2017b). After all, as Hudson et al. (2006) pointed out, a healthy ecosystem can be considered one that is rich in parasites.

With about 25,000 described species and a cosmopolitan distribution, digenean trematodes belong to one of the most diverse and widespread groups of metazoan parasites on the planet (Esch et al. 2002). Trematodes have complex life cycles, with molluscs, primarily gastropods, serving as essential first intermediate hosts. Aside from vertebrates, which serve as obligate final hosts, a wide range of invertebrates and vertebrates act as second intermediate hosts (Figure 1). While the second intermediate hosts are infected actively by penetrating larval stages (cercariae), the infection of the final host usually involves trophic transmission. As a result, trematodes are intricately linked to the food web and energy flow throughout ecosystems. Furthermore, knowledge of the presence of specific trematodes in a habitat can provide precious information about the presence of the parasite's required hosts (Hechinger and Lafferty 2005; Byers et al. 2010), implying that trophically transmitted parasites in particular can act as cross-taxon surrogates for the presence of their hosts (Marcogliese et al. 2003; Moore et al. 2020).

Fortunately, issues, such as parasite extinction and conservation have recently received more attention (e.g., Spencer and Zuk 2016; Doughtery et al. 2016; Carlson et al. 2020; Kwak et al. 2020; Votýpka et al. 2020). In fact, a global plan for their protection and inclusion in monitoring programmes has even been developed (Carlson et al. 2020). In addition, the taxonomic group "parasites" is now also listed in the table of knowledge gaps (IPBES 2019b) which could raise hope for more intensive research and conservation measures regarding parasites.

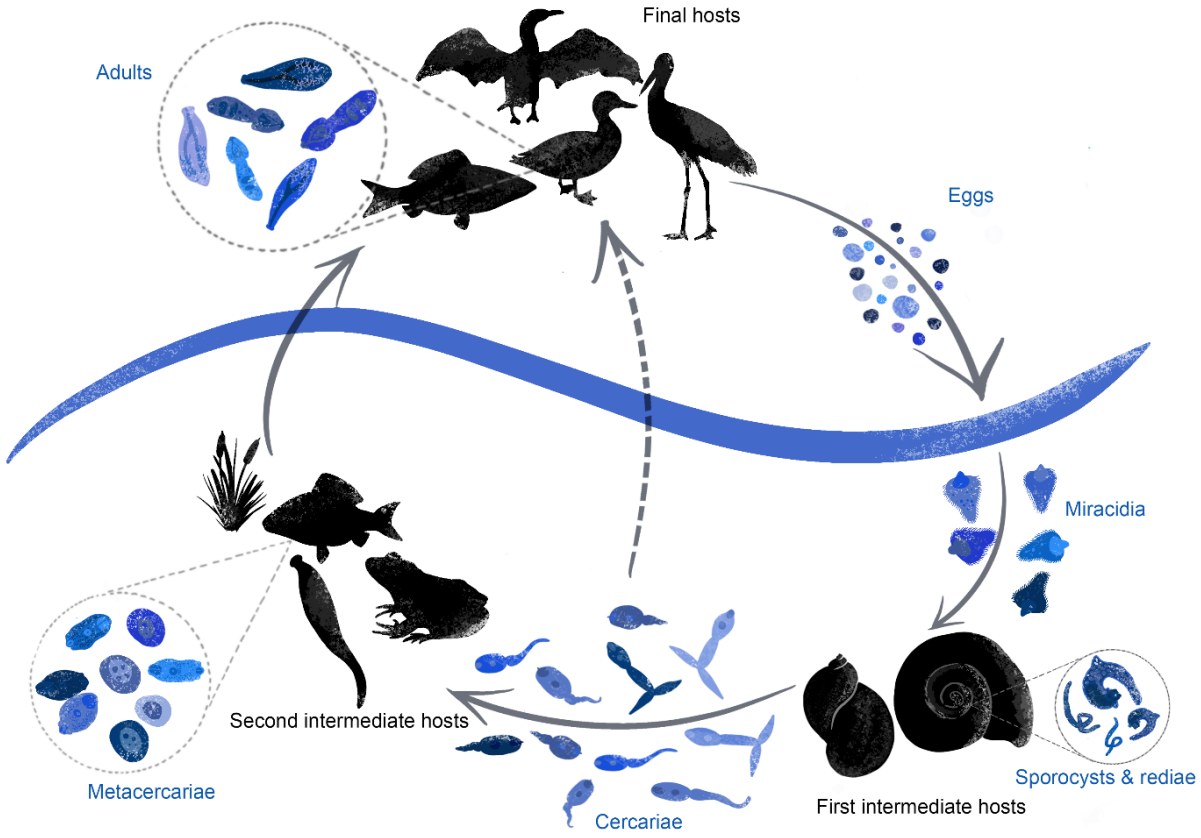


Figure 1 The life cycle of most trematodes includes two intermediate hosts and a final host. Most trematode species reside in the intestine of the final host during the adult stage and reproduce sexually there. The eggs are usually excreted via faeces or urine and thus enter aquatic ecosystems. Depending on the species, the miracidia first have to mature in the eggs before hatching. In some cases, they hatch immediately after entering the water body. This fimbriated larval stage is able to swim and actively search for its first intermediate host. Infection takes place by penetration of the skin or by oral ingestion of the eggs. Within the snail, the miracidia develop into sporocysts with considerable growth in length. Depending on the trematode species, either daughter sporocysts or daughter rediae develop. These migrate into the midgut gland and produce further generations of sporocysts or rediae and eventually the second larval stage, the cercariae. Several thousand cercariae are produced per snail over a period of days or weeks via asexual reproduction. They leave the snail through the skin in search of their second intermediate host, where they develop into metacercariae. The second intermediate host must now be consumed by the final host (e.g., waterfowl) to complete the life cycle. In some cases, as with Schistosomes, the cercariae infect their final host directly via penetration through the skin.

Parasites are extremely vulnerable to extinction due to their way of life. On the one hand, this risk is caused by direct factors, such as climate change, the introduction of invasive species

and environmental pollution (Carlson et al. 2020; Cizauskas et al. 2017). For example, parasites often react more sensitive to harmful environmental influences than their hosts. Free-swimming stages, such as cercariae, are particularly sensitive to toxic substances (Sures 2001; Sures et al. 2017b). On the other hand, they are threatened by indirect causes, such as coextinction, i.e. the extinction of their hosts (Dunn et al. 2009; Lafferty et al. 2012; Carlson et al. 2017; Cizauskas et al. 2017). Although the relevance of parasites in ecosystems has been increasingly acknowledged (e.g., Thomas et al. 1999; Hudson et al. 2006; Lefèvre et al. 2009; Watson et al. 2017; Vannatta and Minchella 2018), we still face the issue that knowledge about their distribution, life cycle and taxonomic relationships is relatively incomplete (Poulin and Morand; Poulin 2014; Sures et al. 2017a; Jorge and Poulin 2018). And that is precisely the core problem: considering the important functions parasites fulfil in ecosystems and how little we know about them, it becomes apparent that without this knowledge we are actually unable to fully grasp and understand these systems.

Overall, this knowledge gap applies to several organizational levels. The most obvious of these levels is the study of parasite diversity itself. Apart from the fact that we cannot say exactly how many parasite species exist (Polin and Morand 2004; Sures et al. 2017a), identifying those we have already discovered is a time-consuming process that requires a substantial amount of expertise and experience. This is further exacerbated by the scarcity of well-trained taxonomists, resulting in slow progress in descriptions and investigation of their taxonomic relationships (Poulin and Morand 2004). However, as knowledge in the field of parasitology advances, one aspect becomes very clear: the closer you look, the more diversity you find (Poulin and Morand 2004). This may be demonstrated, for example, in the increasing number of detected cryptic species and species complexes, of which there are several examples nowadays. For instance, the eye fluke *Diplostomum mergi* was for a long time considered to be a generalist species until it was discovered that it is in fact a species complex whose members are quite host-specific with respect to their final host (Selbach et al. 2015). Likewise, *Neopetasiger* spp. (Selbach et al. 2014), *Echinostoma revolutum* species complex (Georgieva et al. 2013, 2014), and *Plagiorchis* spp. (Zikmundová et al. 2014; Soldánová et al. 2017) are further examples. When we zoom out from the level of the parasites and take a glance at the hosts, we can observe that there is also a paucity of knowledge on certain hosts or taxonomic groups. This includes not only the first and second intermediate hosts, but also the final hosts,

which are exclusively vertebrates and hence have a very limited availability for parasitological investigations due to their often protected status. For a different reason, but similarly disillusioning, is the state of knowledge on parasites of invertebrates. Invertebrates and their diversity are typically less extensively explored than vertebrates. Consequently, we know less about their parasites in general, as evidenced by the fact that for many trematodes, we may only know the adult stage that parasitizes vertebrates (especially those, which are commonly investigated, e.g., for medical reasons), rather than the accompanying larval stages often parasitizing invertebrates (Poulin and Morand 2004).

If we take a further step back and again zoom out a little more to the ecosystem level, we find a similar pattern. Knowledge about parasites and their diversity is patchy and unevenly distributed. As a general rule, our understanding of parasites is higher in temperate regions than in tropical areas (Poulin and Morand 2004). Regardless of this pattern, some of the ecosystems that tend to be poorly studied have been very well explored due to the efforts of individual research groups. For example, the group of researchers led by T. Cribb has detected a large proportion of the trematodes known to parasitize Australian fish (Poulin and Morand 2004). Other ecosystems, however, are particularly accessible, which facilitates faunistic studies of parasites (e.g., Faltýnková and Haas 2006; Grabner et al. 2017; Soldánová et al. 2017; Selbach et al. 2020). On the other hand, certain habitats have received relatively little attention in parasite surveys due to their poor accessibility and associated sampling effort, or due to their rarity and associated conservation status. In Europe, this is especially true for natural and protected areas, as these ecosystems are becoming increasingly scarce and sometimes hard to access. Due to limitations and protective measures, assessing parasite biodiversity in such regions is fraught with various obstacles and necessarily entails a considerable administrative burden.

Although there is increasing research on the diversity, community structure and distribution of trematode communities in man-made and anthropogenically influenced freshwater systems (e.g., Faltýnková 2005; Żbikowska 2007; Schwelm et al. 2018; Selbach et al. 2020), our knowledge of parasites in natural systems is often still limited. This remains a significant barrier, as knowledge on trematode diversity in protected areas may provide the best estimate of the parasites' natural state in these environments and regions, which may be used

to evaluate changes in trematode community composition in extensively altered ecosystems. In the absence of this basis for comparison, it will remain difficult to anticipate whether and how parasites will respond to continuing anthropogenic habitat alterations and climate change and how these changes will influence the ecosystem processes regulated by parasites. In the light of the rapidly progressing biodiversity crisis the lack of this basic knowledge may also imply that numerous parasite species become extinct before they could even be discovered, let alone examined and documented thoroughly (Sures et al. 2017a). Conversely, this also entails that we are massively missing out on knowledge of species and their important functional roles in ecosystems.

This thesis, therefore, aims to explore the yet unknown trematode diversity in European freshwater ecosystems at different organizational levels from individual parasite taxa, to host communities, to the ecosystem level. In detail, I hypothesize to uncover new parasite diversity at each of these levels, i.e., (i) cryptic parasite diversity and exploration of species boundaries within a selected trematode genus, (ii) parasite diversity within a neglected snail host community, and (iii) species richness and diversity of parasites in a protected near natural ecosystem. Accordingly, the thesis consists of 3 papers/chapters that will explore each of these objectives in detail.

3. Material and methods

3. Material and methods

This chapter provides a general overview of the sampling sites, the field work, and the workflow in the laboratory. More detailed descriptions of materials and methods of the individual studies are presented in the respective chapters.

Study area

In order to investigate the trematode diversity and ecology at different water bodies, a total of 11 sample sites were examined for aquatic snails at three riparian systems (Ruhr, Lippe, and Rhine) in North Rhine-Westphalia (Fig. 2). In the following, the different water bodies and sampling sites are briefly characterised.

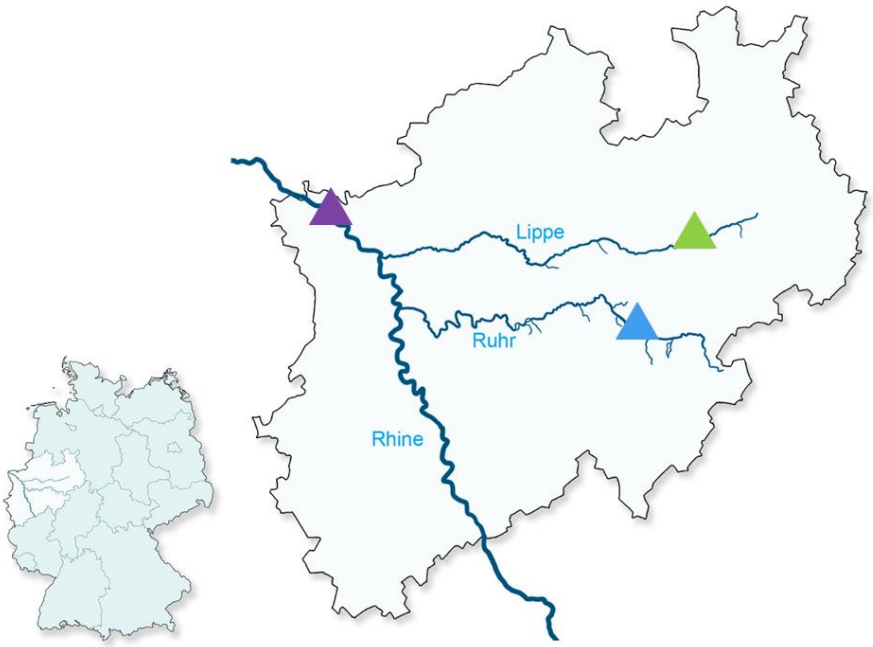


Figure 2 Map of Germany and the federal state of North Rhine-Westphalia indicating sampling locations along the rivers Ruhr, Lippe and Rhine. The sampling location of the river Ruhr is marked with a blue triangle, the sampling location at the river Lippe is marked with a green triangle and the sampling location at the river Rhine is marked with a purple triangle.

Sampling locations at the river Ruhr, river Lippe and river Rhine

The sampling location of the river Ruhr belong to the upper part of the Ruhr and is located near the city of Arnsberg (sites "Binnerfeld"), North Rhine-Westphalia. In total, aquatic gastropods were collected at four sites at the river section "Binnerfeld" (B0: 51°26'24.4"N 7°58'35.8"E; B1: 51°26'25.9 "N 7°57'50.9"E; B2: 51°26'53.2"N 7°57'09.5"E; B3: 51°26'57.7"N 7°57'05.8"E) (Figure 3a-d).

The sampling sites of the river Lippe are located near the city of Lippstadt. Due to regular flooding events, the main river is periodically connected to its floodplain. The area is managed and monitored by the ABU Soest and has been under nature conservation since the year 1993 (Bezirksregierung Arnsberg 2010). The Lippe and its floodplain are a species-rich area including rare and endangered species, e.g., the burbot (*Lota lota*) or the river nerit (*Theodoxus fluviatilis*) (Bezirksregierung Arnsberg 2010). The Lippe floodplain, in particular, is home to Taurus cattle and Konik horses, which ensure a diversely structured semi-open landscape through their grazing activity. In total, four sampling sites (K0: 51°39'44.1"N 8°10'23.9"E; K1: 51°39'42.3"N 8°13'49.3"E; K2: 51°39'41.5"N 8°14'18.1"E; K3: 51°39'41.8"N 8°14'19.2 were studied (Figure 3e-g).

The sampling sites of the lower river Rhine are located near the urban area of Rees. The water body Bienener Altrhein is a tributary of the oxbow lake of the river Rhine. Until 1800, the Rhine was largely unaffected. During industrialisation, however, dyking, canalisation, and thus the decoupling of backwaters from the main watercourse occurred. The Bienener Altrhein nature reserve is part of the Rhine floodplains between Rees and Emmerich, which are among the few remaining natural floodplains in Europe. They form a unique system of oxbow lakes that has been protected since 1969. Various measures (e.g., partial desilting, acquisition, and extensification of adjacent agricultural land) have been taken to preserve this area as natural as possible and to prevent siltation and increased nutrient input (Brühne and Schabert 2005). It is a very bird-rich area, where many migratory birds, like geese, swans, or ducks, rest and breed. Among them are rare and endangered bird species, such as the black tern (*Chlidonias niger*) (Vossmeyer 2009). Two of the sampling sites are located at the main watercourse (R1: 51°47'59.2 "N 6°21'46.3 "E; R3: 51°48'37.1 "N 6°21'23.4 "E), the third sampling site is a groundwater-fed pond in the adjacent floodplain (R2: 51°49'07.0 "N 6°20'26.8 "E) (Fig. 3h-j).

Figure 3 Overview of all 11 sampling sites: a: sampling site B0; b: sampling site B1; c: sampling site B2 d: sampling site B3; e: sampling sites K1 and K2; f: sampling site K3; g: sampling site K0; h: sampling site R1; i: sampling site R2; j: sampling site R3

Sample collection and examination

During monthly collections aqutic gastropods of 20 species belonging to 8 families were collected and examined for trematode infections. Sampling sites were sampled monthly, but not all of them over the same period due to restrictions caused by nature conservation measures, e.g., protection of breeding birds, but also due to seasonal occurence of snails and time management. A detailed overview of the sampling periods and the respective sampling sites can be found in Figure 4. In 2016-2017, all snail species present were collected, while in 2019 only one species, *Ampullaceana balthica*, was collected specifically. Snails were collected

by hand and by sieve from driftwood, stones and floating and submerged aquatic vegetation within a time frame of 30 minutes per site. In the laboratory, the shell width and height of all snails were measured [mm] using a digital calliper following Glöer (2002) and sorted by species and sampling site. Individual snails were placed in separate containers of filtered river water and kept in laboratory conditions (20 °C) under a light source to stimulate the emergence of cercariae. After the day of sampling, each container was examined daily for three consecutive days under a stereomicroscope for the presence of cercariae. Snails that did not show infections with trematodes during this period were dissected and examined for pre-patent infections (rediae/sporocysts). Additionally, 15 individuals of the Eurasian minnow *Phoxinus phoxinus* were sampled via electrofishing in June 2019 at the River Ruhr. All fish were dissected in the laboratory and examined for the presence of metacercariae in the brain. Cercariae, sporocysts, rediae, and metacercariae were fixed in molecular ethanol for DNA isolation and sequencing and preserved in a 4% formaldehyde solution for morphological analyses (see Fig. 5).

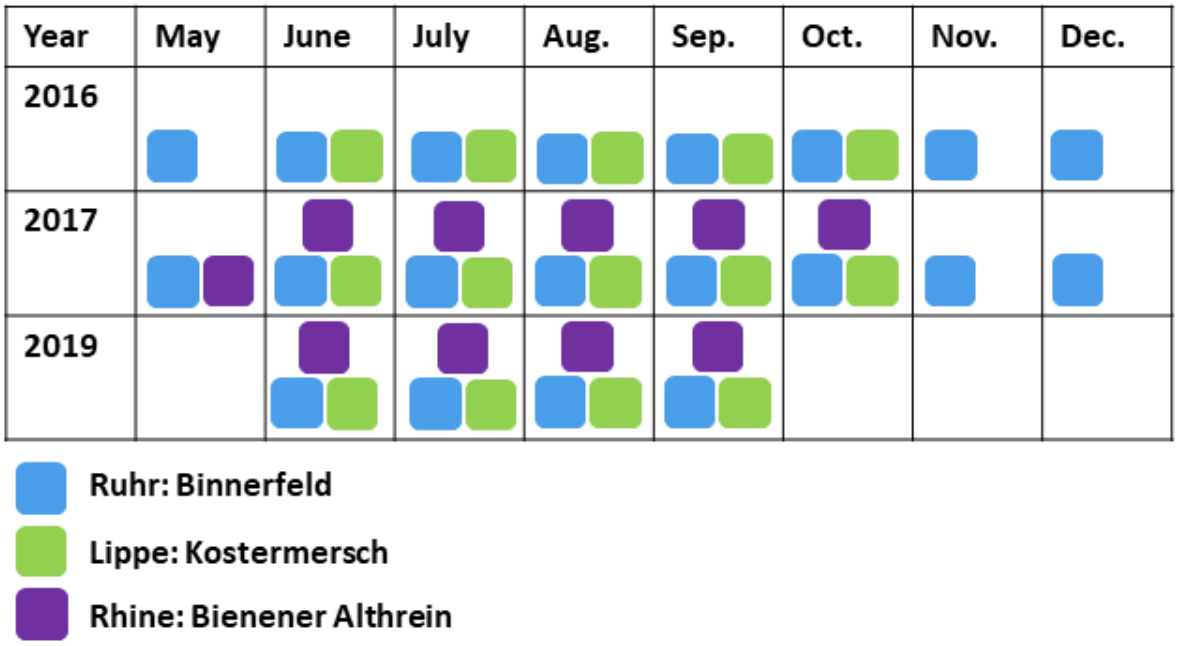


Figure 4 Overview of samplings carried out at the individual water bodies between the years 2016 and 2019.

Trematode identification

Morphological identification

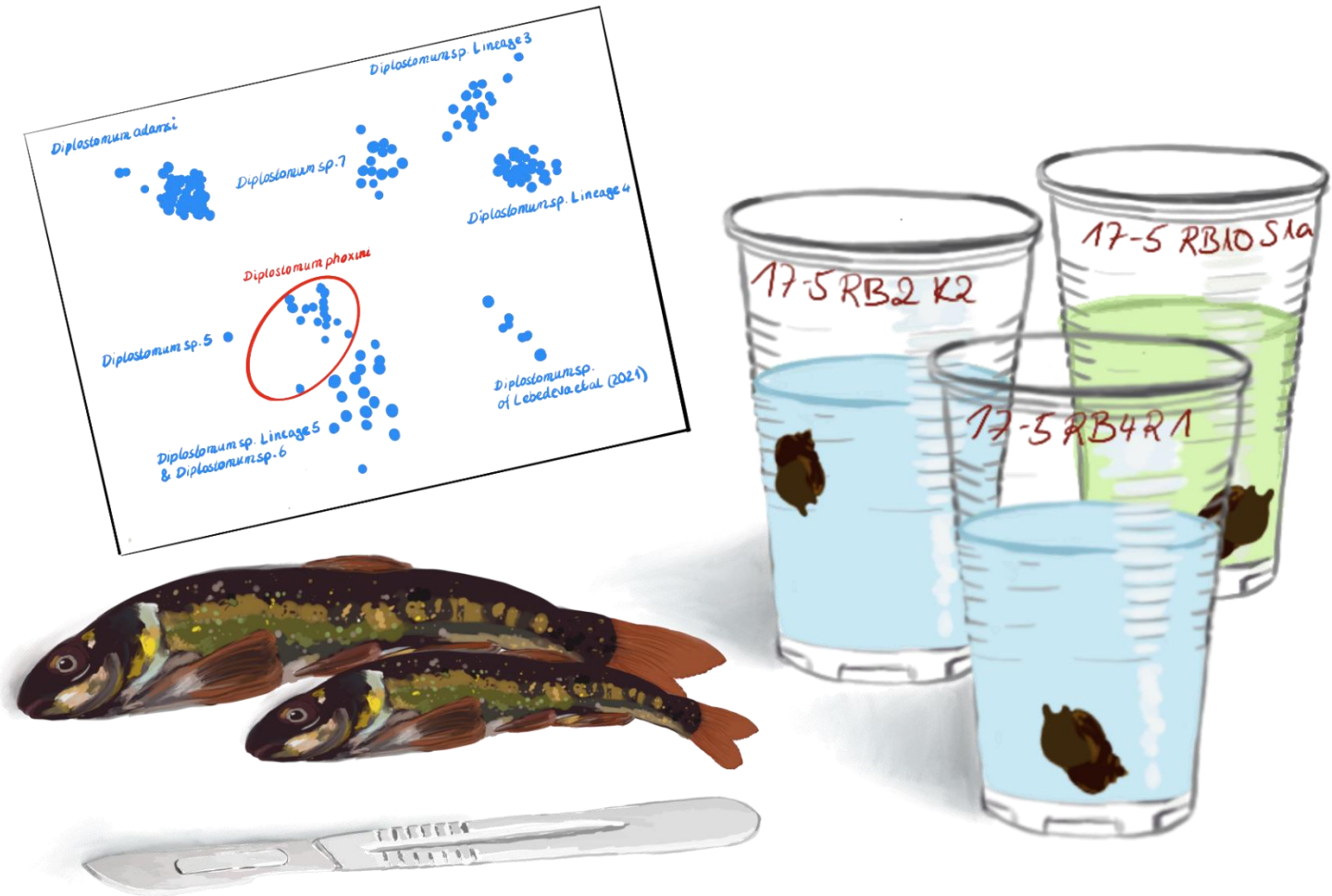
Trematode larval stages were identified alive using light-microscopy (Olympus BX51). Cercariae were identified to the species level based on the morphological descriptions and appropriate identification keys or other relevant primary sources (e.g., Arvy and Buttner 1954; Hugghins 1954; Rees 1957; Zdun 1961; Dönges 1969; Bykhovskaya-Pavlovskaya and Kulakova 1971; Našincová 1992; Našincová and Scholz, 1994; Faltýnková et al. 2007, 2008; Selbach et al. 2014; Selbach et al. 2015; Kudlai et al. 2015). Morphology of cercariae was studied on live and fixed specimens. Live photomicrographs were taken with an Olympus UC30 digital camera for measurements and further identification. For documentation and measurements of the snail hosts photomicrographs of the snail shell were taken with a Keyence VHX5000 microscope.

Molecular identification

For further investigation of specimens that were not able to be accurately identified and for species belonging to taxonomically problematic groups with cryptic diversity, e.g., *Echinostoma* spp. (Georgieva et al. 2013, 2014) and *Diplostomum* spp. (Selbach et al. 2015), trematode material was fixed in molecular grade ethanol and hot and cold 4 % formaldehyde solution for molecular and morphological studies, respectively. Additionally, foot tissue from infected snails was fixed in molecular grade ethanol for molecular analysis and identification of the host. DNA isolation was performed following a modified salt precipitation protocol after Sunnucks and Hales (1996) and Grabner et al. (2015). Target gene fragments (*cox*1, 28S, ITS1-5.8S-ITS2, ITS2, and *nad*1) were chosen based on preliminary identification of cercariae and were amplified through polymerase chain reaction (PCR). PCR products were purified and sent to a commercial sequencing company. For species identification, each sequence was compared to sequences available in GenBank by using the Basic Local Alignment Search Tool (BLAST).

Figure 5 Workflow for sampling and identification of trematode infections in snails. a: Collecting snails; b: Separating snails in individual containers with additional light sources; c: Inspecting each snail for released cercariae under binoculars; d: Free-swimming cercariae in the water column (larvae marked with red arrows); e: Sporocysts and prepatent cercariae from dissected snail under binoculars; f: Cercaria under light microscopy; g: Identification of trematode species using morphological and/or molecular tools.

4. Chapter I
Taxonomy and morphology of a selected trematode species



Parasitology

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Molecular and morphological characterisation of *Diplostomum phoxini* (Faust, 1918) with a revised classification and an updated nomenclature of the species-level lineages of *Diplostomum* (Digenea: Diplostomidae) sequenced worldwide

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Research Article

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Abstract

We characterised morphologically and molecularly *Diplostomum phoxini* (Faust, 1918) based on cercarial isolates from the snail *Ampullaceana balthica* (L.) (Gastropoda: Lymnaeidae) and metacercariae from the Eurasian minnow, *Phoxinus phoxinus* (L.) (Cypriniformes: Leuciscidae), and provided molecular evidence for the identification of the snail intermediate host. Phylogenetic analyses based on the cytochrome *c* oxidase subunit 1 (*cox*1) gene depicted 44 molecularly characterised species and genetically distinct lineages of *Diplostomum*, and resulted in: (i) a re-identification/re-classification of 98 isolates plus *D. baeri* sampled in North America; (ii) re-definition of the composition of the *D. baeri* species complex which now includes nine molecularly characterised species/lineages; (iii) re-definition of the composition of the *D. mergi* species complex which now includes seven molecularly characterised species/lineages; and (iv) an updated nomenclature for the molecularly characterised species-level lineages of *Diplostomum*.

Introduction

The application of molecular tools for characterisation and phylogenetic analyses has greatly advanced our understanding of the diversity, taxonomy, systematics and phylogeny of virtually all major groups of parasitic worms. Molecular data have become a 'must-have' characteristic not only in species discovery and delineation but also in large-scale biodiversity inventories, and ecological and evolutionary research. This is especially true for the trematode subclass Digenea, parasitic flatworms representing a remarkable example of the diversity of complex life-cycles among the Metazoa (Minelli and Fusco, 2010), which involve alternation of generations and a diversity of phenotypes in the sequential hosts in the life-cycle.

The digenean genus *Diplostomum* von Nordmann, 1832 (Diplostomidae) has received increased attention in recent years. Intensive sampling and molecular analyses predominantly of the larval stages of *Diplostomum* spp. from their intermediate hosts, freshwater lymnaeid snails and fishes, have resulted in delineation of more than 40 species/species-level lineages (Locke *et al.*, 2010*a*, 2010*b*, 2015, 2020; Georgieva *et al.*, 2013; Blasco-Costa *et al.*, 2014; Faltýnková *et al.*, 2014; Pérez-del-Olmo *et al.*, 2014; Selbach *et al.*, 2015; Kudlai *et al.*, 2017; Soldánová *et al.*, 2017; Gordy and Hanington, 2019; Hoogendoorn *et al.*, 2020; Lebedeva *et al.*, 2021). Complete mitochondrial genomes have been characterised for four species, *Diplostomum spathaceum* (Rudolphi, 1819) and *D. pseudospathaceum* Niewiadomska, 1984 (see Brabec *et al.*, 2015), *D. ardeae* Dubois, 1969 (see Locke *et al.*, 2020) and *Diplostomum baeri* Dubois, 1937 (see Landeryou *et al.*, 2020).

However, the number of named molecularly characterised species remains low because of the difficulties in gathering adult worms from their definitive hosts (fish-eating birds) and the virtual lack of taxonomic expertise in identification of the larval stages. These include *D. spathaceum* (Rudolphi, 1819) (type-species), *D. ardeae* Dubois, 1969, *D. baeri sensu* Galazzo *et al.* (2002), *D. huronense* (La Rue, 1927), *D. indistinctum* (Guberlet, 1922), *D. lunaschiae* (Locke, Drago, Núñez, Rangel e Souza & Takemoto, 2020), *D. pseudospathaceum* Niewiadomska, 1984 and *D. parviventosum* Dubois, 1932. Phylogenetic analyses have depicted two species complexes among the prevailing unnamed species-level lineages. The *D. mergi* complex comprises one named species and three species-level lineages (*D. parviventosum*; *D. mergi* Lineages 2 and 3 of Georgieva *et al.* (2013); and *D. mergi* Lineage 4 of Selbach *et al.* (2015)), and the *D. baeri* complex *sensu* Blasco-Costa *et al.* (2014) comprises one named species and seven species-level lineages (*D. baeri sensu* Galazzo *et al.* (2002),

Diplostomum sp. Lineages 3–5 of Blasco-Costa *et al.* (2014), *Diplostomum* sp. 2 of Moszczynska *et al.* (2009), and *Diplostomum* spp. 5–7 of Locke *et al.* (2010*a*)). Of these, seven lineages have been sequenced and morphologically characterised in Europe based on larval isolates (Blasco-Costa *et al.*, 2014; Faltýnková *et al.*, 2014; Selbach *et al.*, 2015; Lebedeva *et al.*, 2021).

In a study of larval digenean communities in the snail host *Ampullaceana balthica* (L.) (Gastropoda: Lymnaeidae) in the River Ruhr drainage, we collected a number of cercarial isolates which at first glance resembled morphologically the known cercariae of the *D. baeri* species complex. However, sequencing of the cytochrome *c* oxidase subunit 1 (*cox*1) mitochondrial gene indicated high similarity with two isolates of *Diplostomum phoxini* (Faust, 1919) collected in Norway (Soldánová *et al.*, 2017). Therefore, we sampled the specific second intermediate host of this species, *Phoxinus phoxinus* (L.) (Cypriniformes: Leuciscidae), and sequenced the metacercariae recovered from the brain of the fish. Here, we provide molecular and morphological characterisation of *D. phoxini*, one of the few species of *Diplostomum* exhibiting strict host specificity to the second intermediate host (*P. phoxinus*), and molecular evidence for the identification of its first intermediate host (*A. balthica*). Phylogenetic analyses revealed changes in the composition of the *D. baeri* and *D. mergi* species complexes, and resulted in a re-classification of a large number of sequenced isolates. Finally, we compare the prevalence of *D. phoxini* and the molecularly characterised species/lineages of the *D. baeri* complex in the lentic and lotic aquatic habitats of Europe.

Materials and methods

Sample collection and examination

A total of 1599 *Ampullaceana balthica* (Gastropoda: Lymnaeidae; formerly often reported as *Radix balthica*) were collected and examined for trematode infections during spring (May), summer (June, July, August), autumn (September, October, November) and winter (December) in 2016 and 2017 and from May to September in 2019. Snails were collected at three sampling sites at the River Ruhr at Neheim (North Rhine-Westphalia, Germany): B0 (51°26′24.4″N, 7°58′35.8″E); B1 (51°26′25.9″N, 7°57′50.9″E) and B2 (51° 26′53.2″N, 7°57′09.5″E). All snails were collected by hand and using a strainer from stones, driftwood and macrophytes, or picked directly from the sediment in shallow, slow-moving parts of the river. In the laboratory, snails were measured (shell width and height) and placed separately in containers with filtered river water under a light source to stimulate the emergence of cercariae. All containers were checked under light microscope for three consecutive days for the presence of cercariae in the water column. On the fourth day, all snails were dissected and examined for the presence of prepatent infections (sporocysts) (as described, e.g. in Selbach *et al.*, 2015; Schwelm *et al.*, 2018). Additionally, 15 specimens of the Eurasian minnow *Phoxinus phoxinus* (L.) were sampled *via* electrofishing at one site of the River Ruhr at Arnsberg (B4) (51° 24′04.3″N, 8°04′01.1″E) in June 2019. In the laboratory, all fish were identified using Kottelat and Freyhof (2007), dissected and investigated for the presence of metacercariae in the brain.

Cercariae and metacercariae were fixed in molecular grade ethanol for DNA isolation and sequencing, and in 4% formaldehyde solution for morphological analyses (scanning electron microscopy, SEM). Cercariae studied by SEM were cleaned and subsequently post-fixed in 1% osmium tetroxide for 2 h, washed in 0.1 M phosphate buffer, dehydrated in an ethanol series, critical-point dried, sputter-coated with gold and examined and photographed with a scanning electron microscope (Hitachi 4100 FE Ltd., Tokio, Japan) at 20 kV at the Central Service for Experimental Research (SCSIE), University of Valencia, Spain. Foot tissue from snails was fixed in molecular grade ethanol for molecular identification.

Morphological data

Trematode larval stages were identified live using light microscopy (Olympus BX51, Tokyo, Japan). Cercariae and metacercariae were identified to the species level based on the morphological descriptions of Arvy and Buttner (1954), Rees (1957) and Dönges (1969*a*, 1969*b*). Series of detailed light microscopy photographs of cercariae and metacercariae of *D. phoxini* were taken with a digital camera (Olympus UC30, Tokyo, Japan) attached to the light microscope and all visible features were recorded. Descriptions of the cercariae are based on examination of live material and digital photomicrographs from both, light microscopy and SEM. Measurements were taken with the program ImageJ 1.47v (available from https://imagej.nih.gov/ij/download.html) and are given in micrometres as the range followed by the mean in parentheses. The following abbreviations were used in the description of the cercaria: AOW, anterior organ width; BL, body length; FL, furca length; TSL, tail stem length; VSW, ventral sucker width.

Molecular data

Total genomic DNA (gDNA) was isolated from ethanol-fixed snail tissue, pooled samples of 10–15 cercariae or single metacercariae by placing the samples in 200 μL of a 5% suspension of deionised water and Chelex®, containing 0.1 mg mL^{-1} proteinase K, followed by incubation at 56°C for 3 h, boiling at 90°C for 8 min, and centrifugation at 14 000× *g* for 10 min. Polymerase chain reaction (PCR) amplification was carried out using 2× MyFi Mix (Meridian Bioscience, Cincinnati, USA), 8 pmol of each primer and *c.*50 ng of gDNA in a total volume of 20 μL. Partial fragments of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene, the nuclear 28S rRNA gene and the complete ITS1-5.8S-ITS2 gene cluster (ITS2 only for *A. balthica*) were sequenced for the snail host and larval stages of *D. phoxini* using the primers and cycling conditions listed in Online Resource Table S1.

PCR amplicons were purified using QIAquick PCR purification kit (Qiagen Ltd, Hilden, Germany) following the manufacturer's instructions. PCR fragments were sequenced directly with ABI BigDye chemistry (ABI Perkin-Elmer, UK), alcohol-precipitated and run on an ABI Prism 3730XL DNA analyser using the primers listed in Online Resource Table S1.

Newly generated and published sequences were aligned with MAFFT v.7 (Kuraku *et al.*, 2013; Katoh *et al.*, 2019). The *cox*1 sequences were aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code (translation table 9; Telford *et al.*, 2000) for parasite isolates and the invertebrate mitochondrial code (translation table 5) for snail host isolates; the alignments contained no insertions or deletions.

Molecular identification/delimitation of the snail and parasite samples was achieved using neighbour-joining (NJ) analyses of Kimura 2-parameter distances for the *cox*1 alignments conducted with MEGA v.7 (Kumar *et al.*, 2016); nodal support was estimated using 1000 bootstrap replicates. Genetic distances (uncorrected p-distance) were calculated with MEGA v.7. Non-metric multidimensional scaling (NMDS) plot was generated with Primer v.6 software (Anderson *et al.*, 2008) to visualize the raw pairwise distances between species/lineages of the *D. baeri* species complex. Unique haplotypes were identified with DnaSP (Rozas *et al.*, 2003) against the recently published sequences for *D. phoxini* by Lebedeva *et al.* (2021).

Table 1. Summary data for isolates of *Diplostomum phoxini* and *Ampullaceana balthica* from the River Ruhr at Neheim (Germany) used for generation of the new *cox*1, ITS1-5.8S-ITS2 and 28S rDNA (domains D1–D3) sequences

Species	Site	Isolate	Life-cycle stage	GenBank ID (*cox*1/ITS1-5.8S-ITS2/28S)
D. phoxini ex *A. balthica*	B1	DphoAbal1	C	MZ615631
	B0	DphoAbal2	C	MZ615632
	B1	DphoAbal3	C	MZ615633
	B0	DphoAbal4	C	MZ615634/MZ616379[a]
	B0	DphoAbal5	C	MZ615635
	B2	DphoAbal6	C	MZ615636
	B2	DphoAbal7	C	MZ615637
D. phoxini ex *P. phoxinus*	B4	DphoPpho1	M	MZ615638/MZ616381/MZ616380
	B4	DphoPpho2	M	MZ615639/MZ616382[b]
A. balthica	B1	AbalRuhr1	A	MZ615629/MZ616378[c]/MZ616383
	B0	AbalRuhr1	A	MZ615630

Abbreviations: A, adult; C, cercaria; M, metacercaria.
[a]28S.
[b]ITS1-5.8S-ITS2.
[c]ITS2 only.

Species relationships within *Diplostomum* were assessed using Bayesian inference (BI) analysis of *cox*1 data. Prior to analysis, the best-fitting model of nucleotide substitution (HKY + Γ + I) was estimated based on the Bayesian information criterion (BIC) using jModelTest v. 2.1.4 (Darriba *et al.*, 2012). BI analysis was carried out with MrBayes v. 3.2.7 (Ronquist *et al.*, 2012) on the CIPRES Science Gateway v.3.3 (Miller *et al.*, 2010) using Markov chain Monte Carlo searches on two simultaneous runs of four chains for 10^7 generations, sampling trees every 10^3 generations. The 'burn-in' determined by stationarity of lnL assessed with Tracer v.1.5 (http://beast.bio.ed.ac.uk/Tracer) was set for the first 25% of the trees sampled, and a consensus topology and nodal support estimated as posterior probability values (Huelsenbeck *et al.*, 2001) were calculated from the remaining trees. Phylogenetic trees were visualised and finalised in FigTree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/.)).

Results

Prevalence of Diplostomum phoxini *in the intermediate hosts*

A total of 1599 *Ampullaceana balthica* representing 41 distinct individual samples (i.e. collected at a given place and date, as per the definition of Bush *et al.*, 1997) were collected in the River Ruhr at Neheim from May to December during 2016, 2017 and 2019, and examined for prepatent and patent infections with *Diplostomum* spp. Infections with *D. phoxini* were detected from July to November (prevalence range: 3.3–13.6%, see Online Resource Table S2 for details) with the largest number of infected snails and greatest prevalence being recorded in September (five samples). Although large samples were examined in May (12.v.–22.v.; $n = 255$) and June (7.vi.–25.vi.; $n = 250$), no snails infected with *D. phoxini* were found (see Online Resource Table S2).

All dissected *P. phoxinus* ($n = 15$) were infected with large numbers of metacercariae of *D. phoxini* ($n > 100$) located in the optical lobes of the brain.

Molecular identification of the snail host

Representative *cox*1 (591–610 nt; $n = 2$), 28S (1048 nt; $n = 1$) and ITS2 (402 nt; $n = 1$) sequences were generated for the snail host *A. balthica*. The new *cox*1 sequences differed at two nucleotide (nt) positions (0.03%). The new 28S rDNA sequence was identical with a sequence for *A. balthica* originally identified as *R. ovata* (EF417136; see Sonnenberg *et al.*, 2007) and differed at a single-nucleotide position from an isolate originating from Russia (MH168039; Aksenova *et al.*, 2018). ITS2 sequence comparisons revealed differences at 0–3 nt positions between the present isolate and the data for *A. balthica* available on GenBank; the new ITS2 sequence was identical with sequences for a total of 55 isolates of *A. balthica* originating from Belgium, Iceland, Norway and the UK.

Molecular identification of the snail host was further carried out on a *cox*1 alignment corresponding to the clade representing the subfamily Amphipepleinae Pini, 1877 in Aksenova *et al.* (2018) (36 species; 43 sequences; 636 nt) using *Galba truncatula* (O.F. Müller) (Lymnaeidae) as the outgroup. As shown in the NJ tree in Online Resource Fig. S1, the two newly generated *cox*1 sequences fell within the strongly supported clade of *Ampullaceana* spp. and clustered with five sequences for *A. balthica* originating from Europe and Asia with high support, thus confirming their identification based on morphology and the reclassification of *Radix balthica* to *Ampullaceana* (see Aksenova *et al.*, 2018).

Molecular characterization of Diplostomum phoxini

Partial *cox*1 sequences (349–407 nt) of *D. phoxini* were generated for a total of nine isolates (seven cercarial and two metacercarial), representing eight haplotypes (Table 1). Genetic divergence between seven cercarial and one metacercarial isolate and the two isolates of *D. phoxini* sequenced from Norway (see Soldánová *et al.*, 2017) ranged between 0% and 1.2% (0–5 nt difference), whereas one newly sequenced metacercarial isolate (MZ615639) exhibited considerable divergence (2.2–3.1%, 9–12 nt difference) in the comparisons with the remaining isolates of *D. phoxini* from River Ruhr. A comparison with the recently published sequences for *D. phoxini* from *P. phoxinus* in Finland and Russia (Lebedeva *et al.*, 2021) revealed an overall range for genetic divergence of 0–1.3%, excluding the most divergent haplotype sequenced from River Ruhr (MZ615639) and one most divergent haplotype sequenced from River Varzuga, Russia (MT982208: 3.5–4.8%; the upper limit represents the divergence between these two most divergent haplotypes). A total of 15 haplotypes were identified among the isolates sampled in Europe, including six novel haplotypes from the present material, six haplotypes

Table 2. Percent interspecific genetic divergence (p-distance model) for *D. phoxini* compared with the species/lineages of the *D. baeri* species complex based on all *cox*1 sequences available on GenBank (retrieved on 29 June 2021)

Species/Lineage	*n*	Divergence (%)
D. adamsi (syn. *D. baeri sensu* Galazzo *et al.*, 2002)	979	8.7–11.6
Diplostomum sp. 5 of Locke *et al.* (2010*a*)	11	8.8–9.5
Diplostomum sp. 6 of Locke *et al.* (2010*a*)	44	8.3–10.0
Diplostomum sp. 7 of Locke *et al.* (2010*a*)	176	7.5–9.6
Diplostomum sp. Lineage 3 of Blasco-Costa *et al.* (2014)	451	9.1–12.3
Diplostomum sp. Lineage 4 of Blasco-Costa *et al.* (2014)	473	8.7–11.3
Diplostomum sp. Lineage 5 of Blasco-Costa *et al.* (2014)	286	6.9–9.2
Diplostomum sp. of Lebedeva *et al.* (2021)	144	9.1–11.1

Abbreviation: n, number of pairwise comparisons

from the material of Lebedeva *et al.* (2021) and one haplotype (KY513185) reported from Norway by Soldánová *et al.* (2017). One haplotype (MZ615634) was shared with an isolate ex *P. phoxinus* from Lake Ovre Heimdalsvatnet, Norway (KY513186; Soldánová *et al.*, 2017) and an isolate ex *P. phoxinus* from River Uksa, Russia (MT982204; Lebedeva *et al.*, 2021) and one haplotype was represented by two cercarial isolates from the River Ruhr (MZ615631 and MZ615632). Genetic divergence between *D. phoxini* and the species of the *D. baeri* complex ranged between 6.9% and 12.3% (Table 2).

Additionally, two partial 28S (1214–1223 nt) and two complete ITS1-5.8S-ITS2 (1046–1049 nt) rDNA sequences were generated for representative cercarial and metacercarial isolates of *D. phoxini* (Table 1). The newly generated 28S sequences were identical and differed at six positions from the published sequence for *D. phoxini* by Olson *et al.* (2003).

Morphological characterisation of Diplostomum phoxini

Description of the cercaria

[Based on 40 live specimens; (Figs 1–3; Table 3 and Online Resource Table S3)] Body elongate-oval, 138–154 × 37–50, shorter than tail stem (TSL/BL = 1.34–1.62). Anterior organ elongate-oval, 52–59 × 27–31. Ventral sucker subspherical, with small undulating membrane (Figs 2 and 3), just post-equatorial, 30–34 × 30–33; width exceeding width of anterior organ (AOW/VSW = 0.84–0.94). Mouth opening ventro-subterminal; prepharynx long, narrower in anterior organ; pharynx round to elongate-oval, muscular, 11–14 × 12–16, followed by short oesophagus bifurcating anterior to ventral sucker; intestinal caeca well developed, terminating almost at posterior extremity of body. Penetration gland-cells two pairs with fine granular content, similar in size, posterior to ventral sucker, overlap caeca, posterior pair not reaching extremities of caeca; ducts open antero-laterally to mouth, two on either side. Anlagen of reproductive organs a compact mass of small cells just anterior to excretory vesicle. Tail stem 212–226 long, 29–37 wide at base, nearly as long as furcae, 212–239 long, 13–24 wide at base (TSL/FL = 0.95–1.06), with six pairs of caudal bodies with slightly irregular margins along excretory duct. Furcae 212–239 long, 13–24 wide at base, without fin-folds. Excretory vesicle small, V-shaped, with round stem; caudal excretory duct passes through tail stem; excretory pores at mid-length of furcae.

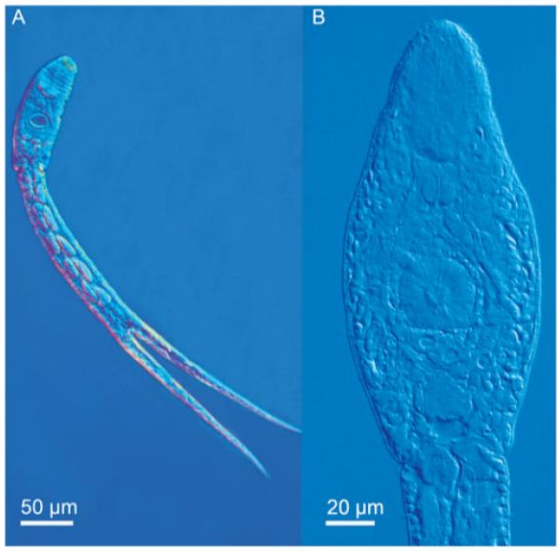

Fig. 1. Cercaria of *Diplostomum phoxini* ex *Ampullaceana balthica* (light microscopy). A, Resting position; B, Cercarial body.

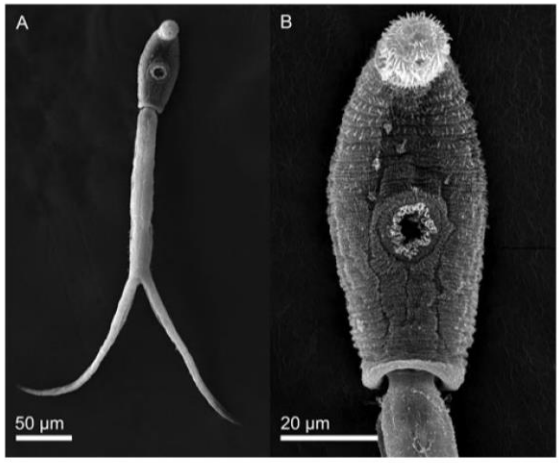

Fig. 2. Cercaria of *Diplostomum phoxini* ex *Ampullaceana balthica* (scanning electron microscopy), ventral view. A, Entire cercaria; B, Cercarial body.

Body armature: Pre-oral spines arranged in a single median group of 10 spines in two rows, anterior row comprised of four spines; two central larger than the lateral; lateral groups of pre-oral spines lacking. Post-oral spines more robust than spines on body, in seven alternate rows encircling body to about mid-level of anterior organ; spines in first two rows much larger than remaining spines, all of similar size. Wide zone of smaller, less dense, irregularly dispersed spines present posterior to post-oral spines. Transverse rows of spines 8, extending to about mid-level of ventral sucker ventrally. Rows 1–5 complete ventrally; rows 6–8 incomplete ventrally; only row 1 complete dorsally. Two ventro-lateral non-confluent fields of smaller spines present in posterior body third. Ventral sucker armed with two rows of spines (*c.* 40 spines per row). Tail stem and furcae armed with minute spines; spines along tail stem in two ventral and two dorsal bands with two medio-lateral bands consisting of small, irregularly dispersed scale-like spines. Spines on furcae in one medial band laterally, consisting of 1–3 scale-like spines, size and density of spines decreasing distally.

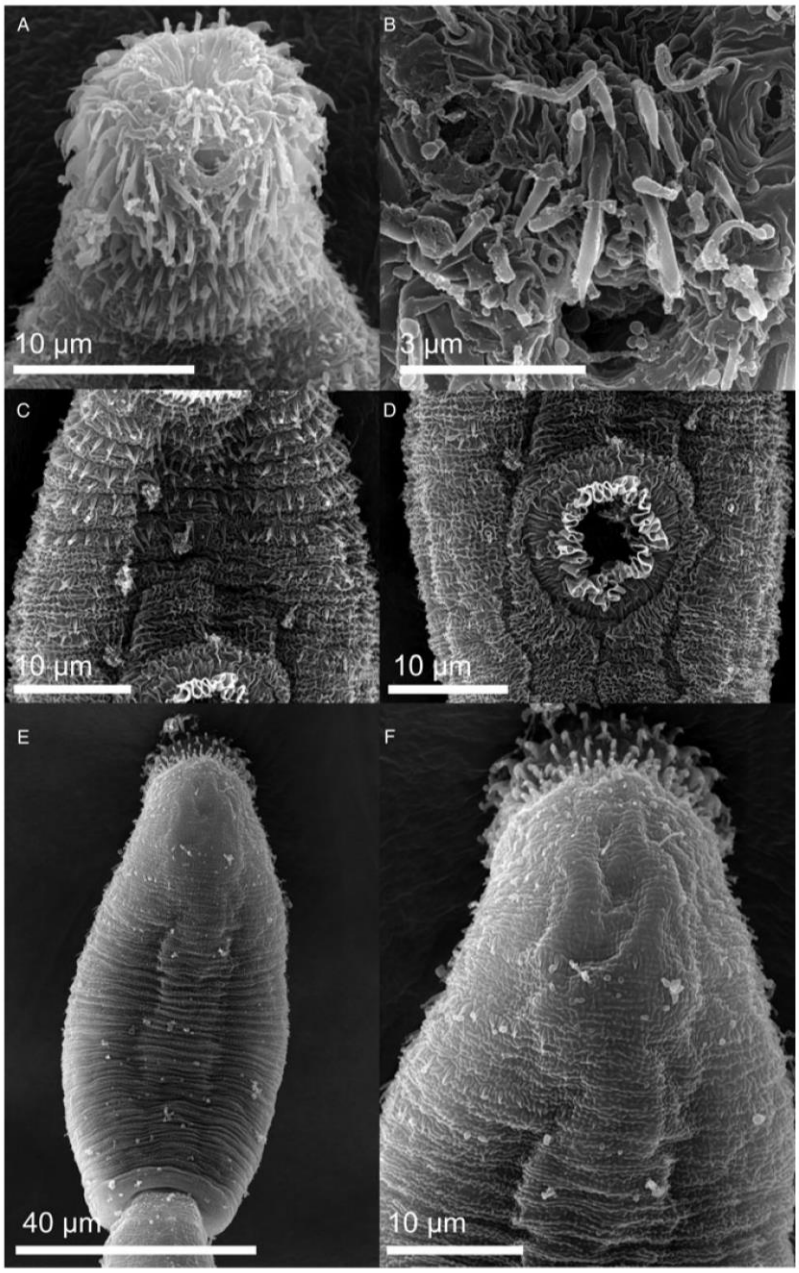

Fig. 3. Cercaria of *Diplostomum phoxini* ex *Ampullaceana balthica* (scanning electron microscopy), ventral view. A, Anterior organ, ventral view; B, Pre-oral spines, apical view; C, Transverse rows of tegumental spines on the body, ventral view; D, Ventral sucker with a well-developed undulating membrane, ventral view; E, Cercarial body, dorsal view; F, Anterior part of cercarial body, dorsal view at a higher magnification.

Resting position: Tail stem straight, body slightly bent ventrally.

Description of the metacercaria

[Based on 20 live specimens from the optical lobes of the brain of *Phoxinus phoxinus*; Fig. 4; Online Resource Table S4] Body elongate-oval, 326–411 (358), with maximum width just anterior to ventral sucker, 145–227 (186). Oral sucker subterminal, subspherical, 38–56 × 36–50 (49 × 44). Pseudosuckers two, contractile, small-sized, 28–42 × 20–40 (34 × 30). Prepharynx indistinct; pharynx muscular, elongate-oval, 18–39 × 12–22 (31 × 18); oesophagus very short, bifurcates close posterior to pharynx; intestinal caeca narrow, encroach holdfast organ and terminate blindly at mid-level of excretory vesicle. Ventral sucker subspherical, 34–48 × 43–54 (43 × 47), similar in size to oral sucker or slightly larger [VSW/OSW = 1.0–1.1 (1.1)], at mid-body length or slightly posterior. Holdfast organ massive, 47–95 × 78–102 (71 × 91), bi-partite with median slit, transversely oval, contiguous with ventral sucker and excretory vesicle. Excretory vesicle large, conspicuous, V-shaped; reserve excretory system of diplostomid type; excretory concretions predominantly large, 345–579 (454) in number, grouped into two lateral and one median fields. Hindbody short, 24–53 (42).

Remarks

The present detailed descriptions expand the known range of variation of the metrical features and provide morphological detail that will facilitate the morphological identification of the larval stages of *D. phoxini*. The cercaria of *D. phoxini* resembles

Table 3. Comparative data for the cercariae of *Diplostomum phoxini* and species of the *D. baeri* species complex

Species/Feature	*D. phoxini*				*D. baeri* Dubois, 1937	*Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014)
Source	Present study	Arvy and Buttner (1954)	Rees (1957)	Dönges (1969*a*)	Niewiadomska and Kiselienė (1994)	Faltýnková *et al.* (2014)
Relation BL-TSL-FL	BL < TSL = FL	BL < TSL > FL	BL < TSL > FL	BL < TSL > FL	BL < TSL < FL	BL < TSL = FL
Relation VSW/AOW	VSW > AOW	VSW > AOW	VSW > AOW	VSW > AOW	VSW = AOW	VSW < AOW
No. of pre-oral spines in the median group	10	–	5	–	7–11	8
No. of pre-oral spines in each lateral group	Absent	–	Absent	–	Absent	Absent
No. of post-oral rows of spines	7	–	7	–	7–9	5–6
No. of transverse rows of spines on body	8 (rows 1–5 complete ventrally, rows 6–8 incomplete ventrally; only row 1 complete dorsally)	–	8 (rows 1–5 complete ventrally, rows 6–8 incomplete ventrally; only rows 1–2 complete dorsally)	8	10	9
No. of spine rows on ventral sucker	2	–	2	2	3	3
No. of spines on ventral sucker	40 per row (*c.*80 in total)	28	36 per row (*c.*72 in total)	48 per row (*c.*96 in total)	90–130	38 per row (*c.*120 in total)
Penetration gland-cells	Large, do not cover ends of caeca	Small, do not cover ends of caeca	Large, do not cover ends of caeca	–	Large, cover ends of caeca	Large, cover ends of caeca
Spines on tail stem	Present	–	–	–	Absent	Present
Spines on furcae	Present	–	–	–	Absent	Present
Resting position	Tail stem straight	–	Tail stem straight	Tail stem straight	Tail stem straight	Tail stem straight

the known cercariae of the *D. baeri* species complex in the lack of the lateral group of pre-oral spines and the resting position with a straight tail stem but differs in the following species-specific features: (i) BL < TSL ⩾ FL; (ii) VSW > AOW; (iii) eight transverse rows of spines on the body; (iv) two rows of spines on the ventral sucker; (v) penetration gland-cells not covering ends of caeca (Table 3). Although the metrical data exhibit overlapping ranges for some features, the cercaria of *D. phoxini* can be distinguished from the cercaria of both *D. baeri sensu* Niewiadomska and Kiselienė (1994) and *Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014) in having on average smaller body, shorter tail and furcae, a narrower apical organ; the tail in *D. phoxini* is also much longer than the body (Faltýnková *et al.*, 2014; see Online Resource Table S3 for details).

The metacercaria of *D. phoxini* exhibits overlapping ranges for the metrical data with *Diplostomum* sp. Lineages 3–5 of Blasco-Costa *et al.* (2014) but the means for the latter species-level lineages are greater (Online Resource Table S4; see also Faltýnková *et al.*, 2014). Comparisons with the metacercariae measured by Lebedeva *et al.* (2021) revealed an overall agreement for the metrical data except for the somewhat smaller body dimensions and the greater number of excretory concretions (Online Resource Table S4). Both differences are due to the fact that Lebedeva *et al.* (2021) examined fixed material; this may have led to misinterpretations of excretory concretions.

Parasitism in a specific second intermediate host (*P. phoxinus*) can be also used to distinguish *D. phoxini* from *Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014), the only species of the *D. baeri* complex with a European distribution which was also recorded in the brain of *Gasterosteus aculeatus*.

Phylogenetic analyses

The newly generated *cox*1 sequences were analysed together with all published sequences for *Diplostomum* spp. (1203 sequences; 407 nt). The neighbour-joining analysis depicted 44 species/species-level lineages of *Diplostomum* with typically maximum or very high support (see Table 4 and Online Resource Fig. S2); these included seven taxa represented by singletons: *Diplostomum* sp. 5 and *Diplostomum* sp. 8 of Locke *et al.* (2010*a*); *Diplostomum* sp. 11 of Locke *et al.* (2015);

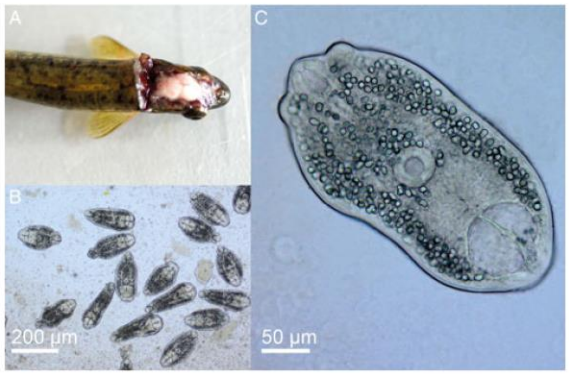

Fig. 4. *Phoxinus phoxinus* (A) and live metacercariae of *Diplostomum phoxini* (B, C).

Diplostomum sp. A of Gordy and Hanington (2019), *Diplostomum* spp. A, B and C of Kudlai *et al.* (2017).

The sequences for *D. phoxini* clustered in a strongly supported reciprocally monophyletic lineage associated with three lineages of the *D. baeri* species complex *sensu* Blasco-Costa *et al.* (2014): *Diplostomum* spp. 5 and 6 of Locke *et al.* (2010*a*), and *Diplostomum* sp. Lineage 5 of Blasco-Costa *et al.* (2014). The sequences for the second lineage of *Diplostomum* sp. from the brain of *P. phoxinus* discovered in Mongolia by Lebedeva *et al.* (2021) also formed a strongly supported monophyletic clade (Online Resource Fig. S2).

Bayesian inference phylogenetic reconstruction for representatives of the genus *Diplostomum* (currently comprising 44 species/species-level lineages, see Table 4) depicted a composition of the *D. baeri* species complex similar to that in Blasco-Costa *et al.* (2014) (Fig. 5). The only differences are the addition of *D. phoxini* and *Diplostomum* sp. of Lebedeva *et al.* (2021) and the exclusion of *Diplostomum* sp. 2 of Moszczynska *et al.* (2009). There was support for a close association with the *D. baeri* complex for 5 lineages: *Diplostomum* spp. 2, 12, 18 and 19 of Locke *et al.* (2015) forming a cluster, albeit with poor support, plus the singleton ex *Rana pipiens* (*Diplostomum* sp. 11). Within the *D. baeri* species complex, there was a strongly supported sister-group relationship between: (i) *Diplostomum* sp. Lineage 5 of Blasco-Costa *et al.* (2014) and *Diplostomum* sp. 6 of Locke *et al.* (2010*a*); and (ii) *D. baeri sensu* Galazzo *et al.* (2002) and *Diplostomum* sp. 5 of Locke *et al.* (2010*a*).

The present phylogenetic hypothesis for *Diplostomum* spp. depicted two additional well supported clusters: a group of 10 lens-infecting species/lineages containing the type-species of the genus, *D. spathaceum*; and the species/lineages of the *D. mergi* species complex *sensu* Selbach *et al.* (2015) which also included one additional lineage from Japan (see Komatsu *et al.*, 2019) and two singletons from River Danube in Slovakia (see Kudlai *et al.*, 2017) (Fig. 5). The relationships of the remaining species/lineages remained unresolved.

By means of raw pairwise interspecific divergence, the species/lineages of the *D. baeri* species complex appear well differentiated except for the two loose groups indicated by ellipses in the NMDS plot (Fig. 6) comprising *D. phoxini* and *Diplostomum* sp. Lineage 5 of Blasco-Costa *et al.* (2014) + *Diplostomum* sp. 6 of Locke *et al.* (2010*a*) (range for the latter two lineages: 3.5–5.5%) (see ranges for *D. phoxini* in Table 2). Importantly, comparisons of the isolates of *D. baeri sensu* Galazzo *et al.* (2002) sampled in North America with the species/lineages of the *D. baeri* species complex revealed levels of genetic divergence (7.7–15.3%, see Table 5 for details) within the range reported for distinct species/lineages of *Diplostomum* (4.2–16.4%, see Georgieva *et al.*, 2013; 4.3–14.7%, see Selbach *et al.*, 2015). The intraspecific divergence for these isolates was low (0–1.3%; based on 3872 pairwise comparisons).

Re-classification and an updated nomenclature for Diplostomum *spp.*

The present phylogenetic analyses helped update the nomenclature of a total of 144 sequenced isolates of *Diplostomum* spp. (see Table 4 for a summary and Online Resource Table S5 for a detailed list of all isolates; these are also indicated in the tree in Online Resource Fig. S2). Of these, we updated the species/lineage definitions with links to the GenBank accession numbers for 46 isolates (indicated in blue in Online Resource Fig. S2 and Table S5) and re-classified 98 isolates (indicated in red in Online Resource Fig. S2 and Table S5) assigned to 11 lineages as follows:

(i) *D. adamsi* (syn. *D. baeri sensu* Galazzo *et al.*, 2002): reported as "*Diplostomum baeri* LIN2" by Gordy and Hanington (2019) and annotated on GenBank as "*Diplostomum baeri* complex sp. LIN2"and "*Diplostomum* aff. *baeri* LIN2";
(ii) *D. mergi* Lineage 4 of Selbach *et al.* (2015): annotated as *D. mergi* on GenBank by Dang (unpublished sequence); this lineage is also not correctly annotated by Selbach *et al.* (2015);
(iii) *D. spathaceum*: reported and annotated on GenBank as *D. paracaudum* by Behrmann-Godel (2013); this isolate has been re-classified by Georgieva *et al.* (2013);
(iv) *Diplostomum* sp. 3 of Moszczynska *et al.* (2009): reported and annotated on GenBank as *D. baeri* by Ubels *et al.* (2018);
(v) *Diplostomum* sp. 4 of Moszczynska *et al.* (2009): reported and annotated on GenBank as *D. baeri* by Ubels *et al.* (2018);
(vi) *Diplostomum* sp. 13 of Locke *et al.* (2015): reported and annotated on GenBank as "*Diplostomum* sp. C" by Gordy and Hanington (2019);
(vii) *Diplostomum* sp. 18 of Locke *et al.* (2015): reported and annotated on GenBank as "*Diplostomum* sp. B" by Gordy and Hanington (2019);
(viii) *Diplostomum* sp. Lineage 3 of Blasco-Costa *et al.* (2014): reported as *D. baeri* by Landeryou *et al.* (2020);
(ix) *Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014): reported and annotated on GenBank as *D. baeri* by Behrmann-Godel (2013) (assigned to the "perch" lineage or "*D. baeri* " by Georgieva *et al.*, 2013); reported as "*D. baeri* 2" and annotated on GenBank as *D. baeri* by Rahn *et al.* (2016);
(x) *Diplostomum* sp. Lineage 6 of Blasco-Costa *et al.* (2014): reported as "*D.* Lineage 6" but annotated on GenBank as *Diplostomum* sp. 6 by Rahn *et al.* (2016); and
(xi) *Diplostomum* sp. Clade Q: reported and annotated on GenBank as *D. mergi* by Behrmann-Godel (2013); these isolates have been re-classified by Georgieva *et al.* (2013).

Discussion

Diplostomum phoxini is a well-delimited species which differs from all described species of *Diplostomum* in the morphology of the adult stage, the strict host specificity to the second intermediate hosts (*P. phoxinus*) and the specific location in the fish brain. Nevertheless, in light of the expansive development of molecular studies on *Diplostomum* spp. and the current uncertainty in linking sequence data from larval stages to named species of this genus, it is desirable to describe the sequenced forms and thus anchor the molecular data to morphological reference (see e.g. Blasco-Costa *et al.*, 2014; Faltýnková *et al.*, 2014;

Table 4. Species and species-level lineages of *Diplostomum* with a re-identification of some isolates (GenBank data as of 29 June 2021; see the neighbour-joining tree based on all available sequences in Online Resource Fig. S2 and Online Resource Table S5 for details)

Species/Lineage	Reference	Distribution	Re-identified isolates [Reference]	Isolates with updated nomenclature [Reference]
D. ardeae Dubois, 1969	Locke *et al.* (2015, 2020)	Canada, Puerto Rico		
D. adamsi (syn. *D. baeri sensu* Galazzo *et al.*, 2002)	Moszczynska *et al.* (2009); Locke *et al.* (2010a, 2010*b*, 2015); Ubels *et al.* (2018)	Canada, USA	*Diplostomum baeri* complex sp. LIN2 (isolates MGC1740, MGC1824, MGC1861, MGC2242) ex *Ladislavella elodes* (Canada) (MH368850-MH368853) [Gordy and Hanington, 2019]	
			Diplostomum aff. *baeri* LIN2 (isolate MGC190) ex *Ladislavella elodes* (Canada) (KT831353) [Gordy and Hanington, 2019]	
D. huronense (La Rue, 1927)	Moszczynska *et al.* (2009); Locke *et al.* (2010*a*, 2010*b*, 2015)	Canada		
D. indistinctum (Guberlet, 1922)	Moszczynska *et al.* (2009); Locke *et al.* (2010*a*, 2010*b*, 2015; Gordy *et al.* (2016)	Canada		
D. lunaschiae Locke, Drago, Núñez, Rangel e Souza & Takemoto, 2020	Locke *et al.* (2020)	Argentina, Brazil		
D. mergi Lineage 2 of Georgieva *et al.* (2013)	Georgieva *et al.* (2013); Selbach *et al.* (2015); Kudlai *et al.* (2017); Locke *et al.* (2015)	China, Germany, Slovakia, Hungary		*D. mergi* ex *Radix auricularia* (Germany) (JX986874-JX986876) [Georgieva *et al.*, 2013][a]
				D. mergi ex *Radix auricularia* (Germany) (KR149513-KR149523) [Selbach *et al.*, 2015][b]
D. mergi Lineage 3 of Georgieva *et al.* (2013)	Georgieva *et al.* (2013); Selbach *et al.* (2015)	Germany		*D. mergi* ex *Salmo trutta* and *Gobio gobio* (Germany) (JX986877-JX986885) [Georgieva *et al.*, 2013][c]
				D. mergi (isolates RaHe17-RaHe19) ex *Radix auricularia* (Germany) (KR149524-KR149527) [Selbach *et al.*, 2015][d]
D. mergi Lineage 4 of Selbach *et al.* (2015)	Selbach *et al.* (2015)	China, Germany	*D. mergi* (China) (KY271543) [Dang (unpublished)]	*D. mergi* (isolate RaHe20) ex *Radix auricularia* (Germany) (KR149528) [Selbach *et al.*, 2015][e]
D. parviventosum Dubois, 1932[f]	Selbach *et al.* (2015)	Germany		*D. mergi* (isolate RAH1) ex *Radix auricularia* (Germany) (JX986873) [Georgieva *et al.*, 2013][g]
D. phoxini (Faust, 1919)	Soldánová *et al.* (2017); Lebedeva *et al.* (2021); present study	Finland, Germany, Norway, Russia		
D. pseudospathaceum Niewiadomska, 1984	Georgieva *et al.* (2013); Behrmann-Godel (2013); Pérez-del-Olmo *et al.* (2014); Locke *et al.* (2015); Kudlai *et al.* (2017); Enabulele *et al.* (2018)	Czech Republic, Germany, Hungary, Poland, Romania, Slovakia, Spain, UK		
D. spathaceum (Rudolphi, 1819)	Georgieva *et al.* (2013); Blasco-Costa *et al.* (2014); Pérez-del-Olmo *et al.* (2014); Locke *et al.* (2015); Kudlai *et al.* (2017); Dang *et al.* (unpublished)	China, Croatia, Czech Republic, Germany, Hungary Iceland, Iraq, Italy, Poland, Romania, Slovakia, Spain	*D. paracaudum* (isolate RA155) ex *Radix auricularia* (Germany) (JQ639176) [Behrmann-Godel, 2013]	

(*Continued*)

Table 4. (*Continued.*)

Species/Lineage	Reference	Distribution	Re-identified isolates [Reference]	Isolates with updated nomenclature [Reference]
Diplostomum sp. 1 of Moszczynska *et al.* (2009)	Moszczynska *et al.* (2009); Locke *et al.* (2010*a*, 2010*b*, 2015); Rudko *et al.* (2018); Gordy and Hanington (2019); Ubels *et al.* (2018)	Canada, USA		
Diplostomum sp. 2 of Moszczynska *et al.* (2009)	Moszczynska *et al.* (2009); Locke *et al.* 2010*a*, 2010*b*)	Canada, USA		
Diplostomum sp. 3 of Moszczynska *et al.* (2009)	Moszczynska *et al.* (2009); Locke *et al.* (2010*a*, 2010*b*, 2015); Gordy *et al.* (2016); Gordy and Hanington (2019); Ubels *et al.* (2018)	Canada, USA	*D. baeri* (isolate 49D-R2) ex *Perca flavescens* on GenBank but the host is given as *Luxilus cornutus* in Fig. S1 (USA) (MF142178) [Ubels *et al.*, 2018]	
Diplostomum sp. 4 of Moszczynska *et al.* (2009)	Moszczynska *et al.* (2009); Locke *et al.* (2010*a*, 2010*b*), Gordy and Hanington (2019); Rudko *et al.* (2018); Ubels *et al.* (2018)	Canada, USA	*D. baeri* (isolate 55D-T1) ex *Luxilus cornutus* on GenBank but the host is given as *Perca flavescens* in Fig. S1 (USA) (MF142161) [Ubels *et al.*, 2018]	
Diplostomum sp. 5 of Locke *et al.* (2010a)	Locke *et al.* (2010*a*)	Canada		
Diplostomum sp. 6 of Locke *et al.* (2010a)	Locke *et al.* (2010*a*, 2015)	Canada		
Diplostomum sp. 7 of Locke *et al.* (2010a)	Locke *et al.* (2010*a*, 2015)	Canada		
Diplostomum sp. 8 of Locke *et al.* (2010a)	Locke *et al.* (2010*a*)	Canada		
Diplostomum sp. 9 of Locke *et al.* (2010a)	Locke *et al.* (2010*a*, 2015)	Canada		
Diplostomum sp. 10 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada		
Diplostomum sp. 11 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada		Diplostomidae gen. SL sp. 1 SAL-2010 (isolate Di.BR.Bo.Rp.2.1) ex *Rana pipiens* (Canada) (HM064650) [Locke *et al.*, 2010*b*]
Diplostomum sp. 12 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada, USA		
Diplostomum sp. 13 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada, USA	*Diplostomum* sp. C MAG-2016 ex *Ladislavella elodes* (Canada) (KT831360, KT831378, KT831382) [Gordy and Hanington, 2019]	
			Diplostomum sp. C MAG-2019 ex *Ladislavella elodes* and *Planorbella trivolvis* (Canada) (MH368933-MH368941) [Gordy and Hanington, 2019]	
Diplostomum sp. 14 of Locke *et al.* (2015)	Locke *et al.* (2015); Hoogendoorn *et al.* (2020)	China, Iraq, South Africa		
Diplostomum sp. 15 of Locke *et al.* (2015)	Locke *et al.* (2015)	China		
Diplostomum sp. 16 of Locke *et al.* (2015)	Locke *et al.* (2015); Hoogendoorn *et al.* (2020)	Iraq, South Africa		
Diplostomum sp. 17 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada		
Diplostomum sp. 18 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada	*Diplostomum* sp. B MAG-2019 (isolate MGC2308) ex *Ladislavella elodes* (Canada) (MH368932) [Gordy and Hanington, 2019]	
Diplostomum sp. 19 of Locke *et al.* (2015)	Locke *et al.* (2015)	Canada, USA		*Diplostomum* sp. BOLD ACK9826 ex *Osmerus mordax* (Canada) (KM538089) [Van Steenkiste *et al.* (2015)]

(*Continued*)

Table 4. (*Continued.*)

Species/Lineage	Reference	Distribution	Re-identified isolates [Reference]	Isolates with updated nomenclature [Reference]
Diplostomum sp. Lineage 2 of Blasco-Costa *et al.* (2014)[h]	Blasco-Costa *et al.* (2014); Faltýnková *et al.* (2014)	Iceland		
Diplostomum sp. Lineage 3 of Blasco-Costa *et al.* (2014)[h]	Blasco-Costa *et al.* (2014); Faltýnková *et al.* (2014); Soldánová *et al.* (2017)	Germany, Iceland, Norway, UK	*D. baeri* (isolates LF1-LF10) ex *Salmo trutta* (UK) (MT311204-MT311213) [Landeryou *et al.*, 2020]	*D. baeri* ex *Salmo trutta* and *Gobio gobio* (Germany) (JX986859-JX986872) [Georgieva *et al.*, 2013][i]
Diplostomum sp. Lineage 4 of Blasco-Costa *et al.* (2014)[h]	Blasco-Costa *et al.* (2014); Faltýnková *et al.* (2014); Locke *et al.* (2015); Kuhn *et al.* (2015); Soldánová *et al.* (2017)	Germany, Iceland, Italy, Norway, Romania, UK	*D. baeri* ex *Perca fluviatilis* (Germany) (JQ639180-JQ639195) [Behrmann-Godel, 2013][j]	
			D baeri (isolates Diplob2UistGa01-Diplob2UistGa04) ex *Gasterosteus aculeatus* (UK) (KX037874-KX037877) [Rahn *et al.*, 2016]	
Diplostomum sp. Lineage 5 of Blasco-Costa *et al.* (2014)[h]	Blasco-Costa *et al.* (2014); Faltýnková *et al.* (2014); Locke *et al.* (2015); Soldánová *et al.* (2017)	Iceland, Norway		
Diplostomum sp. Lineage 6 of Blasco-Costa *et al.* (2014)[h]	Blasco-Costa *et al.* (2014); Faltýnková *et al.* (2014); Kuhn *et al.* (2015); Soldánová *et al.* (2017)	Iceland, Norway, UK	*Diplostomum* sp. 6 AKR-2016 ex *G. aculeatus* ((isolates Diplolin6ICEGa02-Diplolin6ICEGa05; Diplolin6UistGa01-Diplolin6UistGa35) (Iceland, UK) (KX037902-KX037915; KX140051-KX140055) and *P. pungitius* (isolates Diplolin6UistPp01-Diplolin6UistPp03) (UK) KX037878-KX037880) [Rahn *et al.*, 2016]	
Diplostomum sp. A of Gordy and Hanington (2019)	Gordy and Hanington (2019)	Canada		
Diplostomum sp. A of Kudlai *et al.* (2017)	Kudlai *et al.* (2017)	Slovakia		
Diplostomum sp. B of Kudlai *et al.* (2017)	Kudlai *et al.* (2017)	Slovakia		
Diplostomum sp. C of Kudlai *et al.* (2017)	Kudlai *et al.* (2017)	Slovakia		
Diplostomum sp. of Chibwana *et al.* (2013)	Chibwana *et al.* (2013); Hoogendoorn *et al.* (2020)	Nigeria, South Africa		
Diplostomum sp. (Japan) of Komatsu *et al.* (2019)	Komatsu *et al.* (2019)	Japan		
Diplostomum sp. Clade Q	Georgieva *et al.* (2013); Pérez-del-Olmo *et al.* (2014); Selbach *et al.* (2015); Locke *et al.* (2015)	Germany, Spain	*Diplostomum mergi* (RR43, RR45, RA97) ex *Rutilus rutilus* and *Radix auricularia* (Germany) (JQ639177-JQ639179) [Behrmann-Godel, 2013]	
Diplostomum sp. of Lebedeva et al. (2021)	Lebedeva et al. (2021)	Mongolia		

[a]*D. mergi* Lineage 2 ("*D. mergi* 2" and "Clade 3-2") in Georgieva *et al.* (2013)
[b]*D. mergi* Lineage 2 in Selbach *et al.* (2015) and Kudlai *et al.* (2017)
[c]*D. mergi* Lineage 3 ("*D. mergi* 3" and "Clade 3-3") in Georgieva *et al.* (2013)
[d]*D. mergi* Lineage 3 in Selbach *et al.* (2015)
[e]*D. mergi* Lineage 4 in Selbach *et al.* (2015)
[f]Member of the *D. mergi* species complex, indicated as *D. mergi* Lineage 1 in Georgieva *et al.* (2013)
[g]*D. mergi* Lineage 1 ("*D. mergi* 1" and "Clade 3-1") in Georgieva *et al.* (2013)
[h]Lineages discovered in Iceland and characterised molecularly and morphologically by Blasco-Costa *et al.* (2014) and Faltýnková *et al.* (2014), respectively
[i]*D. baeri* Lineage 1 ("*D. baeri* 1", "Clade 4-1" or "trout lineage") in Georgieva *et al.* (2013)
[j]*D. baeri* Lineage 2 ("*D. baeri* 2", "Clade 4-2" or "perch lineage") in Georgieva *et al.* (2013)

Pérez-del-Olmo *et al.*, 2014; Selbach *et al.*, 2015). Our study thus anchors the molecular data to detailed descriptions of the larval stages and provides molecular evidence for the identification of the first intermediate host, *A. balthica*. Furthermore, the phylogenetic analyses resulted in: (i) a re-identification/re-classification of 98 isolates including *D. baeri sensu* Galazzo *et al.* (2002); (ii)

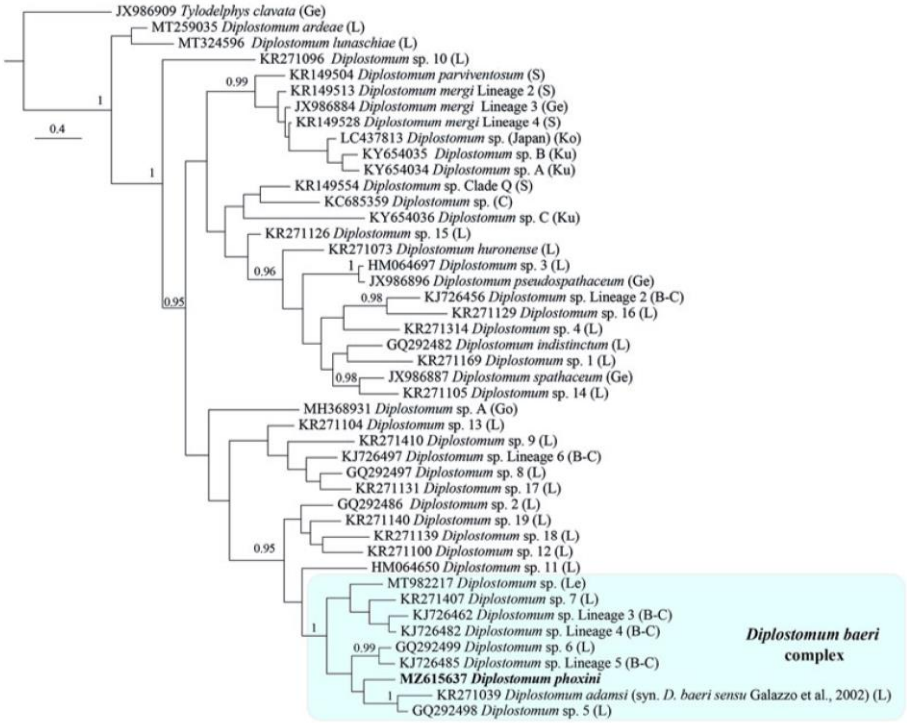

Fig. 5. Phylogram from Bayesian inference (BI) analysis of the *cox*1 sequence alignment (407 nt) for 44 species/species-level lineages of *Diplostomum*. Outgroup: *Tylodelphys clavata*. Nodal support is given as posterior probabilities; only values ⩾ 0.95 are shown. The scale-bar indicates the expected number of substitutions per site. The shaded rectangle indicates the content of the *Diplostomum baeri* species complex inferred from the present study. *Abbreviations*: B-C, Blasco-Costa *et al.* (2014); C, Chibwana *et al.* (2013); Ge, Georgieva *et al.* (2013); Go, Gordy and Hanington (2019); L, Locke *et al.* (2010*a*, 2010*b*, 2015, 2020); Le, Lebedeva *et al.* (2021); Ko, Komatsu *et al.* (2019); Ku, Kudlai *et al.* (2017); S, Selbach *et al.* (2015).

re-definition of the composition of the *D. baeri* species complex which now includes nine molecularly characterised species/lineages, i.e. *D. adamsi* (syn. *D. baeri sensu* Galazzo *et al.*, 2002), *D. phoxini*, *Diplostomum* sp. Lineages 3–5 of Blasco-Costa *et al.* (2014), *Diplostomum* spp. 5–7 of Locke *et al.* (2015), and *Diplostomum* sp. of Lebedeva *et al.* (2021); (iii) re-definition of the composition of the *D. mergi* species complex which now includes seven molecularly characterised species/lineages, i.e. *D. parviventosum*, *D. mergi* Lineages 2 and 3 of Georgieva *et al.* (2013), *Diplostomum mergi* Lineage 4 of Selbach *et al.* (2015), *Diplostomum* spp. A and B of Kudlai *et al.* (2017), and *Diplostomum* sp. of Komatsu *et al.* (2019); and (iv) an updated nomenclature for the molecularly characterised species-level lineages of *Diplostomum*.

Prevalence and life-cycle of Diplostomum phoxini

The summarised data for the prevalence of the molecularly characterised species/lineages of *Diplostomum* in the snail intermediate hosts in Europe (Table 6) indicate that prevalence is generally low when estimated from pooled samples (0.7–4.7%), whereas the prevalence estimated from distinct individual samples (i.e. as per the definition of Bush *et al.*, 1997) ranges between 1.0 and 13.6% but is typically greater than 3.0%. The prevalence of *D. phoxini* in *A. balthica* studied in the River Ruhr fell within the latter range but with values greater than 3.0% in all distinct samples and a maximum prevalence of 13.6% recorded to date for *Diplostomum* spp. in Europe (Table 6; see also Online Resource Table S2 for details).

Although most of the populations of *Diplostomum* spp. originate from lentic aquatic habitats (lakes, reservoirs, ponds) it is worth noting that both studies with prevalence estimated for multiple distinct samples originating from lotic waterbodies (River Ruhr in Germany, present study; River Veude in France, see Arvy and Buttner, 1954) revealed a high prevalence range of *D. phoxini* in both *A. balthica* and *R. auricularia*. This is in contrast with the expectation that flow conditions in the aquatic habitat affect digenean dispersal (Radke *et al.*, 1961) with lentic habitats guaranteeing accelerated transmission rates (Soldánová and Kostadinova, 2011).

The data from our longitudinal study of the prevalence in *A. balthica* indicate that significant and consistent foci of infection with *D. phoxini* exist in the River Ruhr. This is further strengthened by the narrow transmission window estimated for *D. phoxini* in the riverine habitats studied. In Germany, the life span of *A. balthica* is estimated as one year, with copulation and egg-laying occurring in March (Glöer, 2002). We assume that in the River Ruhr juveniles hatch in April and the first patent infections in the new generation develop during June. This is supported by the fact that although large samples of snails were examined at all sites in May and June, the first patent infections with *D. phoxini* were registered as early as July. Our data thus indicate a transmission window of six months (June to November) with infection restart in each new snail generation.

Metacercariae of *D. phoxini* can survive in a minnow brain for up to five years and are accumulated by their fish hosts (Dönges, 1969*b*). This explains the maximum prevalence of 100% and high abundance of *D. phoxini* recorded in *P. phoxinus* host as reported previously (e.g. Arvy and Buttner, 1954; Rees, 1955, 1957). The longevity and accumulation of metacercariae in fish counteract the narrow transmission window for the larval stages and ensure the existence of a reservoir for maintenance of the infection with

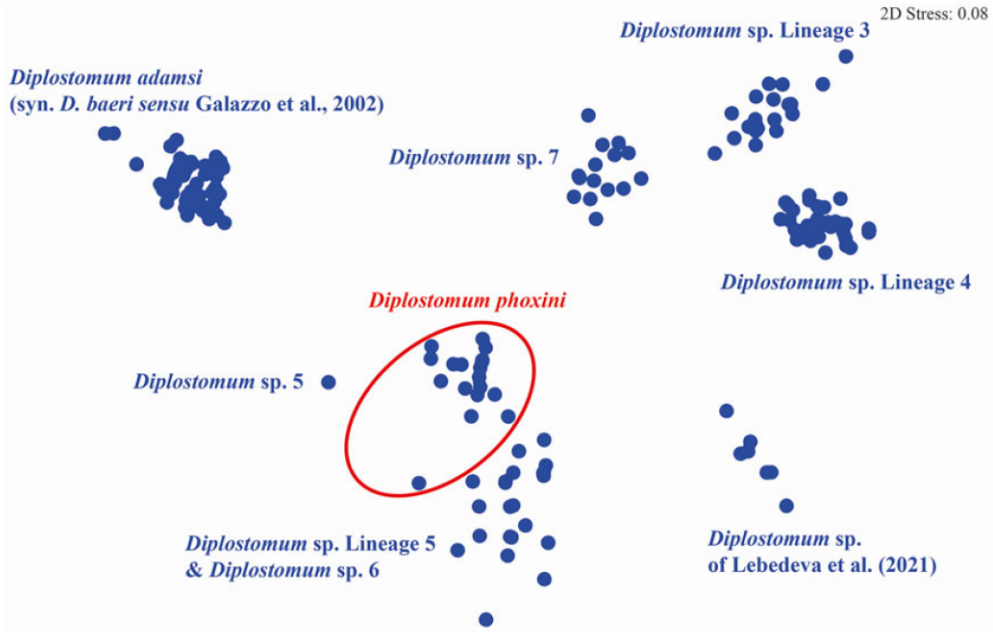

Fig. 6 Non-metric multidimensional scaling ordination plot derived from the raw pairwise distances (p-distance) calculated for the species/lineages of the *D. baeri* complex based on the *cox*1 dataset.

Table 5. Percent interspecific genetic divergence (p-distance model) for *D. adamsi* (syn. *D. baeri sensu* Galazzo *et al.*, 2002) sampled in North America compared with the species/lineages of the *D. baeri* species complex based on all *cox*1 sequences available on GenBank (retrieved on 29 June 2021)

Species/Lineage	*n*	Divergence (%)
D. phoxini	1602	8.7–12.1
Diplostomum sp. 5 of Locke *et al.* (2010*a*)	89	7.7–9.2
Diplostomum sp. 6 of Locke *et al.* (2010*a*)	356	9.8–13.5
Diplostomum sp. 7 of Locke *et al.* (2010*a*)	1424	9.0–12.0
Diplostomum sp. Lineage 3 of Blasco-Costa *et al.* (2014)	3649	10.3–14.5
Diplostomum sp. Lineage 4 of Blasco-Costa *et al.* (2014)	3827	11.2–15.3
Diplostomum sp. Lineage 5 of Blasco-Costa *et al.* (2014)	1780	9.3–13.1
Diplostomum sp. of Lebedeva *et al.* (2021)	712	10.2–12.9

Abbreviation: *n*, number of pairwise comparisons

this species in the River Ruhr. However, the definitive host of *D. phoxini* is still poorly known since the only natural infection has been reported in *Mergus merganser* L. (the host of *D. pelmatoides* (Dubois, 1932), a synonym of *D. phoxini*). The distribution of this bird species in Germany is generally confined to the wintering areas around the coasts of the Baltic Sea (between October –November and March–April) and there is an isolated declining breeding population in Bavaria (Keller, 2009).

Recent observations, however, suggest an increase in the number of breeding pairs of *M. merganser* along the River Ruhr, that might contribute to the infection foci in *A. balthica* along the river.

Shigin (1986, 1993) reported the existence of intense and persistent foci of infection with *D. phoxini* in fish populations of waterbodies where *Mergus* spp. are practically lacking. Studies on the life-cycle indicate that *D. phoxini* is not highly specific to its definitive host as adult flukes containing eggs and mature sperm have been obtained from both avian and mammalian hosts. Adults of *D. phoxini* have been raised experimentally in ducklings of *Anas platyrhynchos* (see Arvy and Buttner, 1954; Dönges, 1969*a*, 1969*b*; Erasmus, 1969), *Cairina moschata domestica* (see Arvy and Buttner, 1954), *L. argentatus* (see Berrie, 1960) and laboratory mice (Berrie, 1960; Shigin, 1986, 1993), but not in *L. ridibundus* (see Dönges, 1969*a*); a very rapid rate of development in ducklings (3–5 days post-infection) has been observed (Rees, 1955; Berrie, 1960; Dönges, 1969*a*, 1969*b*; Erasmus, 1969). This rapid rate of adult development and compatibility with anatid and mammalian hosts, in association with a possibility of parasite-induced changes in fish behaviour and mortality at high intensity of infection levels, tend to support the hypothesis that purely facultative ichthyophages such as aquatic rodents (Shigin, 1986, 1993) and/or *A. platyrhynchos* (see Miroshnichenko and Sten'ko, 1983) may also act as definitive hosts of *D. phoxini*.

Diplostomum baeri *species complex*

Including in the phylogenetic analysis *D. phoxini* and the additional 26 species-level lineages molecularly characterised during 2015–2021 resulted in a change of the composition of the *D. baeri* species complex with the inclusion of *D. phoxini* and *Diplostomum* sp. of Lebedeva *et al.* (2021) and the exclusion of *Diplostomum* sp. 2 of Moszczynska *et al.* (2009) which was associated with three North American lineages (*Diplostomum* spp. 12, 18 and 19 of Locke *et al.*, 2015) sequenced recently by Locke *et al.* (2015).

Metacercariae of all species/lineages of the *D. baeri* species complex represent non-lens-dwelling forms recovered from the eye vitreous humour and retina and the brain of the fish hosts. The microhabitat within the fish outside the lens utilised by the metacercariae of *Diplostomum* spp. is an important species characteristic (Shigin, 1986) and defining the exact location of the

Table 6. Comparative data for the prevalence of *D. phoxini* and molecularly characterised species/lineages of *Diplostomum* spp. in intermediate snail hosts examined in Europe

Species	Host	Prevalence (%)	Locality	Source
D. phoxini	*Ampullaceana balthica*	3.3–13.6	River Ruhr, Germany	Present study
	Radix auricularia (as *Lymnaea auricularia*)	4.0–5.0	River Veude, France	Arvy and Buttner (1954)
	Peregriana peregra (as *Lymnaea pereger*)	3.9	Lake Fron Goch, UK	Rees (1957)
	Ampullaceana balthica (as *Lymnaea peregra ovata*)	0.9[a]	River Nagold, Germany	Dönges (1969*a*, 1969*b*)
	Peregriana peregra (as *Lymnaea peregra*)	3.4	Lake Fron Goch, UK	Bibby and Rees (1971)
Diplostomum sp. Lineage 2	*Ampullaceana balthica* (as *Radix peregra*)	0.7[b]	Lake Raudavatn, Iceland	Faltýnková *et al.* (2014)
Diplostomum sp. Lineage 4	*Ampullaceana balthica* (as *Radix peregra*)	2.8[b]	Lake Nordic House, Iceland	Faltýnková *et al.* (2014)
Diplostomum sp. Lineage 6	*Ampullaceana balthica* (as *Radix peregra*)	4.7[b]	Lake Nordic House, Iceland	Faltýnková *et al.* (2014)
D. parviventosum	*Radix auricularia*	3.1–7.1	Hengsteysee, Germany[c]	Selbach *et al.* (2015)
	Ampullaceana lagotis (as *Radix lagotis*)	0.4–1.5	Most Lake, Czech Republic	Vyhlídalová and Soldánová (2020)
D. mergi Lineage 2	*Radix auricularia*	2.1–6.7	Hengsteysee, Germany[c]	Selbach *et al.* (2015)
D. mergi Lineage 2	*Radix auricularia*	2.2–10.7	Sorpetalsperre, Germany[c]	Selbach *et al.* (2015)
D. mergi Lineage 3	*Radix auricularia*	1.0–3.1	Hengsteysee, Germany[c]	Selbach *et al.* (2015)
D. mergi Lineage 4	*Radix auricularia*	1.0	Hengsteysee, Germany[c]	Selbach *et al.* (2015)
D. mergi species complex	*Ampullaceana lagotis* (as *Radix lagotis*)	0.4–9.6[d]	Most Lake, Czech Republic	Vyhlídalová and Soldánová (2020)
D. spathaceum	*Radix auricularia*	2.1–4.1	Hengsteysee, Germany	Selbach *et al.* (2015)
	Ampullaceana lagotis (as *Radix lagotis*)	3.0–4.2	Most Lake, Czech Republic	Vyhlídalová and Soldánová (2020)
Diplostomum sp. Clade Q	*Radix auricularia*	3.6	Hengsteysee, Germany[c]	Selbach *et al.* (2015)

[a]Overall prevalence for pooled samples taken during 1959–1965.
[b]Data from pooled samples.
[c]Water reservoirs of the River Ruhr catchment area in North Rhine-Westphalia.
[d]Pooled data for all lineages of the *D. mergi* species complex.

metacercariae can facilitate identification/differentiation as illustrated by Blasco-Costa *et al.* (2014) who sequenced and differentiated morphologically two species-level lineages (*Diplostomum* sp. Lineage 3 from the vitreous humour of the eye and *Diplostomum* sp. Lineage 5 from the eye retina) in the salmonids *Salmo trutta* L. and *Salvelinus alpinus* (L.) and two lineages (*Diplostomum* sp. Lineage 4 from the eye retina and brain and *Diplostomum* sp. Lineage 6 from the retina) in the gasterosteid *Gasterosteus aculeatus* L.

Unfortunately, Locke *et al.* (2010*a*) made no distinction between the sub-retinal space, retina and vitreous humour of the eye "because metacercariae in these sites often detach in frozen material". This applies to six species/lineages for which additional clarification of the metacercarial microhabitat in fish is required based on examination of unfrozen material: *D. baeri sensu* Galazzo *et al.* (2002); *Diplostomum* sp. 2 of Moszczynska *et al.* (2009); and *Diplostomum* spp. 5–9 of Locke *et al.* (2010*a*). Locke *et al.* (2015) applied the division of "lens" *vs* "non-lens (eye)" for these species and for five additional lineages (*Diplostomum* spp. 12, 13, 17–19). Overall, there is conflicting information for the location of the metacercariae between the two large inventories of Locke *et al.* (2010*a*) and Locke *et al.* (2015) and between the text and the supplementary data of Locke *et al.* (2015) for 12 isolates of one lineage (*Diplostomum* sp. 2 of Moszczynska *et al.* (2009)) and nine isolates of six lineages, respectively (highlighted in red in Online Resource Table S5). Regarding the lineages of the *D. baeri* species complex, conflicting information for isolate microhabitats in the fish hosts has been provided for one isolate (KR271039) of *D. baeri sensu* Galazzo *et al.* (2002) and five isolates of *Diplostomum* sp. 7 of Locke *et al.* (2010*a*) (KR271398, KR271399, KR271402, KR271404, KR271407) (see Online Resource Table S5; Locke *et al.*, 2015).

What is D. baeri *sensu Galazzo* et al. *(2002)?*

In the *Guide to the Parasites of Fishes of Canada*, Gibson (1996) provided a key for the metacercariae of seven species of *Diplostomum*, including three non-lens-dwelling forms: *D. scudderi* (Olivier, 1941) Dubois, 1966 (syn. *Diplostomulum baeri eucaliae* Hoffman & Hundley, 1957) from the brain or retina of gasterosteids; *D. baeri bucculentum* Dubois & Rausch, 1948 from the retina or vitreous humour of the eye of salmonids; and *D. adamsi* Lester & Huizinga, (1977) from the retina of *Perca flavescens* (Mitchill).

Galazzo *et al.* (2002) developed experimentally adults in *Larus delawarensis* fed metacercariae from the "vitreous humour" of *P. flavescens* collected in the St Lawrence River near Montreal, Canada. These authors found a substantial differentiation (3.8%,

23 nt positions) in the ITS1 rDNA region between the specimens sequenced in North America and Europe and concluded that the two forms are not conspecific. However, Galazzo *et al.* (2002) used the name *D. baeri* for their experimentally developed adults. Locke *et al.* (2010*a*) generated *cox*1 sequences "from archived DNA of three vouchered adult specimens" studied by Galazzo *et al.* (2002) and from eight additional adult specimens and 64 metacercariae from the "vitreous humour" of *P. flavescens* collected in Canada. These authors also used the name *D. baeri* based on the sequence matching with the adults identified by Galazzo *et al.* (2002).

All recent molecular phylogenies indicate that the North American lineage named as *D. baeri* by Galazzo *et al.* (2002) and Locke *et al.* (2010*a*, 2010*b*, 2015) and the species-level lineages of the *D. baeri* complex are genetically distinct (Georgieva *et al.*, 2013; Blasco-Costa *et al.*, 2014; Faltýnková *et al.*, 2014; Selbach *et al.*, 2015; Soldánová *et al.*, 2017) and the present analyses strongly support this (Figs 5, 6; Table 4; Online Resource Fig. S2). Therefore, there is no justification for perpetuating use of this name for the North American lineage from *P. flavescens* and *Larus* spp. Compared with the original description of *D. baeri* based on specimens from Europe, the experimentally obtained material measured and illustrated by Galazzo *et al.* (2002) differs in having: a much larger body with a longer and narrower forebody and a substantially longer and narrower hindbody; an oral sucker much larger than pharynx (mean OSW/PHW = 1.5 *vs* oral sucker slightly larger than pharynx; OSW/PHW = 0.96–1.26) that is also equal to ventral sucker (mean VSW/OSW = 1.01 *vs* oral sucker slightly smaller than ventral sucker in *D. baeri*). Additionally, the anterior margins of the vitelline fields reach to the level of ventral sucker in the material described by Galazzo *et al.* (2002) whereas they extend anteriorly to ventral sucker up to mid-distance between pharynx and ventral sucker in *D. baeri* (see Dubois, 1970).

Unfortunately, Galazzo *et al.* (2002) did not compare their material with the description of *D. adamsi*, the only species with metacercariae known to develop in *P. flavescens* in Canada and North America in general (see Gibson, 1996; Zelmer and Arai, 1998). The life-cycle of *D. adamsi* was completed experimentally by Lester and Huizinga (1977) using *Lymnaea stagnalis* (L.) and *Ladislavella elodes* (Say) as the first intermediate hosts, *P. flavescens* as the only susceptible host out of five fish species tested, and *Larus argentatus* Pontoppidan as the experimental definitive host. In addition to the detailed descriptions of the life-cycle stages of *D. adamsi*, these authors provided histological and scanning electron microscopy evidence for the microhabitat of the metacercariae in *P. flavescens*, i.e. "in the peripheral retina, in a cavity between the photoreceptor cells and the pigment epithelium".

The solution for the confusion with the identification of *D. baeri sensu* Galazzo *et al.* (2002) comes from the detailed histological study of Ubels *et al.* (2018) clearly showing that infection with *D. baeri sensu* Galazzo *et al.* (2002) is confined to tissues associated with the eye retina (choroidal vasculature) of *P. flavescens*; these authors also generated sequence data for the metacercariae from the retinal tissues of *P. flavescens*. As shown in Online Resource Fig. S2, these sequences clustered with the sequences from the same fish host and *Larus* spp. in the studies of Galazzo *et al.* (2002), Moszczynska *et al.* (2009) and Locke *et al.* (2010*a*, 2010*b*, 2015). All of the above considerations clearly suggest that the metacercariae originating from *P. flavescens* and sequenced by these authors and by Ubels *et al.* (2018) represent the retinal form *D. adamsi*. The reclassification of the sequences labelled as "*Diplostomum baeri* complex sp. LIN2" and "*Diplostomum* aff. *baeri* LIN2" by Gordy and Hanington (2019) (see above) provides molecular evidence that, in agreement with the original description of *D. adamsi*, the snail *L. elodes* acts as the first intermediate host of this species. The introduction of *D. adamsi* as the only plausible identification for the lineage *D. baeri sensu* Galazzo *et al.* (2002) sequenced by Galazzo *et al.* (2002), Locke *et al.* (2010*a*, 2015) and Gordy and Hanington (2019) does not require changing the name of the *D. baeri* species complex as there is a number of lineages within it awaiting taxonomic scrutiny.

Re-classification and an updated nomenclature for Diplostomum *spp.*

Based on the present phylogenetic analyses, an updated nomenclature was applied and a large number of isolates of *Diplostomum* spp. published before 29 June 2021 was re-identified/re-classified (Table 4, Fig. 5, Online Resource Table S5, Fig. S2). The present re-classification revealed new linkages between life-cycle stages for three species/lineages, i.e. *D. adamsi*, *Diplostomum* sp. 13 of Locke *et al.* (2015) and *Diplostomum* sp. 18 of Locke *et al.* (2015). Cercarial isolates of these forms were sequenced from *L. elodes* in Canada by Gordy and Hanington (2019) (see Table 4 and Online Resource Table S5).

Here, we would like to highlight two cases with relevance to the data for the *D. baeri* complex discussed above. Landeryou *et al.* (2020) used *cox*1 and ITS sequences to identify the metacercariae from the vitreous humour of *Salmo trutta* collected in Scotland and used for characterisation of the mitochondrial genome of a species they believed to be *D. baeri*. However, these authors selected for their analysis *cox*1 sequences for just two lineages of the *D. baeri* species complex, i.e. *D. baeri sensu* Galazzo *et al.* (2002) from North America and *Diplostomum* sp. Lineage 3 of Blasco-Costa *et al.* (2014) (the "trout clade" of the *D. baeri* species complex *sensu* Georgieva *et al.*, 2013). Although the *cox*1 sequences of Landeryou *et al.* (2020) clearly fell within the clade of *Diplostomum* sp. Lineage 3 of Blasco-Costa *et al.* (2014) (the "trout clade" of Georgieva *et al.*, 2013), they named the species as "*D. baeri*". Our analysis revealed that the material sequenced by Landeryou *et al.* (2020) in fact belongs to and should be referred to as *Diplostomum* sp. Lineage 3 of Blasco-Costa *et al.* (2014) (Table 4, Online Resource Table S5 and Fig. S2).

Ubels *et al.* (2018) reported as *D. baeri* two sequences from metacercariae ex *P. flavescens* and *Luxilus cornutus* (Mitchill) collected in Douglas Lake, Michigan, USA. However, there is a conflict with host annotation in their paper and the Supplementary Fig. S1 provided by these authors (see Table 4). Whichever the host, our analysis clearly showed that the sequence MF142178 belongs to *Diplostomum* sp. 3 of Moszczynska *et al.* (2009) and the sequence MF142161 belongs to *Diplostomum* sp. 4 of Moszczynska *et al.* (2009).

The nomenclature of the genetic lineages of *Diplostomum* is in a state of flux since scientific names for 35 species-level lineages of *Diplostomum* have not yet been suggested. Identification to the species level *via* linking the genetic and morphological data for these lineages will be a long process and some lineages will remain unidentified for indefinite time. Locke *et al.* (2015) highlighted the problems associated with name discrepancies in the publications *vs* GenBank annotations for the expanding number of molecularly delineated species-level lineages within the Diplostomidae. Whilst we agree with their criticisms, we should like to highlight that the publication should be the leading source for the identification and host/microhabitat data for the newly sequenced isolates and the precise linking to GenBank sequences (and their annotations) should be part of the publication. In an ideal world with a centralised system for registering the lineage number sequence, the numbering system would be effective (as

suggested by Locke *et al.*, 2015) but this is not the case; the same applies to a lettering system, e.g. there are pairs of lineages currently labelled as A, B and C (see Kudlai *et al.*, 2017; Gordy and Hanington, 2019).

Lineage 'names' (labels) are not species binomens and thus no compliance with the International Code of Zoological Nomenclature is required. However, it would be wise to follow Code's principles of homonymy and priority of publication to ensure that the 'name' of each genetic lineage is unique and distinct, and that the oldest available 'name' is used for already characterised lineages. The uniqueness is ensured by consistently using the 'name' in association with the reference of the first publication, e.g. *Diplostomum* sp. 1 of Moszczynska *et al.* (2009), *Diplostomum* sp. A of Gordy and Hanington (2019), *Diplostomum* sp. A of Kudlai *et al.* (2017), *Diplostomum* sp. Lineage 2 of Blasco-Costa *et al.* (2014) or simply *Diplostomum* sp. of Chibwana *et al.* (2013) (see Table 4 for all updated lineage labels).

The updated data on the nomenclature and distribution for molecularly characterised species/lineages of *Diplostomum* based on our global analysis provided in Table 4 indicate that, in spite of the accumulation of sequences from recent studies, current distribution of *Diplostomum* spp. is the result of uneven sampling effort and suggest our knowledge of the species and genetic diversity in this group is still rudimentary in Africa, Asia and South America. Thus, nearly half of the molecularly characterised species/lineages (21; 48%) have only been recorded in North America. Of these, 12 (57%) taxa, including four singletons, have only been recorded in Canada. Nearly a third of the species/lineages (12 taxa, 27%, including 3 singletons) have only been recorded in Europe and there are fewer molecular records from Asia (eight taxa, including three also found in Europe: *D. spathaceum*; *D. mergi* Lineage 2 of Georgieva *et al.* (2013); *D. mergi* Lineage 4 of Selbach *et al.* (2015)) and Africa (three taxa, including two also found in Asia: *Diplostomum* spp. 14 and 16 of Locke *et al.* (2015)). Just one species has been characterised molecularly in South America.

Finally, the present updated synopsis of *Diplostomum* species/lineages highlights an important caveat for enhancing the knowledge of the diversity of *Diplostomum* spp. in fish hosts, i.e. the virtual lack of sequences for metacercariae of salmonid and gasterosteid hosts from North America. Currently, only four sequences are available from these host groups, three sequences from metacercariae in salmonids, two for *Diplostomum* sp. 7 and one for *Diplostomum* sp. 9, and a single sequence for *Diplostomum* sp. 13 of Locke *et al.* (2015) (possibly *D. scudderi* (Olivier, 1941)) from *G. aculeatus*. We predict that, similar to the current situation in Europe, focused sampling of gasterosteids and salmonids with a careful identification of the location of the non-lens-dwelling metacercariae will reveal a number of additional species/lineages of the *D. baeri* complex in North America. Furthermore, precise identification of the microhabitat in salmonid hosts anchored to novel morphological and sequence data may help assess the status of *D. baeri bucculentum* Dubois & Rausch, 1948 and distinguish it from the retinal form reported from salmonoids in Canada (see Gibson, 1996) and from the European lineages molecularly and morphologically characterised by Blasco-Costa *et al.* (2014) and described by Faltýnková *et al.* (2014). Sequencing of metacercariae from salmonids and gasterosteids will also provide additional data for testing the hypothesis for North America being an ancestral area for the *D. baeri* species complex (Blasco-Costa *et al.*, 2014) and shed light on the evolution of this group.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001372

Data. The data supporting the findings of this study are available within the article and its supplementary materials. All newly generated sequences were deposited in the GenBank database under the following accession numbers: MZ615631-MZ615639 (*cox*1, *D. phoxini*); MZ616379 and MZ616380 (28S, *D. phoxini*); MZ616381 and MZ616382 (ITS1-5.8S-ITS2, *D. phoxini*); MZ615629 and MZ615630 (*cox*1, *A. balthica*); MZ616383 (28S, *A. balthica*); and MZ616378 (ITS2, *A. balthica*). Raw data are available on request from the corresponding author [JS].

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Conflict of interest. The authors declare that they have no conflicts of interest.

Ethical standards. All applicable institutional, national and international guidelines for the care and use of animals were followed. The necessary permit for collecting and euthanizing fish was obtained prior to sampling (No. 51.3.1-6.2 Bezirksregierung Arnsberg).

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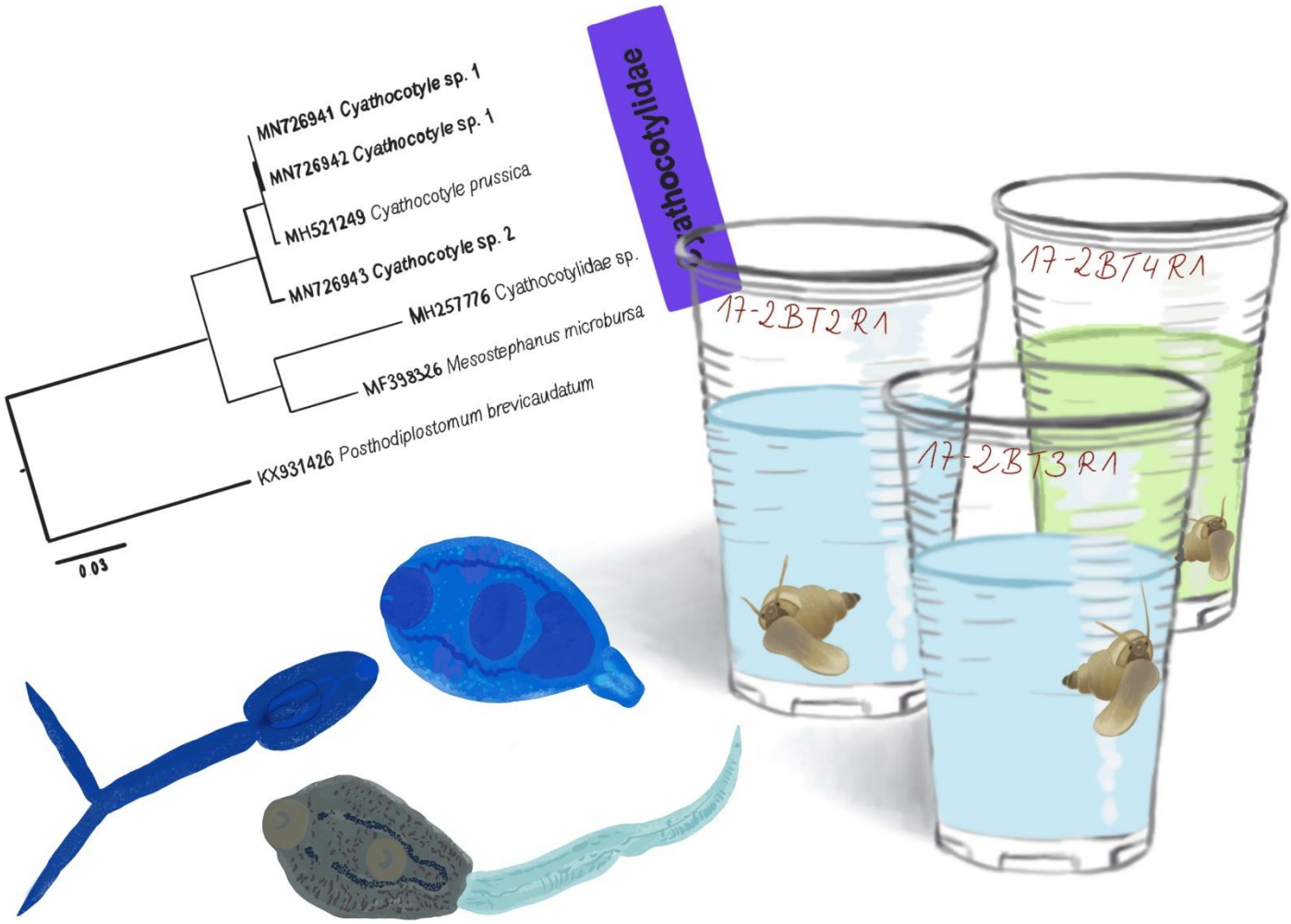
5. Chapter II
Taxonomy and morphology of trematodes from a neglected host



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High parasite diversity in a neglected host: larval trematodes of *Bithynia tentaculata* in Central Europe

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Abstract

Bithynids snails are a widespread group of molluscs in European freshwater systems. However, not much information is available on trematode communities from molluscs of this family. Here, we investigate the trematode diversity of *Bithynia tentaculata*, based on molecular and morphological data. A total of 682 snails from the rivers Lippe and Rhine in North Rhine-Westphalia, Germany, and 121 *B. tentaculata* from Curonian Lagoon, Lithuania were screened for infections with digeneans. In total, *B. tentaculata* showed a trematode prevalence of 12.9% and 14%, respectively. The phylogenetic analyses based on 55 novel sequences for 36 isolates demonstrated a high diversity of digeneans. Analyses of the molecular and morphological data revealed a species-rich trematode fauna, comprising 20 species, belonging to ten families. Interestingly, the larval trematode community of *B. tentaculata* shows little overlap with the well-studied trematode fauna of lymnaeids and planorbids, and some of the detected species (*Echinochasmus beleocephalus* and *E. coaxatus*) constitute first records for *B. tentaculata* in Central Europe. Our study revealed an abundant, diverse and distinct trematode fauna in *B. tentaculata*, which highlights the need for further research on this so far understudied host–parasite system. Therefore, we might currently be underestimating the ecological roles of several parasite communities of non-pulmonate snail host families in European fresh waters.

Introduction

With about 25,000 species and a cosmopolitan distribution, digenetic trematodes constitute one of the most diverse and ubiquitous groups of parasites on the planet (Esch *et al.*, 2002). Despite their complex life history with a wide variety of vertebrate definitive hosts, including fish, amphibians, reptiles, birds and mammals, this group shares a unifying character: practically all species require molluscs (usually gastropods) as first intermediate hosts. Due to their complex interaction with their hosts and their wide distribution and abundance, trematodes have been studied in a wide range of ecological contexts. For example, trematodes have been shown to make up a large proportion of an ecosystem's biomass (Kuris *et al.*, 2008; Preston *et al.*, 2013; Soldánová *et al.*, 2016), contribute significantly to the energy flow within ecosystem (Thieltges *et al.*, 2008), function as structuring forces in food webs (Lafferty *et al.*, 2008; Thieltges *et al.*, 2013) and can affect host populations by influencing host mortality, fecundity, growth and behaviour (Mouritsen & Jensen, 1994; Marcogliese, 2004; Lagrue & Poulin, 2008; Rosenkranz *et al.*, 2018). Moreover, they can serve as useful bioindicators to assess environmental conditions and changes due to their intricate life cycles (e.g. Lafferty, 1997; Huspeni & Lafferty, 2004; Vidal-Martínez *et al.*, 2010; Shea *et al.*, 2012; Nachev & Sures, 2016). Altogether, there is increasing awareness that trematodes are important ecosystem components that require our attention in order to fully understand the complex interactions and dynamics in ecosystems.

In freshwater systems, snails of the families Lymnaeidae and Planorbidae play a key role in the life cycle of trematodes. In Europe, members of both families serve as important first intermediate hosts to a wide variety of digenean trematodes, with 87 and 92 described species, respectively, which accounts for more than 85% of the described trematode species from gastropod hosts from this region (Faltýnková *et al.*, 2016; Schwelm *et al.*, 2018). Both groups are well-studied model host–parasite systems in terms of their diversity, ecological function and their role as infectious agents (e.g. Faltýnková & Haas, 2006; Faltýnková *et al.*, 2007, 2008, 2016; Soldánová *et al.*, 2010, 2013, 2017; Novobilský *et al.*, 2014; Horák *et al.*, 2015; Selbach *et al.*, 2015a, b). Consequently, detailed identification keys (Faltýnková *et al.*, 2007, 2008; Selbach *et al.*, 2014) as well as accessible molecular vouchers (e.g. Georgieva *et al.*,

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2013, 2014; Selbach *et al.*, 2014, 2015b; Zikmundová *et al.*, 2014; Soldánová *et al.*, 2017) are available for these parasite taxa, which enable studies to accurately identify trematodes and asses their ecological role. Moreover, the life cycles of many trematodes are known in detail (Brown *et al.*, 2011 and references therein), which allows conclusions to be drawn about the presence or absence of free-living organisms in ecosystems (Byers *et al.*, 2010).

In contrast to the detailed knowledge about lymnaeid and planorbid host-trematode systems, the role of bithyniid snails and other non-pulmonate snails, such as Hydrobiidae, Melanopsidae, Neritidae, Valvatidae and Viviparidae, as first intermediate hosts for trematodes in Central Europe remains largely unexplored. Although snails of these families are known to host digenean trematodes and these data were included in some faunistic surveys (e.g. Cichy *et al.*, 2011; Faltýnková *et al.*, 2016), these studies were mostly focused on lymnaeid and planorbid hosts. The faucet snail, *Bithynia tentaculata*, which is common and widespread throughout Europe, has established itself as a non-indigenous species in North America (Mills *et al.*, 1993; Duggan *et al.*, 2003; Bachtel *et al.*, 2019). *Bithynia tentaculata* is highly tolerant towards salinity and temporal droughts and occurs in most waterbodies throughout Europe (Glöer, 2002; Welter-Schultes, 2012). With 32 trematode species according to Cichy *et al.* (2011) and 14 species according to Faltýnková *et al.* (2016) known from *B. tentaculata*, it represents the most species-rich snail–parasite assemblage among the group of non-pulmonate freshwater snails (formerly known as 'Prosobranchia') in Europe. However, due to the lack of focussed faunistic studies on trematode communities in this host species, with the exception of individual studies investigating selected parasite groups (e.g. Serbina, 2005; Kudlai *et al.*, 2015, 2017), the number of trematode species known from *B. tentaculata* may well grossly underestimate the true diversity of this host–parasite system.

A serious obstacle for most freshwater ecologists and parasitologists encountering trematodes of the snail families Bithynidae, Hydrobiidae, Melanopsidae, Neritidae, Valvatidae and Viviparidae is the lack of morphological and molecular information for species identification. Unlike for planorbid and lymnaeid snails, there are no keys for larval trematodes parasitizing snails from these families, and morphological descriptions are often restricted to adult stages (e.g. Gibson *et al.*, 2002; Tkach *et al.*, 2003; Jones *et al.*, 2005; Bray *et al.*, 2008; Besprozvannykh *et al.*, 2017; Kudlai *et al.*, 2017). Moreover, existing literature is often not available in English (e.g. Našincová, 1992; Ataev *et al.*, 2002; Serbina, 2005; Besprozvannykh, 2009), which also exacerbates the investigation of this parasite–host system. These obstacles lead to a further bias towards well-studied species, such as *Lymnaea stagnalis*, *Radix* spp. and *Planorbarius corneus*, while other snail species continue to remain overlooked and avoided in the assessments of the ecological role of trematodes. It is, therefore, important to characterize the trematode fauna in *B. tentaculata*, and thus facilitate further studies on the ecological role of this host–parasite system, as is possible for lymnaeid and planorbid snails. Moreover, some of the trematodes utilizing *Bithynia* spp. are important pathogens of wildlife that can have serious impacts on migrating birds (e.g. Herrmann & Sorensen, 2009; Roy & St-Louis, 2017; Bachtel *et al.*, 2019), which further highlights the need for a better understanding of this host and its parasite fauna.

Here, we assess the diversity of the larval trematodes of *B. tentaculata* in Central Europe and provide molecular and morphological reference material to fill this gap in our knowledge. With this study, we also hope to draw more attention to this essential and largely overlooked parasite–host system and promote further studies on this group.

Material and methods

Sample collection

In total, 682 snails of the species *B. tentaculata* were collected and examined for trematode infections during monthly collections in 2016 and 2017. Snails were collected at four sampling sites at the River Lippe in summer and autumn in 2016 and 2017 (K4: 51° 39′44.1″N, 8°10′23.9″E; K1: 51°39′42.3″N, 8°13′49.3″E; K2: 51° 39′41.5″N, 8°14′18.1″E; K3: 51°39′41.8″N, 8°14′19.2″E) and at two sampling sites at the lower River Rhine (R1: 51°47′59.2″N, 6°21′46.3″E; R3: 51°48′37.1″N, 6°21′23.4″E) and one pond of its adjacent floodplain (R2: 51°49′07.0″N, 6°20′26.8″E) in spring, summer and autumn in 2017 in North Rhine-Westphalia, Germany (fig. 1). Additionally, 121 *B. tentaculata* were collected from the Curonian Lagoon near the village of Juodkrante, Lithuania (55°35′38″N, 21°7′57″E) in June 2013. Snails from Germany were identified using the identification keys of Glöer (2002) and Welter-Schultes (2012).

All snails were collected with strainers or hand-picked from sediments, stones, macrophytes and floating vegetation from the riverside or along the littoral zone of the pond. In the laboratory, snails were placed in individual cups with filtered river water at 20°C and exposed to a light source to induce the emergence of cercariae. Each cup was screened for the presence of cercariae three times over three consecutive days after sampling under a stereomicroscope. Snails that did not shed cercariae during this time period were dissected and examined for prepatent infections (rediae/sporocysts). To obtain isolates for molecular analyses, cercariae, rediae and sporocysts were pooled from one single infected snail host and fixed in molecular-grade ethanol. Additionally, cercariae were fixed in 4% formaldehyde solution for measurements of fixed material. For documentation and measurements of the snail hosts, photomicrographs of the snail shell were taken with a Keyence VHX5000 microscope (Osaka, Japan). Foot tissue from infected snails was fixed in molecular-grade ethanol for molecular analysis and identification.

Morphological analyses

Larval stages were preliminarily identified under an Olympus BX51 microscope (Tokyo, Japan) using morphological descriptions of Našincová (1992) and Bykhovskaya-Pavlovskaya & Kulakova (1971) and other relevant publications (e.g. Heinemann, 1937; Zdun, 1961; Našincová & Scholz, 1994; Kudlai *et al.*, 2015). Preliminary identification was made to the family or genus level. Morphology of cercariae was studied on live and fixed specimens. Series of photomicrographs were taken for collected isolates with an Olympus UC30 digital camera (Tokyo, Japan) for measurements and further identification. Measurements were taken from the digital images using cellSens 1.16 Life Science image software (https://www.olympus-lifescience.com/en/software/cellsens). Measurements are in micrometres (µm) and are presented as a range, followed by a mean in parentheses.

Molecular sequencing

DNA isolation was performed following a modified salt precipitation protocol after Sunnucks & Hales (1996) and Grabner *et al.*

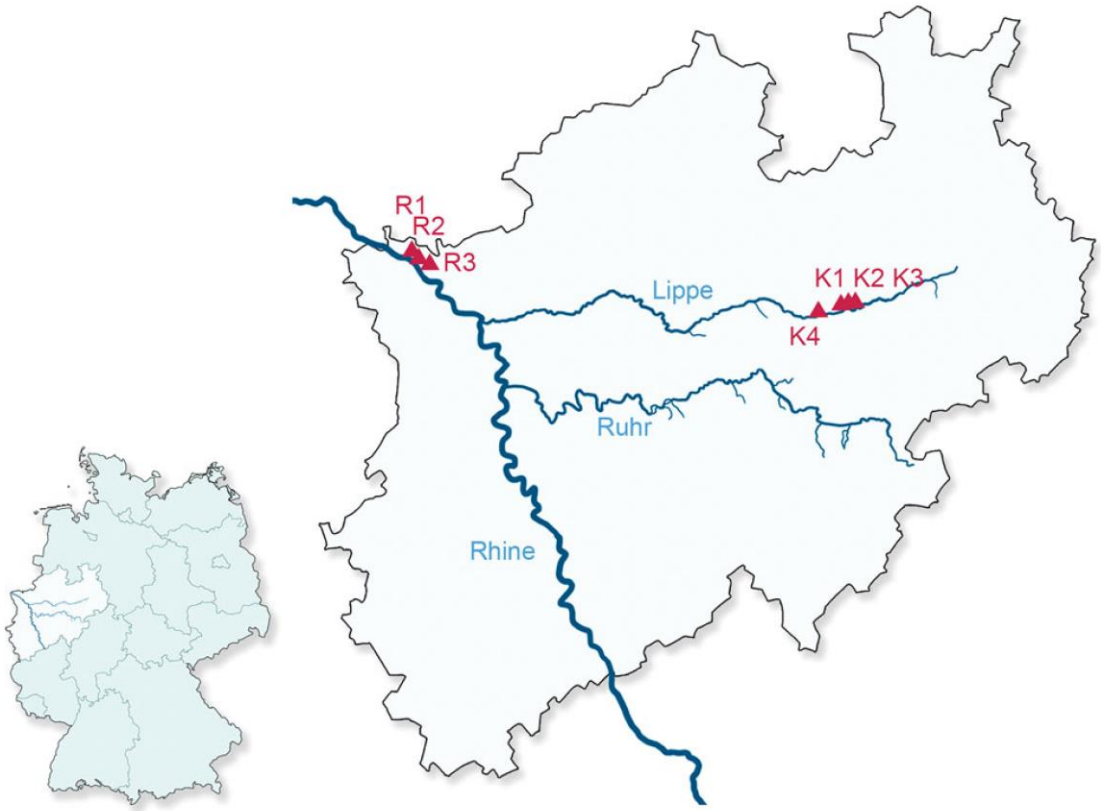

Fig. 1. Map of Germany and the federal state of North Rhine-Westphalia indicating sampling sites along the rivers Lippe and Rhine. Sampling sites are marked with red triangles.

(2015). To each sample, 600 µl TNES Buffer and 10 µl proteinase K solution were added. Trematode samples were incubated at 50° C for two to three hours depending on the quantity of the sample material. Snail tissue samples were incubated overnight at 35°C. In order to precipitate the proteins, 170 µl of 5 M sodium chloride was added, followed by vortexing and centrifuging for 5 min at 20,000×g at room temperature. The supernatant was transferred into a new reaction tube and centrifuging was repeated. The supernatant was again transferred into a new reaction tube, the pellet was discarded and 800 µl of 99% ice-cold ethanol was added to the supernatant and mixed by repeated inverting. The solution was centrifuged at 20,000×g for 15 min at 4°C. In order to purify the sample, 180 µl of 70% ethanol was added after the supernatant was discarded. The sample was centrifuged for 15 min at 20,000×g at 4°C, the ethanol was discarded and the pellet air-dried. The DNA pellet was dissolved in 100 µl TE buffer.

Target gene fragments were chosen based on preliminary identification of cercariae to the family level and were amplified via polymerase chain reaction (PCR) (table 1) following the corresponding protocols (Folmer *et al.*, 1994; Cribb *et al.*, 1998; Galazzo *et al.*, 2002; Kostadinova *et al.*, 2003; Tkach *et al.*, 2003). Tissue of snails was also used for DNA extraction and PCR amplification using the primers and protocols by Folmer *et al.* (1994). PCR products were purified using purification kits (GATC Biotech, Constance, Germany). The original PCR primers and the internal primers for 28S were used for sequencing (table 1). Contiguous sequences were assembled and edited in Geneious ver. 11 (https://www.geneious.com). All sequences were submitted to GenBank under accession numbers MN720141–MN720149; MN723852–MN723854; MN726941–MN726975; and MN726988–MN727001. For species identification, each sequence was compared with sequences available in GenBank by using the Basic Local Alignment Tool (BLAST).

Phylogenetic analyses

The newly generated sequences were aligned with sequences available in GenBank according to the trematode family and gene amplified (supplementary table S1). Sequences were aligned with MUSCLE (Edgar, 2004) implemented in Geneious ver. 11. A total of eight alignments for nine families were analysed. Outgroup selection was based upon the molecular phylogenies of Olson *et al.* (2003), Tkach *et al.* (2016), Kanarek *et al.* (2017) and Hernández-Orts *et al.* (2019). Phylogenetic trees for each dataset were constructed with Bayesian inference (BI) and maximum likelihood (ML) analyses on the CIPRES portal (Miller *et al.*, 2010) and the ATGC bioinformatics platform, respectively. The Akaike Information Criterion implemented in jModelTest 2.1.1 (Guindon and Gascuel, 2003; Darriba et al. 2012) was used to determine the best-fit nucleotide substitution model for each dataset. These were the general time reversible model, with estimates of invariant sites and gamma distributed among-site rate variation (GTR + I + G) for six alignments:

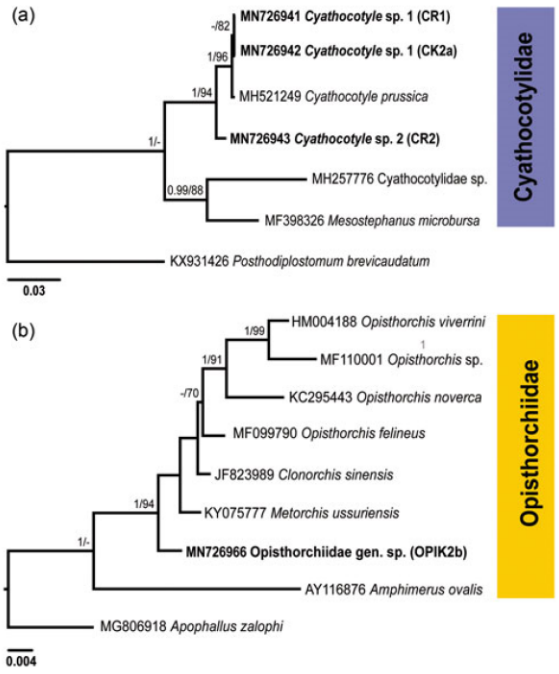

Fig. 2. Phylogenetic trees for Cyathocotylidae (a) and Opisthorchiidae (b) based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

Echinochasmidae and Psilostomidae (28S), Psilostomidae (28S), Notocotylidae (28S), Pleurogenidae and Prosthogonimidae (28S and internal transcribed spacer 2 (ITS2)), and Opecoelidae (28S); and (GTR + I) for two alignments: Cyathocotylidae (28S) and Opisthorchiidae (28S). BI analyses were performed using MrBayes v3.2.6 (Ronquist *et al.*, 2012). Markov chain Monte Carlo chains were run for ten million generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the 'burnin' parameter at 2500 for six alignments: Echinochasmidae and Psilostomidae (28S), Psilostomidae (28S), Notocotylidae (28S) and Pleurogenidae and Prosthogonimidae (28S and ITS2). Markov chain Monte Carlo chains were run for three million generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the 'burnin' parameter at 750 for two alignments: Cyathocotylidae (28S) and Opisthorchiidae (28S). ML analyses were performed using PhyML version 3.0 (Guindon *et al.*, 2010) with a non-parametric bootstrap validation based on 100 replicates. Trees were visualized using the FigTree ver. 1.4 software (http://tree.bio.ed.ac.uk/software/figtree/). One *cox*1 sequence alignment was analysed for the snails. The genetic divergence among taxa was estimated using uncorrected *p*-distances with the program MEGA version 6 (Tamura *et al.*, 2013).

Results

General observations

A total of 12.9% of all *B. tentaculata* from Germany showed larval trematode infections. Snails collected in Lithuania showed an overall prevalence of 14%. Phylogenetic and BLAST analyses based on 55 novel sequences for 36 isolates recovered from *B. tentaculata* collected in Germany and Lithuania (table 2) demonstrated high diversity of digeneans, including 20 species belonging to ten families: Cyathocotylidae, Echinochasmidae, Lecithodendriidae, Lissorchiidae, Notocotylidae, Opecoelidae, Opisthorchiidae, Pleurogenidae, Prosthogonimidae and Psilostomidae.

Six partial *cox*1 sequences were generated from isolates of *B. tentaculata* sampled in all German localities (MN720141–MN720146). The sequence difference between the isolates was 0–0.2% (1 nucleotide (nt) difference), thus confirming their conspecificity. Molecular identification of the snail isolates was achieved by comparing our sequences with sequences for *B. tentaculata* in GenBank. A BLAST search analysis indicated a 86% coverage and 98% of similarity with two isolates of *B. tentaculata* from Germany (AF445334) (Hausdorf *et al.*, 2003) and North America (JX970605) (Wilke *et al.*, 2013); and a 89% coverage and 92% of similarity with one isolate from Croatia (AF367643) (Wilke *et al.*, 2001). Snails from the Lithuanian system were identified morphologically.

Systematics

Superfamily: Diplostomoidea Poirier, 1886
Cyathocotylidae Mühling, 1898

Molecular results

In total, four snails from three localities were infected with cercariae belonging to the family Cyathocotylidae (prevalence: River Lippe: 0.2%; River Rhine: 4%). Sequences for the partial 28S rRNA gene and entire ITS1-5.8S-ITS2 gene cluster were generated for one isolate per locality.

Both BI and ML analyses of the Cyathocotylidae based on 28S rDNA alignment included novel sequences and those retrieved from GenBank (fig. 2a; supplementary table S1), and resulted in trees with similar topologies. Sequences for the isolates CR1 and CK2a clustered with the sequence for *Cyathocotyle prussica* Mühling, 1896 with a strong support. A single isolate (CR2) formed a branch basal in the clade of *Cyathocotyle* spp.

The two identical 28S rDNA sequences (isolates CR1 and CK2a) differed from the sequence for *C. prussica* (MH521249) by 0.2% (2 nt) and from the sequence for the isolate CR2 by 1.4% (17 nt). The ITS1-5.8S-ITS2 sequences for the isolates CR1 and CK2a were identical and differed from the sequence for the isolate CR2 by 3.7% (48 nt). A BLAST analysis based on ITS1-5.8S-ITS2 data revealed two closely related sequences of the Cyathocotylidae available in GenBank. These were *Holostephanus dubinini* Vojtek & Vojtkova, 1968 (AY245707) and *C. prussica* (MH521249) (supplementary table S1). However, the isolates CR1 and CK2a differed from *C. prussica* by 0.2% (3 nt) and from *H. dubinini* by 3.8% (49 nt), whereas the isolate CR2 differed from the same species by 3.6% (47 nt) and 3.5% (45 nt), respectively. Based on the results of molecular analyses, the isolates CR1 and CK2a were identified as *Cyathocotyle* sp. 1 and isolate CR2 as *Cyathocotyle* sp. 2.

Systematics

Cyathocotyle Muhling, 1896

Cyathocotyle *sp. 1*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Table 1. PCR primers for gene-fragments used in the study.

Gene fragment	Primer name	Nucleotide sequence	Source
28S	Digl2	5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach *et al.* (2003)
	1500R	5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach *et al.* (2003)
	ECD2[a]	5′-CTTGGTCCGTGTTTCAAGACGGG-3′	Tkach *et al.* (2003)
	300F[a]	5′-CAAGTACCGTGAGGGAAAGTTG-3′	Tkach *et al.* (2003)
ITS1-5.8S-ITS2	D1F	5′-AGGAATTCCTGGTAAGTGCAA-3′	Galazzo *et al.* (2002)
	D2R	5′-CGTTACTGAGGGAATCCTGGT-3′	Galazzo *et al.* (2002)
ITS2	3S	5′-GGTACCGGTGGATCACGTGGCTAGTG-3′	Morgan & Blair (1995)
	ITS.2	5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′	Cribb *et al.* (1998)
*cox*1	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer *et al.* (1994)
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al.* (1994)
*nad*1	NDJ11	5′-AGATTCGTAAGGGGCCTAATA-3′	Kostadinova *et al.* (2003)
	NDJ2a	5′-CTTCAGCCTCAGCATAAT-3′	Kostadinova *et al.* (2003)

[a]Internal primers. PCR conditions were followed as described in the source papers.

Localities. River Rhine (R1), River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726941, MN726942); ITS1-5.8S-ITS2, two replicates (MN723852, MN723853).

Description

(Measurements from eight fixed specimens.) Furcocercous cercariae (fig. 3a, b) with colourless, opaque, elongate-oval body, 192–226 × 116–147 (205 × 130). Entire body surface covered with minute spines. Tail with furcae; tail stem 267–300 (288) long with maximum width 45–61 (54). Tail stem longer than body [tail stem/body length ratio 1:0.67–0.78 (1:0.71)]. Furcae 229–255 (245) long with maximum width 31–39 (35). Furcae without finfold. Tail stem/furcal length ratio 1:0.80–0.78 (1:0.85). Tips of furcae form contractile processes. Anterior organ terminal, elongate-oval, 36–49 × 29–36 (40 × 32) with several rows of spines (10–12). Prepharynx distinct, pharynx small, elongate-oval, 9–13 × 9–10 (11 × 9); oesophagus short, bifurcating in first third of body, intestinal caeca well developed, conspicuous and wide with lobate walls, terminating blindly at posterior extremity of body. Ventral sucker absent. Penetration gland-cells numerous, small, pear-shaped, posterior to anterior organ and lateral to prepharynx and pharynx, with narrow ducts opening subterminally at the anterior end of anterior organ. Excretory commissures forming two conspicuous loop-like structures. Genital primordium in one group of compact small cells anterior to excretory vesicle. Excretory vesicle thin-walled, transversely oval.

Cyathocotyle *sp. 2*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726943); ITS1-5.8S-ITS2, one replicate (MN723854).

Description

(Measurements from ten fixed specimens.) Furcocercous cercariae (fig. 3c) with colourless, opaque, elongate-oval body, 151–176 × 93–120 (165 × 107). Entire body surface covered with minute spines. Several rows (7–8) of small postoral spines at anterior body part. Tail with furcae; tail stem 164–207 (182) long with maximum width at base 39–53 (45). Tail stem longer than body [tail stem/body length ratio 1:0.93–0.97 (0.91)]. Furcae lance-shaped, 135–163 (150) long, maximum width 28–41 (35), with small triangular fins, forming contractile processes. Tail stem/furcal length ratio 1:0.74–0.90 (1:0.82). Anterior organ terminal, elongate-oval, 29–36 × 20–30 (32 × 24). Prepharynx distinct; pharynx small, elongate-oval, muscular, 9–13 × 6–10 (11 × 9), conspicuous; oesophagus very short, indistinct, bifurcation just postpharyngeal. Intestinal caeca well developed, with slightly lobate walls, terminating blindly at posterior extremity of body. Ventral sucker absent. Penetration gland-cells numerous, small, pear-shaped, posterior to anterior organ. Main collection ducts without refractile excretory granules. Excretory commissures forming two loop-like structures. Genital primordium in one group of compact medium-sized cells anterior to excretory vesicle. Excretory vesicle thin-walled, transversely oval.

Remarks

The morphological features of cercariae of both species are consistent with the morphology of cyathocotylid cercariae according to Ginetsinskaya & Dobrovolskij (1968) and Niewiadomska (1980). To date, cyathocotylid cercariae of nine species have been reported from *B. tentaculata* in Europe: *Cyathocotyle bithyniae* Sudarikov, 1974, *C. bushiensis* (Khan, 1962), *C. prussica* Muhling, 1896; *Holostephanus cobitidis* Opravilova, 1968, *H. curonensis* (Szidat, 1933), *H. dubinini* Vojtek & Vojtkova, 1968, *H. luehei* Szidat, 1936, *H. volgensis* (Sudarikov, 1962) and *Prohemistomum vivax* (Sonsino, 1892).

The cercariae of *Cyathocotyle* sp. 1 and *Cyathocotyle* sp. 2 show several distinctive features that allow the separation of the two species. Cercariae of *Cyathocotyle* sp. 1 differ by body size [length: 192–226 (205) vs. 151–176 (165); width: 116–147 (130) vs. 93–120 (107)], number of rows of postoral spines (10–12 vs. 7–8), shape of furcae (more slender with a sharp tip vs. more compact with small triangular fins), size of furcae [length: 229–255 (245) vs. 135–163 (150); width: 31–39 (45) vs. 39–53 (35)] and tail

Table 2. Summary data for isolates collected from *Bithynia tentaculata* and used for generation of novel sequences.

		GenBank accession number			
Species	Isolate	28S	ITS1-5.8S-ITS2	ITS2	*nad*1
Family Cyathocotylidae Mühling, 1898					
Cyathocotyle sp. 1	CR1	MN726941	MN723852		
	CK2a	MN726942	MN723853		
Cyathocotyle sp. 2	CR2	MN726943	MN723854		
Family Echinochasmidae Odhner, 1910					
Echinochasmus coaxatus Dietz, 1909	ECR1	MN726944			MN720147
Echinochasmus bursicola (Creplin, 1837)	EBR1	MN726945			
Echinochasmus sp. 1	E2R1	MN726946			
	E1CB	MN726947			
Echinochasmus sp. 2	E1K2a	MN726948			MN720148
Family Psilostomidae Looss, 1900					
Sphaeridiotrema sp.	SR2	MN726949			MN720149
Psilostomidae gen. sp. 1	PS1R1	MN726950			
	PS1R2	MN726951			
	PS1K2a	MN726952			
	PSCB	MN726953			
Psilostomidae gen. sp. 2	PS2R2	MN726954			
Family Lissorchiidae Poche, 1926					
Asymphylodora sp.	ATK2b	MN726955			
Family Notocotylidae Lühe, 1909					
Notocotylus sp.	N11K0	MN726956			
	N12K0	MN726957			
Notocotylidae gen. sp.	N2K0	MN726958			
	N2K2b	MN726959			
Family Opecoelidae Ozaki, 1925					
Sphaerostoma sp.	O11K2a	MN726960		MN726988	
	O1K1	MN726961		MN726989	
	O1K2b	MN726962		MN726990	
	O12K2a			MN726991	
	O13K2a	MN726963			
Opecoelidae gen. sp.	O2K2a	MN726964		MN726992	
Family Lecithodendriidae Lühe, 1901					
Lecithodendrium linstowi (Dollfus, 1931)	LLK2a	MN726965		MN726993	
Family Opisthorchiidae Looss, 1899					
Opisthorchiidae gen. sp.	OPIK2b	MN726966			
Family Pleurogenidae Looss, 1899					
Parabascus duboisi (Hurkova, 1961)	PBK2b	MN726967		MN726994	
Pleurogenidae gen. sp. 1	PL11K2a	MN726968		MN726995	
	PL12K2a	MN726969		MN726996	
Pleurogenidae gen. sp. 2	PL2R1	MN726970		MN726997	
Family Prosthogonimidae Lühe, 1909					

(*Continued*)

Table 2. (*Continued.*)

Species	Isolate	GenBank accession number			
		28S	ITS1-5.8S-ITS2	ITS2	*nad*1
Prosthogonimus ovatus Rudolphi, 1803	PO1K2b	MN726971		MN726998	
	PO2K2b	MN726972			
	PO1R1	MN726973		MN726999	
	PO2R1	MN726974		MN727000	
	POK2a	MN726975		MN727001	

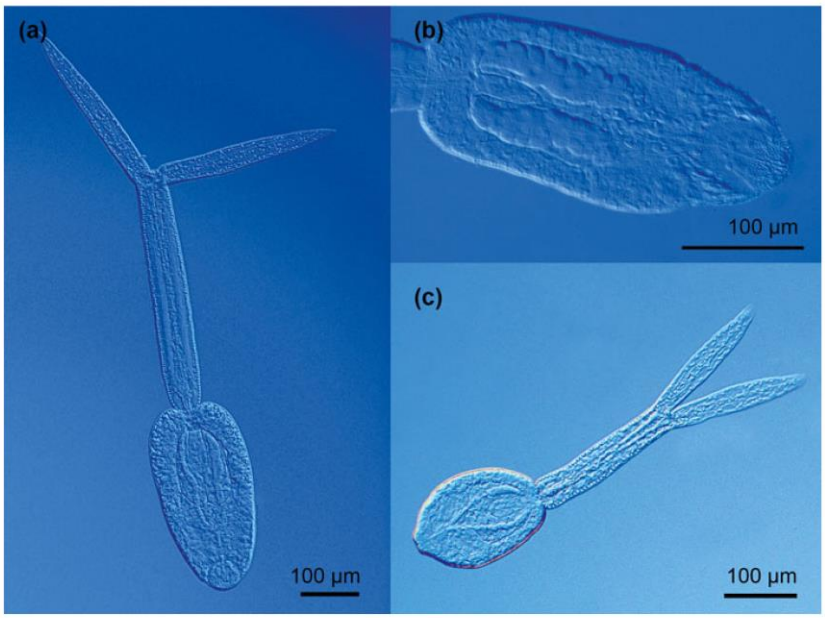

Fig. 3. Photomicrographs of live cercariae of the trematode family Cyathocotylidae. (a) *Cyathocotyle* sp.1; (b) *Cyathocotyle* sp.1, details of body spines and penetration gland-cells; (c) *Cyathocotyle* sp. 2.

stem/furcal length ratio [1:0.80–0.89 (1:0.85) vs. 1:0.74–0.90 (1:0.82)]. The oesophagus of the cercariae of *Cyathocotyle* sp. 2 is much shorter than in *Cyathocotyle* sp. 1 and it bifurcates just behind the pharynx (fig. 3a, b). The intestinal caeca are well developed in both species, but the cercariae of *Cyathocotyle* sp. 1 show more conspicuous ceaca with wide and lobate walls, whereas the ceaca of the cercariae of *Cyathocotyle* sp. 2 exhibit narrower and straighter walls.

General morphology of cercariae of *Cyathocotyle* sp. 1 and *Cyathocotyle* sp. 2 corresponds well to cercariae of *C. bithyniae*, *C. prussica* and *C. bushiensis* described by Niewiadomska (1980), Kanev (1984) and Khan (1962), respectively. The cercariae of *Cyathocotyle* sp. 1 resemble cercariae of *C. prussica* as described by Kanev (1984) in the absence of the small triangular fins on the tip of furca. However, cercariae of *Cyathocotyle* sp. 1 have larger body [length: 192–226 (205) vs. 136–169; width: 116–147 (130) vs. 71–105], higher limits for the length and width of the anterior organ [length: 36–49 (40) vs. 32–45; width: 29–36 (32) vs. 26–32], smaller pharynx [9–13 (11) × 9–10 (9) vs. 14 × 14], shorter and narrower tail [length: 267–300 (288) vs. 400–500; width: 45–61 (54) vs. 56–65] and higher low limits for the length and width of furcae [length: 229–255 (245) vs. 208–234; width: 31–39 (35) vs. 26–39].

The cercariae of *Cyathocotyle* sp. 1 differ from the cercariae of *C. bithyniae* by larger body [length: 192–226 (205) vs. 161–187; width: 116–147 (130) vs. 102–119], longer tail [267–300 (288) vs. 192–204], shorter and wider anterior organ [length: 29–36 (32) vs. 37–44; width: 36–49 (40) vs. 30–39] and by the absence of the small triangular fins at the ends of the tip of furca. In addition, cercariae of *Cyathocotyle* sp. 1 bear a small elongate-oval pharynx, whereas the pharynx of *C. bithyniae* was described as fairly large and muscular [9–13 × 9–10 (11 × 9) vs. 11–17 × 10–15]. The differences between the cercariae of *Cyathocotyle* sp. 1 and *C. bushiensis* as described by Khan (1962) include: body width [116–147 (130) vs. 90–93 (92), respectively], size of the furca [229–255 (245) × 31–39 (35) vs. 200–216 (209) × 13–20 (17)], anterior organ [36–49 (40) × 29–36 (32) vs. 43–46 (44) × 33–36 (38)] and pharynx [9–13 (11) × 9–10 (9) vs. 16 × 16].

The present cercariae identified as *Cyathocotyle* sp. 2 appeared most similar to the cercariae of *C. bithyniae* and *C. bushiensis* based on the presence of the small triangular fins at the ends of the tail furcae, but differ from cercariae of *C. bithyniae* as described by Niewiadomska (1980) by the lower limits for body length [151–176 (165) vs. 161–187], lower low limits for body width [93–120 (107) vs. 102–119], lower low limits for tail length [164–207 (182) vs. 192–204], higher limits for the width of furca

[28–41 (35) vs. 20–34] and shorter anterior organ [20–30 (32) vs. 37–44]. Additionally, the cercariae of *Cyathocotyle* sp. 2 possess a smaller pharynx than the cercariae of *C. bithyniae* [9–13 (11) × 6–10 (9) vs. 11–17 × 10–15].

Cercariae of *Cyathocotyle* sp. 2 differ from *C. prussica* as described by Kanev (1984) by larger body [length: 151–176 (165) vs. 136–169; width: 93–120 (107) vs. 71–105], lower low limits for length and width of the anterior organ [length: 29–36 (32) vs. 32–45; width: 20–30 (24) vs. 26–32], smaller pharynx [length: 9–13 (11) vs. 14; width: 6–10 (9) vs. 14], shorter and narrower tail [length: 164–207 (182) vs. 400–500; width: 39–53 (45) vs. 56–65] and shorter furcae [135–163 (150) vs. 208–234].

The cercariae of *Cyathocotyle* sp. 2 differ from cercariae of *C. bushiensis* as described by Khan (1962) by smaller body dimensions except the width of body [93–120 (107) vs. 90–93 (92), respectively] and furca [28–41 (35) vs. 13–20 (17)].

Systematics

Superfamily: Echinostomatoidea Looss, 1899
Echinochasmidae Odhner, 1910 and Psilostomidae Looss, 1900

Molecular results

Infection with the cercariae of the families Echinochasmidae and Psilostomidae was detected in nine snails from two localities in the River Rhine and one locality at the River Lippe, and in two snails collected in the Curonian Lagoon (prevalence: Echinochasmidae: River Lippe, 0.2%; River Rhine, 4%; Curonian Lagoon, 0.8%; Psilostomidae: River Lippe, 0.2%; River Rhine, 6.7%; Curonian Lagoon, 13.2%). Partial 28S rDNA sequences were generated for all collected isolates ($n = 11$); *nad*1 sequences were successfully generated for three isolates (fig. 4; supplementary fig. S1; table 2).

Sequences of partial 28S rDNA obtained in this study were aligned with the available sequences for echinoschasmids ($n = 15$) and psilostomids ($n = 13$) from GenBank (supplementary table S1). Two species of the Himasthlidae, *Acanthoparyphium spinulosum* Johnston, 1917 and *Himasthla limnodromi* Didyk & Burt, 1997 were used as the outgroup based on the topologies in the phylogenetic tree of the Echinostomatoidea published by Tkach *et al.* (2016). Both BI and ML analyses yielded a similar topology, with two main clades representing the two families, Echinochasmidae and Psilostomidae. The newly generated sequences fell into two distinct and strongly supported clades within each family. Two isolates, ECR1 and EBR1, collected from the River Rhine were identical with the sequences for *Echinochasmus coaxatus* Dietz, 1909 (KT956928) ex *Podiceps nigricollis* and *E. bursicola* (Creplin, 1837) (KT956938) ex *Ardea alba* from Ukraine (Tkach *et al.*, 2016), respectively (fig. 4; supplementary table S1). The three remaining isolates within the Echinochasmidae represent two unidentified species of *Echinochasmus*. *Echinochasmus* sp. 1 (E2R1 and E1CB) clustered with the sequence for *Echinochasmus milvi* Yamaguti, 1939 (KT873319) from Russia, and *Echinochasmus* sp. 2 (E1K2a) with *E. beleocephalus* (Linstow, 1873) (KT956929) ex *A. alba* from Ukraine and *E. japonicus* Tanabe, 1926 (JQ890579) ex *Gallus gallus* from Vietnam. The interspecific divergence between *Echinochasmus* sp. 1 and *E. milvi* was 1.5% (58 nt), and between *Echinochasmus* sp. 2 and *E. beleocephalus* and *E. japonicus* was 0.2% (2 nt) and 0.6 (7 nt), respectively.

Six isolates (SR2, PS1R1, PS1R2, PS1K2a, PSCB and PS2R2) represented by three species fell within the clade for the Psilostomidae. One isolate (SR2) collected from the River Rhine was identical to the isolate for *Sphaeridiotrema* sp. ex *B. tentaculata* (KT956958) from Lithuania (fig. 5; supplementary table S1). Five remaining isolates formed a strongly supported clade with four of them (PS1R1, PS1R2, PS1K2a and PSCB) representing the same species, whereas the fifth isolate (PS2R2) was distinct. The sequences for these five isolates did not show affiliation to any of the psilostomid genera included in the analyses. The interspecific divergence between the two species was 1.8% (20 nt). Based on these results, both species were identified to the family level as Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2.

Additional analyses were conducted for the Psilostomidae in order to include sequences of the three species of the genus *Psilotrema* Odhner, 1913 available in GenBank (supplementary table S1). The sequences for these species were not included in the main analyses due to their short length (759 nt). In these analyses, the isolates for Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 clustered in a clade with representatives of the genus *Psilotrema*, *P. oschmarini*, *P. simillium* and *P. acutirostris*, while Psilostomidae gen. sp. 1 appeared to be conspecific with *P. oschmarini* (see supplementary fig. S1). Based on this result, both species – Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 – may belong to the genus *Psilotrema*. However, at this stage, we refrain from identifying cercariae in our material as *P. oschmarini* due to the results being based on a short dataset and the lack of morphological vouchers for the sequences in GenBank.

Systematics

Echinochasmidae Odhner, 1910
Echinochasmus Dietz, 1909

Echinochasmus coaxatus *Dietz, 1909*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726944); *nad*1, one replicate (MN720147).

Description

(Measurements from 11 fixed specimens.) Gymnocephalous cercariae (fig. 5a). Body colourless, oval, with maximum width slightly anterior to ventral sucker, 103–124 × 68–102 (115 × 86). Tegument thick, tegumental spines absent. Collar poorly developed. Collar spines absent. Tail simple, muscular, contractile, 72–115 (92) long, with maximum width at base 22–33 (29), slightly shorter than body [tail/body length ratio 1:1.12–1.35 (1:1.25)]. Oral sucker subterminal, muscular, subspherical, 23–32 × 28–36 (29 × 31). Ventral sucker subspherical, just postequatorial, 26–33 × 23–31 (30 × 27). Oral/ventral sucker width ratio 1:0.90–1.14 (1:1.03). Prepharynx distinct, pharynx spherical, muscular, 6–10 × 5–10 (8 × 7). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells few, rounded, with rhabditiform contents. Excretory vesicle bipartite; anterior part transversely oval, at level of posterior margin of body, posterior part transversely oval, at junction of body and tail; main collecting ducts wide, dilated between mid-level of pharynx and level of posterior margin of ventral sucker, containing large dark refractile excretory granules of different size (12 on each side).

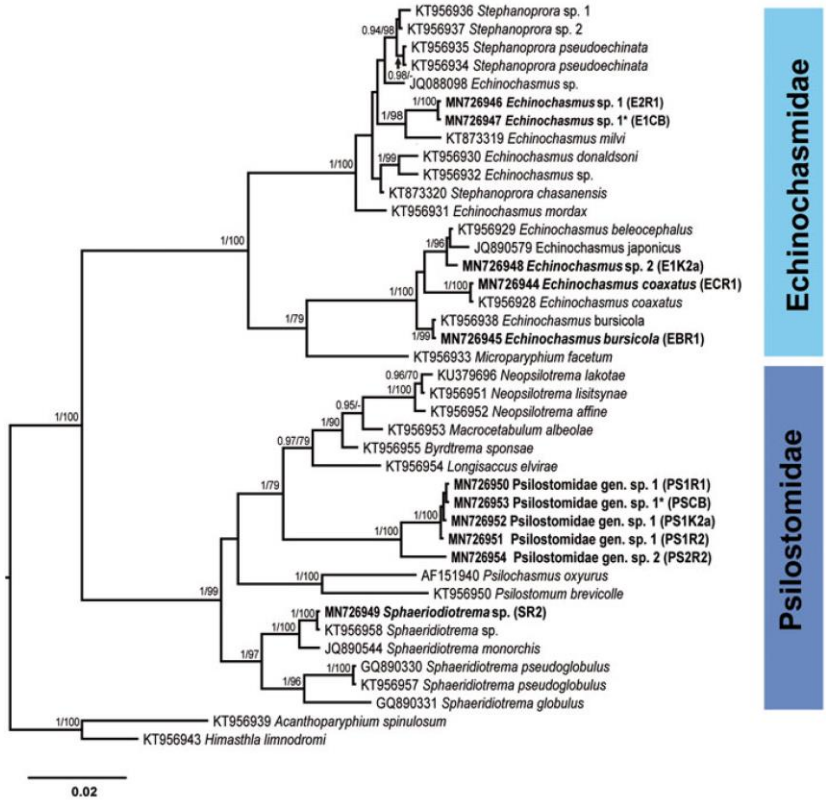

Fig. 4. Phylogenetic tree for Echinochasmidae and Psilostomidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold. Sequences obtained in Curonian Lagoon indicated by asterisk.

Echinochasmus bursicola *(Creplin, 1837)*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726945).

Description

(Measurements from ten fixed specimens.) Gymnocephalous cercariae (fig. 5b). Body colourless, oval, with maximum width slightly anterior to ventral sucker, 110–132 × 71–105 (120 × 88). Tegument thick, tegumental spines absent. Collar well developed. Collar spines absent. Tail simple, muscular, contractile, 83–101 (93) long, with maximum width at base 31–39 (36), shorter than body [tail/body length ratio 1:1.18–1.42 (1:1.29)]. Oral sucker subterminal, muscular, transversely oval, 25–37 × 31–40 (30 × 35). Ventral sucker subspherical, just postequatorial, 27–38 × 25–37 (33 × 31). Oral/ventral sucker width ratio 1:0.90–1.27 (1:1.10). Prepharynx long, distinct, pharynx subspherical, muscular, 7–10 × 6–9 (9 × 7). Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells few, rounded, with rhabditiform contents. Excretory vesicle bipartite; anterior part transversely oval, at level of posterior margin of body, posterior part transversely oval, at junction of body and tail; main collecting ducts narrow, containing large dark refractile excretory granules of different size (seven on each side).

Echinochasmus *sp. 1*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany; Curonian Lagoon (CB), Lithuania.

Representative DNA sequences. 28S rDNA, two replicates (MN726946, MN726947).

Description

(Measurements from seven fixed specimens.) Gymnocephalous cercariae (fig. 5c). Body colourless, oval, with maximum width at level of ventral sucker, 105–136 × 74–125 (123 × 95). Tegument thick, brownish, tegumental spines absent. Collar unincisive. Collar spines absent. Tail simple, leaf-like, brownish, muscular, contractile, with few randomly arranged subspherical concretions, 115–170 (141) long with maximum width at base 27–82 (53), longer than body [tail/body length ratio 1:0.74–0.96 (1:0.87)]. Oral sucker subterminal, subspherical 28–40 × 28–35 (32 × 31). Ventral sucker subspherical, 25–34 × 26–33 (30 × 29). Oral/ventral sucker width ratio 1:0.78–1.06 (1:0.94). Prepharynx indistinct, pharynx elongate-oval, muscular, 12–16 × 6–10 (13 × 8). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells numerous, with rhabditiform contents, on both sides anterior to ventral sucker. Excretory vesicle bipartite; anterior part smaller, transversely oval, at level of posterior margin of body, posterior part larger, transversely oval, at junction of body and tail; main collecting ducts wide, dilated between level of pharynx and anterior

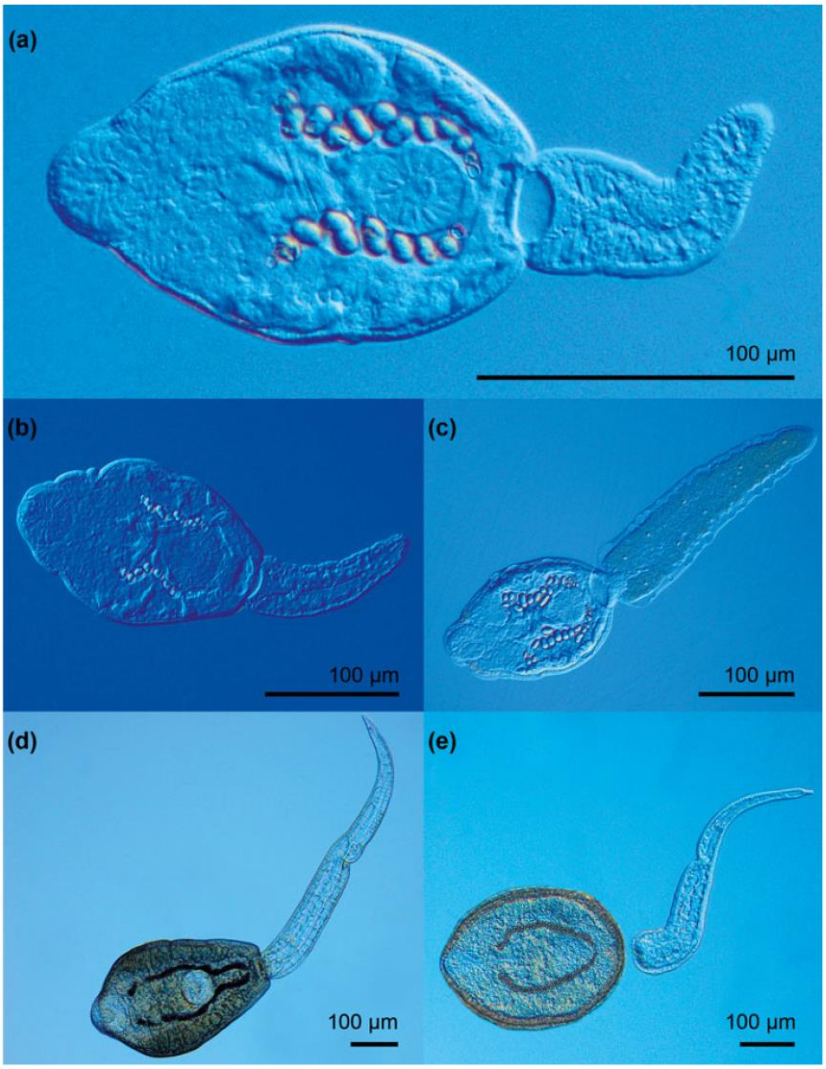

Fig. 5. Photomicrographs of live trematode cercariae of the families Echinochasmidae and Psilostomidae. (a) *Echinochasmus coaxatus*; (b) *Echinochasmus bursicola*; (c) *Echinochasmus* sp. 1; (d) Psilostomidae gen. sp. 1; (e) Psilostomidae gen. sp. 2.

margin of ventral sucker, narrowing posteriorly and anteriorly, containing dark refractile excretory granules of different size (13–15 large ones on each side plus several small ones at posterior and anterior ends).

Echinochasmus *sp. 2*

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726948); *nad*1, one replicate (MN720148).

Description (no photomicrograph available)

(Measurements from seven fixed specimens.) Gymnocephalous cercariae. Body colourless, oval, with maximum width at level of ventral sucker, 98–133 × 66–84 (117 × 75). Tegument thick, tegumental spines absent. Collar poorly developed. Collar spines absent. Tail simple, muscular, contractile, 83–123 (102) long, with maximum width at base 23–31 (28), slightly shorter than body [tail/body length ratio 1:0.96–1.30 (1:1.15)]. Oral sucker subterminal, subspherical 27–37 × 26–36 (31 × 33). Ventral sucker spherical, muscular, 22–31 × 22–31 (28 × 28). Oral/ventral sucker width ratio 1:0.71–1.03 (1:0.90). Prepharynx indistinct, pharynx elongate-oval, muscular, 7–10 × 5–8 (9 × 7). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells numerous, with rhabditiform contents, on both sides anterior to ventral sucker. Excretory vesicle transversely oval at junction of body and tail; main collecting ducts wide, containing large, dark refractile excretory granules of different size (19 on each side).

Remarks

Cercariae of both identified species of *Echinochasmus* – *E. coaxatus* and *E. bursicola* – have been previously reported from *B. tentaculata* (Karmanova, 1973, 1974). The life cycle of *E. coaxatus*

was described by Karmanova (1974) in the Astrakhan Nature Reserve, Russia. Cercariae were found in *B. tentaculata* and metacercariae were found in the freshwater fishes of the families Cyprinidae and Percidae, collected in the River Volga. Cercariae of *E. coaxatus* were also reported from *Radix auricularia* in the lake Gołdapiwo, Poland, by Wiśniewski (1957). General morphology of cercariae found in our study corresponded well to the description for cercariae of *E. coaxatus* by Karmanova (1974), except in the number of the refractile excretory granules per collecting duct (12 vs. 13–14, respectively). The differences in the metrical data for body [114–184 vs. 103–124 (115)] and tail lengths [123–140 vs. 72–115 (92)] may be due to the different fixation methods. Karmanova (1974) did not indicate whether the measurements were taken from live or fixed cercariae.

Cercariae of *E. bursicola* were previously described by Karmanova (1973) from *B. tentaculata* collected in the Lower Volga, Russia. Although the method of fixation was not specified, the present cercariae differ from cercariae described by Karmanova (1973) by a shorter [110–132 (120) vs. 129–340, respectively] and narrower body [71–105 (88) vs. 104–110], shorter tail [83–101 (93) vs. 114–300] and the number of the refractile excretory granules per collecting duct (seven vs. six).

Cercariae of both *Echinochasmus* sp. 1 and *Echinochasmus* sp. 2 differ from cercariae of *Echinochasmus* sp. reported from *Lithoglyphus naticoides* (Pfeiffer, 1928), as described by Stanevičiūtė *et al.* (2008) in Lithuania, by smaller dimensions for all morphological characters, in particular by smaller body (105–136 × 74–125 vs. 98–133 × 66–84 vs. 240–280 × 136–144) and much smaller tail (115–170 × 27–82 vs. 83–123 × 23–31 vs. 1120–1360 × 132), respectively.

Further identification of *Echinochasmus* sp. 1 and *Echinochasmus* sp. 2 to the species level requires the sequences of the adults from the definitive hosts, which are typically fish-eating birds and rarely mammals (Tkach *et al.*, 2016).

Systematics

Psilostomidae Looss, 1900
Sphaeridiotrema Odhner, 1913

Sphaeridiotrema *sp.*

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726949); *nad*1, one replicate (MN720149).

Description

Cercariae of *Sphaeridiotrema* sp. were found in one snail at Locality R2 in the River Rhine. The species of the cercariae was identified based on the results of molecular analyses. No morphological data were obtained for cercariae of this species.

Psilostomidae gen. sp. 1

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K2), River Rhine (R1, R2), Germany; Curonian Lagoon (CB), Lithuania.

Representative DNA sequences. 28S rDNA, four replicates (MN726950–MN726953).

Description

(Measurements from 11 fixed specimens.) Gymnocephalous cercariae (fig. 5d). Body dark, elongate-oval, with maximum width at level of posterior margin of pharynx, 243–314 × 218–267 (273 × 239). Tegument thick, tegumental spines not observed. Collar and collar spines absent. Tail simple, muscular, contractile, 454–495 (476) long, with maximum width at base 72–82 (76), longer than body [tail/body length ratio 1:0.51–0.66 (1:0.57)]. Oral sucker subterminal, subspherical, muscular, 56–75 × 52–71 (65 × 63). Ventral sucker subspherical, just postequatorial, 58–82 × 68–82 (71 × 72). Oral/ventral sucker width ratio 1:0.89–1.26 (1:1.09). Prepharynx indistinct, pharynx subspherical, muscular, 17–22 × 14–22 (19 × 17). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells numerous on both sides posterior to oral sucker. Main collecting ducts narrow, containing numerous small dark refractile excretory granules of similar size. Main collecting ducts forming small lobes with an accumulation of slightly larger excretory granules posterior to oral sucker. Excretory vesicle transversely oval, at posterior margin of body.

Psilostomidae gen. sp. 2

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726954).

Description

Cercariae of Psilostomidae gen. sp. 2 (fig. 5e) were found in one snail at Locality R2 in the River Rhine. Cercariae were identified based on the results of molecular analyses. No morphological data were obtained for cercariae of this species.

Remarks

To date, *B. tentaculata* was reported as the first intermediate host for six species of psilostomids from three genera: (i) *Psilochasmus*: *Psilochasmus oxyurus* (Creplin, 1825) in Poland (Wiśniewski, 1958); (ii) *Psilotrema*: *Psilotrema oligoon* (Linstow, 1887) in the UK (Pike, 1968), *P. simillimum* (Mühling, 1898) in Bulgaria (Samnaliev, 1981) and *P. spiculigerum* (Mühling, 1898) in Bulgaria, Russia, Ukraine and the UK (Zdun, 1961; Bykhovskaya-Pavlovskaya & Kulakova, 1971; Frolova, 1975; Samnaliev *et al.*, 1977; Morley & Lewis, 2006); and (iii) *Sphaeridiotrema*: *Sphaeridiotrema globulus* (Rudolphi, 1814) in Bulgaria, Finland, Russia and the UK (Szidat, 1937; Selinheimo, 1956; Bykhovskaya-Pavlovskaya & Kulakova, 1971; Kanev & Vasilev, 1984; Morley & Lewis, 2006) and *Sphaeridiotrema* sp. in Lithuania (Tkach *et al.*, 2016).

Sphaeridiotrema sp. in our material appeared to be conspecific to the species that was previously reported from Lithuania (Tkach *et al.*, 2016). Both isolates were identified only to the genus level and require sequences of adults from the definitive hosts (water birds), to complete identification to the species level.

General morphology of cercariae of Psilostomidae gen. sp. 1 resemble morphology of cercariae of *P. oligoon* described by Pike (1968), *P. simillimum* described by Samnaliev (1981) and *P. spiculigerum* described by Bykhovskaya-Pavlovskaya & Kulakova (1971) and Samnaliev *et al.* (1977). However, cercariae of Psilostomidae gen. sp. 1 differ from cercariae of *P. oligoon* by wider body [218–267 (239) vs. 165–235 (196)], longer tail

[454–495 (476) vs. 261–461 (370)] and smaller pharynx [length: 17–22 (19) vs. 30–45 (39); width: 14–22 (17) vs. 22–39 (29)]; and from cercariae of *P. simillimum* by larger body [length: 243–314 (273) vs. 150–208 (178); width: 218–267 (239) vs. 92–135 (109)], larger oral [length: 56–75 (65) vs. 30–48 (38); width: 52–71(63) vs. 30–45 (37)] and ventral suckers [length: 68–82 (72) vs. 25–40 (32); width: 58–82 (71) vs. 28–40 (31)] and larger tail [length: 454–495 (476) vs. 288–350 (333); width: 72–82 (76) vs. 38–55 (46)]. Cercariae of Psilostomidae gen. sp. 1 differ from *P. spiculigerum* described by Bykhovskaya-Pavlovskaya & Kulakova (1971) in having larger low limits for body width (218–267 vs. 165–249), tail length (454–495 vs. 388–499), tail width (72–82 vs. 52–78), smaller low and high limits for oral sucker width (52–71 vs. 65–78) and smaller pharynx [length: 17–22 vs. 44–49; width: 14–22 vs. 26–39]. It also differs from *P. spiculigerum* described by Samnaliev *et al.* (1977) by larger body [length: 243–314 (273) vs. 204–242 (221); width: 218–267 (239) vs. 130–155 (138)], larger oral [length: 56–75 (65) vs. 50–62 (53); width: 52–71 (63) vs. 56–62 (56)] and ventral suckers [length: 68–82 (72) vs. 34–50 (41); width: 58–82 (71) vs. 37–56 (45)], smaller pharynx [length: 17–22 (19) vs. 19–31 (23); width: 14–22 (17) vs. 25–31 (28)] and larger tail [length: 454–495 (476) vs. 324–342 (336); width: 72–82 (76) vs. 43–56 (49)]. The above comparisons demonstrate that Psilostomidae gen. sp. 1 may represent a species of the genus *Psilotrema*, but is not conspecific with *P. oligoon*, *P. simillimum* or *P. spiculigerum*. Further identification of Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 to the species level requires the sequences of the adults from the definitive hosts, which are mainly birds and mammals (Kostadinova, 2005).

Systematics

Superfamily: Monorchioidea Odhner, 1911
Lissorchiidae Magath, 1917
Asymphylodora Looss, 1899

Asymphylodora *sp.*

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K3), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726955).

Molecular results

Cercariae of *Asymphylodora* sp. were found in one snail in the River Lippe (prevalence: 0.2%). Partial 28S rDNA sequences were generated from one isolate (table 2). The partial 28S rDNA sequence for *Asymphylodora* sp. obtained in the present study was compared to the sequences of *Asymphylodora perccotti* Besprozvannykh, Ermolenko & Atopkin, 2012 (FR822715 −FR822731) ex *Perccottus glenii* Dybowski, 1877 from Russia (Besprozvannykh *et al.*, 2012), the only sequences for this genus currently available in GenBank. The sequence divergence between our isolate and 17 isolates for *A. perccotti* ranged between 2.7% and 2.8% (31−32 nt).

Description

The species of the cercariae was identified based on the results of molecular data. No morphological data were obtained for cercariae of this isolate.

Systematics

Superfamily: Pronocephaloidea Looss, 1899
Notocotylidae Lühe, 1909

Molecular results

Infection with the cercariae of the family Notocotylidae was detected in nine snails from four localities in the River Lippe (prevalence: 1.5%). Partial 28S rDNA sequences were generated for four isolates (fig. 6; table 2) and aligned with 16 sequences for species of the Notocotylidae available in GenBank (supplementary table S1). Members of the families Opisthotrematidae, Rhabdiopoeidae and Labicolidae were used as the outgroup based on the topologies in the phylogenetic tree of the Digenea published by Olson *et al.* (2003). The results of phylogenetic analyses demonstrated that two isolates preliminarily identified as *Notocotylus* sp. (N11K0 and N12K0) clustered within a clade comprising *Notocotylus* spp., demonstrating the close affinity to the isolate of *Notocotylus attenuatus* (Rudolphi, 1809) (AF184259), the type species of the genus *Notocotylus*, collected from *Aythya ferina* in Ukraine (Tkach *et al.*, 2001). Sequences for two isolates of *Notocotylus* sp. from our study were identical and differed from *N. attenuatus* by 0.4% (3 nt). The sequences for two other notocotylid isolates (N2K0 and N2K2b) from *B. tentaculata* collected in the River Lippe were identical and formed a basal branch to the clade consisting of *Notocotylus* spp. and *Catatropis* spp., albeit without support (fig. 6). The taxonomic identity of these two isolates was not justified based on the phylogenetic analyses and we, thus, provide the identification for this species only to the family level, as Notocotylidae gen. sp. The sequence divergence between two notocotylid species recorded in our study was 2.9% (23 nt).

Systematics

Notocotylus Diesing, 1839

Notocotylus *sp.*

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K4), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726956, MN726957).

Description

No morphological data were obtained for cercariae of these isolates since the infections were prepatent.

Notocotylidae gen. sp

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K3, K4), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726958, MN726959).

Description

No morphological data were obtained for cercariae of these isolates since the infections were prepatent.

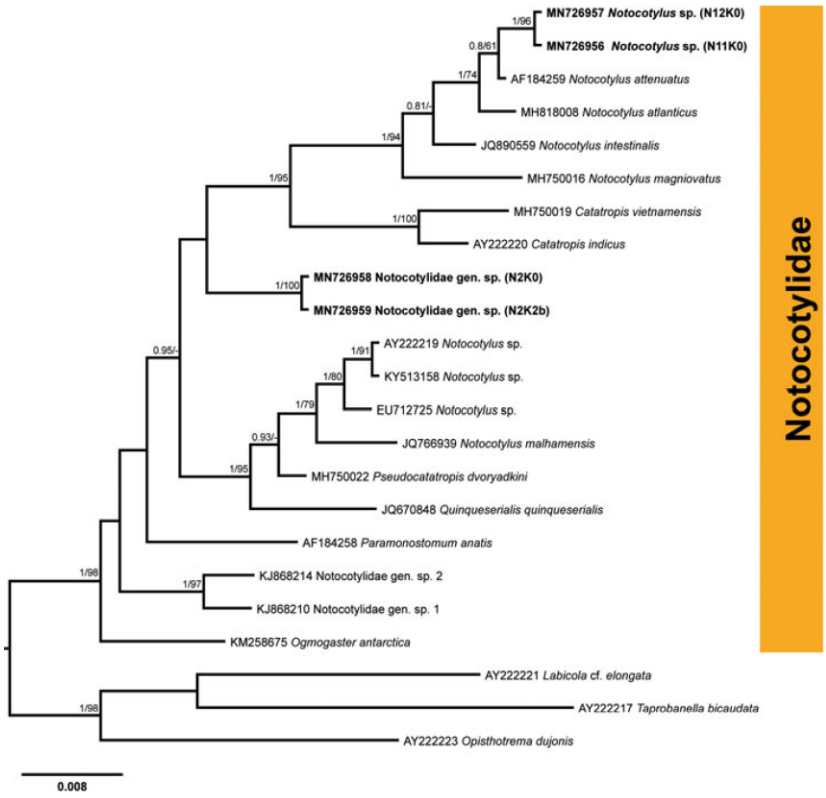

Fig. 6. Phylogenetic tree for Notocotylidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

Remarks

Members of the family Notocotylidae are reported to utilize lymnaeids, planorbids and a variety of other snail families in their life cycles (Filimonova, 1985). To date, six species – namely, *N. attenuatus*, *N. ponticus*, *N. parviovatus*, *N. imbricatus*, *Notocotylus* sp. and *Catatropis verrucosa* – were reported to develop in *B. tentaculata* in Europe (Bock, 1982; Filimonova, 1985 and references therein; Morley *et al.*, 2004). Further identification of the two species collected from *B. tentaculata* in the River Lippe to the species level requires the sequences of adult worms from the definitive hosts, which are mammals and birds (Filimonova, 1985).

Systematics

Superfamily: Allocreadioidea Looss, 1902
Opecoelidae Ozaki, 1925

Molecular results

Infection with cercariae of the family Opecoelidae was detected in 45 snails from four localities in the River Lippe (prevalence: 7.4%). Sequences for the partial 28S rDNA and ITS2 region were generated for six isolates (fig. 7; table 2). Comparative sequence analyses of 28S and ITS2 datasets revealed the presence of two species of the family Opecoelidae in our material. Five sequences of partial 28S rRNA gene (table 2) were aligned with seven GenBank sequences for species of the Opecoelidae known to parasitize freshwater fish (supplementary table S1). A species of the Opecoelidae, *Buticulotrema thermichthysi* Bray, Waeschenbach, Dyal, Littlewood & Morand, 2014, was used as the outgroup based on the topologies in the phylogenetic tree of the Opecoelidae published by Martin *et al.* (2019). The results of the phylogenetic analyses demonstrated a close affinity of the four isolates (O11K2a, O1K1, O1K2b and O13K2a) with *Sphaerostoma bramae* (Müller, 1776) (MH161435) collected from *Abramis brama* in Russia (Sokolov *et al.*, 2019). The sequences for our isolates were identical and differed from the sequence of *S. bramae* by 0.2% (3 nt), which is considered as interspecific variation and, thus, this species was identified as *Sphaerostoma* sp. The intraspecific divergence between the isolates of *Sphaerostoma* sp. (O11K2a, O1K1, O1K2b and O12K2a) within the ITS2 dataset was 0.2% (1 nt).

The remaining isolate (O2K2a) collected in the River Lippe, representing a second opecoelid species in our material, formed a branch basal to a clade consisted of *Sphaerostoma* spp. and *Plagiocirrus* spp. The 28S rDNA sequence of this species differed from the sequence of *Sphaerostoma* sp. by 5.9% (71 nt), from *S. bramae* by 5.6% (68 nt) and from *Plagiocirrus* spp. by 7.1–7.2% (85–87 nt), whereas the ITS2 sequence differed from the sequence of *Sphaerostoma* sp. by 4.7–4.9% (21–22 nt). Based on the results, we identified this species only to the family level as Opecoelidae gen. sp.

Sphaerostoma sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K1, K2, K3), Germany.

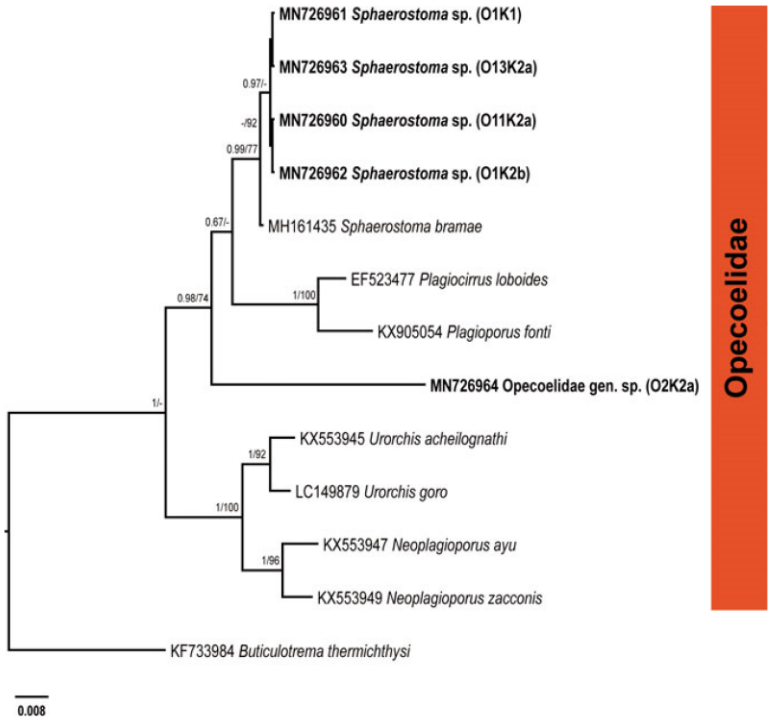

Fig. 7. Phylogenetic tree for Opecoelidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

Representative DNA sequences. 28S rDNA, four replicates (MN726960–MN726963); ITS2, four replicates (MN726988–MN726991).

Description

(Measurements from ten fixed specimens.) Microcercous cercariae (fig. 8a, b). Body colourless, elongate-oval, 207–285 × 110–132 (246 × 118), with maximum width at level just anterior to ventral sucker. Tegument thick, smooth. Tail reduced to small stump (cotylocercous), 22–42 (28) long with maximum width at base, 35–42 (40). Posterior half of the tail comprising glandular structures. Oral sucker large, subterminal, muscular, subspherical, 39–53 × 42–60 (49 × 50) armed with a small, simple stylet, 6–12 × 2–4 (8 × 3) dorsal to mouth opening. Ventral sucker subspherical, equatorial, 53–79 × 60–69 (68 × 64), larger than oral sucker, opening surrounded by one row of minute spines. Oral/ventral sucker width ratio 1:1.08–1.61 (1:1.39). Prepharynx long, pharynx distinct, elongate-oval, 20–25 × 15–19 (22 × 17). Caeca indistinct. Cystogenous gland-cells numerous, widespread throughout body. Four pairs of small penetration gland-cells, at level of prepharynx. Excretory vesicle broader anteriorly, heart-shaped.

Opecoelidae gen. sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726964); ITS2, one replicate (MN726992).

Description (no photomicrograph available)

No morphological data were obtained for cercariae of this isolate since the infections were prepatent.

Remarks

To date, one species of the Opecoelidae, *S. bramae*, has been reported in *B. tentaculata* in Europe: in Denmark (as *Cercaria micrura*; Wesenberg-Lund, 1934), Finland (Wikgren, 1956), Lithuania (Petkevičiūtė *et al.*, 1995), the Netherlands (as *C. micrura*; Keulen, 1981), Russia (as *C. micrura*; Bykhovskaya-Pavlovskaya & Kulakova, 1971), Ukraine (as *C. micrura*; Zdun, 1961) and the UK (Pike, 1967). Comparative sequence analysis suggested *Sphaerostoma* sp. of the present study to be close but not conspecific with *S. bramae*. Morphology of cercariae of *Sphaerostoma* sp. corresponds well to cercariae of *S. bramae* described by Wikgren (1956), Wesenberg-Lund (1934), Pike (1967) and Bykhovskaya-Pavlovskaya & Kulakova (1971). However, our cercariae differ from cercariae of *S. bramae* as described by Wesenberg-Lund (1934) by lower maximum of the body length (207–285 vs. 255–450), body width (110–132 vs. 85–135) and shorter tail [22–42 (28) vs. 45–50]; and from cercariae as described by Pike (1967) by shorter body [207–285 (246) vs. 261–418 (299)] and smaller oral [39–53 (49) × 42–60 (50) vs. 54–61 (57) × 50–59 (55)] and ventral suckers [53–79 (68) × 60–69 (64) vs. 63–81 (68) × 68–84 (73)]. Further identification of *Sphaerostoma* sp. and Opecoelidae gen. sp. to the species level requires the sequences of the adults from the definitive hosts, freshwater fish.

Systematics

Superfamily: Opisthorchioidea Looss, 1899
Opisthorchiidae Looss, 1899

Molecular results

Cercariae of the family Opisthorchiidae were found in one snail in the River Lippe (prevalence: 0.2%). A partial 28S rDNA sequence

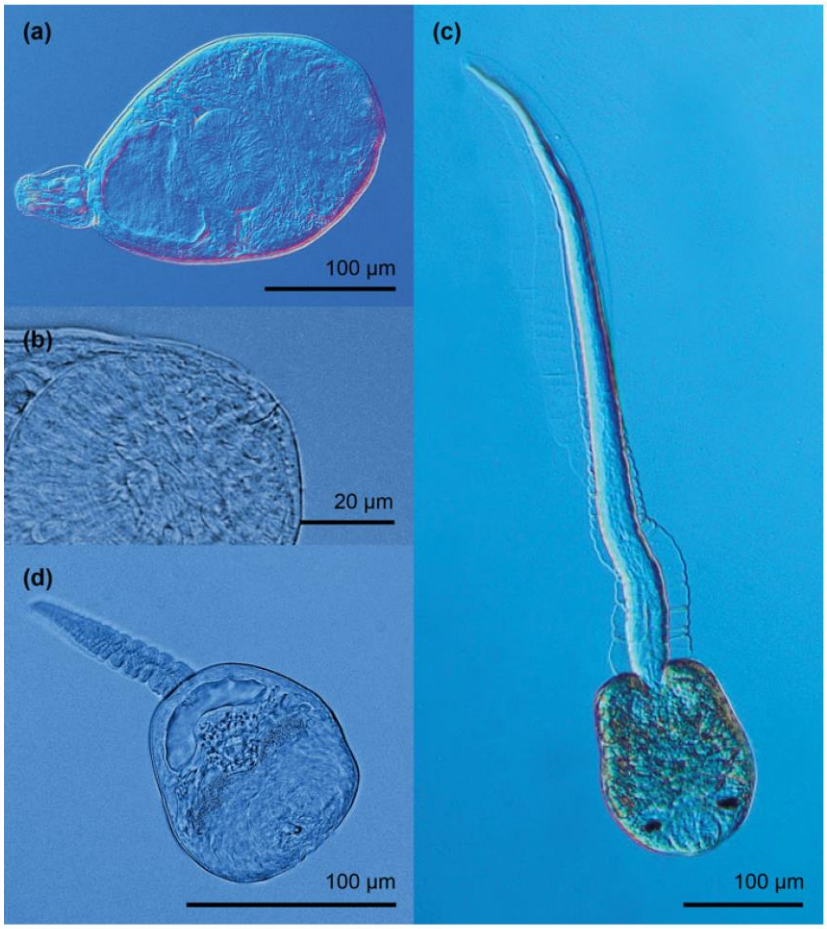

Fig. 8. Photomicrographs of live cercariae of the trematode families Opecoelidae, Opisthorchiidae and Lecithodendriidae. (a) *Sphaerostoma* sp.; (b) *Sphaerostoma* sp., stylet; (c) Opisthorchiidae gen. sp.; (d) *Lecithodendrium linstowi*.

was generated for one isolate (fig. 2b; table 2) and aligned with sequences of seven species belonging to the family Opisthorchiidae available in GenBank (supplementary table S1). *Apophallus zalophi* Price, 1932 (Heterophyidae) was used as the outgroup based on the topologies in the phylogenetic tree of the Opisthorchioidea published by Hernández-Orts *et al.* (2019). In phylogenetic analyses of the Opisthorchiidae (fig. 2b), the novel sequence formed a branch at a basal position in the low supported clade consisting of *Opisthorchis* spp., *Clonorchis sinensis* (Cobbold, 1875) and unidentified species of *Metorchis*. Within this clade, our isolate demonstrated the lowest level of sequence divergence relative to the isolate of *Metorchis ussuriensis* (KY075777) (0.8%, 9 nt) and the highest level of divergence to the isolate of *Opisthorchis* sp. (MF110001) (2.3%, 25 nt). Based on the results of molecular analyses, cercariae were identified to the family level as Opisthorchiidae gen. sp.

Opisthorchiidae gen. sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K3), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726966).

Description (fig. 8c)

(Measurements from 11 fixed specimens.) Pleurolophocercariae (fig. 8c) with elongate-oval body 143–191 × 89–118 (169 × 101), with brown pigment. Tegument thick with minute spines in anterior part. Tail simple, longer than body, 437–494 (459) × 24–40 (33) with dilatation in anterior part and conspicuous finfold present in the posterior two thirds of tail, with maximum width 11–22 (16). Tail/body length ratio 1:0.31–0.42 (1:0.37). Crescent-shaped eye-spots two, with black pigment, large, 13–18 (15) long and maximum width 7–10 (9), mediolateral at level of prepharynx. Oral sucker subterminal, subspherical, 29–39 × 28–36 (34 × 33). Ventral sucker inconspicuous, rudimentary, subspherical 13–25 × 11–23 (19 × 17), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.38–0.74 (1:0.56). Cystogenous gland-cells large, nucleated, posterior to eye-spots. Preacetabular penetration gland-cells six pairs, with long ducts, dilated anteriorly, opening at the anterior margin of oral sucker. Excretory vesicle thick-walled, transversely oval.

Remarks

Four species of the family Opisthorchiidae – *Metorchis bilis* (Braun, 1890), *M. intermedius* Heinemann, 1937, *M. xanthosomus* (Creplin, 1846) and *Metorchis* sp. – have been reported from *B. tentaculata* in Europe (Zdun, 1961; Bykhovskaya-

Pavlovskaya & Kulakova, 1971; Cichy *et al.*, 2011). Based on sequence data analyses, the present cercariae may belong to the genus *Metorchis*. Morphologically, cercariae collected from *B. tentaculata* in the River Lippe resemble cercariae of *Metorchis intermedius* Heinemann, 1937 reported from the same snail host in the Curonian Lagoon by Bykhovskaya-Pavlovskaya & Kulakova (1971). However, cercariae in our material differ by having a larger body [143–191 (169) × 89–118 (101) vs. 135–170 × 78–81] and longer tail [437–494 (459) × 24–40 (33) vs. 350–390 × 26]. The present cercariae differ from *Metorchis* sp. as described by Zdun (1961) by smaller body [143–191 × 89–118 (169 × 101) vs. 200–320 × 32–70] and oral sucker [29–39 × 28–36 (34 × 33) vs. 48 × 48].

Systematics

Superfamily: Microphalloidea Ward, 1901
Lecithodendriidae Lühe, 1901
Lecithodendrium Looss, 1896

Lecithodendrium linstowi *(Dollfus, 1931)*

Molecular results

Cercariae of *L. linstowi* were found in one snail in the River Lippe (prevalence: 0.2%). Sequences for the partial 28S rDNA and ITS2 region were generated for one isolate (table 2). Newly generated 28S rDNA sequence appeared identical to sequence of *L. linstowi* (AF151919) obtained from an adult collected from *Nyctalus noctula* in Ukraine (Tkach *et al.*, 2000) and sequence of *L. linstowi* (MF498821) obtained from cercariae from *Radix balthica* in the UK (Enabulele *et al.*, 2018). The sequence for ITS2 region from the present study showed 99% similarity to those of *L. linstowi* (JF784190 and KJ934792) from *Pipistrellus pipistrellus* in England (Lord *et al.*, 2012) and *N. noctula* from Ukraine (Kudlai *et al.*, 2015), and 94% similarity with the sequence of *L. linstowi* (MF498820) from *R. balthica* (Enabulele *et al.*, 2018).

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726965); ITS2, one replicate (MN726993).

Description

(Measurements from 11 fixed specimens.) Xiphidiocercariae (fig. 8d). Body colourless, elongate-oval, 118–148 × 67–85 (132 × 75). Tegument thin, covered with minute spines. Tail simple, 55–63 (64) long, with maximum width at base 16–23 (21), shorter than body. Tail/body length ratio 1:1.84–2.31 (1:2.06). Oral sucker subspherical, subterminal, muscular, 29–37 × 28–35 (33 × 30) armed with large stylet, 15–17 (16) long with maximum width at base 5–7 (6). Stylet with dilatation 1–2 (2). Virgula absent. Ventral sucker spherical, equatorial, 14–24 × 14–24 (19 × 18), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.42–0.73 (1:0.58). Prepharynx indistinct, pharynx small, elongate-oval, 8–12 × 5–9 (10 × 7). Caeca indistinct. Penetration gland-cells three pairs, anterolateral to ventral sucker, filled with dark, granular secretory material. Excretory vesicle thin-walled, V-shaped.

Remarks

Digenean trematodes of the family Lecithodendriidae infect insectivorous vertebrates (most prominently bats, occasionally birds), using aquatic insect larvae as second intermediate hosts and usually snails of the group formerly known as 'prosobranchia' as first intermediate hosts (Enabulele *et al.*, 2018). However, cercariae of *L. linstowi* were also reported from *R. auricularia* collected in the Queen's River, England (Enabulele *et al.*, 2018). Adults were found in a wide range of bat species, e.g. *Myotis daubentonii* (Kuhl) in Germany (Gottschalk, 1970), in *N. noctula* (Schreber) in Ukraine (Tkach *et al.*, 2000; Kudlai *et al.*, 2015) and in *P. pipistrellus* (Schreber) in Spain and the UK (Esteban *et al.*, 2001; Lord *et al.*, 2012). Morphology of cercariae found in our study corresponded well to the description of the cercariea of *L. linstowi* by Enabulele *et al.* (2018). However, the present cercariae differ in having a shorter body [78–116 (88) vs. 118–148 (132)] and smaller oral [19–14 (11) × 9–15 (11) vs. 29–37 (33) × 28–35 (30)] and ventral suckers [10–13 (11) × 8–14 (9) vs. 14–24 (19) × 14–24 (18)].

Systematics

Pleurogenidae Looss, 1899 and Prosthogonimidae Lühe, 1909

Molecular results

Infection with cercariae belonging to the families Pleurogenidae and Prosthogonomidae was detected in nine snails from three localities (prevalence: Pleurogenidae: River Lippe, 0.5%; River Rhine, 1.3%; Prosthogonomidae: River Lippe, 0.7%; River Rhine, 1.3%). Sequences for the partial 28S rDNA ($n = 9$) and ITS2 region ($n = 8$) were generated for the isolates from all localities (fig. 9; table 2). The 28S rDNA sequences were aligned with the sequences for pleurogenids ($n = 13$) and prosthogonomids ($n = 5$) available in GenBank. Two species of the family Microphallidae were used as the outgroup based on the topologies in the phylogenetic tree of the Microphalloidea published by Kanarek *et al.* (2017) (fig. 9; supplementary table S1). Both BI and ML analyses yielded similar topology with two main clades corresponding to the Pleurogenidae and Prosthogonomidae. The sequence of the isolate (PBK2b) collected from the River Lippe (K3) appeared to be identical to the sequence of *Parabascus duboisi* ex *M. daubentonii* from Ukraine (AY220618) (Tkach *et al.*, 2003). The isolate (PL2R1) collected from *B. tentaculata* in the River Rhine clustered with *Leyogonimus polyoon* (KY752116) from *Fulica atra* collected in Poland (Kanarek *et al.*, 2017). The sequence divergence between two species was 2% (23 nt). This isolate was identified to the family level as Pleurogenidae gen. sp. 2. The two isolates (PL11K2a and PL12K2a) collected from the River Lippe (K2) clustered with pleurogenid species from the genera *Brandesia*, *Candidotrema*, *Pleurogenes*, *Pleurogenoides* and *Prosotocus*, and identified only to the family level as Pleurogenidae gen. sp. 1.

The 28S rDNA sequences of the remaining five isolates (PO1K2b, PO2K2b, PO1R1, PO2R1 and POK2a) collected from the River Rhine (R1) and from the River Lippe (K2 and K3) were identical with the sequence of *Prosthogonimus ovatus* from *Pica pica* collected in Ukraine (AF151928) (Tkach *et al.*, 2000) (fig. 9b; supplementary table S1).

Sequences of the ITS2 region for pleurogenids and prosthogonomids obtained in this study were aligned with sequences of prosthogonomids available in GenBank (supplementary table S1). Sequences of the four isolates (PO1K2b, PO1R1, PO2R1 and POK2a) clustered with the sequence of *P. ovatus* (KP192722) from *A. ferina* collected in the Czech Republic (Heneberg *et al.*, 2015). The four remaining isolates (PBK2b, PL2R1, PL11K2a and PL12K2a) identified as the members of

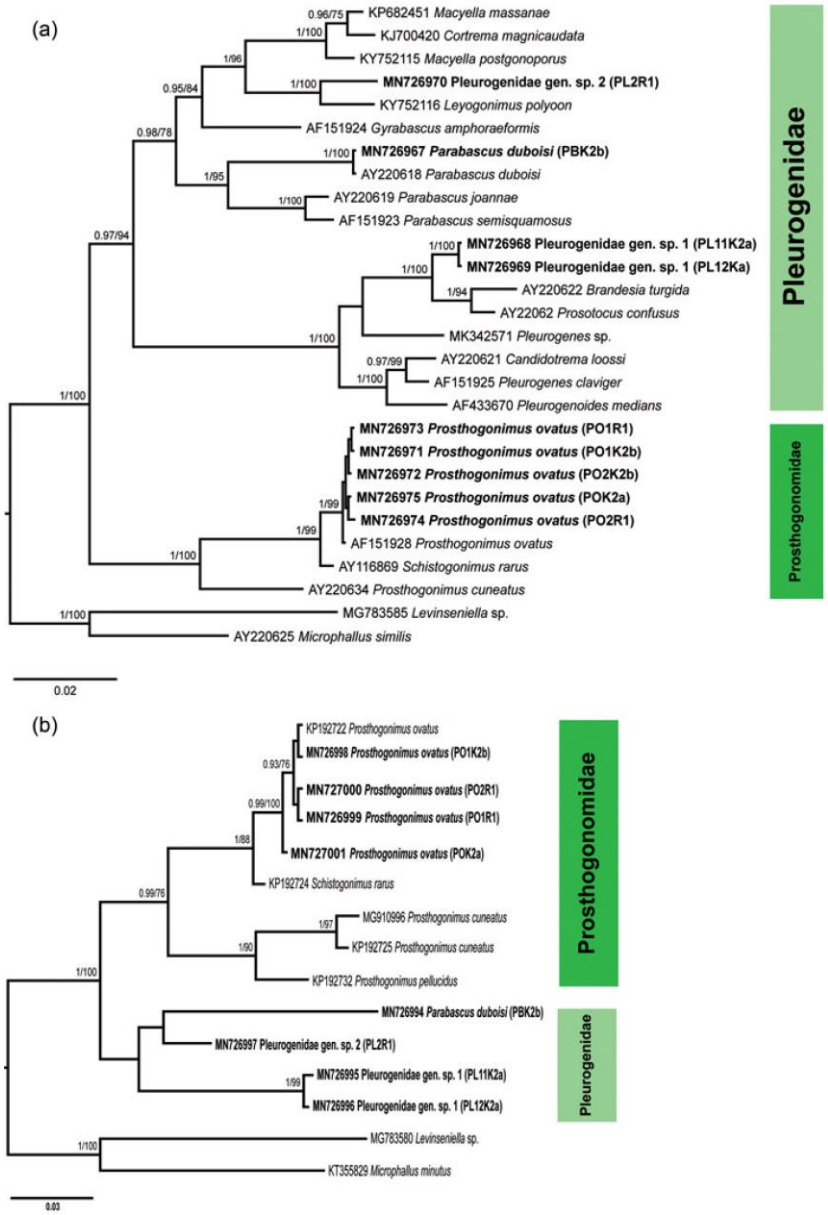

Fig. 9. Phylogenetic tree for Pleurogenidae and Prosthogonimidae based on the partial sequences of the 28S rRNA gene (a) and the internal transcribed spacer 2 (ITS2) region (b). Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

the family Pleurogenidae based on 28S rDNA analyses clustered within a nearly supported clade (fig. 9a).

Systematics

Pleurogenidae Looss, 1899
Parabascus Looss, 1907

Parabascus duboisi *(Hurkova, 1961)*

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K3), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726967); ITS2, one replicate (MN726994).

Description

No morphological data were obtained for cercariae of this isolate since the infection was prepatent.

Remarks

The life cycle of *P. duboisi* is unknown and our finding is the first to report *B. tentaculata* serving as the first intermediate host for this species. *Parabascus duboisi* is known to parasitize, among other bats, those of the genera *Eptesicus*, *Miniopterus*, *Myotis*, *Pipistrellus* and *Rhinolophus* (Sharpilo & Iskova, 1989).

Pleurogenidae gen. sp. 1

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, two replicates MN726968, MN726969); ITS2, two replicates (MN726995, MN726996).

Description

(Measurements from ten fixed specimens.) Virgulate xiphidiocercariae (fig. 10a, b). Body colourless, oval, 82–115 × 69–94 (92 × 82). Tail simple, 41–48 (45) long with maximum width at base 14–20 (17), longer than body. Tail/body length ratio 1:1.82–2.56 (1:2.04). Oral sucker subterminal, elongate-oval, 32–40 × 21–28 (37 × 25), armed with small stylet 14–20 (16) long with maximum width 3–5 (4). Stylet in anterior part of oral sucker, with anterior dilatation of blade. Small pyriform virgula organ in posterior part of oral sucker. Ventral sucker subspherical, 14–19 × 11–16 (16 × 14), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.38–0.51 (1:0.43). Prepharynx long, pharynx spherical, 8–11 × 7–10 (9 × 8). Caeca indistinct. Few medium-sized fat inclusions in body parenchyma. Cystogenous gland-cells numerous, widespread throughout body. Four pairs of penetration gland-cells, anterolateral to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

Pleurogenidae gen. sp. 2

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726970); ITS2, one replicate (MN726997).

Description

(Measurements from seven fixed specimens.) Virgulate xiphidiocercariae (fig. 10c, d). Body colourless, elongate oval, 120–154 × 77–109 (135 × 90). Tail simple, contractile, 77–150 (105) long with maximum width 21–27 (25), slightly longer than body. Body/tail length ratio 1:1.14–1.47 (1:1.10). Oral sucker subspherical, subterminal, 31–39 × 30–36 (35 × 33), armed with small stylet, 13–19 (16) long with maximum width 4–6 (5). Stylet in anterior part of oral sucker, with anterior dilatation of blade. Virgula organ large, bilobed, in posterior part of oral sucker. Ventral sucker subspherical, equatorial, 21–25 × 16–25 (23 × 21), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.60–0.71 (1:0.66). Prepharynx long, pharynx distinct, close to virgula organ, oval, 10–12 × 12–16 (11 × 14). Caeca indistinct. Numerous small fat inclusions in body parenchyma. Penetration gland-cells four pairs, posterolateral to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

Remarks

Bithynia tentaculata is known to be the intermediate hosts for three species of the Pleurogenidae – *Pleurogenes claviger* (Rudolphi, 1819), *Pleurogenoides medians* (Olsson, 1876) and *Pleurogenoides* sp. – in the Czech Republic (Žďárská, 1963), Germany (Palm, 1966), Lithuania (Bykhovskaya-Pavlovska & Kulakova, 1971), Poland (Grabda-Kazubska, 1971), Russia (Frolova, 1975) and Ukraine (Zdun, 1961).

The cercariae of Pleurogenidae gen. sp. 1 and Pleurogenidae gen. sp. 2 show some distinctive features that distinguish the two species. Differences between cercariae of Pleurogenidae gen. sp. 1 and Pleurogenidae gen. sp. 2 comprise the size of body [body length: 82–115 (92) vs. 120–154 (135); width: 69–94 (82) vs. 77–109 (90), respectively], the size and shape of virgula (small pyriform vs. large bilobed), position of the penetration gland-cells (anterolateral to the ventral sucker vs. posterolateral to the ventral sucker), the length of tail [41–48 (45) vs. 77–150 (105)] and the width of tail [14–20 (17) vs. 21–27 (25)]. Both species possess distinct fat inclusions in the body parenchyma, which are medium-sized and low in numbers in Pleurogenidae gen. sp. 1 and small and numerous in Pleurogenidae gen. sp. 2.

The cercariae of both species in the present study differ from cercariae of *P. claviger* as described by Grabda-Kazubska (1971), *P. medians* as described by Chernogorenko (1983) and *Pleurogenoides* sp. as described by Palm (1966) in having much smaller dimensions for all morphological characters.

Systematics

Prosthogonimidae Lühe, 1909
Prosthogonimus Lühe, 1899

Prosthogonimus ovatus *Rudolphi, 1803*

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K2, K3), River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, five replicates (MN726971–MN726975); ITS2, four replicates (MN726998–MN727001).

Description

(Measurements from 11 fixed specimens.) Xiphidio cercariae (fig. 10e, f). Body colourless, elongate-oval, 104–126 × 60–79 (115 × 69). Tail simple, 57–86 (72) long with maximum width 19–26 (23), shorter than body. Body/tail length ratio 1:1.44–1.75 (1:1.60). Oral sucker subspherical, subterminal, 23–35 × 20–33 (30 × 29) armed with small stylet, 14–20 (18) long with maximum width 4–6 (5). Stylet in anterior part of oral sucker with thickening and incomplete wall at basis and anterior dilatation of blade. Ventral sucker subspherical, equatorial, 18–32 × 17–30 (22 × 20), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.60–1.07 (1:0.73). Prepharynx indistinct. Pharynx spherical, muscular, 7–11 × 6–10 (9 × 8). Caeca indistinct. Four pairs of large, overlapping conspicuous penetration gland-cells anterior to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

Remarks

Three species of *Prosthogonimus* – namely, *P. cuneatus*, *P. ovatus* and *Prosthogonimus* sp. – have been reported to parasitize *B. tentaculata* in Europe: in the Netherlands (Boddeke, 1960), Germany (Palm, 1966), Lithuania (Bykhovskaya-Pavlovskaya & Kulakova, 1971), Russia (Ginetsinskaya & Dobrovolskij, 1968; Sharpilo & Iskova, 1989; Serbina, 2005), Ukraine (Sergienko, 1972; Sharpilo & Iskova, 1989) and the UK (Probert, 1965; Morley & Lewis, 2006). The morphology of cercariae found in our study corresponded well to the description of the cercariae of *P. ovatus* by Boddeke (1960). The only feature that differs in the present cercariae is the number of penetration gland-cell pairs (four vs. three). However, this difference might be due to a miscount in

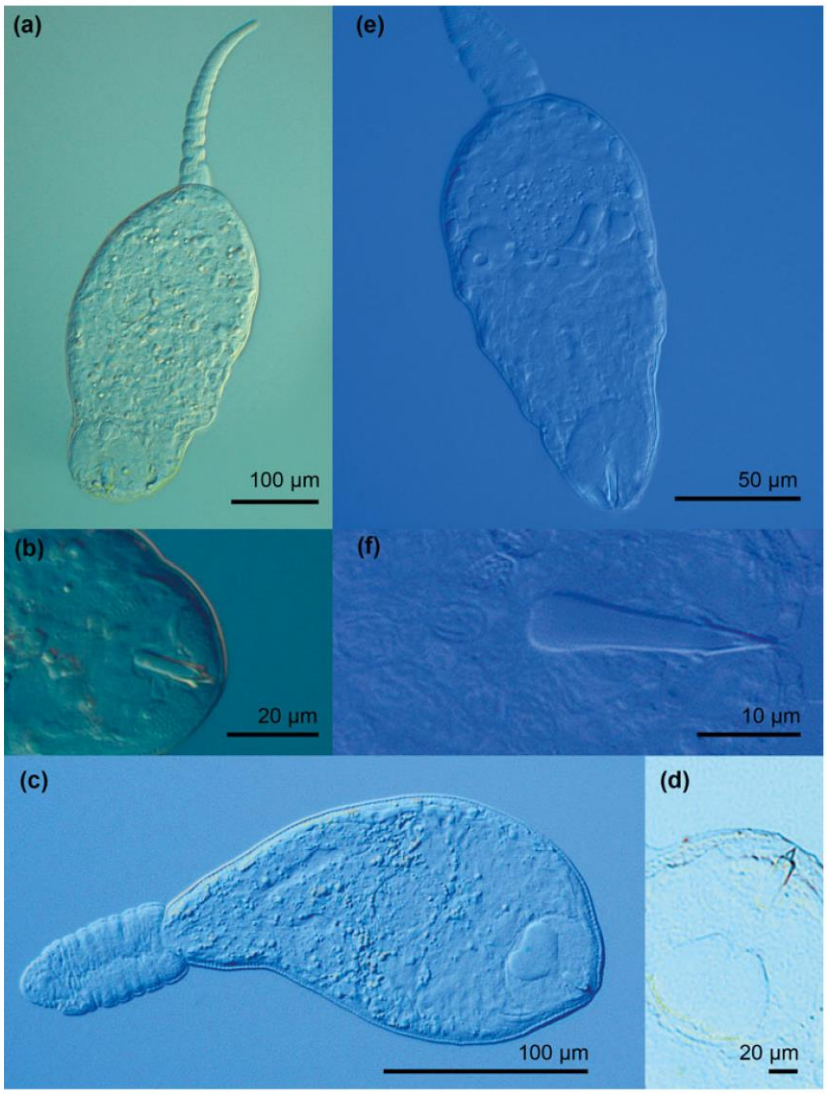

Fig. 10. Photomicrographs of live cercariae of the trematode families Pleurogenidae and Prosthogonomidae. (a) Pleurogenidae gen. sp. 1; (b) Pleurogenidae gen. sp. 1, stylet; (c) Pleurogenidae gen. sp. 2; (d) Pleurogenidae gen. sp. 2, virgula organ and stylet; (e) *Prosthogonimus ovatus*; (f) *P. ovatus*, stylet.

the previous description, as penetration gland-cells can be overlapping and hard to count.

Discussion

This study examined the parasite diversity in the faucet snail *B. tentaculata* in Central European fresh waters. To the best of our knowledge, this is the first broad faunistic survey investigating the trematode fauna in *B. tentaculata* in Central Europe and providing a combined morphological and molecular dataset. The study reveals a high trematode diversity of 20 species belonging to ten families and a high overall prevalence of infection of 12.9% in this snail host.

Not surprisingly, the larval trematode community of *B. tentaculata* shows little overlap with that of lymnaeids and planorbids, highlighting the distinctive host–parasite associations of pulmonate and non-pulmonate snails. Only one (*L. linstowi*) out of 20 species has also been recorded from lymnaeid and planorbid snail hosts (Enabulele *et al.*, 2018). Bithynids are the typical hosts of *L. linstowi*, and the single finding of this species in *Radix* sp. most probably represents an accidental infection. The species has only been reported in a single lymnaeid host, so it is reasonable to consider it as an accidental infection. Looking at the faunistic overlap of trematodes at the family level, we find a slightly different picture. Out of ten recorded families, six (Lecithodendriidae, Lissorchidae, Notocotylidae, Opecoelidae, Pleurogenidae, Psilostomidae) are also known to occur in lymnaeids and planorbids (Faltýnková *et al.*, 2007, 2008, 2016; Cichy *et al.*, 2011; Enabulele *et al.*, 2018). On the other hand, species of the families Cyathocotylidae, Echinochasmidae, Prosthogonomidae and Opisthorchiidae seem to be strictly host-specific to non-pulmonate freshwater molluscs as first intermediate hosts. Some species, such as *E. beleocephalus* and *E. coaxatus*, have been recorded from *B. tentaculata* only in Russia

(Frolova, 1975; Karmanova, 1975), so our records constitute the first record for Central Europe.

Echinochasmus sp. 1 and Psilostomidae gen. sp. 1 are the only species that were both detected in Germany and Lithuania. The definitive hosts of echinochasmids and psilostimids are typically birds and mammals (Kostadinova, 2005; Tkach *et al.*, 2016). Birds are especially mobile, so the occurrence of the two species in both countries can be easily explained by seasonal migration. However, since the sampling effort in Germany was much higher (682 vs. 121 *B. tentaculata*), we would expect more trematode species to be present in Curonian Lagoon, which our limited survey did not detect.

Overall, this diverse and distinctive trematode community of *B. tentaculata*, and the high prevalence of infection, reveal the important role of this snail species as a first intermediate host for trematodes in European freshwater ecosystems. Similar to other well-studied host–parasite systems, *B. tentaculata* supports a parasite community that presumably fulfils vital and central ecological functions, ranging from contributing to ecosystem diversity, structuring food webs or serving as ecosystem engineers (Thomas *et al.*, 1999; Mouritsen & Poulin, 2002; Lafferty *et al.*, 2008; Hatcher *et al.*, 2012; Dunne *et al.*, 2013). Moreover, since parasites can also serve as indicators of the local diversity and trophic interactions of free-living organisms (Hechinger *et al.*, 2007; Byers *et al.*, 2010; Shea *et al.*, 2012), the distinct trematode communities of *B. tentaculata* offer valuable insights into local habitat conditions.

One interesting example of the indication of local diversity and trophic interaction using digenean trematodes might be the detection of *P. duboisi* and *L. linstowi*. On the basis of our findings, we can infer the presence of bats in the studied habitat, as both are known to parasitize bats, e.g. the Daubenton's bat *M. daubentonii* (Gottschalk, 1970; Esteban *et al.*, 2001; Tkach *et al.*, 2003). The Daubenton's bat feeds on aquatic insects and insects with aquatic larvae, such as Lepidoptera, Diptera and Trichoptera, and it is, therefore, highly dependent on water sources. It hunts over standing or slow-moving water bodies and takes its prey from the water surface (Krapp, 2011 and references therein). Based on the finding of *P. duboisi* and *L. linstowi*, we are able to make inferences about the presence of bats at the studied habitat and the trophic relations between aquatic insects, which most probably serve as second intermediate hosts for the detected parasite species (Sharpilo & Iskova, 1989; Kudlai *et al.*, 2015; Enabulele *et al.*, 2018) and its final bat host.

The current study was limited by the lack of relevant sequences for many trematode families in GenBank. Consequently, a major proportion of our isolates could only be identified to the genus or family level. Moreover, the complete life cycle of many trematodes parasitizing *B. tentaculata* have not yet been elucidated (see Kudlai *et al.*, 2015) and remain unclear. Such obstacles impede and exacerbate extensive studies on the diversity, the ecological role and the influence of digeneans on food webs. Therefore, it is important to extend and compile morphological data of cercariae also from non-pulmonate snails, obtain more molecular isolates of adult specimens to facilitate molecular identification and clarify the still unknown life cycles of many trematode species. The present study can be seen as an important step in compiling morphological and molecular data on the digenean parasite fauna of bithyniids. Among the 20 digenean species, we are able to present the characteristics (measurements and/or photomicrographs) for 14 taxa. With the present host–parasite list we hope to foster parasitological research on parasites of understudied snail families.

Taken together, our findings and the limitations we encountered demonstrate unambiguously that our knowledge of the studied parasite–host system remains limited and large-scale studies focussing on non-pulmonate freshwater snails are lacking. This fits the overall trend of a currently highly patchy research effort on parasite diversity, which not only prevents a full inventory of parasite biodiversity but also impedes predictions of their role in ecosystems (Jorge & Poulin,2018). Our study revealed an abundant and diverse trematode fauna in *B. tentaculata*, which highlights the need for further research on this host–parasite system. Therefore, we might currently be underestimating the ecological roles and impacts of parasite communities of non-pulmonate freshwater snails in European fresh waters. In order to fully comprehend the numerous and often central roles these parasites play in aquatic ecosystems, we need to better understand such understudied host–parasite systems.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19001093

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Conflicts of interest. None.

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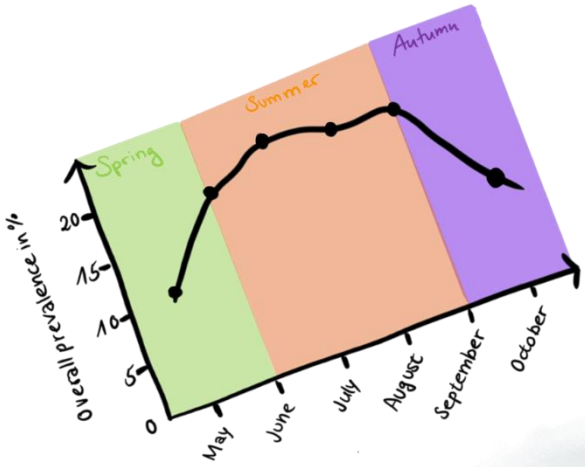
6. Chapter III

Trematode ecology: species richness and diversity of trematodes in a protected natural habitat



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Rare inventory of trematode diversity in a protected natural reserve

Jessica Schwelm[1,4]✉, Christian Selbach[2,4], Jenia Kremers[1] & Bernd Sures[1,3]

In the face of ongoing habitat degradation and the biodiversity crisis, natural reserves are important refuges for wildlife. Since most free-living organisms serve as hosts to parasites, the diverse communities in protected areas can be expected to provide suitable habitats for a species-rich parasite fauna. However, to date, assessments of parasite diversity in protected nature reserves are rare. To expand our knowledge of parasite communities in natural habitats, we examined 1994 molluscs belonging to 15 species for trematode infections in a central European natural reserve. The parasitological examination revealed an overall prevalence of 17.3% and a total species richness of 40 trematode species. However, the parasite diversity and prevalence did not differ markedly from trematode communities in non-protected environments, which might be partly explained by a dilution effect caused by a high number of non-host organisms in our study system. The proportion of complex and long life cycles of parasites in the present study is high, indicating complex biotic interactions. We conclude that life cycle complexity, in addition to parasite diversity and trematode species richness, can provide valuable information on ecosystem health and should therefore be considered in future studies.

Anthropogenic activity has a direct impact on most of the Earth's ecosystems. Globally, human activities have altered a large part of the terrestrial area[1], the oceans[2] and freshwater habitats[3]. The latter are among the most affected and threatened ecosystems suffering from multiple stressors[3]. The ongoing freshwater biodiversity crisis is gaining increasing awareness as a major environmental problem, as exemplified by implementation of the European Water Framework Directive (WFD)[4]. Mainly due to habitat degradation, an increasingly large number of species is threatened by extinction[5,6]. More frequent and more extreme weather events, such as droughts, storms or floods in the context of the global climate change, deterioration of freshwater quality, and the rapidly growing global population will further intensify this problem[7–10]. Natural reserves and protected areas are therefore becoming increasingly important as reservoirs and retreat areas for aquatic organisms, including rare and endangered species which are considered worthy of protection by society and politics[11,12]. These groups of organisms are well documented in many systems (e.g.[13,14]) and are used, for instance, as indicator species for the environmental conditions of ecosystems[15,16].

Free-living organisms may serve as hosts for a wide range of parasitic species. Since most free-living animals are assumed to host at least one parasite species, a correspondingly high diversity of parasites must be expected in natural reserves or protected areas. Parasitism is considered as one of the most successful and widespread life forms, and estimates of parasite contributions to global species diversity range from 30 to 50%[17–19]. Although parasites are often overlooked (or ignored) in nature conservation approaches and discussions, they play crucial roles in every ecosystem, influencing and shaping it in various ways. For instance, parasites have been shown to act as ecosystem engineers[20], shaping and regulating host population dynamics[21–23] as well as predator prey interactions[24,25] by influencing host growth, mortality, fitness and behaviour[26–28]. Parasites therefore have to be considered as important structuring forces in food-webs[29,30]. Furthermore, they account for a large part of the biomass of ecosystems and contribute significantly to the energy flow within those systems[31–34]. Due to their complex life cycles, parasites may also function as bioindicators to determine environmental conditions and changes[35–37].

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With about 25,000 described species and a cosmopolitan distribution, digenean trematodes represent one of the most diverse and widespread groups of metazoan parasites on the planet[38]. Trematodes have complex life cycles, with molluscs, mostly gastropods, playing a key role as first intermediate hosts. Besides vertebrates as obligate final hosts, a broad spectrum of invertebrates and vertebrates serve as second intermediate hosts. Due to the trophic transmission of many trematode species, they are particularly interwoven into the food-web and energy flow within ecosystems. Moreover, knowledge of the occurrence of specific trematodes in a habitat can provide valuable information on the presence of the hosts required by the parasite (see e.g.[39]), meaning that trophically transmitted parasites in particular can act as cross-taxon surrogates for the presence of their hosts[40].

Encouragingly, topics such as parasite extinction and conservation have recently gained more attention (e.g.[41–44]). Even a global plan for their protection and incorporation in monitoring programs has been proposed lately[42]. Due to their lifestyle, parasites are especially vulnerable and threatened with extinction, either when they are directly affected[42] by factors such as climate change or invasive species[45], or indirectly via the extinction of their hosts[46,47]. Although the importance of parasites in ecosystems has been increasingly recognised (e.g.[20–22,48,49]), we still face the problem that knowledge about the distribution of many parasite taxa is fairly incomplete[50]. In contrast to well-studied "man made" and anthropogenically influenced freshwater systems (e.g.[51–54]), our knowledge of these parasites in natural systems is often still limited. This is especially true for protected areas, since such habitats are increasingly rare and usually not easily accessible. Due to restrictions and protection measures, assessments of parasite biodiversity in such areas encounter various obstacles and are inevitably associated with high administrative burdens. This shortcoming remains a fundamental obstacle, as the knowledge on trematode diversity in protected areas might represent the best approximation of a natural status of the parasites in these regions, which can be used as a basis to assess changes in trematode community composition in heavily modified ecosystems. In the absence of this basic information, it will also be difficult to predict whether and how parasites will react to the ongoing anthropogenic-driven habitat alterations and how these reactions will affect ecosystem processes.

We hypothesize that freshwater natural reserves serve as a home to diverse, species-rich and well-connected communities of digenean trematodes, reflecting the system's free-living species richness. We predict parasites in natural reserves to (i) show species-rich communities that (ii) predominantly contain parasite species with complex life cycles (i.e. more hosts involved in the life cycle) and (iii) a high proportion of trematodes parasitizing rare host taxa. The aim of the present study is to assess the diversity of trematodes in a protected European freshwater natural reserve and to relate these data to other well-studied freshwater systems.

Results

Occurrence of snails and trematodes. A total of 1994 snails was collected at three different sites during monthly samplings from May to October 2017 and from May to September in 2019 in the natural reserve "Bienener Altrhein" in North Rhine-Westphalia, Germany (Fig. 1). Fifteen snail species belonging to seven families were sampled (Bithynidae, Hydrobiidae, Lymnaeida, Physidae, Planorbidae, Valvatidae, Viviparidae). Nine of these species harboured larval trematodes. The lymnaeid *Ampullaceana balthica* (former name: *Radix balthica*), which was collected in 2017 and 2019, was the most frequently collected snail species (n = 574), followed by the planorbids *Planorbis planorbis* (n = 309) and *Planorbarius corneus* (n = 228) (Table 1).

Parasitological examination of molluscs revealed an overall prevalence of 17.3% and a total species richness of 40 digenean trematode species. Distinct seasonal variations occurred among the sampling periods (χ^2 test, Df = 5, n = 1,994, $\chi^2 = 21.7$, $p < 0.001$), with overall prevalence peaking in the summer months compared to spring and early autumn (Fig. 2). In total, 15 trematode families were identified. The three most species-rich parasite families in our study represent the Echinostomatidae (10 species), Diplostomidae (5) and Strigeidea (4). The most abundant trematode species detected were *Echinoparyphium recurvatum* (found 64 times), *Petasiger radiatus* (60), *Cotylurus* sp. (45), *Echinostoma revolutum* (38) and *Hypoderaeum conoideum* (19) (Fig. 3).

With 13 trematode species, *A. balthica* showed the highest species richness among the snail hosts studied, followed by *Bithynia tentaculata, P. corneus* and *Lymnaea stagnalis* with 11, 10 and 9 species, respectively. The remaining snail hosts harboured only few species (Table 1). The relative species richness is on average four trematode species per snail species (Table 2).

Trematode life cycle complexity. Based on a literature survey, the life cycles of the trematode species identified in this study were reconstructed. Our investigation revealed that the proportional occurrence of detected digenean trematodes with a shorter life cycle (i.e. one intermediate host) accounts for 15%, compared to 85% with longer life cycles (i.e. two or three intermediate hosts) (Fig. 4). Species of the genera *Asymphylodora*, *Notocotylus*, *Sanguinicola* and *Trichobilharzia* are the main representatives that require only one intermediate host. Species belonging to the genera *Asymphylodora* and *Sanguinicola* utilise fish as final hosts, whereas species of the genera *Notocotylus* and *Trichobilharzia* parasitize waterfowl (Table 3). No significant association between sampling location and life cycle length were detected (Fisher's exact test, $p = 0.648$).

Host taxa. An extensive literature search was conducted to compile an overview of all recorded trematode species in their second intermediate and final host groups (Table 3). We reviewed final host specificity to detect trematode species that use rare and/or protected host species. Additionally, we looked at host specificity regarding the first intermediate host, for which trematodes usually exhibit high specificity[55]. With a few exceptions, individual trematode species were mainly found in one snail host species or in different species of the same family. The exceptions include, for instance, *P. radiatus or E. recurvatum*, which were detected in five and four snail species from three families, respectively. A large proportion of digeneans parasitize waterfowl (37.5%), in

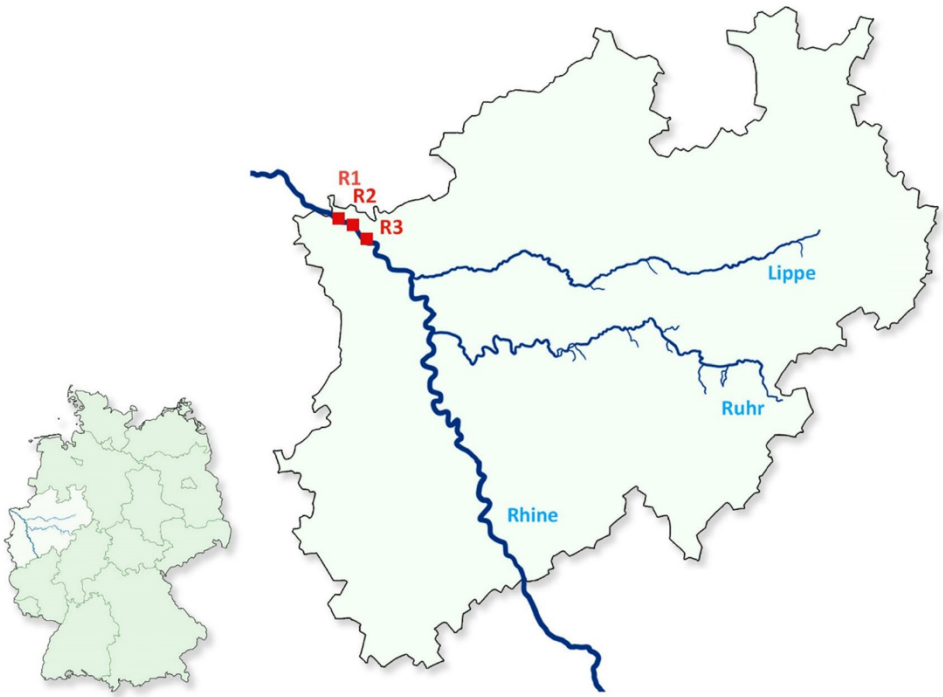

Figure 1. Map of Germany (left) and the federal state of North Rhine-Westphalia (right) indicating the sampling sites at the natural reserve Bienener Altrhein. Sampling sites are marked with red squares.

Collected mollusc species	Total no. of snails	Total no. of infections	Overall prevalence of infection (%)	No. of harboured trematode species
Bithynidae				
Bithynia tentaculata	75	14	18.7	11
Hydrobiidae				
Potamopyrgus antipodarum	49	–	–	–
Lymnaeidae				
Lymnaea stagnalis	160	32	20	9
Ampullaceana balthica	574	183	31.9	13
Stagnicola palustris	22	13	59.1	1
Physidae				
Aplexa hypnorum	87	–	–	–
Physa acuta	143	1	0.7	1
Planorbidae				
Anisus vortex	137	4	2.9	1
Bathyomphalus contortus	16	–	–	–
Ferrissia fragilis	15	–	–	–
Gyraulus albus	16	–	–	–
Planorbarius corneus	228	65	28.5	10
Planorbis planorbis	309	22	7.1	4
Valvatidae				
Valvata piscinalis	141	11	7.8	5
Viviparidae				
Viviparus viviparus	22	–	–	–
Total	1994	345	17.3	40

Table 1. Overview of collected mollusc species, number and prevalence of infection, and the number of harboured trematode species.

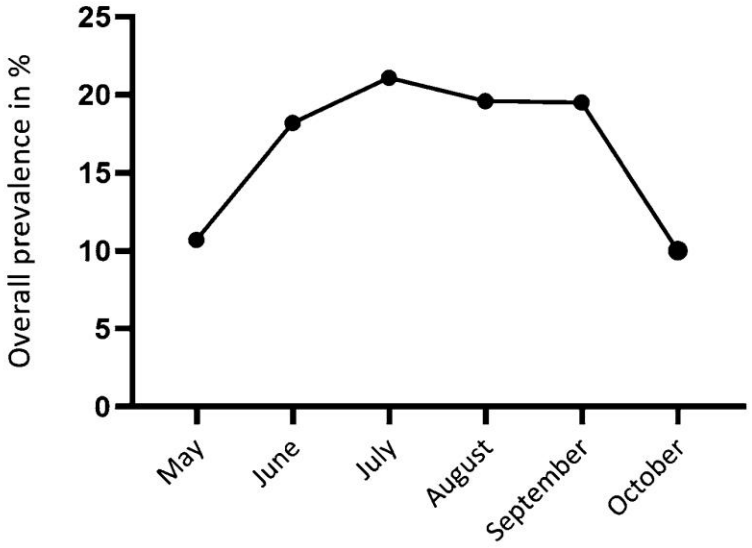

Figure 2. Seasonal overall trematode prevalence pooled from all collected snails in 2017 and 2019.

most cases anatid birds. Both, fish-eating birds and a very unspecific group consisting of mammals, birds and amphibians each account for almost 18% of hosts (Fig. 5).

Discussion

Our present knowledge of parasite diversity is still very limited and reveals major gaps in our understanding of the distribution of parasites in different ecosystems, especially in natural freshwaters. Due to this incomplete knowledge of parasite diversity, predictions about how anthropogenic environmental changes will affect parasite communities in the future remain difficult. The present study therefore presents a valuable and rare inventory of trematode diversity in a protected and natural aquatic system and is, to the best of our knowledge, the first evaluation of trematode communities in a natural freshwater reserve. While we can confirm our hypothesis that the studied freshwater nature reserve supports diverse and species-rich trematode communities, no significant differences in parasite life cycle complexity could be shown in comparison to other habitats, and the patchy data basis on host specificity of digenean trematodes hinders accurate conclusions on the role of rare host taxa in our study system.

With 40 trematode taxa in 1994 snails, the trematode species richness in our system ranks at the top compared to similar faunistic surveys from freshwater systems[51,52,54,56,57]. However, due to the rather high number of snail host species analyzed in the present study (15 species), of which some hosts harboured only few or no parasites, our study area shows a rather medium to low average species richness per host species with four trematode species per snail species. Moreover, with a prevalence of 17.3%, the natural reserve investigated in the present study is among the lower range, while the overall trematode prevalence in comparable studies ranged from 13.2[56] to 35.1%[52]. In our system, the snail hosts belonging to the two families Lymnaeidae and Planorbidae harboured the vast majority of trematodes we have identified (92%). This is in line with other broad faunistic surveys (e.g.[57–62]), which indicate that these two snail host families show a particular high prevalence and species richness and can therefore be considered key host groups in aquatic systems[54,63]. Since these trematode-rich key host taxa seem to be prevalent in heavily modified waterbodies, e.g. man-made reservoirs[54] or fish ponds[52], we do not find trematode communities with an extraordinary high species richness in the natural reserve compared to other systems, as initially expected.

Overall, with higher free-living diversity in restored habitats and natural reserves, not only does the diversity of suitable trematode hosts increase but also that of non-host organisms which can act as potential diluters of parasites. High free-living diversity can result in various dilution mechanisms, e.g. an encounter reduction of parasites and suitable hosts, a transmission reduction, in which fewer parasites successfully infect a host, or a reduction of reproduction, in which infected hosts produce fewer infectious stages[64]. Experiments under laboratory as well as under natural conditions have shown that dilution effects particularly affect parasites with complex life cycles and with vulnerable larval stages, such as trematodes and other helminths[64–66]. Due to their short-lived and fragile transmission stages (miracidia and cercariae), trematodes are particularly susceptible to reduction by predation or physical disruptions[67–69]. Furthermore, a high density and diversity of non-host species, decoy hosts or alternative hosts, especially benthic organisms, may decrease the probability of transmission stages finding suitable hosts[65]. Miracidia in particular show a high host specificity for their mollusc intermediate hosts[55], so that high mollusc diversity is likely to reduce trematode transmission to target hosts, while parasite species richness might be less impacted by the diversity of vertebrates, for which trematodes show lower host specificity[64]. The

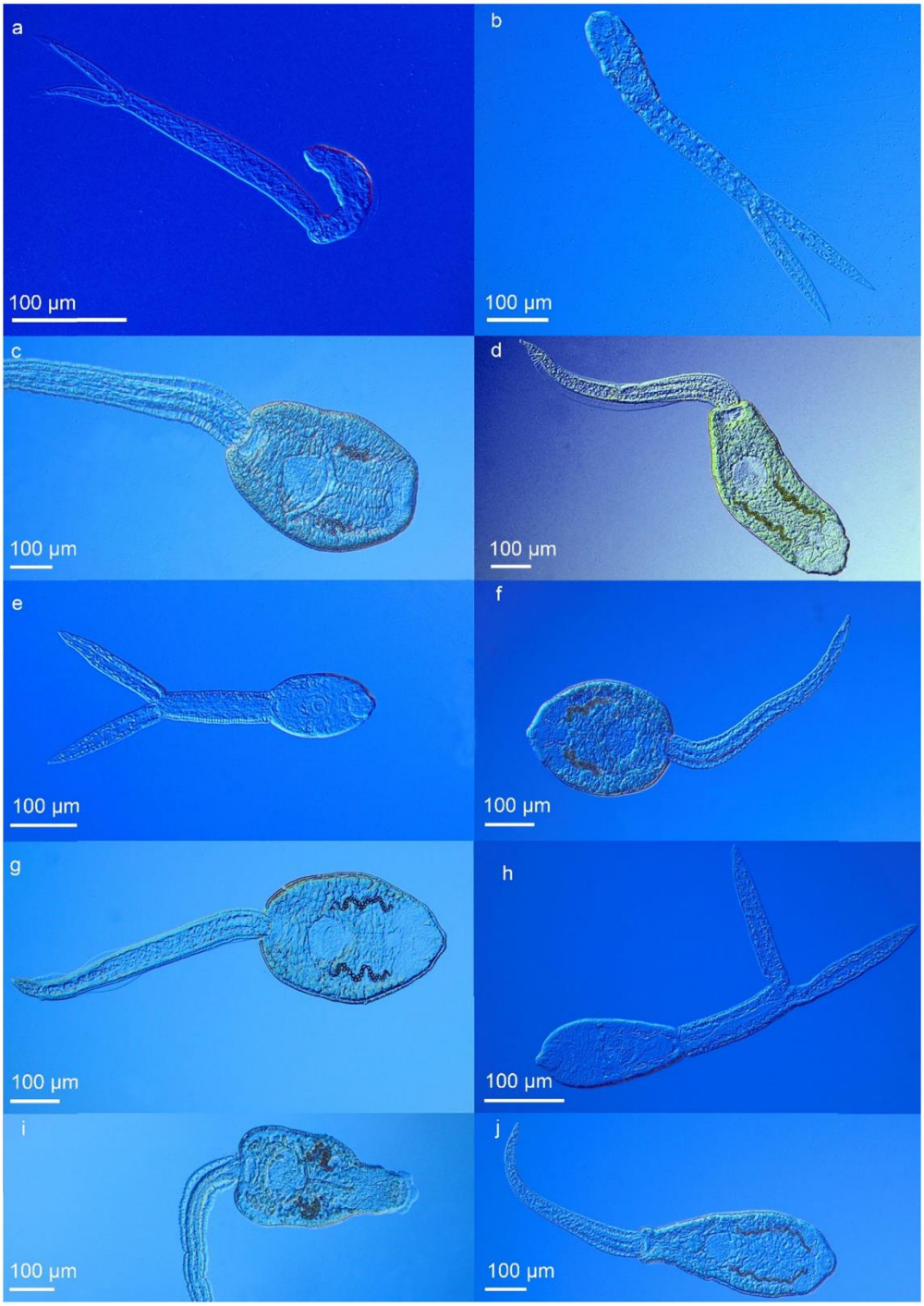

Figure 3. Microphotographs of live cercariae of the 10 most prevalent species. (**a**) *Sanguinicola inermis* (found 7 times). (**b**) *Tylodelphis excavata* (8). (**c**) *Echinoparyphium aconiatum* (10). (**d**) *Moliniella anceps* (14). (**e**) *Australapatemon burti* (16). (**f**) *Hypoderaeum conoideum* (19). (**g**) *Echinostoma revolutum* (38). (**h**) *Cotylurus* sp. (45). (**i**) *Petasiger radiatus* (60). (**j**) *Echinoparyphium recurvatum* (64).

Sampling sites	Country	No. of snails species	No. of snails species showing infections	No. of snail sampled	No. of trematode species	Relative species richness	Overall prevalence (%)	Reference
Central European natural reserve	Germany	15	9	1994	40	4.4	17.3	Present study
Northern European lake	Norway	3	2	1007	15	7.5	21.5	[57]
Northern American lakes	Canada	5	5	13,179	39	7.8	13.5	[56]
Central European fish ponds	Czech Republic	11	11	2584	27	2.5	15.2	[51]
Central European lakes	Poland	6	6	10,527	25	4.2	35.1	[52]
Central European reservoirs	Germany	6	6	5347	36	6.0	19.6	[54]

Table 2. Overview of snail host species, trematode species richness and prevalence from comparable well-studied fresh water systems in Europe and North America.

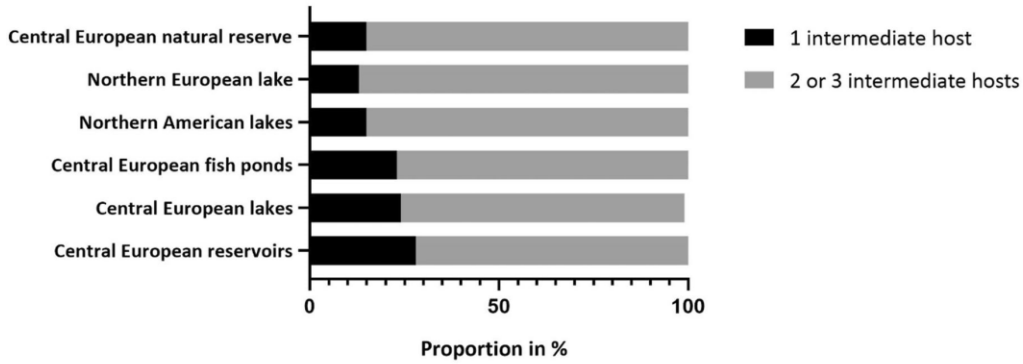

Figure 4. Proportion of trematode species with one intermediate host and two or more intermediate hosts for the studied Central European natural reserve in comparison to five freshwater systems with varying degree of anthropogenic impact.

species rich and abundant mollusc fauna detected in our study system (15 species) could thus represent one of the main diluters, causing a lower parasite species richness than initially expected. However, the dilution effect of free-living species diversity on parasitism is not universal and may depend on various factors, such as the individual species composition in a particular habitat, the characteristics of certain host-parasite assemblages, or the scale of the study[65]. To further explore such essential concepts in host-parasite ecology, comparable studies from various aquatic systems and habitats with different species compositions will be required.

However, comparing parasite diversity and functional ecology across different ecosystem often remains hindered and limited by a patchy knowledge of parasite taxonomy, host-specificity, and by dissimilar sampling and study approaches in different regions. For instance, some of the available freshwater studies assessing parasite diversity are broad faunistic surveys, in which many snail hosts, including those with low parasite diversity (e.g. *Potamopyrgus antipodarum*, *Physa acuta*, *Aplexa hypnorum*), were sampled[51] (present study). Other studies, in contrast, specifically targeted selected species-rich key trematode hosts in the habitat[54,57]. In addition, we find different degrees of resolution regarding parasite identification across studies. Some assessments have focused intensively on selected taxonomic parasite groups using morphological and molecular approaches to discover cryptic species diversity within problematic trematode lineages. Selbach et al.[54], for example, focused intensively on the genera *Diplostomum* and *Neopetasiger*, whereas Soldánová et al.[57] examined the species complex in the genus *Plagiorchis* in detail, resulting in the discovery of a high number of species in these groups. Altogether, such differences need to be considered when comparing parasite diversity in different systems, and when using parasites as bioindicators to determine environmental conditions and changes over time and between habitats[35–37].

Moreover, for many taxa, data on host specificity of trematodes in their second intermediate and final hosts are still too scarce, and many life cycles of parasites are not fully known[70]. The decreasing parasitological taxonomic expertise and the limited number of large-scale studies of vertebrate final hosts are major obstacles in elucidating many parasite life cycles[19]. For instance, parasites considered to be host-specific might be able to infect a wider host range, as host specificity data are usually based on a limited number of samples[71]. On the other hand, taxa considered generalists might be much more host specific. For example, *Diplostomum mergi* was long considered a generalist species until it was recently discovered to constitute a species complex whose individual members are highly host-specific in their final host[72]. Accordingly, the available literature information on final hosts is

Trematode family and species	First intermediate host*	Second intermediate host	Final hosts (literature data)	Final hosts (classification for analysis)	No. of required hosts	References
Cyathocotylidae						
Cyathocotylidae gen sp.	BT	Fishes, amphibians, aquatic invertebrates	Reptiles, birds, mammals	Reptiles, birds, mammals	3	85
Cyathocotyle sp. 1	BT	Fishes, amphibians, aquatic invertebrates	Reptiles, birds	Reptiles, birds, mammals	3	85
Cyathocotyle sp. 2	BT	Fishes, amphibians, aquatic invertebrates	Reptiles, birds	Reptiles, birds, mammals	3	85
Diplostomidae						
Alaria sp.	PP	Frogs, amphibians	Carnivores	Mammals	3 or 4[a]	86
Diplostomum pseudospathaceum	LS	Fishes	Fish-eating birds	Fish-eating birds	3	61,87
Diplostomum sp.	AB	Fishes	Fish-eating birds	Fish-eating birds	3	88
Hysteromorpha triloba	AV	Fishes	Fish-eating birds: cormorants, herons, grebes	Fish-eating birds	3	61,88
Tylodelphis excavata	PC	Amphibians	Birds: storks, herons, birds of prey	Birds	3	61,87
Echinochasmidae						
Echinochasmus coaxatus	BT	Fishes	Birds, fish-eating birds: grebes	Fish-eating birds	3	89
Echinochasmus bursicola	BT	Fishes	Fish-eating birds: herons	Fish-eating birds	3	89,90
Echinochasmus sp. 1	BT	Unknown	Fish-eating birds	Fish-eating birds	3	89
Echinostomatidae						
Echinoparyphium aconiatum	LS, AB	Molluscs, tadpoles	Anatidae	Waterfowl	3	87
Echinoparyphium recurvatum	LS, AB, PP, VP	Molluscs, tadpoles	Anatidae	Waterfowl	3	87
Echinostoma revolutum	LS, AB	Molluscs	Anatidae	Waterfowl	3	87
Echinostoma sp.	PC	Molluscs, bivalves, planarians, tadpoles	Waterfowl	Waterfowl	3	87
Hypoderaeum conoideum	AB	Molluscs	Waterfowl	Waterfowl	3	87
Moliniella anceps	AB, SP	Molluscs	Rallidae	Birds	3	87
Moliniella sp.	VP	Molluscs	Rallidae	Birds	3	87
Neoacanthoparyphium echinatoides	VP	Molluscs	Birds, Anatidae	Waterfowl	3	91
Neopetasiger sp. 3	PP	Tadpoles, Fishes: Cyprinids	Grebes	Fish-eating birds	3	92
Petasiger radiatus	LS, AB, PA, PC, PP	Fishes	Cormorants	Fish-eating birds	3	61,87,92
Lissorchiidae						
Asymphylodora sp.	VP	None (direct life cycle)	Fishes	Fishes	2	93
Notocotylidae						
Notocotylus attenuatus	LS; PC	None (cercariae encyst on vegetation)	Anatidae	Waterfowl	2	87
Notocotylus ephemera	PC	None (cercariae encyst on vegetation)	Anatidae	Waterfowl	2	87
Omphalometridae						
Rubenstrema sp.	PC	Insect larvae	Mammals	Mammals	3	87
Plagiorchiidae						
Plagiorchis elegans	LS; PC	Molluscs insect larvae, freshwater crustaceans	Various birds, mammals	Reptiles, birds, mammals	3	87,94
Plagiorchis neomidis	AB	Molluscs, insect larvae, freshwater crustaceans	Various birds, mammals: Soricidae	Reptiles, birds, mammals	3	94
Pleurogenidae						
Pleurogenidae gen sp. 2	BT	Unknown	Waterfowl: Rallidae	Birds		95
Prosthogonomidae						
Prosthogonimus ovatus	BT	Insect larvae	Birds	Birds	3	96
Psilostomidae						
Sphaeriodiotrema sp.	BT	Molluscs	Waterfowl	Waterfowl	3	97
Psilostomidae gen sp. 1	BT	Unknown	Birds, mammals	Reptiles, birds, mammals	3	98
Psilostomidae gen sp. 2	BT	Unknown	Birds, mammals	Reptiles, birds, mammals	3	98
Sanguinicolidae						
Sanguinicola inermis	AB	None (direct life cycle)	Fishes: Cyprinids	Fishes	2	61,87
Continued						

Trematode family and species	First intermediate host*	Second intermediate host	Final hosts (literature data)	Final hosts (classification for analysis)	No. of required hosts	References
Sanguinicola sp.	VP	None (direct life cycle)	Fishes	Fishes	2	61,87
Schistosomatidae						
Trichobilharzia szidati	LS	None (direct life cycle)	Anatid birds	Waterfowl	2	87
Strigeidae						
Australapatemon burti	AB; VP	Leeches	Anatid birds	Waterfowl	3	87
Australapatemon sp.	AB	Unknown	Anatid birds	Waterfowl	3	99
Cotylurus cornutus	LS	Leeches, Molluscs	Anatid birds	Waterfowl	3	87
Cotylurus sp.	PC	Leeches, Molluscs	Anatid birds	Waterfowl	3	87,99
Telorchiidae						
Opisthioglyphe ranae	AB	Amphibians, tadpoles	Amphibians: Anura	Amphibians	3	87

Table 3. Summary of the recorded trematode species in all collected mollusc species and their second intermediate and final host groups. *BT: *Bithynia tentaculata*; LS: *Lymnaea stagnalis*; AB: *Ampullaceana balthica*; SP: *Stagnicola palustris*; PA: *Physa acuta*; AV: *Anisus vortex*; PC: *Planorbarius corneus*; PP: *Planorbis planorbis*; VP: *Valvata piscinalis*. [a]*Alaria* spp. develop in a three-host life cycle with an interjectional mesocercarial stage between the cercarial and the metacercarial stage. This life cycle can be extended by paratenic hosts.

often designated in rather broad categories, so it may appear that many parasites are not particularly specific with respect to their final host taxa. This is also reflected in the results of our study, which show that most of the parasites detected seem to parasitize very common and widespread host taxa, e.g., waterfowl or fish-eating birds.

Keeping the above-mentioned limitations in mind, the results from our study allow some valuable comparisons with host-parasite communities from other habitats and ecosystems. Even the rather broad final host categories of parasite communities can reveal information on distinctive free-living communities utilized by the parasites in different aquatic ecosystems that represent a characteristic final host composition. For example, despite the close geographic proximity, similar study approaches as well as similar reported trematode species richness and prevalence, the results of Selbach et al.[54] and from the present study reveal fundamentally different final host structures in these waterbodies (see Fig. 5a vs b). Moreover, the parasites' life cycle complexity, i.e. the number of hosts required to complete the full life cycle, can serve as an indicator to assess biotic interactions in ecosystems. While parasite species with longer life cycles that rely on trophic transmission can indicate longer food chains and thus more complex biotic interactions in a habitat, a higher share of less complex parasite life cycles can indicate ecosystems that are more severely impacted[73]. Assessments from marine systems and estuarine marshes using parasite communities as bioindicators could show contaminated or only recently restored habitats to contain mostly parasites with short life cycles with only one intermediate host, or direct transmission pathways[74,75]. The natural reserve of the present study is predominantly characterized by trematode species with long life cycles that require two or three intermediate hosts (85% of all species, see Fig. 4). Although the statistical analysis did not reveal any significant differences between the studies compared, the complex life cycles and diverse final host groups of the trematode communities in the present study suggest a diverse and trophically well-connected free-living fauna in the protected nature reserve adjacent to the River Rhine. These long chains of host-parasite connections can indicate stable community structures that provide the basis for ecosystem resilience and persistence[21,76]. Overall, in addition to the diversity of parasites that can indicate ecosystem health[21], their functional ecology and life histories can provide valuable information on interspecific interactions in these habitats.

In summary, we investigated a protected freshwater ecosystem that provides a valuable model system for free-living and parasite diversity. However, our study also highlights the patchy and heterogeneous data availability, especially with regard to final hosts specificity, which makes it difficult to draw conclusions on the functional diversity and compare parasite communities across various habitats. We therefore encourage future studies on host-parasite communities in natural and protected aquatic systems as well as taxonomic research in this field that will allow a better resolution of cryptic trematode diversity and host specificity for future comparisons. Ultimately, understanding and predicting how global climate change and other anthropogenic pressures will affect complex and fragile freshwater ecosystems and their biotic communities will require a thorough awareness of the parasite communities and their ecological roles that too often remain overlooked in environmental assessments and conservation approaches.

Materials and methods

In total, 1648 snails of 15 species belonging to seven families were collected and examined for trematode infections during monthly collections in spring (May), summer (June–August) and autumn (September, October) in 2017. Additionally, 346 snails of the species *A. balthica* were sampled monthly from May to September in 2019. To allow comparability, the same sampling effort (time spent sampling) was applied at each sampling site, during each sampling trip, and for all snail host species. Snails were identified to the species level using the identification keys of Glöer[77] and Welter-Schultes[78]. Snails were collected with hand-nets from the submersed vegetation or picked by hand from sediments, stones, deadwood and macrophytes along the shoreline of the oxbow or the littoral zone of the pond. All snails were taken to the laboratory, placed in individual beakers filled with filtered

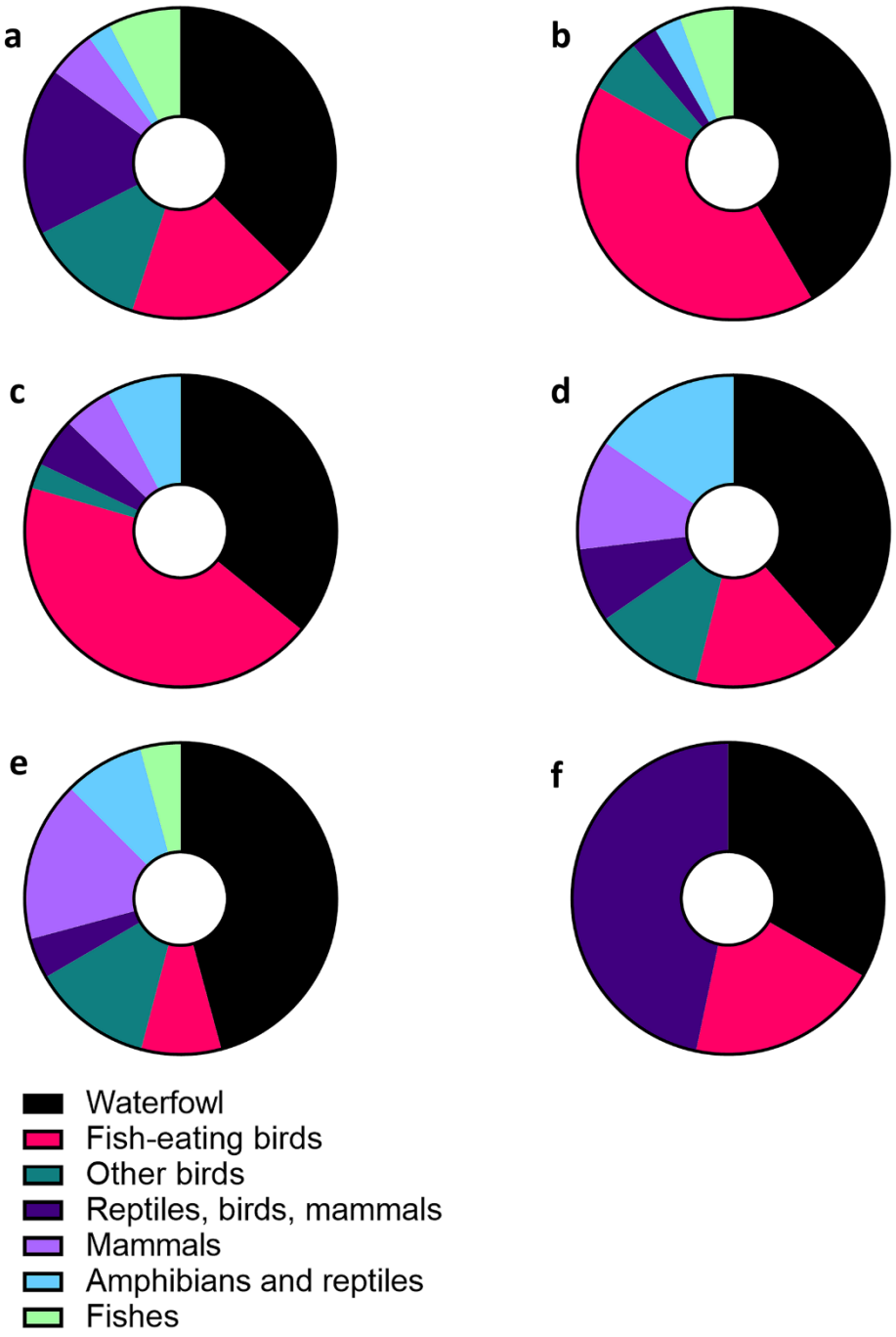

Figure 5. Proportion of final hosts. (**a**) Central European natural reserve. (**b**) Central European reservoirs. (**c**) Northern American lakes. (**d**) Central European fish ponds. (**e**) Central European lakes. (**f**) Northern European lake.

river water at 20 °C room temperature and exposed to artificial light to induce the emission of cercariae. After the day of sampling, every beaker was screened daily for three consecutive days under a stereomicroscope for the presence of cercariae. Snails that did not show an infection with trematodes during this time were dissected and examined for prepatent infections (rediae/sporocsysts).

Study site. All snails were collected at two sampling sites at the lower River Rhine (R1: 51° 47′ 59.2″ N 6° 21′ 46.3″ E; R3: 51° 48′ 37.1″ N 6° 21′ 23.4″ E) and one groundwater-fed pond of its adjacent floodplain (R2: 51° 49′ 07.0″ N 6° 20′ 26.8″ E) in North Rhine-Westphalia, Germany (Fig. 1). Until the year 1800, the Rhine river was largely unaffected. During industrialisation, however, dyking, canalisation and thus the decoupling of backwaters from the main watercourse occurred. The natural reserve Bienener Altrhein is part of the Rhine floodplains between Rees and Emmerich, which are among the few remaining natural floodplains in Europe. They form a unique system of oxbow floodplains, and have been protected since 1969. Various measures have been taken to preserve this area as natural as possible[79].

Morphological analyses. Cercariae were identified alive under a light microscope (Olympus BX51) based on identification keys[54,59,60] and morphological descriptions and other relevant publications (e.g.[70,80–82]). For documentation and further identification photomicrographs of visible features were taken for collected species with an Olympus UC30 digital camera attached to the light microscope.

Molecular analyses. Cercariae were molecularly spot-checked. DNA was isolated from pooled fixed cercariae by salt precipitation according to Grabner et al.[83]. PCR products were purified (my-budget PCR/Gel-purification kits; Biobudget Technologies, Krefeld) and sent for sequencing (GATC Biotech, Constance). Sequences were compared to GenBank entries by Blast-searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis. Trematode prevalence (p) was calculated separately for parasite assemblages in all infected snail species as the proportion of infected host individuals in relation to the total number of host individuals in a population ($p = n_{inf}/N \times 100$, where n_{inf} is the number of infected snails of one species and N are all snails of one species in a sampled population) following Bush et al.[84]. The overall prevalence was calculated for all parasites in all snail species as the proportion of all infected host individuals in relation to the total number of all host individuals ($Op = a_{inf}/S \times 100$, where a_{inf} is the number of infected snails of all species and S are all snails collected). Species richness was calculated as the total number of trematode species in all host individuals. The relative species richness (S_r) was calculated for parasite assemblages in all infected snail species as a quotient of the number of trematode species in relation to the total number of infected snail host species studied ($S_r = t/M$, where t is the number of detected trematode species and M are all infected snail species sampled).

Statistical analyses. Statistical analyses were performed with the open-source software Rstudio (version 2021.09.0, Rstudio Inc.) based on R (version 4.1.1, R Core Team; (www.r-project.org)). A χ^2 test followed by a post-hoc pairwise test of independence with Bonferroni correction was applied to compare overall trematode prevalence between sampling months. While a Fisher's exact test was employed to assess differences in the proportions of parasite species with one and two or three intermediate hosts between individual studies.

Data availability

The raw data that support the findings of this study are available from the corresponding author, [J.S.], upon reasonable request.

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Author contributions
Names given as initials B.S., J.K., C.S. and J.S. B.S. and C.S. conceived the study and supervised the project. J.S. and J.K. carried out the sampling, J.S. carried out the data analyses and J.S., B.S. and C.S. wrote the manuscript. B.S. and C.S. oversaw the analyses and writing, and reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests
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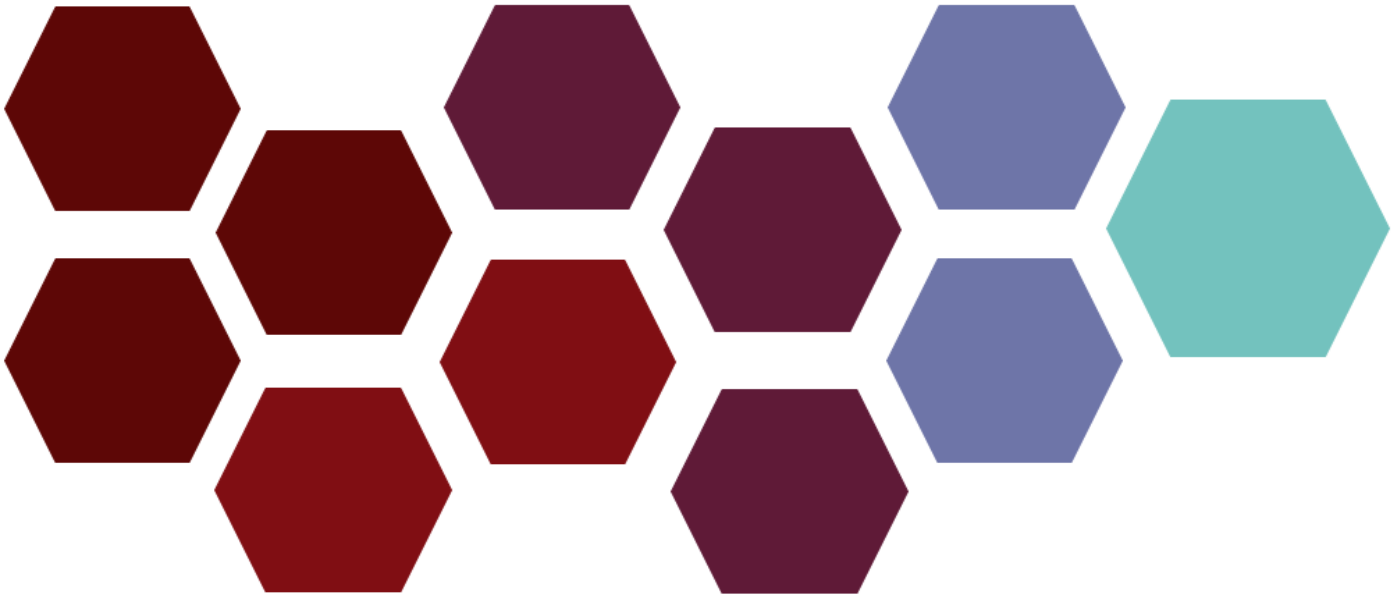
7. General discussion



7. General discussion

Today, there is overwhelming evidence that biodiversity concerns us all and that its protection and conservation should be of high priority (IPBES 2019b; Diaz et al. 2019; Bradshaw et al. 2021). Not only for its own sake, but also because the well-being and survival of future generations depend on it. We are in the midst of a biodiversity crisis, maybe even at the beginning of the sixth mass extinction, the extent and impacts of which are momentous (Barnosky et al. 2011; Ceballos et al. 2015, 2017; Andermann et al. 2020). Biodiversity is important, and it is rapidly dwindling, with implications that will drastically affect life on this planet (Bradshaw et al. 2021). Along with the consequences of the ongoing climate change, the biodiversity crisis is one of the Anthropocene's most pressing issues (Svenning et al. 2016; Albert et al. 2021). Although these insights can be challenging and frightening by themselves, we should be aware that they may only be a rather simplified view on the transformation process that might lie ahead. Conservation and species protection projects and measures, as well as biodiversity assessments, such as habitat restoration, rewilding, translocation, and reintroduction, almost exclusively target free-living organisms (e.g., Biron et al. 2004; Batson et al. 2015; Svenning et al. 2016; Jepson 2016; Het et al. 2018; Lorenz et al. 2018; Dumeier et al. 2020; Mata et al. 2021; Zajicek et al. 2021). However, most free-living species are most likely hosts to at least one parasite species (Marcogliese 2003), many of which have not been described yet (Singh 2002; Poulin and Morand 2004; Barnosky et al. 2011; Mora et al. 2011). Accordingly, we may be largely underestimating how many species have gone extinct, are on the brink of extinction, or will become extinct in the near future (Dirzo and Raven 2003; Joppa et al. 2010). Could it be the case that parasites are even more affected by the biodiversity crisis than free-living species? This is suggested by the fact that parasites are not only threatened by direct factors (Carlson et al. 2020), such as environmental pollution (Sures 2001; Sures et al. 2017b), climate change or invasive species (Carlson et al. 2017), but also indirectly via the extinction of their hosts (Lafferty et al. 2012; Dunn et al. 2009, 2013; Cizauskas et al. 2017). Their hidden lifestyle, their relatively small size, the effort involved in their study, as well as the missing of taxonomic expertise, undermine their discovery, inclusion in food web assessments and result in a general poorer resolution of (taxonomic) diversity when compared to free-living organisms (Poulin and Morand 2004; Lafferty et al. 2008). Therefore, it is not

surprising that experts in the field believe many species are yet unknown to us (Poulin and Morand 2004). Since the study of parasites has the potential to significantly contribute to our understanding of biodiversity, food web dynamics, and ecosystems (Marcogliese 2003), it is important to include them in our concept of biodiversity and study their important functional roles in ecosystems thoroughly.

To contribute to this knowledge and shed some light, especially on the aspect of parasite diversity, the diversity of digenean trematodes at different organizational levels has been examined in this thesis. Specifically, the aims were to i) investige parasite diversity at the species level of a cryptic trematode genus; ii) assess trematode diversity in a previously neglected host-parasite system; and iii) uncover species richness and diversity in a protected freshwater ecosystem.

In detail, the individual published studies of this thesis reveal exceptional and novel insights into parasite diversity at different levels. At the individual or species level, *Diplostomum phoxini* was characterised morphologically and molecularly and analyses that resulted in a re-identification/re-classification of several isolates as well as the re-definition of the *D. baeri* and the *D. mergi* species complexes were provided (Schwelm et al. 2021a; Chapter I). Likewise, at the host group level, trematode diversity was emphasized in the faucet snail *Bithynia tentaculata*, which comprises 20 species (10 families), demonstrating a unique species composition when compared to well-studied snail host families, such as Lymnaeidae and Planorbidae (Schwelm et al. 2020; Chapter II). Similarly, at the ecosystem level, the investigated fifteen snail species revealed a high trematode species richness of 40 species in a central European natural reserve, with a high proportion of species with complex life cycles (Schwelm et al. 2021b; Chapter III).

In the following, I will briefly highlight the main findings of the individual studies of this thesis to discuss aspects, such as taxonomic resolution, the reconstruction of life-cycles, the role of parasites in food webs, ecosystem health and point out challenges and gaps in our knowledge and, last but not least, present ideas for future research.

Taxonomic resolution

The capacity to estimate parasite diversity and extinction rates, as well as monitor potential zoonotic diseases or invasive pathogens, depends on precise parasite identification (Poulin

2011). For a long time, parasites were seen as negligible members of ecosystems, and parasite diversity was vastly underestimated (Poulin 2011). Advances in optical instruments, such as light microscopy and scanning electron microscopy have led to progress in the identification processes of parasites, but improved microscopy technologies alone are sometimes not enough when dealing with cryptic diversity. Cryptic species are genetically separate but morphologically highly similar sibling species, which are often sympatric (Poulin and Morand 2004; Poulin 2011). In many cases, a parasite species thought to be capable of parasitizing a few closely related hosts has been revealed to be a complex of related but genetically distinct and highly host specific species (Poulin and Morand 2004), as it is for example the case for the *D. mergi* species complex (see Selbach et al. 2015). As demonstrated by a study of Poulin (2011), current estimates of the diversity of helminth parasites should actually be doubled and in the case of trematodes even tripled due to cryptic diversity. As the name indicates, cryptic diversity is difficult to identify using just morphological examination methods. Therefore, the advent of molecular examination tools was a significant breakthrough for parasitology. Although, parasitology, as a scientific discipline, appears to be slower to adopt innovative molecular techniques than other disciplines (Selbach et al. 2019), it is clear that molecular tools for species identification have led to enormous knowledge gains in the field of parasitology and has enhanced our estimates of parasite biodiversity (Poulin and Morand 2004; Poulin 2011; Pérez-Ponce de León and Poulin et al. 2018).

Within this thesis, it has been demonstrate that the application of molecular methodologies has significantly contributed to the clarification of species relationships. No cryptic diversity could be detected for *D. phoxini* within the isolates studied here or in the analysis involving isolates retrieved from GenBank. Nevertheless, isolates of cercariae obtained from *A. balthica* and metacercariae obtained from *P. phoxinus* could be matched, underlining literature data that suggest a high host specificity to the second intermediate host (Ballabeni and Ward 1993). Moreover, two species complexes were redefined based on our findings. The redefinition of the *Diplostomum* species complexes is one of many examples demonstrating that taxonomy is a dynamic field of study, in which the state-of-the-art is regularly updated and altered in response to new discoveries. Taxonomic groupings are repeatedly dismantled and re-sorted, and species are allocated to different genera based on new (molecular) findings (e.g., Kostadinova and Gibson 2001; Kostadinova et al. 2003; Tkach et al. 2016; Heneberg et al.

2018). It is also evident that we are still in the process of classifying and describing the great diversity of parasites, including many cryptic species that have been co-evolving with their host for a long time.

The trematode community in the neglected snail host species *B. tentaculata* was examined and the attempt to initially identify the discovered species morphologically proved to be impractical due to a lack of identification literature, such as that available for lymnaeids or planorbids (e.g., Faltýnková et al. 2007, 2008; Selbach et al. 2014). Applying molecular methods, we were able to identify 20 distinct species in our study. However, due to a lack of relevant sequences for several trematode families in GenBank, a substantial number of isolates could only be identified to the genus or family level. This highlights the importance of expanding and compiling morphological and molecular data from cercariae from (non-pulmonate) snails, as well as obtaining more morphological and molecular isolates of adult specimens. Matching isolates of larval stages to those of adults would benefit the molecular identification of digenean trematode larval stages (Georgieva et al. 2013). Obtaining sequences of reliably identified adult stages can be a straightforward and efficient way of identifying larval stages and thus deduce full life cycles (Criscione et al. 2005; Pérez-Ponce de León and Nadler 2010; Georgieva et al. 2013). However, in many cases (e.g., endangered or rare species), obtaining adults is quite difficult. Therefore, parasitologists should be dedicated to conducting morphological species discrimination at the larval level and providing sufficiently detailed descriptions accordingly. Hence, in this chapter, we not only present sequences, but also morphometric data and profound morphological descriptions for a wide variety of digenean trematodes from this neglected host. Because of the increasing number of genetic studies and the ambiguity in linking sequence data from larval stages to identified species, it is preferable to characterize the sequenced species and therefore relate the molecular data to morphological reference data (e.g., Georgieva et al. 2013; Heneberg et al. 2018; Schwelm et al. 2020; 2021a). Hence, both studies (Chapter I and Chapter II) anchor molecular data with detailed descriptions of larval stages. This holistic approach also facilitates the detection of potential misidentifications and prevents the re-designation of previously described larval stages as new species, resulting in unwanted duplication. The absence of a standardized naming system sometimes leads to further confusion and duplication, particularly among cryptic species, such as *Diplostomum* spp. As a result, it would be beneficial

to follow the rules of the code of homonymy and previous publishing to ensure that the "name" of each genetic linage is distinct and unique (Schwelm et al. 2021, Chapter I).

Life cycle reconstruction

The issue of life cycle reconstruction is closely linked to the question of taxonomic resolution and certainty, and is a prominent topic in all three chapters of this thesis. Since parasites can serve as indicators of free-living diversity and trophic interactions (Hechinger et al. 2007; Byers et al. 2010; Shea et al. 2012), exploring trematode communities and reconstructing their life cycles offers valuable insights into local habitat conditions. However, many trematode life cycles are insufficiently known and have yet to be thoroughly unraveled (Poulin and Morand 2004; Kudlai et al. 2015; Sures et al. 2017a).

The complexity of trematode life cycles, which typically include several morphologically distinct stages, complicates reconstruction. But also other factors, such as strict regulations for parasitological studies of vertebrates, and dwindling taxonomic expertise (Marcogliese 2003; Poulin and Morand 2004), contribute to the fact that many parasite-host systems remain unknown. In this thesis, not only the first, but also the second intermediate fish host of *D. phoxini* was investigated. The European minnow, *Phoxinus phoxinus* is commonly infected with *D. phoxini* metacercariae (Dezfuli et al. 2007). The metacercariae are known to be unevenly distributed throughout the brain and aggregate in immunologically privileged areas (Barber and Crompton 1997; Dezfuli et al. 2007), which are related to sensory function, motor activity, and vision. The damage of these brain tissue regions has a high potential to influence flight reactions to predators (Barber and Crompton, 1997; Barber et al. 2000). For instance, Dönges (1969) observed that infected minnows had a higher respiratory rate and jumped to the "shore" more frequently than uninfected individuals. Moreover, it is generally known that species of *Diplostomum* manipulate the behaviour of their hosts to increase the probability of transmission to the next host (e.g., Crowden and Broom 1980; Seppälä et al. 2004, 2008).

Although this species is exceptionally well-studied and shows high host specificity for its second intermediate fish host (Ballabeni and Ward 1993), questions regarding the life cycle remain open. The only natural infection of a final host has been reported so far in *Mergus merganser* (Sitko and Rzad 2014), but experimental studies on the life cycle indicate that *D. phoxini* is not very specific to its final host, as adult flukes containing eggs and mature

sperm were obtained from both birds and mammals (e.g., Arvy and Buttner 1954; Berrie 1960; Shigin 1993), with a rapid development of the larvae to the adult stage, especially in birds (Rees 1955; Berrie 1960). Taken together with the behavioural alteration in the second fish host caused by the metacercariae, it is reasonable to assume that *D. phoxini* does not exhibit a high host specificity for its final host and that it is more crucial that *P. phoxinus* is on the diet of the species in question (Schwelm et al. 2021; Chapter I).

Although, only a limited number of infected snails in the investigated area could be detected, *P. phoxinus* showed a prevalence of 100 %, which emphasizes the effectiveness of the dispersal strategies of the cercariae and the important influence that *A. balthica* takes on food web interactions and connectivity as a keystone species in that specific habitat.

With the parasites gathered from *B. tentaculata,* some life cycles could be reconstructed, which helped to gain insights in the local free-living diversity and trophic interactions within the systems studied. Thus, the presence of *Parabascus duboisi* and *Lecithodendrium linstowi* represents an exciting example. Since both species are known to parasitize bats, such as the Daubenton's bat *Myotis daubentonii* (Gottschalk 1970; Esteban et al. 2001; Tkach et al. 2003), the presence of bats in the studied region could be deduced. Based on the diet of this bat species, it is feasible to predict which second intermediate hosts *P. duboisi* and *L. linstowi* are likely to utilise. The Daubenton's bat feeds on aquatic insects and insects with aquatic larvae, such as Lepidoptera, Diptera, and Trichoptera, and it is extremely reliant on water supplies. It hunts over still or slow-moving water, catching its prey on the water's surface (Krapp 2011 and references therein; Schwelm et al. 2020). Therefore, it can be reasonably concluded that *P. duboisi* and *L. linstowi* most likely use aquatic larvae as second intermediate hosts (Sharpilo and Iskova, 1989; Kudlai et al. 2015; Enabulele et al. 2018). Another example is the finding of *Echinochasmus bursicola.* This species of the family Echinochasmidae is known to parasitize herons (Ardeidae) (Kostadinova and Gibson 2001; Tkach et al. 2016). Some species of herons are widespread in Germany, like the grey heron (*Ardea cinerea*, 24,000 – 30,000 breeding pairs), whereas some species, such as the purple heron (*A. purpurea*, 40-50 breeding pairs) are extremely rare (Gedeon et al. 2014). Since this trematode species is known from various species of herons (e.g., *A. cinerea, A. purpurea, A. alba* (Kostadinova and Gibson 2001; Tkach et al. 2016)), this example does not allow us to identify the final host at the species level, but

it does indicate the presence of herons and provides valuable insights into the trophic connections within the studied habitat.

The reconstruction of life cycles and their subsequent analysis was also one of the main interests in the study, which is presented in Chapter III. Based on the parasite communities in the habitat studied, the aim was to reconstruct life cycles in order to draw conclusions about the free-living diversity. On the one hand, species like *Plagiorchis elegans,* a very common and ubiquitous species in Central Europe, with a high variety of final hosts, including mammals, reptiles, and birds, were detected (Brown et al. 2011). Obviously, no precise statements regarding the diversity of free-living species in an ecosystem can be made based on such vague information. On the other hand, species, such as *P. duboisi* and *L. linstowi* have also been discovered, about which quite precise information is available on their required final hosts. However, those representative examples demonstrate quite well the patchy and heterogeneous data situation and highlight the need for further research, especially at the taxonomic level, before we can make valid estimates of free-living diversity in ecosystems based on parasites. The elucidation of life cycles could also be supported by alternative and less invasive investigation approaches, such as coproscopic examination of parasite eggs in faecal samples, as it is done in veterinary medicine for the detection of parasites (e.g., Kajugu et al. 2015).

Food web connectivity

Through their complex life cycles, usually involving multiple hosts with the final host often becoming infected via trophic transmission, parasites create additional connections within food webs, contributing to their stability, resilience, and connectivity (Marcogliese 2003; Lafferty et al. 2006, 2008). As food webs are networks of trophic interactions among species (Quevedo et al. 2009), these networks frequently incorporate nutrient and energy flows across habitats, which can impact trophic dynamics and food web stability (Vanni 2002). Cross-habitat links in food webs (habitat coupling) are common in many biomes and are frequently linked to predator and prey movements (Polis et al. 1997). Parasitism, as another form of interspecific relationship, also contributes to habitat coupling (Marcogliese 2003; Selbach et al. in press). Moreover, parasites can reveal direct predator-prey interactions in a system when trophic transmission of the parasite is required to complete the life cycle. In general, all species that use the highly mobile group of birds as final hosts are excellent instances of

habitat coupling between aquatic and terrestrial systems, but the two species previously mentioned, *P. duboisi* and *L. linstowi*, which use bats as final hosts, are also fine examples. The addition of parasites to food webs inherently increases the number of species and linkages and can modify connection density and connectivity (Lafferty et al. 2006). The examples mentioned above highlight what large scale studies have previously demonstrated: Parasites play critical roles in the structure of food webs in terms of chain length, connectivity, and robustness, as well as interaction strength and energy flow (Hernandez and Sukhdeo 2008; Lafferty et al. 2008; Hatcher et al. 2012). Direct comparisons of food webs with and without parasites have demonstrated how including these organisms drastically increases the complexity of such networks and allows more accurate assessments of the impact of any species on the trophic organization (Dunne et al. 2013). However, to successfully incorporate parasites into the study of food webs and their connectivity, a detailed understanding and investigation of parasite life cycles and associated host species or host groups is essential. Without this basic information, we cannot model food webs realistically, which in turn means we cannot fully understand ecosystems either (Figure 6).

Ecosystem health

In line with the the results of this thesis, the frequently revived question of Hudson et al. (2006), whether an ecosystem rich in parasites is a healthy ecosystem, does not appear to be that simple to answer. In contrast to “human health”, which is typically defined as the absence of disease of an individual, the term “ecosystem health”, focuses on the total performance and stability of a community (Hudson et al. 2006). As a result, the concept must incorporate the system's overall performance and persistence. Thus, a healthy ecosystem is one that retains vitality, organization, and resilience (Hudson et al. 2006).

Particularly parasite species that, for example, regulate host population and density through reduction of survival or fecundity (e.g., Marcogliese 2004) or influence ecosystems through host manipulation (Perrot-Minnot et al. 2007; Lafferty and Shaw 2013; Heil 2016) play an important role in the question of ecosystem health. As Lafferty and Shaw (2013) have shown, parasites that infect vertebrates are more likely to affect host response to predation, with parasites that infect the central nervous system appearing particularly likely to affect host behaviour. In this thesis, *D. phoxini* was investigated in its intermediate fish host *P. phoxinus*. The metacercariae accumulate in the brain of the minnow through their life span (Bibby 1972;

Müller 1995). *Diplostomum phoxini* thus probably plays an important key role in the ecosystem studied. The parasite might manipulate the minnows in such a way that they exhibit reduced or modified escape behaviour towards their predators. Considering the high prevalence (100 %) in the studied minnow population, the parasite probably drastically affects the interaction strength between minnows and their predators and thus influences the energy flow in the ecosystem. As demonstrated in the review by Wood and Johnson (2015) the removal of parasites from ecosystem would have far-reaching consequences. In this particular example, it would probably change the community composition, as the minnow would now show "normal" escape behaviour, predators would find less prey, so possibly seek out other foraging grounds. The population of minnows would increase as a result, impacting the population structures of their prey and consequently that of all linked organisms. Even though this line of thought could be continued for a while, it already illustrates the important role that this specific parasite species might play in the stability and productivity of the ecosystem studied. Nevertheless, we must keep in mind that conclusions about ecosystem health based on knowledge about parasites are only possible if their diversity, life cycles and effects on other organisms are well understood.

Simple causal chains

Interestingly, the present study also shows that observations cannot always be explained by simple causal chains — a high diversity of free-living organisms does not necessarily result in a high diversity of parasites, or specific parasite stages. Due to their complex life cycles, parasites are, on the one hand, well suited to study certain ecosystem-relevant aspects, (e.g., food web connectivity). Still, on the other hand, their larval stages, and especially the free-living ones, like cercariae or miracidia, might suffer from predation or other disturbances (Welsh et al. 2014; Gopko et al. 2017; Vielma et al. 2019; Hobart et al. 2021). Hence, interpretation of particular findings might not be straightforward given the underlying interdependencies. Since the results of the ecosystem study (Chapter III) did not necessarily correspond to expectations, i.e., high parasite diversity due to high free-living diversity, it is debatable whether the connection between parasite abundance and the health of an ecosystem is not a little too shortsighted and simplistic. The discovery of this unexpected pattern might be attributable to a variety of factors, but in this case, a dilution effect caused by a large number of non-host snail species was considered as a potential explanation. A high

diversity of free-living organisms also ensures a high diversity of non-host organisms, which can lead to various forms of dilution mechanisms. This includes, for example, a reduction in the number of encounters between parasites and their suitable hosts, a reduction in infection processes, or a reduction in the production of infectious stages (Johnson and Thieltges 2010). Miracidia exhibit a high host specificity for their mollusc intermediate hosts (Gibson and Bray 1994). Therefore, a high mollusc diversity is expected to restrict trematode transmission to target hosts, but parasite species richness may be less influenced by vertebrate diversity, which has a lower host specificity (Johnson and Thieltges 2010). The diverse and rich mollusc fauna seen in the natural reserve might thus be one of the primary diluters, resulting in a lower parasite species richness than predicted (Schwelm et al. 2021b).

Knowledge gaps and future research

The present thesis contributes to our knowledge of digenean trematodes and provides new insights at various diversity levels. However, as already alluded to in the previous paragraphs, some hurdles and obstacles have been encountered in these studies, which are based on knowledge gaps. Therefore, further research in the field of parasitology would be desirable. In order to improve our understanding of the ecological role of parasites and their biodiversity, research should focus primarily on the fields I will now briefly outline.

Due to climate change, biodiversity loss and ongoing globalisation, we find more and more invasive species, which might cointroduce their parasites and can represent serious threats, such as spill over events, to ecosystem stability and health (Poulin et al. 2017; Selbach et al. in press). Therefore, more attention should be given to parasite assemblages of invasive species, such as *Potamopyrgus antipodarum* or *Physella acuta.* Likewise, the gathering of molecular and morphological data from first and second intermediate hosts as well as final hosts is a necessary and important step on the way to the elucidation of life cycles and the improvement of our understanding of food webs and ecosystems. Similarly important is the establishment of large-scale databases and comprehensive reference libraries to apply molecular data effectively and reliably in biodiversity monitoring approaches (Georgieva et al. 2013). Furthermore, it is certainly interesting to consider and explore further methods, such as the use of environmental DNA (eDNA) (Ficetola et al. 2008; Bálint et al. 2018) to test their applicability to the detection and identification of parasites in ecosystems or the use of genome-wide single nucleotide polymorphism data (Weiss et al. 2018) to detect cryptic

species more reliably. Especially the application of eDNA seems to be a non-invasive way of biodiversity assessment, since it is not necessary to remove and dissect organisms (hosts) from their habitats, which is especially important when dealing with rare or endangered species. As freshwater research is extensively concerned with the evaluation and implementation of restoration measures, parasites could certainly serve as additional indicators for measuring restoration success, which was already successfully demonstrated by studies from other systems (Huspeni and Lafferty 2004; Bass and Weis 2008). Parasites could therefore also be a valuable tool in freshwater systems, which should be explored in detail.

Conclusion

In conclusion, the characteristics of digenean trematodes addressed in this dissertation give a thorough and comprehensive overview of these parasites at various levels of diversity, from individuals to communities. Trematodes contribute in a variety of ways to the biotic communities, the energy flow, and the structure of the ecosystems in which they occur. The advancing biodiversity crisis will not only lead to a drastic reduction in the diversity of charismatic megafauna, but it will also cause the loss of its parasites and thereby partially unexplored integral and functional components of ecosystems. Although, the insights we get from this work are only snapshots, the findings open the doors leading to further aspects in parasite taxonomy and ecological parasitology and may prompt the rethinking of research approaches and frameworks of conservation measures. Despite the fact that we still have a long way to go before we obtain a more realistic picture of their vast diversity and their functional roles in ecosystems, we should strive to incorporate parasites in our concept of biodiversity. Therefore, the present study serves as a cornerstone for an essential step towards the inclusion of parasites in ecosystem research and effective biodiversity conservation measures at different levels. Only by fully understanding ecosystems and the multifunctional roles of ***all*** members, will we be able to successfully and sustainably safeguard them (Figure 6).

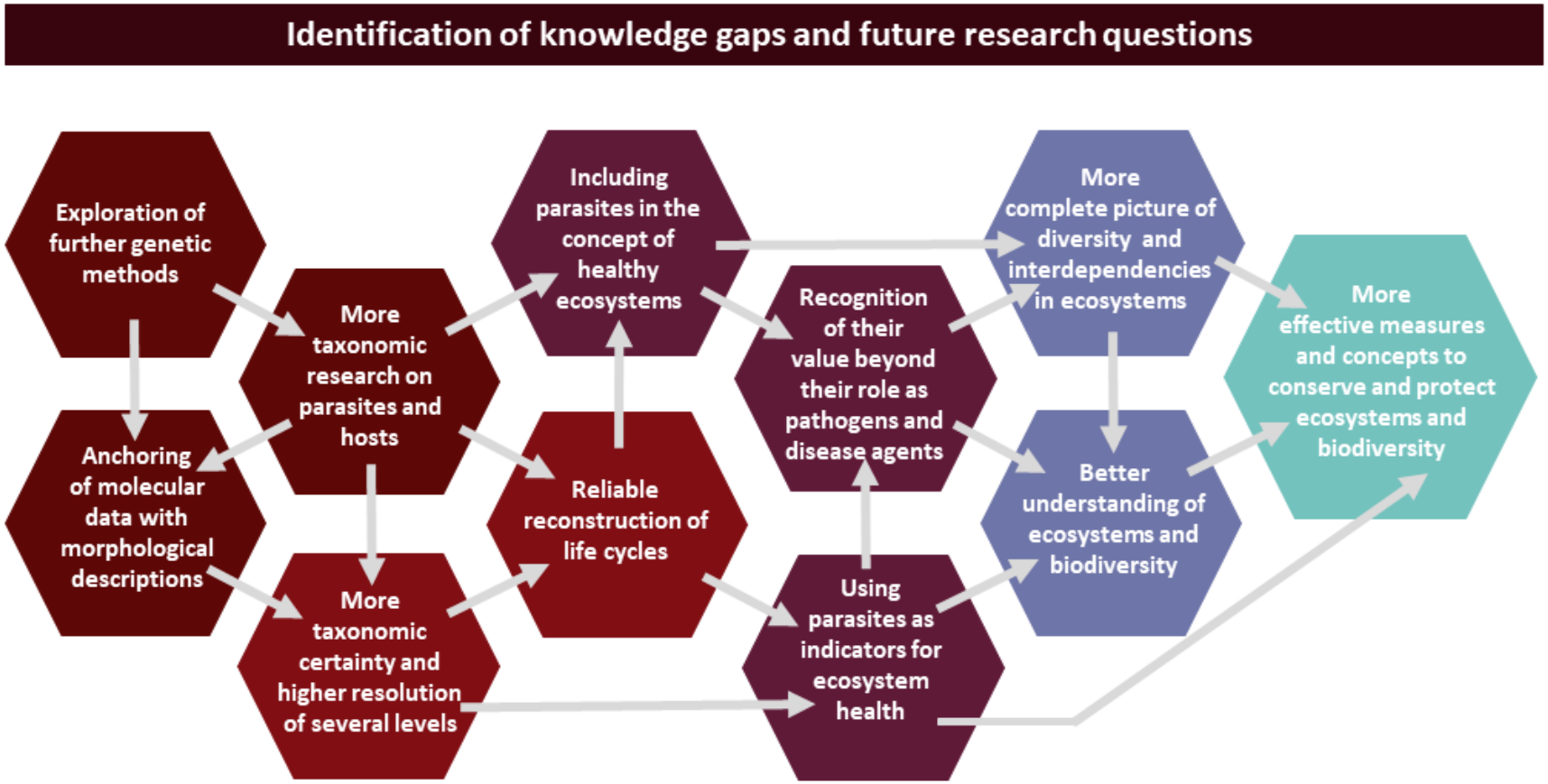


Figure 6 Steps and implications towards the inclusion of parasites in ecosystem research for a better understanding and more effective conservation measures of biodiversity. Parasites are integral and functional components of ecosystems that have coevolved with their hosts for a long time. In order to understand ecosystems, the complex interplay and the occurring interdependencies it is crucial to conduct more taxonomic research, explore new methodologies and combine molecular and morphological data, aiming at a higher taxonomic resolution and certainty. This will allow reliable reconstruction of parasite life cycles and the inclusion of parasites in the concept of healthy ecosystems, improving their reputation in the research community. More detailed information about life cycles will facilitate the use of parasites as indicators for ecosystem health, will lead to a more complete picture and enhance our general understanding of ecosystems, so that more effective conservation and protection measures can be implemented and conducted.

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This reference list includes all references used in the introduction, material and methods as well as in the general discussion. References of the individual articles (Chapters I – III) are provided at the end of the respective articles.

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9. List of Figures

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11. Abbrevations

28s rRNA	28S ribosomal ribonucleic acid
AIC	Akaike information criteria
ANCOVA	analysis of covariance
ANOSIM	analysis of similarities
AB	*Ampullaceana balthica*
AOL	anterior organ length
AOW	anterior organ width
AV	*Anisus vortex*
B0	sampling site Binnerfeld 0
B1	sampling site Binnerfeld 1
B2	sampling site Binnerfeld 2
B3	sampling site Binnerfeld 3
B4	sampling site Binnerfeld 4
BC	*Bathyomphalus contortus*
BI	Bayesian inference
BIC	Bayesian information criterion
BL	body length
BLAST	Basic Local Alignment Search Tool
BT	*Bithynia tentaculata*
BW	body width
C	cercaria
CB	Curonian Lagoon
*cox*1	cytochrome c oxidase subunit 1 gene
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
WFD	European Water Framework Directive
F	forward
FL	furca length
GLM	general linear model
HKY	Hasegawa-Kishino-Yano model
HL	hindbody length
HOL	holdfast organ length
HOW	holdfast organ width
ITS	internal transcribed spacer cluster
ITS1-5.8S-ITS2	see ITS
K0	sampling site Klostermersch 0
K1	sampling site Klostermersch 1
K2	sampling site Klostermersch 2
K3	sampling site Klostermersch 3
L.	Linnaeus
LS	*Lymnaea stagnalis*
M	metacercariae

ML	maximum likelihood
nad1	mitochondrial gene nicotinamide adenine dinucleotide dehydrogenase subunit 1
NJ	neighbour-joining
nt	nucleotide
p	prevalence
PA	*Physa acuta*
PHL	pharynx length
PHW	pharynx width
PC	*Planorbarius corneus*
PCR	polymerase chain reaction
PF	*Physa fontinalis*
PP	*Planorbis planorbis*
PSL	pseudosucker length
PSW	pseudosucker width
R	reverse
R1	sampling site Bienener Altrhein 1
R2	sampling site Bienener Altrhein 2
R3	sampling site Bienener Altrhein 3
S0	sampling site Schellenstein 0
S1	sampling site Schellenstein 1
S2	sampling site Schellenstein 2
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
Seq	sequencing
SP	*Stagnicola palustris*
sp.	species
spp.	species pluralis
TL	tail length
TSL	tail stem length
TSW	tail stem width
TW	tail width
VP	*Valvata piscinalis*
VSL	ventral sucker length
VSW	ventral sucker width

12. Appendices

This appendix contains the additional files to the following publications:

Chapter I[a]

Schwelm, J.*, Georgieva, S.*, Grabner, D., Kostadinova, A. and Sures, B. (2021). Molecular and morphological characterisation of *Diplostomum phoxini* (Faust, 1918) with a revised classification and an updated nomenclature of the species-level lineages of *Diplostomum* (Digenea: Diplostomidae) sequenced worldwide. *Parasitology* **148**, 1648–1664. doi: 10.1017/S0031182021001372.

Online Resource Figure S1. Phylogram from neighbour-joining (NJ) analysis of the *cox*1 sequence alignment for 36 representatives of the subfamily Amphipepleinae (Mollusca: Lymnaeidae)

Online Resource Table S2. Prevalence of *Diplostomum phoxini* in *Ampullaceana balthica* collected during 2016, 2017 and 2019 in the River Ruhr at Neheim (Germany)

Online Resource Table S3. Comparative metrical data for cercariae of species of the *Diplostomum baeri* species complex

Online Resource Table S4. Comparative metrical data for metacercariae of species of the *Diplostomum baeri* species complex

Chapter II

Schwelm, J., Kudlai, O., Smit, N., Selbach, C. and Sures, B. (2020). High parasite diversity in a neglected host: larval trematodes of *Bithynia tentaculata* in Central Europe. *Journal of Helminthology* **94**, doi: 10.1017/S0022149X19001093.

Supplementary Figure S1 Phylogenetic tree for the Psilostomidae based on the partial sequences of the 28S rRNA gene

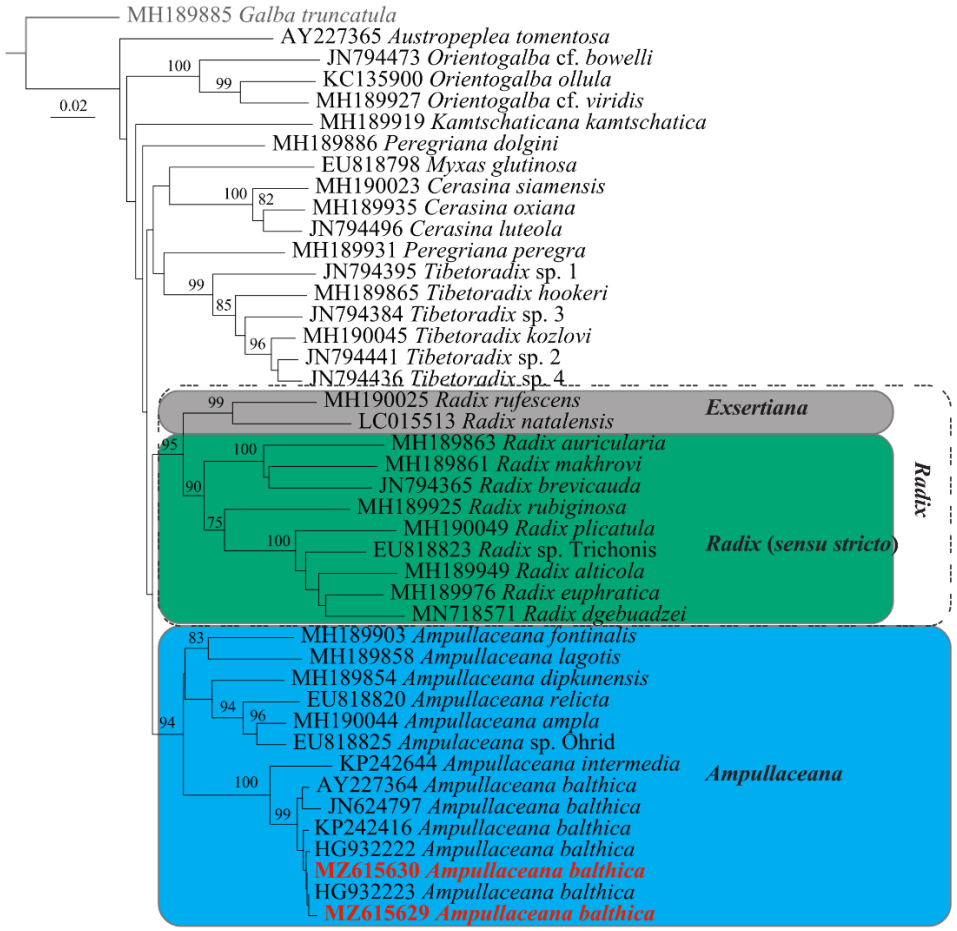
[a] The following additional files are not included in an analogue form in this thesis due to their size and/or format:

Online Resource Figure S2. Phylogram from neighbour-joining (NJ) analysis of the *cox*1 sequence alignment (1,203 sequences; 407 nt) for 44 species/species-level lineages of *Diplostomum*

Online Resource Table S1. Primers used for PCR amplification and sequencing

Online Resource Table S5. Summary data for cox1 sequences for *Diplostomum* spp. retrieved from GenBank

Online Resource Fig. S1 Phylogram from neighbour-joining (NJ) analysis of the *cox*1 sequence alignment (42 sequences; 636 nt) for 36 representatives of the subfamily Amphipepleinae (Mollusca: Lymnaeidae). Outgroup: *Galba truncatula*. Bootstrap support values ≥ 70 are provided above branches. The scale-bar indicates the expected number of substitutions per site



Online Resource Table S2 Prevalence of *Diplostomum phoxini* in *Ampullaceana balthica* collected during 2016, 2017 and 2019 in the River Ruhr at Neheim (Germany)

Site	**May (*n* = 255)**	**June (*n* = 250)**	**July (*n* = 266)**	**August (*n* = 250)**	**September (*n* = 264)**	**October (*n* = 131)**	**November (*n* = 104)**	**December (*n* = 79)**
B0 2016	0 (*n* = 20)	0 (*n* = 21)	0 (*n* = 2)	0 (*n* = 31)	0 (*n* = 28)	0 (*n* = 20)	**4.5 (*n* = 22)**	0 (*n* = 7)
B0 2017	0 (*n* = 28)	0 (*n* = 23)	0 (*n* = 27)	0 (*n* = 21)	**10.0 (*n* = 20)**	0 (*n* = 16)	–	–
B0 2019	0 (*n* = 30)	0 (*n* = 30)	0 (*n* = 30)	0 (*n* = 30)	0 (*n* = 30)	–	–	–
B1 2016	0 (*n* = 30)	0 (*n* = 25)	0 (*n* = 34)	0 (*n* = 40)	**5.7 (*n* = 35)**	**8.7 (*n* = 23)**	0 (*n* = 23)	0 (*n* = 24)
B1 2017	0 (*n* = 33)	0 (*n* = 40)	0 (*n* = 41)	0 (*n* = 31)	0 (*n* = 43)	0 (*n* = 31)	0 (*n* = 35)	0 (*n* = 31)
B1 2019	0 (*n* = 30)	0 (*n* = 30)	**6.7 (*n* = 30)**	**3.3 (*n* = 30)**	**3.3 (*n* = 30)**	–	–	–
B2 2016	0 (*n* = 20)	0 (*n* = 26)	0 (*n* = 33)	0 (*n* = 30)	0 (*n* = 26)	0 (*n* = 20)	0 (*n* = 20)	0 (*n* = 17)
B2 2017	0 (*n* = 40)	0 (*n* = 25)	0 (*n* = 39)	0 (*n* = 7)	**13.6 (*n* = 22)**	0 (*n* = 21)	0 (*n* = 4)	–
B2 2019	0 (*n* = 24)	0 (*n* = 30)	0 (*n* = 30)	0 (*n* = 30)	**3.3 (*n* = 30)**	–	–	–

Online Resource Table S3 Comparative metrical data for cercariae of the "*Diplostomum baeri*" species complex

Species	***Diplostomum phoxini* (Faust, 1918)**				***D. baeri* (Dubois, 1937)**	***Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014)**	
Snail host	***A. balthica***	***L. auricularia***	***L. peregra***	***L. peregra ovata***	***L. ovata, L. auricularia***	***R. peregra***	***R. peregra***
Source	**Present study**	**Arvy and Buttner (1954)**	**Rees (1957)**	**Dönges (1969)**	**Niewiadomska and Kiseliene (1994)**	**Faltýnková *et al.* (2014)**	
	Range (Mean)	**Mean**	**Mean**	**Range (Mean)[1]**	**Range (Mean)**	**Range (Mean)[2]**	**Range (Mean)[3]**
BL	138–154 (143)	125	125	151–178 (159)	185–236 (216)	156–265 (215)	168–214 (189)
BW	37–50 (41)	35	41	45–60 (53)	44–51 (50)	52–82 (68)	38–58 (48)
AOL	52–59 (54)	50	–	56–68 (62)	40–57 (50)	51–82 (62)	41–61 (51)
AOW	27–31 (28)	20	–	22–30 (24)	27–30 (29)	34–44 (39)	26–36 (32)
VSL	30–34 (32)	25	27	19–38 (28)	20–27 (25)	25–33 (29)	22–31 (26)
VSW	30–33 (32)	25	27	19–38 (28)	23–30 (29)	26–37 (31)	22–34 (28)
PHL	11–14	12	–	–	–	10–16 (13)	16–10
PHW	12–16	12	–	–	–	11–15 (13)	
TSL	212–226 (215)	180	200	210–238 (224)	244–281 (261)	220–262 (243)	234–280 (252)
TSW	29–37 (33)	20	24	34–43 (36)	37	31–54 (44)	34–48 (41)
FL	212–239 (226)	150	175	196–226 (210)	251–273 (259)	216–277 (241)	200–259 (233)
AOW/VSW	0.84–0.94 (0.90)	0.80[d]	–	(0.86)[4]	(1.00)[4]	1.16–1.40 (1.26)	1.00–1.30 (1.14)
TSL/BL	1.38–1.69 (1.53)	1.44[d]	1.60[d]	(1.41)[4]	(1.21)[4]	0.91–1.02 (0.96)	1.20–1.43 (1.35)
TSL/FL	0.95–1.03 (0.95)	1.20[d]	1.14[d]	(1.07)[4]	(1.01)[4]	0.91–1.02 (0.96)	0.98–1.19 (1.08)

[1] Heat-killed cercariae
[2] Live cercariae
[3] Fixed cercariae
[4] Calculated from the mean values
Note: Host names as in the original articles

Online Resource Table S4. Comparative metrical data for metacercariae of species of the *Diplostomum baeri* species complex

Species	***D. phoxini* (Faust, 1918)**			***Diplostomum* sp. Lineage 3 of Blasco-Costa *et al.* (2014)**	***Diplostomum* sp. Lineage 4 of Blasco-Costa *et al.* (2014)**	***Diplostomum* sp. Lineage 5 of Blasco-Costa *et al.* (2014)**
Host	***P. phoxinus* (L.)**			***Salmo trutta fario* L., *Salvelinus alpinus* (L.)**	***Gasterosteus aculeatus* L.**	***Salmo trutta fario* L., *Salvelinus alpinus* (L.)**
Source	**Present study**	**Rees (1955)**	**Lebedeva *et al.* (2021)**	**Faltýnková *et al.* (2014)**	**Faltýnková *et al.* (2014)**	**Faltýnková *et al.* (2014)**
Feature	**Range (Mean)[a]**	**Mean[a]**	**Range[b]**	**Range (Mean)[a]**	**Range (Mean)[a]**	**Range (Mean)[a]**
BL	326–411 (358)	344	215–325	447–601 (542)	384–468 (417)	363–501 (450)
BW	145–227 (186)	130	130–190	302–441 (376)	210–309 (252)	215–321 (283)
HL	24–53 (42)	–	–	30–68 (44)	36–66 (49)	Not developed
PSL	28–42 (34)	–	25–40	89–118 (102)	60–67 (64)	49–66 (57)
PSW	20–40 (30)	–	–	32–49 (42)	26–41 (34)	28–35 (31)
OSL	38–56 (49)	46	35–50	51–67 (58)	34–54 (46)	36–45 (41)
OSW	36–50 (44)	41	25–50	46–66 (57)	42–48 (46)	40–61 (50)
PHL	18–39 (31)	–	20–40	29–55 (45)	30–35 (32)	30–37 (33)
PHW	12–22 (18)	–	10–20	20–40 (31)	23–39 (29)	29–40 (33)
VSL	34–48 (43)	43	30–40	41–68 (58)	32–50 (44)	45–61 (54)
VSW	43–54 (47)	50	30–50	68–72 (66)	40–60 (49)	51–64 (58)
HOL	47–95 (71)	–	45–70	128–195 (155)	93–114 (103)	92–146 (126)
HOW	78–102 (91)	–	50–80	162–238 (204)	99–131 (112)	164–188 (181)
No. of excretory concretions	345–579 (454)	–	678–796	450–600	400–450	450–500

[a] Live metacercariae.

[b] Fixed metacercariae.

Abbreviations: BL, body length; BW, body width; HL, hindbody length; PSL, pseudosucker length; PSW, pseudosucker width; OSL, oral sucker length; OSW, oral sucker width; PHL, pharynx length; PHW, pharynx width; VSL, ventral sucker length; VSW, ventral sucker width; HOL, holdfast organ length; HOW, holdfast organ width

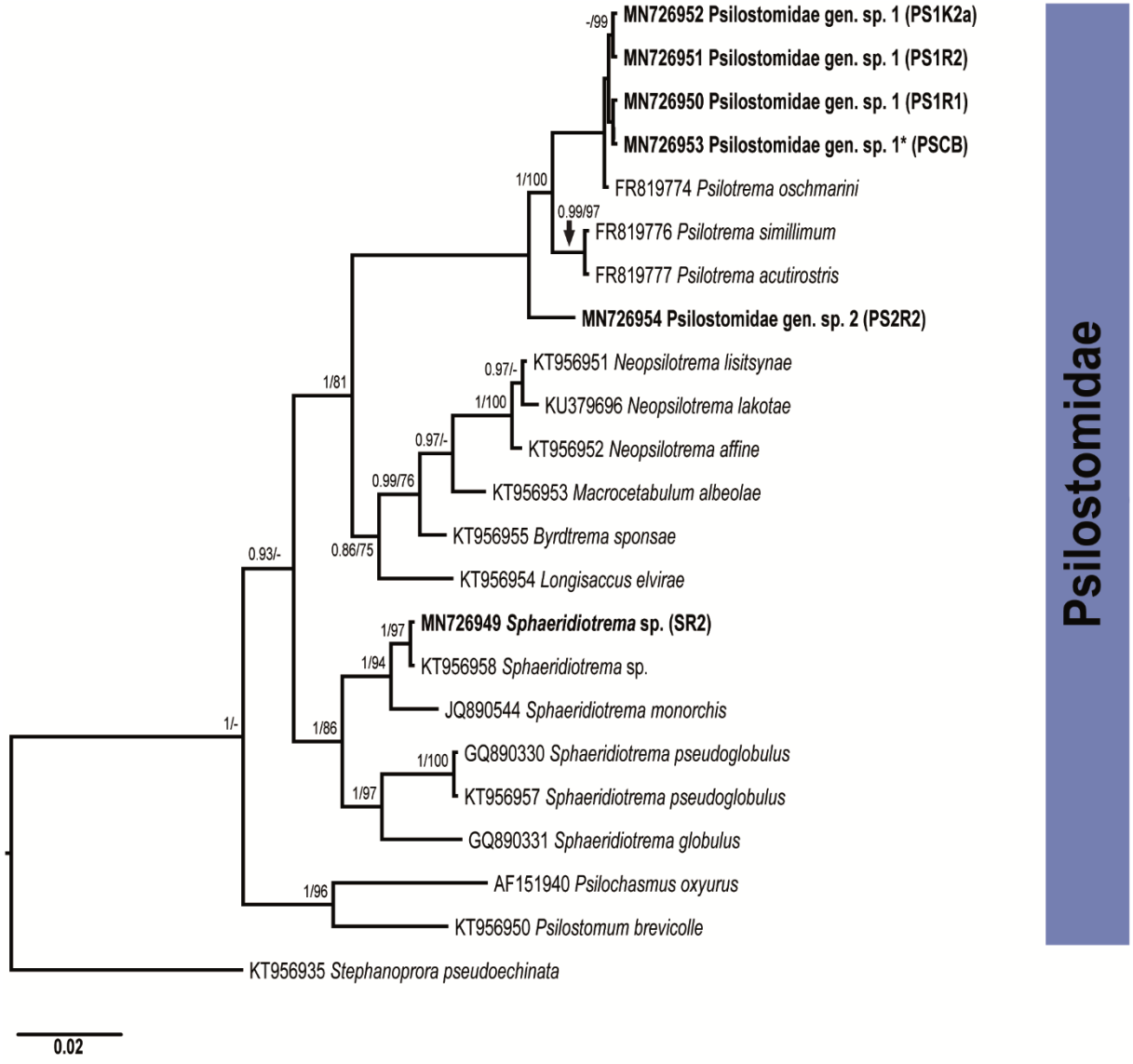
Journal of Helminthology

High parasite diversity in a neglected host: Larval trematodes of *Bithynia tentaculata* in Central Europe

Jessica Schwelm, Olena Kudlai, Nico Smit, Christian Selbach, Bernd Sures

Supplementary Figure S1.

Phylogenetic tree for the Psilostomidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI) followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.



Supplementary Table S1. Summary data for the taxa included in molecular analyses

Taxon	Life-cycle stage[a]	Host	Country	GenBank No. 28S	GenBank No. ITS2	Source
Cyathocotylidae Mühling, 1898						
Cyathocotyle prussica	M	*Gasterosteus aculeatus*	Germany	MH521249	−	Locke et al. (2018)
Mesostephanus microbursa	A	*Sula nebouxii*	Mexico	MF398326	−	Hernández-Mena et al. (2017)
Cyathocotylidae sp.	C	*Clypeomorus batillariaeformis*	Australia	MH257776	−	Huston et al. (2018)
Echinochasmidae Odhner, 1910						
Echinochasmus beleocephalus	A	*Ardea alba*	Ukraine	KT956929	−	Tkach et al. (2016)
Echinochasmus bursicola	A	*Ardea alba*	Ukraine	KT956938	−	Tkach et al. (2016)
Echinochasmus coaxatus	A	*Podiceps nigricollis*	Ukraine	KT956928	−	Tkach et al. (2016)
Echinochasmus donaldsoni	A	*Podilymbus podiceps*	USA	KT956930	−	Tkach et al. (2016)
Echinochasmus japonicus	A	*Gallus gallus*	Vietnam	JQ890579	−	Besprozvannykh et al. (2013)
Echinochasmus milvi	−	not specified	Russia	KT873319	−	Besprozvannykh et al. (2017)
Echinochasmus mordax	A	*Podiceps nigricollis*	Ukraine	KT956931	−	Tkach et al. (2016)
Echinochasmus sp.	R	*Lithoglyphus naticoides*	Lithuania	JQ088098	−	Stanevičiūtė et al. (2015)
Echinochasmus sp.	A	*Anhinga anhinga*	USA	KT956932	−	Tkach et al. (2016)
Microparyphium facetum	A	*Anhinga anhinga*	USA	KT956933	−	Tkach et al. (2016)
Stephanoprora chasanensis	A	*Gallus gallus domesticus*	Russia	KT873320	−	Besprozvannykh et al. (2017)
Stephanoprora pseudoechinata	A	*Chroicocephalus genei*	Ukraine	KT956935	−	Tkach et al. (2016)
Stephanoprora pseudoechinata	A	*Larus fuscus*	USA	KT956934	−	Tkach et al. (2016)
Stephanoprora sp. 1	A	*Gavia immer*	USA	KT956936	−	Tkach et al. (2016)
Stephanoprora sp. 2	A	*Aechmophorus occidentalis*	USA	KT956937	−	Tkach et al. (2016)
Opisthorchiidae Looss, 1899						
Amphimerus ovalis	A	*Trionyx muticus*	USA	AY116876	−	Olson et al. (2003)
Clonorchis sinensis	A	*Homo sapiens*	Vietnam	JF823989	−	Thaenkham et al. (unpublished)
Metorchis ussuriensis	A	"duck"	Russia	KY075777	−	Besprozvannykh et al. (2019)
Opisthorchis felineus	A	"cat"	Russia	MF099790	−	Dao et al. (2017)
Opisthorchis noverca	A	*Sus scrofa domestica*	India	KC295443	−	Tandon et al. (unpublished)
Opisthorchis viverrini	A	*Puntius brevis*	Thailand	HM004188	−	Thaenkham et al. (unpublished)
Opisthorchis sp.	A	"duck"	Vietnam	MF110001	−	Dao et al. (2017)
Notoctylidae Lühe, 1909						
Catatropis indicus	A	*Cairina moschata*	Australia	AY222220	−	Olson et al. (2003)
Catotropis vietnamensis	A	"duck"	Vietnam	MH750019		Izrailskaia et al. (2019)
Ogmogaster antarctica	A	*Balaenoptera borealis*	Argentina	KM258675	−	Fraija-Fernández et al. (2015)
Notocotylus atlanticus	A	*Anas plathyrhynchos*	Russia	MH818008		Gonchar et al. (2019)
Notocotylus attenuatus	A	*Aythya ferina*	Ukraine	AF184259	−	Tkach et al. (2001)

Notocotylus intestinalis	A	*Gallus gallus*	Vietnam	JQ890559	–	Besprozvannykh et al. (2013)
Notocotylus magniovatus		"chicken"	Russia	MH750016		Izrailskaia et al. (2019)
Taxon	**Life-cycle stage[a]**	**Host**	**Country**	**GenBank No. 28S**	**GenBank No. ITS2**	**Source**
Notocotylus malhamensis	A	*Myodes glareolus; Myodes agrestis*	United Kingdom	JQ766939	–	Boyce et al. (2012)
Notocotylus sp.	S	*Lymnaea palustris*	United Kingdom	AY222219	–	Olson et al. (2003)
Notocotylus sp.	C	*Radix balthica*	Norway	KY513158	–	Soldánová et al. (2010)
Notocotylus sp.	C	*Physa gyrina*	USA	EU712725	–	Hanelt (2009)
Paramonostomum anatis	A	*Tringa erythropus*	Ukraine	AF184258	–	Tkach et al. (2001)
Pseudocatatropis dvoryadkini	A	"duck"	Russia	MH750022		Izrailskaia et al. (2019)
Quinqueserialis quinqueserialis	A	*Ondatra zibethicus*	USA	JQ670848	–	Detwiler et al. (2012)
Notocotylidae gen. sp. 1 NZ	C	*Austrolittorina antipodum*	New Zealand	KJ868210	–	O'Dwyer et al. (2014)
Notocotylidae gen. sp. 2 NZ	C	*Austrolittorina antipodum*	New Zealand	KJ868214	–	O'Dwyer et al. (2014)
Pleurogenidae Looss, 1899						
Brandesia turgida	A	*Rana lessonae*	Ukraine	AY220622	–	Tkach et al. (2003)
Candidotrema loossi	A	*Rana ridibunda*	Ukraine	AY220621	–	Tkach et al. (2003)
Cortrema magnicaudata	A	*Hirundo rustica*	Czech Republic	KJ700420	–	Kanarek et al. (2014)
Gyrabascus amphoraeformis	A	*Pipistrellus kuhli*	Ukraine	AF151924	–	Tkach et al. (2000)
Leyogonimus polyoon	A	*Fulica atra*	Poland	KY752116	–	Kanarek et al. (2017)
Macyella massanae	A	*Erithacus rubecula*	Czech Republic	KP682451	–	Kanarek et al. (2015)
Macyella postgonoporus	A	*Dendrocopus major*	Czech Republic	KY752115	–	Kanarek et al. (2017)
Parabascus duboisi	A	*Myotis daubentoni*	Ukraine	AY220618	–	Tkach et al. (2003)
Parabascus joannae	A	*Myotis daubentoni*	Ukraine	AY220619	–	Tkach et al. (2003)
Parabascus semisquamosus	A	*Pipistrellus kuhli*	Ukraine	AF151923	–	Tkach et al. (2003)
Pleurogenes claviger	A	*Rana temporaria*	Ukraine	AF151925	–	Tkach et al. (2000)
Pleurogenes sp.	–	*Limnonectes bannaensis*	China	MK342571	–	Li & Fan et al. (unpublished)
Pleurogenoides medians	A	*Rana lessonae*	Ukraine	AF433670	–	Tkach et al. (2001)
Prosotocus confusus	A	*Rana lessonae*	Ukraine	AY220623	–	Tkach et al. (2003)
Prosthogonimidae Lühe, 1909						
Prosthogonimus cuneatus	A	*Sturnus vulgaris*	Ukraine	AY220634	–	Tkach et al. (2003)
Prosthogonimus cuneatus	A	*Anas platyrhynchos*	Vietnam	–	MG910996	Huynh et al. (unpublished)
Prosthogonimus cuneatus	A	*Anas platyrhynchos*	Czech Republic	–	KP192725	Heneberg et al. (2015)
Prosthogonimus ovatus	A	*Pica pica*	Ukraine	AF151928	–	Tkach et al. (2000)
Prosthogonimus pellucidus	A	*Anas platyrhynchos*	Czech Republic	–	KP192732	Heneberg et al. (2015)
Prosthogonimus ovatus	A	*Aythya ferina*	Czech Republic	–	KP192722	Heneberg et al. (2015)
Schistogonimus rarus	A	*Anas clypeata*	Czech Republic	–	KP192724	Heneberg et al. (2015)
Schistogonimus rarus	A	*Anas querquedula*	Ukraine	AY116869	–	Olson et al. (2003)

Psilostomidae Looss, 1900						
Byrdtrema sponasae	A	*Aix sponsa*	USA	KT956955	−	Tkach et al. (2016)
Longisaccus elvirae	A	*Aix sponsa*	USA	KT956954	−	Tkach et al. (2016)
Macroacetabulum albeolae	A	*Bucephala albeola*	USA	KT956953	−	Tkach et al. (2016)
Taxon	**Life-cycle stage[a]**	**Host**	**Country**	**GenBank No. 28S**	**GenBank No. ITS2**	**Source**
Neopsilotrema affine	A	*Aythya affinis*	USA	KT956952	−	Tkach et al. (2016)
Neopsilotrema lakotae	A	*Aythya americana*	USA	KU379696	−	Kudlai et al. (2016)
Neopsilotrema lisitsynae	A	*Anas crecca*	Ukraine	KT956951	−	Tkach et al. (2016)
Psilochasmus oxyurus	A	*Anas platyrhynchos*	Ukraine	AF151940	−	Tkach et al. (2000)
Psilostomum brevicolle	A	*Haematopus ostralegus*	Ukraine	KT956950	−	Tkach et al. (2016)
Psilotrema acutirostris	A	"chikens"	Russia	FR819777	−	Atopkin (2011)
Psilotrema oschmarini	A	"chikens"	Russia	FR819774	−	Atopkin (2011)
Psilotrema simillimum	A	"chikens"	Russia	FR819776	−	Atopkin (2011)
Sphaeridiotrema globulus	A	"duck"	USA	GQ890331	−	Bergmame et al. (2011)
Sphaeridiotrema monorchis	A	*Gallus gallus*	Vietnam	JQ890544	−	Besprozvannykh et al. (2013)
Sphaeridiotrema pseudoglobulus	A	*Anas platyrhynchos domesticus*	Canada	GQ890330	−	Bergmame et al. (2011)
Sphaeridiotrema pseudoglobulus	A	*Aythya affinis*	USA	KT956957	−	Tkach et al. (2016)
Sphaeridiotrema sp.	C	*Bithynia tentaculata*	Lithuania	KT956958	−	Tkach et al. (2016)
Outgroup						
Acanthoparyphium spinulosum	A	*Pluvialis squatarola*	Ukraine	KT956939	−	Tkach et al. (2016)
Apophallus_zalophi	A	*Callorhinus ursinus*	USA	MG806918	−	Kuzmina et al. (2018)
Himasthla limnodromi	A	*Limnodromus griseus*	USA	KT956943	−	Tkach et al. (2016)
Labicola cf. *elongata*	A	*Dugong dugong*	Australia	AY222221	−	Olson et al. (2003)
Levinseniella sp.	A	*Somateria mollissima v-nigrum*	Russia	MG783585	MG783580	Galaktionov & Blasco-Costa (2018)
Microphallus minutus	C	*Posticobia brazieri*	Australia	−	KT355829	Kudlai et al. (2015)
Microphallus similis	M	*Carcinus maenas*	Northen Ireland	AY220625	−	Tkach et al. (2003)
Opisthotrema dujonis	A	*Dugong dugong*	Australia	AY222223	−	Olson et al. (2003)
Posthodiplostomum brevicaudatum	M	*Perca fluviatilis*	Czech Republic	KX931426	−	Stoyanov et al. (2017)
Taprobanella bicaudata	A	*Dugong dugong*	Australia	AY222217	−	Olson et al. (2003)

[a] Life-cycle stages: A, adult; C, cercaria; M, Metacercariae; R, redia; S, sporocyst, −: not specified

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13. Contributions

Cumulative thesis of Mrs Jessica Schwelm

Schwelm, J.*, Georgieva, S.*, Grabner, D., Kostadinova, A. and Sures, B. (2021). Molecular and morphological characterisation of *Diplostomum phoxini* (Faust, 1918) with a revised classification and an updated nomenclature of the species-level lineages of *Diplostomum* (Digenea: Diplostomidae) sequenced worldwide. *Parasitology* **148**, 1648–1664. doi: 10.1017/S0031182021001372.

***** shared first authorship

Personal contributions: Fieldwork (90%), species identification (50%), drafting (50%), revision (50%) and submission (100%) of the manuscript.

Schwelm, J., Kudlai, O., Smit, N., Selbach, C. and Sures, B. (2020). High parasite diversity in a neglected host: larval trematodes of *Bithynia tentaculata* in Central Europe. *Journal of Helminthology* **94**, doi: 10.1017/S0022149X19001093.

Personal contributions: Fieldwork (90%), species identification (80%), data processing (80%), phylogenetic analysis (20%), drafting (70%), revision (70%) and submission (100%) of the manuscript.

Schwelm, J.*, Selbach, C.*, Kremers, J. and Sures, B. (2021). Rare inventory of trematode diversity in a protected natural reserve. *Scientific Reports* 1–13. doi: 10.1038/s41598-021-01457-2.

Personal contributions: Fieldwork (90%), species identification (80%), (statistical) analysis (100%), drafting (50%), revision (60%) and submission (100%) of the manuscript.

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