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The prevalence of *Fascioloides magna* in *Galba* spp. and *Radix* spp. in the Bavarian and Sumava National Parks

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DECLARATION

I hereby declare that I have authored this thesis independently, that I have not used other than the declared sources / resources and that I have explicitly marked all material which have been quoted either literally or by content from the used sources.

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ABSTRACT IN ENGLISH

Fascioloides magna is a liver fluke that infects game animals and livestock alike, and depends on freshwater snails as intermediate hosts to complete its life cycle. It is native to North America and was introduced to Europe twice, resulting in several enzootic areas with various haplotypes in Italy and the Czech Republic. Since then, the fluke has spread into more countries and is expected to spread further. To investigate the current situation and to determine the prevalence of *F. magna* in the Czech Republic and parts of Germany, 1,847 snails were collected in the Bayerischer Wald and Sumava National Parks over the course of four months. The samples were examined by light microscopy, dissected under the microscope, and subjected to DNA extraction. All samples were screened by targeted PCR using *F. magna* specific primers Nad1 and Cox1. Additionally, trematode primers TremF/R were used to verify the presence of other parasites, such as the human pathogen *Echinostoma revolutum*, in the area. The obtained amplicons were purified for sequencing, and DNA sequences were obtained from both strands by Sanger sequencing. All sequence data were compared to reference sequences from GenBank to determine their origin. Out of all samples, one *Radix labiata* tested positive for *F. magna* resulting in a prevalence of 0.056% in the area of both national parks. Not only does this verify the presence of the fluke in the area, but it also provides new evidence that *R. labiata* is a naturally occurring intermediate host for it. Sequence comparison showed a similarity of 438/439 base pairs to Cox1 haplotype 5 already previously found in the Czech Republic, indicating that the fluke obtained in this study belongs to an established population.

ABSTRACT IN GERMAN

Fascioloides magna ist ein Leberegel, der sowohl Wild- als auch Nutztiere befällt und dabei große Schäden verursachen kann. Er ist auf Süßwasserschnecken als Zwischenwirte angewiesen. Nur so kann er den vollständigen Lebenszyklus durchlaufen. Ursprünglich aus Nordamerika stammend, wurde der Egel mindestens zwei Mal nach Europa eingeschleppt und inzwischen bereits in mehreren Ländern nachgewiesen, darunter auch Italien, Tschechien und Österreich. Das Ziel dieser Arbeit war es, die aktuelle Situation in Tschechien und Deutschland zu erforschen und die dortige Prävalenz von *F. magna* zu ermitteln. Dafür wurden im Laufe von vier Monaten 1847 Schnecken in den Nationalparks Bayerischer Wald und Sumava gesammelt. Die Schnecken wurden seziert, mikroskopisch untersucht, und anschließend wurden Proben für die molekularbiologische Analyse entnommen. Diese wurden der DNA-Extraktion unterzogen, bevor sie mit den *F. magna* spezifischen Primern Cox1 und Nad1 bearbeitet wurden. Die DNA-Fragmente wurden anschließend aufgereinigt. Sequenzen beider Stränge wurden mittels Sanger Sequenzierung erworben. Schließlich wurde die erhaltene Sequenz mit Referenzsequenzen aus der GenBank verglichen, um den Ursprung des identifizierten Egels zu erfahren. Es zeigte sich, dass von allen Proben eine *Radix labiata* positiv auf *F. magna* getestet wurde, was eine Prävalenz von 0,056% bedeutet. Dies bestätigt nicht nur die Anwesenheit des Egels in der Region, sondern liefert einen neuen Beweis, dass die Schneckenspezies als natürlich auftretender Zwischenwirt fungieren kann. Der Vergleich der erworbenen Sequenz mit den Referenzsequenzen zeigte eine Ähnlichkeit von 438/439 Basenpaaren mit dem Cox1 Haplotypen 5 aus Tschechien. Dies deutet darauf hin, dass der identifizierte Leberegel einer bereits in Tschechien etablierten Population angehört und nicht neu eingeschleppt wurde. Zusätzlich verifizierten Untersuchungen mit den Trematoden TremF/R Primern die Anwesenheit weiterer Parasiten, darunter auch *Echinostoma revolutum*.

1. INTRODUCTION

Liver flukes are parasitic worms that occur worldwide and infest the digestive systems of various mammals, including humans. They depend on two or even three hosts to complete their life cycle and go through several stages until reaching the adult form and producing eggs. Once in the environment, the miracidia hatch and infect the intermediate host, mostly freshwater snails, that then release the cercariae. These encyst in the environment and can be transmitted by ingesting raw or undercooked meat or contaminated plants, respectively, depending on species. An infection typically causes abdominal pain, nausea and fever. However, if left untreated, liver flukes can cause serious organ damages, and in some cases, can even lead to death.

Fascioloides magna depends on freshwater snails of the family Lymnaeidae as intermediate hosts to complete its development and only infects animals such as wild deer, sheep, and goats. No cases in humans have been reported to date. It is native to North America and was first introduced to Europe in the 19th century. Since then, it has not only dispersed to several countries, but also adapted to the native animals infecting wildlife as well as farm animals causing substantial economic loss.

1.1. Liver flukes

1.1.1. History

One of the oldest pieces of evidence of liver flukes dates back to around 3,000 BC, and is from Germany where *Fasciola hepatica* eggs were found in human remains as well as bovine skeletal remains (Dittmar and Teegen, 2003). Mas-Coma et al. (2022) reported the presence of *Fasciola gigantica* in the liver of Egyptian mummies, and further state that parasitic infections with both species were common in Roman times and the Middle Ages. The earliest finding of *F. hepatica* eggs in fossilized feces, so-called coprolites, on the Asian continent dates back to around 300 AD in the Zanzan province in Iran (Askari et al., 2018), highlighting the early distribution of liver flukes across several continents. The first written record of the flukes is a manuscript on sheep breeding from 1379 by Jehan de Brie (1879) in France. Although not originally preserved, copies of the manuscript were later printed showing not only the description of symptoms, but also an understanding of the cause of the disease. Centuries later, in 1547, Gabucinus Hieronymus was the first to describe the morphology of the flukes. Outbreaks of infections in livestock in the Netherlands occurred in 1552 (Gemma Cornelius, 1575 in Mas-Coma et al., 2022) and today's Bavaria between 1663 and 1665 (Frommann, 1676 in Mas-Coma et al., 2022). The first finding of *F. hepatica* in the biliary canals of a human was made by Pierre Borel (Mas-Coma et al., 2022) and described in 1715 by Le Clerc followed by the first ever reported infection in a human in Berlin in 1760 (Pallas, 1760 in Mas-Coma et al., 2022). In the 19th century came the breakthrough: German zoologist Leuckart (1881) and British scientist Thomas (1881) simultaneously described the life cycle of *F. hepatica* in 1881 (Mas-Coma et al., 2022) following the death of approximately 3 million sheep in England in 1879 and 1880 (Thomas, 1883). A complete understanding of the life cycle was achieved in 1914. First, Adolpho Lutz discovered that metacercariae lead to infections after feeding them to animals such as goats and rabbits in 1892. This insight was followed by the last missing piece: When working with rabbits, Russian scientist Sinitsin (1914) discovered the migration pattern of the flukes starting in the small intestines and ending in the liver, completing their life cycle (Andrews et al., 2021). In the following years, intensified research on liver flukes was started worldwide. The focus, however, was mainly on *F. hepatica* (Mas-Coma et al., 2022).

As for *Fascioloides magna*, there is not a lot of information available. First described by Bassi in 1875, the species was renamed many times starting with *Distomum magnum*. The species name *F. magna* was introduced by Ward in 1917. The fluke originated from North America and is now found in parts of Europe like the Czech Republic, Slovakia, Austria, and Italy, as well as in North America and Canada (Malcicka, 2015).

1.1.2. Classification

Liver flukes, lung, and intestinal flukes belong to the phylum of Platyhelminthes and the sub-phylum of Neodermata (Figure 1). They are representatives of the Trematoda, a class of 6,000 species known to date (Keiser and Utzinger, 2009). Some of their prominent species are the human pathogens *F. hepatica*, *F. gigantica* and *Echinostoma revolutum*.

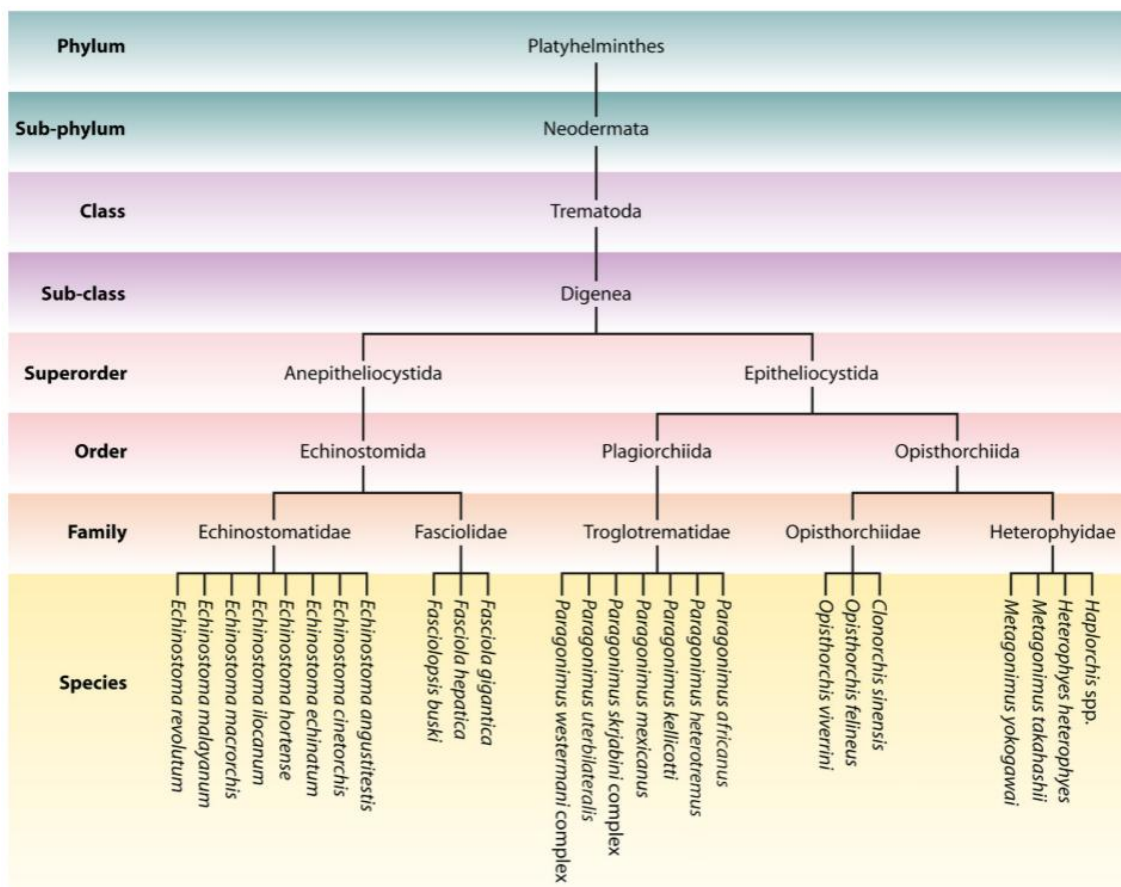


Figure 1. Classification of Trematoda highlighting the different families and species belonging to it. Reproduced with permission from Keiser and Utzinger (2009).

1.1.3. Morphology

The class of Trematoda consists of endoparasites that permanently reside in the internal organs of their host (Gibson et al., 2002). Their bodies are elongated and covered with a tegument that wraps around their entire body and protects them from the immune system of the host. It also helps with the nutrient uptake and secretion (Keiser and Utzinger, 2009). This tegument can be covered in spikes. Trematodes are equipped with typically two suckers for attachment, one in the anterior and one in the posterior part of their body (Gibson et al., 2002), which help to navigate through their hosts (Malcicka, 2015). Most flukes are hermaphroditic, meaning they have both female and male reproductive organs, usually one ovary and two testes that lead into one shared pore. Their caeca are mostly branched and end blindly or lead to other cavities. A pharynx is usually present (Gibson et al., 2002), and they have a one-way gut (Malcicka, 2015).

The family of Fasciolidae shows all of these characteristics. Their bodies are usually flat and can vary in size. They are known for their ‘shoulders’, the part where their bodies grow in width, and they usually have two suckers, the oral slightly smaller than the ventral. They are typically covered in a tegument with spikes, have a pharynx and their caeca vary between simple to branched and end blindly. They are hermaphroditic with usually one ovary and two testes that lead to one shared exit pore (Jones, 2005). These flukes are mostly brown to grey and reach sizes of a couple of centimeters (e.g. five cm for *F. hepatica* and over seven cm for *F. gigantica*) (Schuster, 2002).

F. magna, being a bigger fluke than both human pathogens, reaches a size of up to 10 cm (Figure 2). It has a bilaterally symmetrical body in an oval, leaf-like shape (Schuster, 2002). It has two suckers and is hermaphroditic with symmetrical testes and one ovary on the right side of the body (Jones, 2005). Its eggs are thick-walled and have a coloration from yellow to brown. While a variation in size appears to be normal (Campbell, 1961), Oberhauserová et al. (2010) mention an average length of 150 µm and a width of around 80 µm. Each egg has an operculum, a thicker structure at one pole. When the development is finished and the miracidia are ready to hatch, they release a proteolytic enzyme that loosens the connection between said operculum and the shell which in turn results in hatching. The freshly released

miracidia are cylindric. The anterior end is rounded and harbors the eye-spots, while the posterior end is pointed. The latter is where the reproductive organs are located. The miracidia are covered in cilia that help them move in the water, facilitating the search for their intermediate host (Campbell, 1961).



Figure 2. An adult *F. magna*. Reproduced from Malcicka (2015).

1.1.4. Life cycles

1.1.4.1. Life cycle of *Fasciola hepatica*

The life cycle of *F. hepatica* starts in the common bile duct of its definitive host. There, the flukes live permanently (Dalton, 2021), producing up to 50,000 eggs per day (McManus and Dalton, 2006) that exit the host via feces. Once in the environment, the embryonation occurs. The miracidia hatch and swim through the water in search of their intermediate host, a freshwater snail, that they penetrate via drilling motions. Once inside the host, the miracidia travel through lymph and blood vessels until they reach the digestive gland. They develop into sporocysts and finally into rediae that produce cercariae, which then encyst in the environment (e.g. on grass) and develop into metacercariae. As soon as they are ingested, they excyst in the small intestine. The flukes then penetrate the wall of the intestine migrating into the liver where they grow in size. In the last step, they migrate into the bile duct, where they stay permanently to produce eggs (Dalton, 2021).

1.1.4.2. Life cycle of *Fascioloides magna*

The life cycles of *F. magna* and *F. hepatica* are mostly similar with some minor differences. While *F. hepatica* flukes are located in the bile duct (Dalton, 2021), the adult parasites of *F. magna* live either in pairs or groups encapsulated in pseudocysts in the liver of the definitive host. There, they produce up to 4,000 eggs per day (Malcicka, 2015) (stage 1 in Figure 3). The eggs migrate into the small

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intestine and exit the body through the feces (Halász et al., 2023) – ideally near a waterbody. It is important to note that the eggs will only hatch if the temperature is within a range of 20°C to 34°C. They turn into (ciliated) larvae, the so-called miracidia, a process that can last up to 7 weeks, depending on the environment and the temperature (stage 2). If temperature is too low or too high, the miracidia will not survive. The larvae then swim through the water looking for an intermediate host. In North America, that would be a snail of the genus *Lymnaea*, for example *Lymnaea parva*, while in Europe, it is mostly *Galba truncatula*. However, no matter the continent, the intermediate host is always an aquatic snail. The snails become infected by the larvae and the “asexual phase” begins (stage 3). The larvae migrate into the pulmonary sac, where they develop into sporocysts. These contain germ cells that are necessary for the production of rediae. The rediae however produce cercariae (stage 4) that then leave the host and form cysts on aquatic plants. These cysts are also known as metacercariae, are highly contagious and can infect a wide range of mammals when taken up orally (stage 5) (Malcicka, 2015). Upon ingestion, the metacercariae travel into the intestines where the flukes hatch. They then penetrate through the intestinal wall and migrate to the liver, where they form pseudocysts and start producing eggs (Kralova-Hromadova et al., 2016).

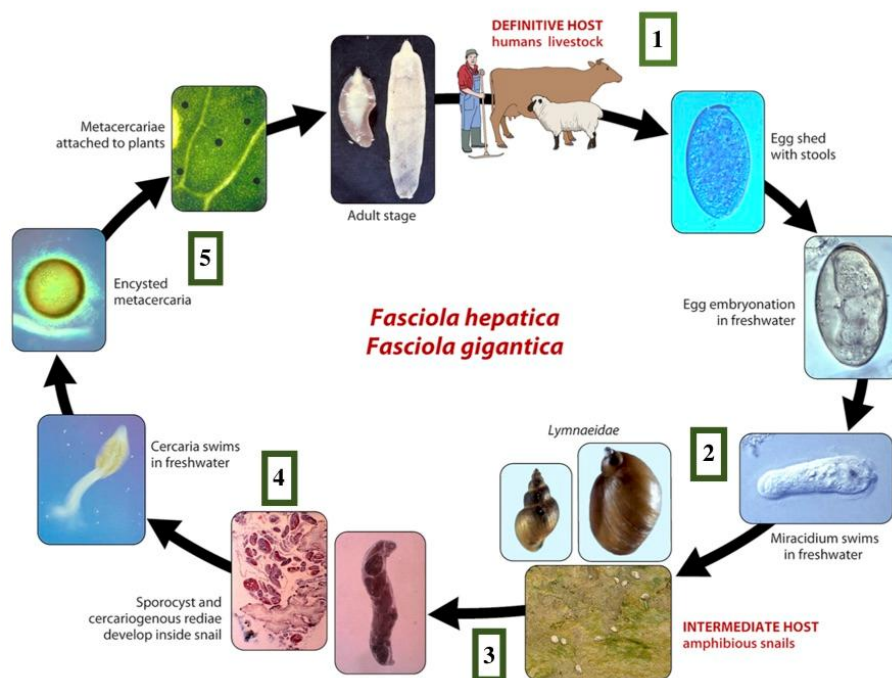


Figure 3. The life cycle of *Fasciola* spp. modified from Mas-Coma et al. (2022). The stages 1-5 highlight the main stages of the cycle: (1) production of eggs in the definitive host, (2) the shedding of eggs and evolution into miracidia in the environment, (3) infection of the intermediate host, (4) the evolution into cercariae inside the host and (5) the encapsulation in the environment.

1.1.5. Diseases in humans

Fasciolosis in humans is caused by two different liver flukes: *F. hepatica* and *F. gigantica*. Known as food/plant-borne disease, the transmission of the fluke occurs through the consumption of metacercariae encysted on plants. The disease is especially common in poorer communities living in close proximity to their livestock, facilitating the transmission of the parasite (Nyindo and Lukambagire, 2015). The infection itself has 2 stages, the (i) migratory and the (ii) chronic phase, and leads to a number of symptoms. During the first phase, the immature parasite enters the host's body and migrates to the ducts connecting small intestines and gallbladder, which can result in fever and abdominal pain. An infection at this stage can be diagnosed via imaging of the liver, as lesions might be visible. After that, the mature parasites start producing eggs initiating the chronic stage. This can last up to 10 years and can lead to more severe symptoms, and may also result in infections of the liver due to bacteria carried by the fluke. It is important to note that symptoms vary from patient to patient, and that asymptomatic cases are possible. Infections of other organs are also not uncommon (Caravedo and Cabada, 2020).

As the disease is typically non-fatal, it is widely neglected and is therefore classified as "neglected tropical disease" (NTD) by the WHO (Nyindo and Lukambagire, 2015). It is estimated that 17 million people are infested worldwide and 180 million are at risk of being infested (Cwiklinski et al., 2016).

1.1.6. Diseases in animals

F. magna is one of the causes of fascioloidosis in animals (Konjević et al., 2021), particularly in deer and livestock such as goats and sheep. Infections in the intermediate hosts, various freshwater snail species, generally show mild progression. Symptoms include growth retardation and castration with more severe infections resulting in death. Infections in the definitive hosts may cause a variety of symptoms: Definitive hosts such as red deer are generally more tolerant to infections. Severe cases can result in reduced quality of antlers and reduced weight gain, blood loss, ruptured livers, and in some cases, death. In dead-end hosts like sheep, goats, or horses, an infection mostly results in death as the immature parasite keeps migrating through the tissue of the liver, thereby destroying it significantly (Malcicka, 2015).

1.2. Intermediate host snails

The Lymnaeidae are a family of freshwater snails that are of great medical and veterinary importance as they act as intermediate hosts for various trematodes including liver flukes. Examples for pathogens are the Echinostomatidae (Class: Trematoda) such as *E. revolutum* and the Fascioloidae (Class: Trematoda) such as *F. magna* (Correa et al., 2010).

1.2.1. Morphology

Snails belonging to the family of Lymnaeidae are a prime example of phenotypic plasticity. As a result of the various environments they populate, populations have morphologically adapted to their specific environmental conditions (e.g. the strength of currents), thus resulting in a big diversity in shell morphology within one species. This creates a challenge to name shared external morphological characteristics (Correa et al., 2010), which becomes apparent in two rather similar species both found in Europe: While *G. truncatula* is rather small and reaches a shell height of 5-8 mm with a width of 4 mm, *Radix labiata*, tends to grow bigger. Their shell reaches a height of 12-20 mm and can have a width of 7-13 mm. Additionally, *G. truncatula* appears brown and rather opaque and consists of 7-8 swirls that are increasingly getting bigger. *R. labiata*, however, appears yellow-brown and the shell is thicker than the one of the *Galba* species. Another big difference is that the *Radix* shell only has 4.5-5 whorls and a significantly bigger opening with a white coloring, the so-called “white lip” (Yildirim et al., 2006).

1.2.2. Distribution

The Lymnaeidae can be found worldwide (Correa et al., 2010) with three genera being particularly important to this study: *Lymnaea*, *Galba* and *Radix*. The genus *Lymnaea* covers all 5 species (*Lymnaea modicella*, *Lymnaea caperata*, *Lymnaea bulimoides*, *Lymnaea parva*, *Lymnaea palustris nuttalliana*) that act as intermediate hosts for *F. magna* in its native habitat. The genus *Galba* includes *G. truncatula* and the genus *Radix* *R. labiata*, both species that are hosts to the liver fluke in Europe. Looking at their distribution, it becomes apparent that the number of host species in Northern America, the fluke’s native habitat, is larger than in Europe. However, it is suspected that with time, this will change and the fluke will become endemic in more areas in Europe and adapt to more new intermediate hosts (Malcicka, 2015).

1.3. *Fascioloides magna* as invasive species

1.3.1. In Europe

F. magna is a species native to North America and was introduced to Europe when interest for hunting grew, increasing the demand for game animals. As a result, animals carrying the fluke were imported into the continent, introducing it for the first time. Since then, the parasite has spread further (Králová-Hromadová et al., 2011), and is now established in various different regions in North America, Canada, and Europe (Figure 4), and is expected to spread even further (Malcicka, 2015).

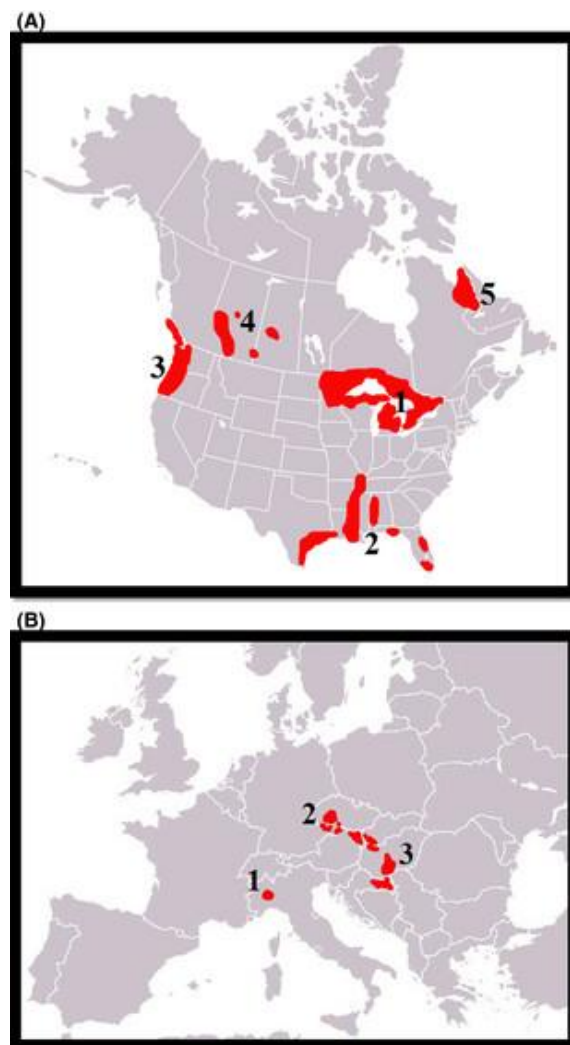


Figure 4. Distribution of *F. magna* in (A) North America and (B) Europe as depicted by Malcicka (2015). Numbers 1-5 highlight areas where the fluke is known to be enzootic.

The first reports of its presence in Europe date back to 1865, when the parasite was first found in Italy. There, fences acted as physical barriers for the infected animals, preventing the fluke from spreading into neighboring countries. The second introduction followed in the early 20th century and started in the Czech Republic. It

is debated if a third introduction occurred separately into the Danube area where the presence of the liver fluke was first reported in 1988 (Králová-Hromadová et al., 2011). However, scientific data indicates that these *F. magna* populations derived from pre-existing hot spots in Europe, most likely from the Czech Republic, contradicting the theory of a third introduction. This is also supported by Sattmann et al. (2014), who state that the Austrian population shows 100% identity to sequences of isolates obtained in the Czech Republic. Nowadays, the liver fluke can be found in more countries, including Croatia, Hungary, and Serbia (Konjević et al., 2021; Nagy et al., 2018; Trailović et al., 2016). Older findings were also reported from Spain and the Polish-German border. However, no recent data are available from these areas (Králová-Hromadová et al., 2011).

1.3.2. In the Bavarian Forest and Sumava National Parks

The Czech Republic is one of the most affected countries by *F. magna* in Europe. There, the first case of a liver fluke infestation was reported in 1910 in fallow deer (Ullrich, 1930 in Králová-Hromadová et al., 2011). In 1932 followed the first case of an infected deer ever reported in Germany (Salomon, 1932 in Novobilský et al., 2007). It is suspected to have originated from the Czech Republic. Since then, the fluke spread further up until the 1960s, when first established enzootic areas in Central and South Bohemia were reported, with a prevalence of up to 80% in local deer. A study in 2007 showed a slow expansion to the south-west of the country indicating a spread towards the German border and the Sumava National Park (Novobilský et al., 2007). This migration was supported by a study from 2012 that examined deer livers from endemic areas and 3,000 snails (1,100 *G. truncatula* and 1,800 *R. labiata*) from all around the country. Additional surveys on regular occurrences of the fluke were distributed to administrative units. The results confirmed the presence of *F. magna* in new areas in the Central, West, and North of the country, and even found its signs in the north-eastern part. All of this shows that the fluke is still adapting to its environment in the Czech Republic (Kasny et al., 2012), and due to the abundance of *G. truncatula* and *R. labiata*, the parasite is assumed to continue to spread further (Novobilský et al., 2007).

1.4. Molecular Phylogeny

In general, molecular phylogeny is a subdiscipline of evolutionary biology that uses sequence data to infer the evolutionary relationships between organisms (Yang and Rannala, 2012).

1.4.1. Genetic markers

Genetic markers are a subgroup of biological markers that can be used to study evolution and genetic diversity. Defined as short sequences of nucleotides, they are based on the DNA sequence and are not directly impacted by the environment which makes them stable and especially reliable. Differences in those sequences occur between species and sometimes populations and are known as polymorphisms, that enable the comparison of organisms (Panchariya et al., 2024). The two most frequently used markers in this study were introduced by Kralova-Hromadova et al. (2008): the cytochrome c oxidase subunit 1 (Cox1) and the nicotinamide dehydrogenase subunit 1 (Nad1). Both are subunits of the mitochondrial DNA and are therefore involved in the production of energy. Complete sequences of both genes were provided in 2008, and regions with the highest conservation were chosen as markers. While the primer pair Cox1F/Cox1R amplifies a 439 bp long fragment of the Cox1 gene (Kralova-Hromadova et al., 2008), the primer pair Nad1F/Nad1R amplifies fragments of a length of 405 bp (Husch et al., 2017). Nowadays, they are well established and used to identify new isolates of liver flukes and their origin. Additionally, they offer a way to compare different populations of *F. magna* to determine their differences (Kralova-Hromadova et al., 2008). LCO1490/COR722b is another but more universal primer pair that targets the cytochrome c oxidase subunit 1 gene. The reason for the frequent use of the gene as a marker is its highly conservative nature in most animals which makes it easier to compare different species and to assess their relationship. The primer pair results in fragments of a length of 658 bp (Folmer et al., 1994; Wilke and Davis, 2000) and can also be used for the identification of snail species. Another commonly used genetic marker is the 18S rDNA that is targeted by the primer pair TremF/TremR and amplifies a 430 bp long fragment (Haider et al., 2012). The marker can be used for the general detection of infections with trematodes.

1.4.2. *Fascioloides magna* haplotypes

Both, Cox1 and Nad1, were used by Králová-Hromadová et al. (2011) to compare *F. magna* sequences from different areas in Europe with those from the American continent to determine their exact origin. In the process, a total of 16 Cox1 haplotypes (CO1-Ha) and 18 Nad1 haplotypes (NA1-Ha) were identified, resulting in 22 concatenated haplotypes (Ha): 15 haplotypes in North America and Canada and 8 haplotypes in Europe showcasing the lower diversity of *F. magna* in Europe. Interestingly, out of those haplotypes, only one was detected on both continents. Haplotype 1 (Ha1) was identified in both Italy (GenBank number: GU599860) and on the American continent, in North America (GenBank number: GU599862) and Canada (GenBank number: GU599861) revealing the western American/ Canadian origin of the haplotype. Whereas haplotypes found in the Czech Republic and Germany are believed to have originated from the south-eastern parts of America. This is also visible in the phylogenetic tree (Figure 5) created by Králová-Hromadová et al. (2011) that highlights the two different groups within the haplotypes. It is also important to note that similarities between the Italian and the northern European populations are generally scarce, indicating different origins. Additionally, Králová-Hromadová et al. (2011) also compared sequences obtained for every haplotype and noted that polymorphism seems to be more common in sequences obtained using Cox1 instead of Nad1. However, 90% of these deviations did not result in changes in the sequence and were therefore trivial.

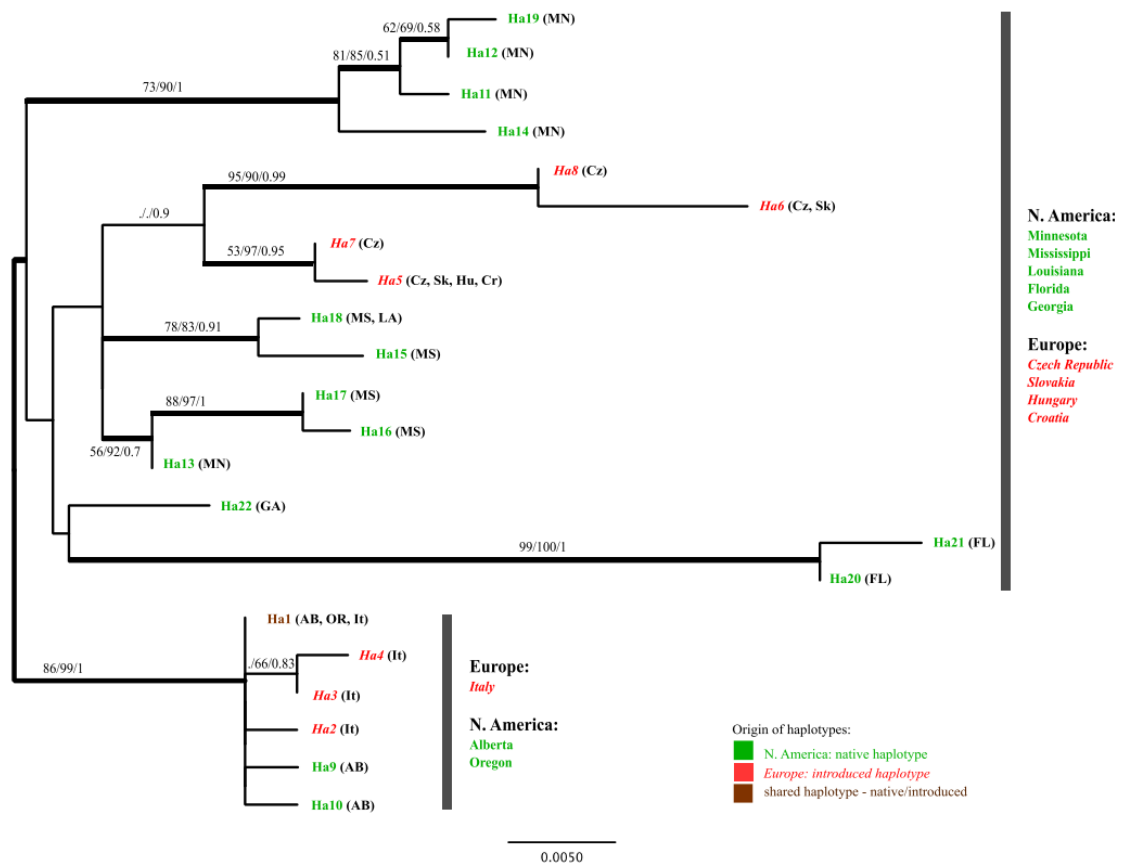


Figure 5. Phylogenetic tree of all 22 concatenated haplotypes of *F. magna* with information on their origin from Králová-Hromadová et al. (2011).

1.4.3. Distribution of haplotypes in Europe

Králová-Hromadová et al. (2011) determined that several Cox1 haplotypes (CO-Ha) can exist in close proximity and observed a maximum of four haplotypes per country. The Czech Republic has CO1-Ha3, CO1-Ha4, and CO1-Ha5, and is therefore the country with the highest number of haplotypes in Europe, followed by Slovakia with CO1-Ha3 and CO1-Ha4. CO1-Ha3 was additionally identified in both Hungary and Croatia. Since this list was updated, the liver fluke has been found in many more countries in Europe (Filip-Hutsch et al., 2022; Haider et al., 2012; Popovici et al., 2024). Information on haplotypes, however, remains scarce. One of the few studies performed on this topic was done by Sattmann et al. (2014) who focused on the *F. magna* populations in Austria. They examined adult flukes as well as rediae extracted from *G. trunctatula* and found sequences with a similarity of 439/439 base pairs to strains of CO1-Ha3 from the Czech Republic (GU599864), Slovakia (GU599865), Hungary (GU599866), and Croatia (GU599867) followed by a

similarity of 425/439 base pairs to CO1-Ha1 from Italy and Canada, supporting the idea of a spreading of the northern haplotypes into the Danube area. A theory that was further corroborated by Sindičić et al. (2022) who examined adult flukes extracted from the livers of red, fallow, and roe deer, as well as wild boar in Croatia. They confirmed the presence of CO1-Ha3 and CO1-Ha4 in the country. One year later, Sindičić et al. (2023) examined adult flukes sampled from deer in Serbia and determined that both haplotypes expanded their territory into the country showcasing the gradual spreading of these haplotypes toward the South of Europe.

1.4.4. Correlation between haplotypes in the Czech Republic and the introduction into Europe

This difference in diversity between countries in Europe and the wide variety of haplotypes in the Czech Republic also points to a long history of *F. magna* in the country. It should be noted that CO1-Ha3 was proven to have the highest abundance worldwide and appears to have originated in North America, even though the exact area was never determined. Králová-Hromadová et al. (2011) suggest that the reason for this could be that the original population simply does not exist anymore, as data indicates a general decline in populations of *F. magna* in North America and Canada. Moreover, it seems likely that it is this haplotype that was first introduced into Europe and then by adapting to the new hosts and environment resulted in the rise of new haplotypes on the continent. As this introduction appears to have taken place in the Czech Republic (Králová-Hromadová et al., 2011), *F. magna* would have had more time to adapt into more haplotypes in the country before spreading into neighboring countries. This could explain the significant difference in variety between the countries that exists nowadays. Juhásová et al. (2016) substantiated this theory by examining adult flukes sampled in North America and Europe and verifying the decrease in diversity along the assumed routes of spreading of the various haplotypes in Europe (e.g. from the Czech Republic along the Danube).

1.4.5. Haplotype networks

The relationships of different isolates of *F. magna* have been visualized in haplotype networks (Figure 6), highlighting several clusters of different haplotypes. Group 1 is depicted in the upper right half of the shown network and consists of all European

haplotypes except the Italian ones and the types from the south-eastern part of North America. This visualizes not only their close relationship but also solidifies the theory that the origin of most of the European *F. magna* populations lies in the south-east of North America. Additionally, this network also highlights the difference in numbers of haplotypes between the Czech Republic and the Danube area (e.g. Austria), which indicates that not only does the population of the latter derive from a pre-existing population in Europe, but also highlights the close relationship between the haplotypes in Central Europe. No central haplotype can be identified in this group. The haplotypes of group 2 on the other hand appear more closely related even if their greater distribution suggests otherwise. The bigger number of them in North America and Canada reveals a larger diversity on the native continent. Additionally, a central haplotype can be identified for this group: Haplotype 1 (Ha1) predominates this group signifying a great number of samples and in turn a high prevalence and appears to be one of the most common haplotypes worldwide (Králová-Hromadová et al., 2011).

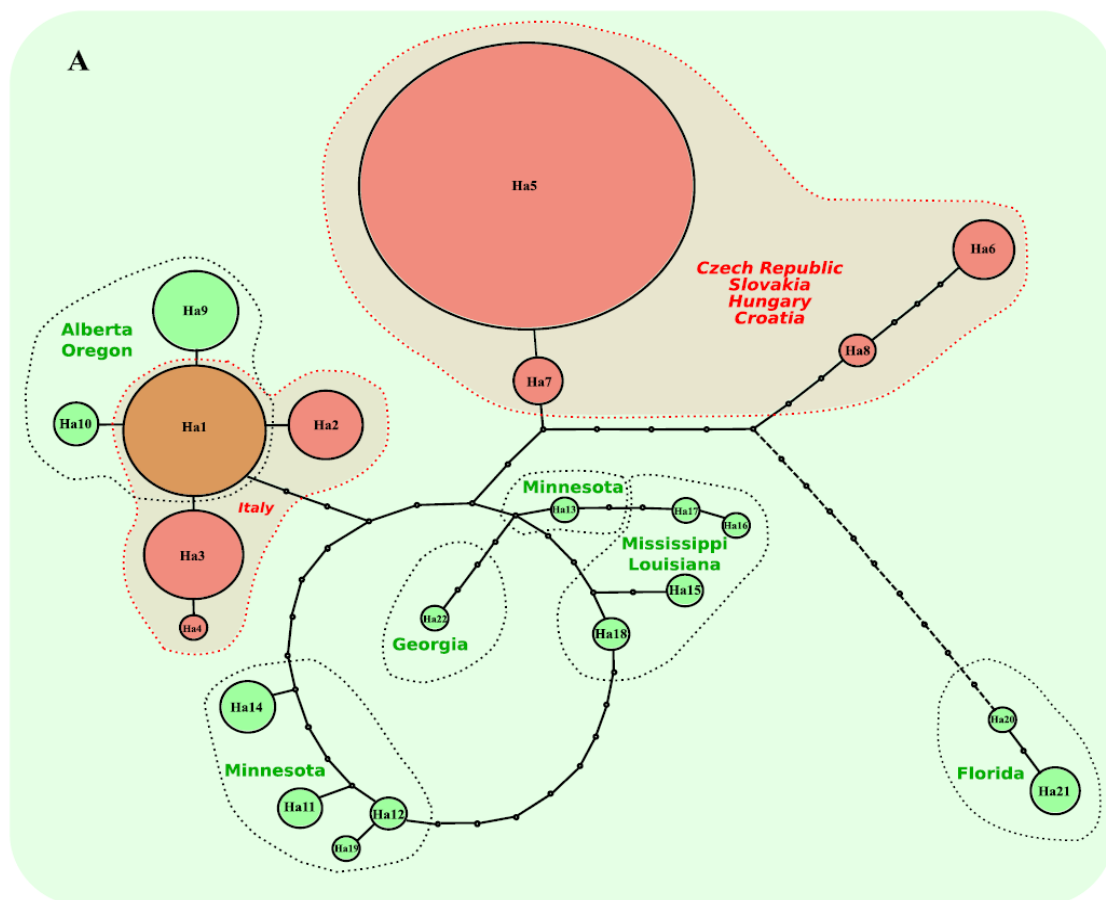


Figure 6. Concatenated haplotype network of *F. magna* based on Cox1 and Nad1 sequences as proposed by Králová-Hromadová et al. (2011).

1.5. Aims

The overall aim of this master's thesis was to assess the prevalence of *F. magna* in the national parks Bayerischer Wald and Sumava at the border between Germany and the Czech Republic. Moreover, this study aimed to evaluate digenean diversity in the intermediate hosts of these parasites.

The specific tasks of this research project were to (i) determine the prevalence of *F. magna* in the Bavarian and Sumava National Parks by collecting and investigating a representative number of intermediate host snails. To achieve this, it was aimed to localize suitable habitats in the area, to monitor the habitats for lymnaeid snails, to establish a host snail collection, to investigate the snails by light microscopy, and to dissect the snails and evaluate their infection status with digenean trematodes. The second task was to (ii) obtain DNA sequence data of liver fluke and other trematode specimens from this area. This is important to document the current diversity and distribution of digenean trematodes in Europe. Additionally, it adds scientific data in a neglected topic. The final task was to (iii) analyze the phylogenetic relationships of the newly collected specimens by comparing them with reference data from the GenBank.

2. METHODS

2.1. Study area and sampling sites

Samples were collected in the area of the Bayerischer Wald and Sumava National Parks in the South-East of Germany and the West of the Czech Republic (Figure 7). Sampling sites were selected for possible habitats of the intermediate host snails following a grid to ensure a complete coverage of the area of both parks. Sampling was performed during the warmer months starting in July and resuming in October.

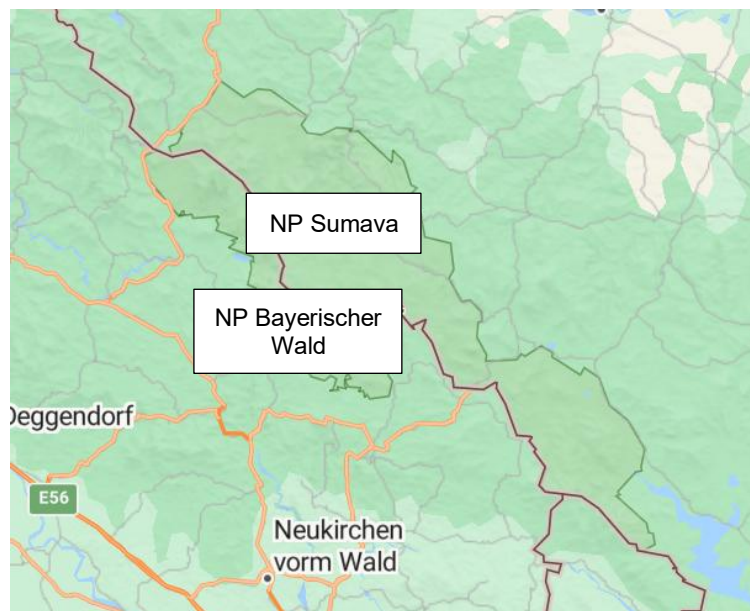


Figure 7. The examined area of both national parks in Germany and the Czech Republic. Orig.

2.2. Sampling procedure

Sampling sites were each linked to a specific coordinate with a distance of one kilometer to the closest points. Upon arrival, each site was examined for possible habitats of the intermediate hosts spending a maximum of ten minutes per sampling site before moving to the next one. The weather, vegetation zone, and temperature on the day of the sampling as well as the duration of the search were documented. Additionally, each area where snails of the species *Galba truncatula*, *Radix labiata*, or *Radix balthica* were found

was given a number for documenting purposes. Each snail was preserved in 80% EtOH until further investigation.

2.3. Morphological screening

Each snail was identified using a Leica Wild M3Z stereo microscope (Leica Camera AG, Wetzlar, Germany). Due to the morphological variety within one species, the identification of exact species was tedious and dependent on general characteristics such as size and number of whorls. Only after the successful identification were the shells carefully removed and the snails examined for any signs of an infection. Positive samples, mostly snails with clearly defined rediae, were stored individually in 80% EtOH. An example of an infected snail is shown in figure 8. Negative samples however were pooled together in groups of up to 9 individual snails, pooling only snails from the same sampling site.

Snails belonging to other genera than *Galba* and *Radix* were not included in the morphological examination. Additionally, for unidentifiable individuals, advice from specialists of the Natural History Museum in Vienna was obtained.



Figure 8. An infected specimen of *Galba truncatula* as seen under the stereo microscope. The arrow points to the visible *Fascioloides magna* rediae. This image was taken with ~10x magnification. Orig.

2.4. DNA extraction

DNA extraction was performed following the tissue protocol of the QIAamp DNA Mini Kit (QIAGEN, Vienna, Austria) handbook. Snails were either pooled in groups of up to 9 snails or examined individually if morphological examination suggested an infection.

The 80% ethanol was removed and 180 µl of ATL buffer added. The samples were mixed by hand and shortly vortexed before adding 20 µl of Proteinase K. They were then incubated at 56°C for up to 3 hours or until the samples were homogenized. Then, 200 µl of Buffer AL were added, the samples vortexed and incubated at 70°C for 10 minutes. 200 µl of 96% ethanol was added and the samples thoroughly mixed by vortexing. Samples were transferred to QIAamp Mini Spin tubes, centrifuged at 6,000 x g for 1 minute before transferring the filters into clean 2 ml Eppendorfer tubes. The supernatant was discarded and 500 µl of AW1 buffer was added to each filter and the samples centrifuged at 6,000 x g for 1 minute. The filters were once again transferred into clean Eppendorf tubes, 500 µl of AW2 buffer was added and the samples centrifuged at 20,000 x g for 3 minutes. After that, the filters were transferred into clean tubes and centrifuged at 20,000 x g for 1 minute. Finally, 100 µl of AE buffer was added. The samples were left to rest for 1 minute at room temperature, centrifuged at 6,000 x g for 1 minute and stored at -20°C.

2.5. Polymerase chain reactions

2.5.1. Protocol set up

2.5.1.1. PCR set up for the identification of trematodes

The basis for the protocol set up for the identification of trematodes was the protocol by Husch et al. (2020). All primer pairs (Table 1) were ordered at MicroSynth (Microsynth AG, Switzerland). They were used at a concentration of 10 µM.

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Table 1. Primers used in this study, their target, sequences, expected length, annealing temperature (AT) and corresponding references.

Primer ID	Target	Sequence	Length	AT	Reference
TremF	18S rDNA	5'-GGT TCC TTA GAT CGT ACA TGC-3'	430 bp	56°C	Haider et al., 2012
TremR	18S rDNA	5'-GTA CTC ATT CGA TTA CGG ACC-3'	430 bp	56°C	Haider et al., 2012
Fm Cox1f	Cox1	5'-GGT CAT GGG GTT ATA ATG A-3'	439 bp	56°C	Kralova-Hromadova et al., 2008
Fm Cox1r	Cox1	5'-ACA GCA TAG TAA TAG CCG C-3'	439 bp	56°C	Kralova-Hromadova et al., 2008
Fm Nad1f	Nad1	5'-TTA TGT GTG TTG TTG TTT TG-3'	405 bp	56°C	Kralova-Hromadova et al., 2008 Husch et al., 2017
Fm Nad1r	Nad1	5'-CTA CAA CAC ATA AAA AAA AG-3'	405 bp	56°C	Kralova-Hromadova et al., 2008 Husch et al., 2017
News2 sense	ITS-2 rDNA	5'-TGT GTC GAT GAA GAA CGC AG-3'	502 bp	54°C	Almeyda-Artigas et al., 2000
Its2Rixo anti	ITS-2 rDNA	5'-TTC TAT GCT TAA ATT CAG GGG-3'	502 bp	54°C	Almeyda-Artigas et al., 2000
LCO1490	Cox1	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	658 bp	50°C	Folmer et al., 1994
COR722b	Cox1	5'-TAA ACT TCA GGG TGA CCA AAA AAT YA-3'	658 bp	50°C	Wilke and Davis, 2000

2.5.1.1.1. TremF/TremR

The preliminary PCRs started with determining the most optimal DNA concentration for this study. For this, three DNA concentrations (1, 3, and 6 µl) of the positive control, in this case F_{mag} 1:3 mixed with H_2O , were used. TremF/R primers (see Table 1) were used to detect trematodes in general. The master mix was prepared, using 5 µl of each: $MgCl_2$ (2.5 µM, Solis BioDyne, Estonia), PCR buffer (Solis BioDyne, Estonia) and both primers (10 µM), 11 µl of sterile ddH₂O, 1 µl dNTP (1.6 µM, Solis BioDyne, Estonia) and 0.25 µl Fire HOT-Polymerase (5 U/µl, Solis BioDyne, Estonia) per sample. To this, 1 to 6 µl of positive control were added. Each sample was filled up to 50 µl with sterile ddH₂O.

The PCR was run in an Eppendorf Mastercycler Nexus Gradient (Eppendorf AG, Hamburg, Germany) with the following cyclor program (Husch et al., 2020): initial denaturation at 95°C for 15 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 2 minutes and extension at 72°C for 3 minutes and ending in a final extension at 72°C for 7 minutes.

As shown in figure 9, the fragment was successfully amplified with all three tested DNA concentrations.

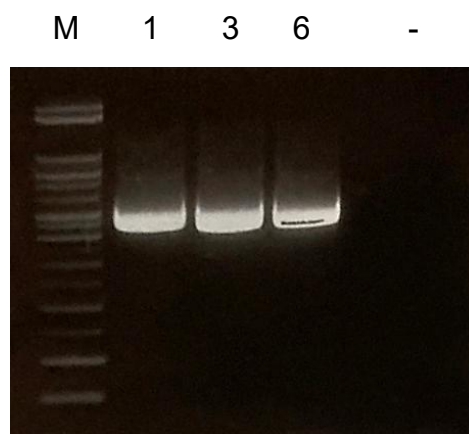


Figure 9. Agarose gel electrophoresis following PCR for the implementation of the protocol using three different concentrations of the positive control and primers TremF/R. (M) Molecular size marker DirectLoad™ Step Ladder, 50bp; (1) DNA in concentration of 1 µl; (3) 3 µl; (6) 6 µl; (-) negative control.

2.5.1.1.2. Cox1F/Cox1R, Nad1F/Nad1R

Next, the primer pairs Cox1F/R and Nad1F/R (Husch et al., 2017) (see Table 1) were used. Although they amplify different genes, both can be used for the identification of trematodes. A comparison was therefore performed to determine the better primer pair for this study. For this, the SolisFAST® Master Mix (Ready to Load, 5x concentrated, Solis BioDyne, Estonia) was used. This contains all needed components except the primers and the DNA template: For each sample, 0.5 µl of both primers FM Cox1f (10 µM) and FM Cox1r (10 µM) or FM Nad1f (10 µM) and FM Nad1r (10 µM) (see Table 1), 5 µl SolisFAST® and 10 µl of sterile ddH₂O were mixed in separate tubes. Next, 4 µl of the positive control was added. Sterile DNase free water was used as a negative control. The samples were spun down to remove any bubbles.

PCRs were performed in an Eppendorf Mastercycler Nexus Gradient (Eppendorf AG, Hamburg, Germany) with the following cycler program: initial denaturation at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 56°C for 10 seconds and extension at 72°C for 1 minute and a final extension at 72°C for 1 minute.

The comparison of primer pairs Cox1F/Cox1R and Nad1F/Nad1R in the concentrations 1 and 0.5 can be found in figure 10. As both showed similarly good amplification, only the primer pair Cox1F/Cox1R was used in the subsequent runs. Additionally, to increase sensitivity and decrease specificity, the annealing temperature was changed to 54°C. The duration of the step in the established protocol was increased to 1 minute.

METHODS

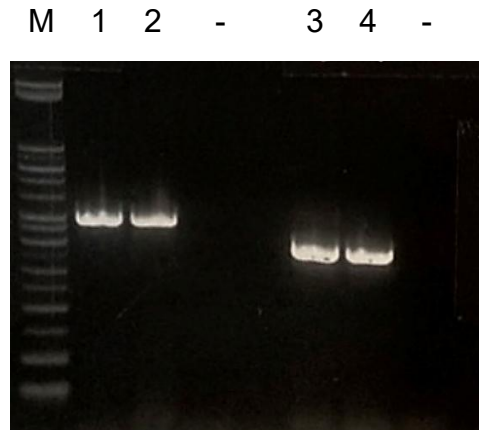


Figure 10. Agarose gel electrophoresis following PCR for the comparison of the efficiency of primer pairs Cox1F/R and Nad1F/R. (M) Molecular size marker DirectLoad™ Step Ladder, 50bp; (1) positive control in a concentration of 1 and the primer pair Cox1F/R; (2) positive control in a concentration of 0.5 and the primer pair Cox1F/R; (3) positive control in a concentration of 1 and the primer pair Nad1F/R; (4) positive control in a concentration of 0.5 and the primer pair Nad1F/R; (-) negative controls.

All samples were stored at 7°C.

2.5.1.2. PCR set up for the identification of snail species

2.5.1.2.1. News2/Its2Rixo

For the identification of snails, the primer pairs News2/Its2Rixo and LCO1490/COR722b were used. The sequences and fragment lengths can be found in table 1. First runs were performed with News2 and Its2Rixo using a master mix that was prepared as described on page 21. PCR started with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 30 seconds, ending in a final extension at 72°C for 7 minutes (Caron et al., 2007).

2.5.1.2.2. LCO1490/COR722b

As the previous protocol did not result in sufficient amounts of amplification for DNA sequencing, the protocol by Wilke and Davis (2000) using different primers LCO1490 and COR722b (Table 1) was used instead. The master mix was prepared in the laboratory (see page 21) before adding the samples. Each sample was then filled up to 50 µl with sterile ddH₂O. PCRs were run in an Eppendorf thermocycler Mastercycler (Eppendorf AG, Hamburg, Germany)

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using the following cycler program. It started with an initial denaturation at 95°C for 1.5 minutes, followed by 36 cycles of denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute and 20 seconds, and extension at 72°C for 1 minute and 10 seconds. The program ended with a final extension at 74°C for 5 minutes (Wilke and Davis, 2000).

In latter runs, the annealing temperature was increased to 50°C and 54°C. The efficacy of this change is visible in figure 11.

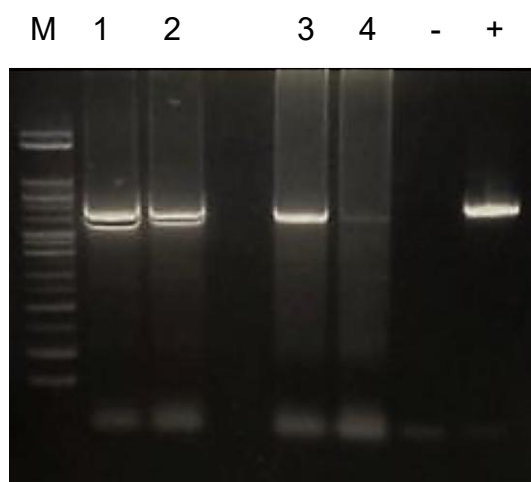


Figure 11. Agarose gel electrophoresis following PCR for the comparison of the efficiency of different annealing temperatures using the LCO1490/COR722b primer pair. (M) Molecular size marker DirectLoad™ Step Ladder, 50b; (1) positive control in a concentration of 1 and annealing temperature of 50°C; (2) positive control in a concentration of 0.5 and annealing temperature of 50°C; (3) positive control in a concentration of 1 and annealing temperature of 54°C; (4) positive control in a concentration of 0.5 and annealing temperature of 54°C; (-) negative control; (+) positive control.

All samples were stored at 7°C.

2.6. Agarose gel electrophoresis and purification

The PCR products were evaluated using agarose gel electrophoresis. In brief, 2 g agarose (Sigma-Aldrich, St. Louis, MO, USA) were mixed with 100 ml 1xTAE (0.04 M Trisacetate & 0.001 M EDTA) to obtain a 2% agarose gel. For this, the mixture was heated up using a magnetic stirrer for mixing. Once it reached the boiling point and turned clear, it was cooled down and 5 µl of GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) was added for visualization of the bands. As for the samples, 20 µl of each PCR product were loaded onto the gel as well as 25 µl of the DirectLoad™ 50 bp DNA Step Ladder marker (Sigma-Aldrich, St. Louis, MO, USA).

Gel electrophoresis was performed at 400 mA and 138 V.

Bands were cut out and purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). First, 300 µl of the Capture buffer type 3 were added to each sample. The samples were homogenized at 60°C for 15 to 20 minutes depending on the weight before transferring them into GFX micro spin columns. They were left to rest for 60 seconds at room temperature, then centrifuged at 16,000 x g for 30 seconds before adding 500 µl wash buffer type 1 to each column. The samples were centrifuged once again at 16,000 x g for 30 seconds. After that, 50 µl of elution buffer type 4 was added to each column. The samples were centrifuged one last time at 16,000 x g for 1 minute.

The supernatant was stored at -20°C.

2.7. DNA sequencing

Sanger sequencing was performed either in the lab or by sending the samples for analysis to MicroSynth (Microsynth AG, Switzerland). For sequencing in the lab, a PCR reaction mix (see Table 2) was prepared: 2 µl AB mix (BigDye™ Terminator v1.1 Cycle Sequencing RR-100, Applied Biosystems, Thermo Fisher Scientific, USA) were mixed with 1 µl of sequencing buffer (BigDye™ Terminator v1.1, v3.1, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA), 2 µl of either primer R or F (10 µM) and 1 to 3 µl of the purified sample. This was filled up to 20 µl with ddH₂O. The sequencing PCR was run in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) with the following program: 30 seconds at 96°C followed by 30 cycles of 10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C. Subsequently, 10 µl of the PCR-products were mixed with 1 µl of sodium acetate and 40 µl of 100% EtOH. This was vortexed and incubated on ice for around 20 minutes before centrifugation at 12,000 x g and 4°C for 30 minutes. The supernatant was then carefully removed. Next, 90 µl of 70% EtOH were added and the sample was then once again centrifuged at 12,000 x g and 4°C for 10 minutes. The EtOH was removed and the lid kept open for around 3 minutes to assure full evaporation of the alcohol. After that, 20 µl of HIDIT™ (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) were added and the lid was opened for another 3 minutes. Finally, the sample was incubated at 94°C for 4 minutes and put on ice for 10 minutes. Samples were transferred into sequencing strips and subjected to DNA sequencing using the Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 2. Reaction set up for sanger sequencing of the Cox1 gene obtained from *F. magna*.

Component	Volume
AB Mix	2 µl
Sequencing Buffer	1 µl
Primer F or R (10 µM)	2 µl
Purified sample	1 or 3 µl
ddH ₂ O	fill up to 20 µl

2.8. DNA sequence analysis

All obtained sequences were compared to reference sequences from GenBank by employing the Basic Local Alignment Search Tool (BLAST) by NCBI and analyzed using ClustralX (Thompson et al., 1997) and GeneDoc (Nicholas et al., 1997). ClustralX was used to create alignments. GeneDoc was used to obtain consensus sequences, and edit the alignments manually to identify homologous positions.

For haplotype identification of the *Fascioloides magna* isolate, the obtained consensus sequence was compared to six different reference sequences. These were chosen by sequence similarity such as Cox1 haplotype 5 (GenBank number: GU599870.1), Cox1 haplotype 4 (GenBank number: GU599868.1) and Cox1 haplotype 3 (GenBank number: GU599864.1) from the Czech Republic. As well as a sequence also from the Czech Republic with no haplotype appointed however mentioned by Králová-Hromadová et al. (2011) (GenBank number: EF534997.1). Additionally, Cox1 haplotype 1 (GenBank number: GU599860.1) from Italy and Cox1 haplotype 6 (GenBank number: GU599871.1) from Canada were added for comparison.

2.9. Use of Artificial Intelligence

The Artificial Intelligence Tool ChatGPT, model GPT-5.5 (OpenAI, 2026) was used for the research of specific literature. This literature was carefully reviewed and only after verification and careful consideration was it used in this study.

3. RESULTS

3.1. Dissection and morphological examination

3.1.1. Identification of snail species and infection rates

Out of the 1,847 samples, 1,098 were identified as *Radix* spp. (59.44%) (841 *Radix labiata*, 144 *Radix fagotes*, 92 *Radix balthica* and 24 *Radix* sp.) and 696 as *Galba truncatula* (37.68%) (Figure 12). The remaining 51 samples were of other genera, including *Succinea*, *Potamopyrgus*, and others (2.76%). One snail was unidentifiable due to its small size (0.054%).

Altogether, 154 of the 1,794 snails identified as either *Radix* spp. (61.23%) or *G. truncatula* (38.77%) were rated as infected by trematode larvae resulting in an infection rate of 8.58%. Out of these 154 snails, 101 were identified as *Radix* spp. and 53 as *G. truncatula* (Figure 13).

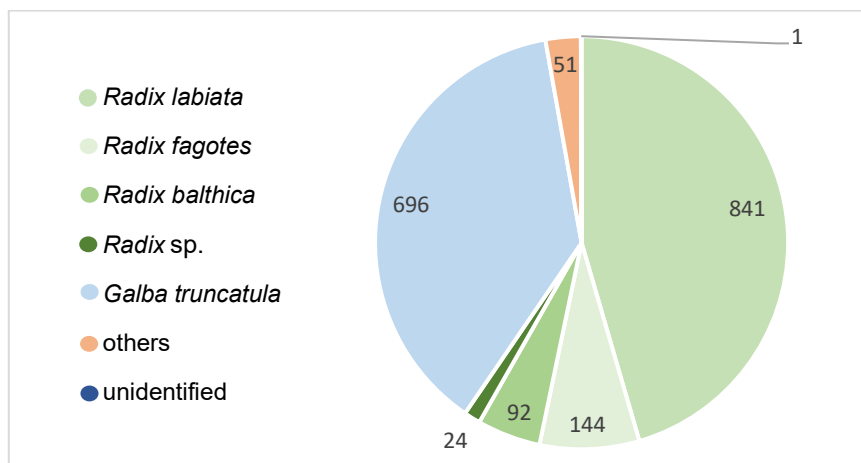


Figure 12. First identification by microscopy. The diagram shows the proportion of *G. truncatula*, *Radix* spp. and other species.

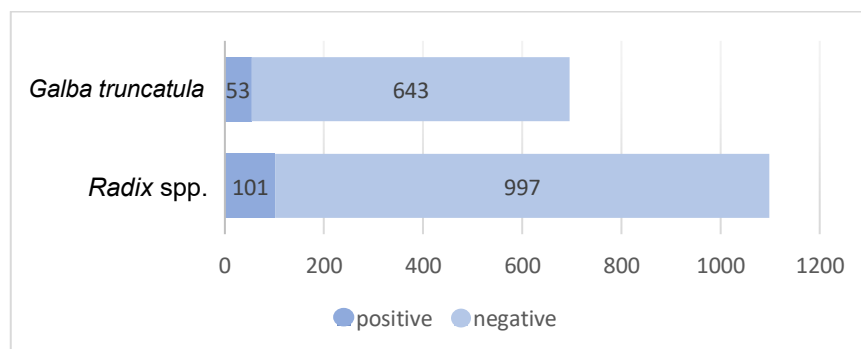


Figure 13. Total number of *G. truncatula* and *Radix* spp. specimens dissected.

3.2. Molecular analysis

3.2.1. Polymerase chain reaction

3.2.1.1. Universal trematode PCRs

The universal trematode PCRs were performed using the primer pair TremF and TremR (Haider et al., 2012) on 154 of the samples rated as infected after a first morphological examination. Previously, the samples were stored individually and therefore not pooled. Out of these, 59 samples had a clear band at the height expected for *F. magna*. Another 33 samples had bands at a height below 400 bp, while 14 samples had bands at a height of over 450 bp. The other 44 samples had no bands and were therefore deemed not infected, resulting in an infection rate of 6.02% (110 out of 1794 samples). An example of an agarose gel after a PCR performed with the primers TremF/TremR is shown in figure 14.

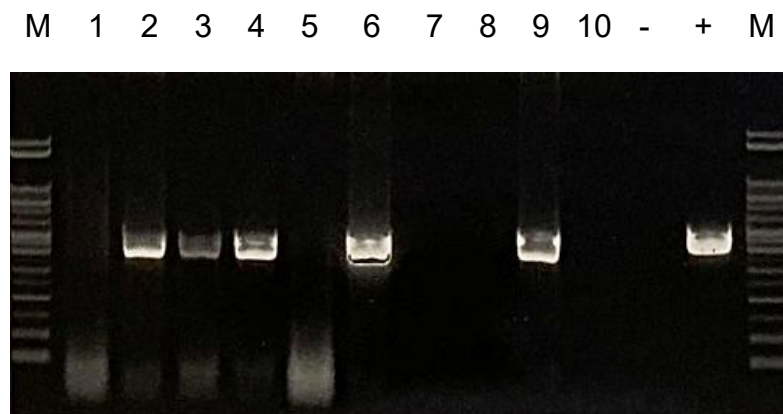


Figure 14. Agarose gel electrophoresis following PCR using TremF/R primer pair in a dilution 1:10. (M) Molecular size marker DirectLoad™ Step Ladder, 50bp; (1-10) samples; (-) negative control; (+) positive control *F. magna*.

3.2.1.2. Specific *Fascioloides magna* PCRs

For a factual confirmation of an infection with *Fascioloides magna*, a PCR using the primers Cox1F and Cox1R was performed. Altogether, out of the 1,794 samples only sample CZ 336_3 from the Czech Republic, belonging to the ones tested positive when using the TremF/TremR primers, had a band at 439 bp (Husch et al., 2017; Kralova-Hromadova et al., 2008) and an additional unspecific band at around 50 bp (Figure 15). The rest of the samples showed no bands at 439 bp but all had an unspecific band at around 50 bp. This

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resulted in a prevalence of 0.056% of *F. magna* in all samples examined. The infection rate of *F. magna* in snails collected in the Czech Republic was determined to be 1.616%.

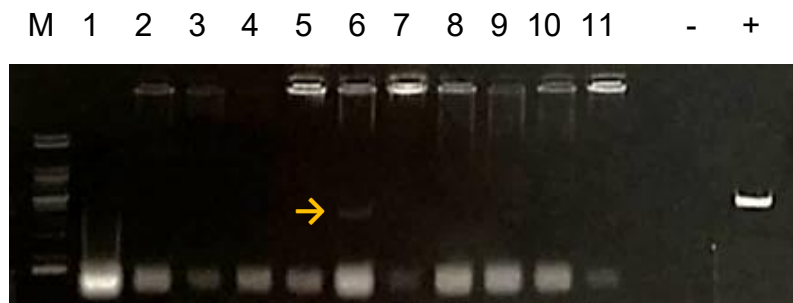


Figure 15. Agarose gel electrophoresis following PCR using Cox1F/R primer pair. (M) Molecular size marker DirectLoad™ Step Ladder, 50bp; (1-11) samples; (-) negative control; (+) positive control *F. magna*. The arrow points to the band at the expected height.

3.2.1.3. Snail PCRs

For confirmation of microscopic species identification, PCR using the primers LCO1490 (forward) (Folmer et al., 1994) and COR722b (reverse) (Wilke & Davis, 2000) was performed on 3 samples: One sample that tested positive for *F. magna* and two that tested positive for *Echinostoma revolutum*. Out of these, only 2 samples had bands at the correct height of 658 bp: Slot 1 and 2 show the bands for the sample infected with *F. magna* in two different concentrations. Slot 3 and 4, however, show the sample infected with *E. revolutum* in two concentrations. No further bands were found (Figure 16).

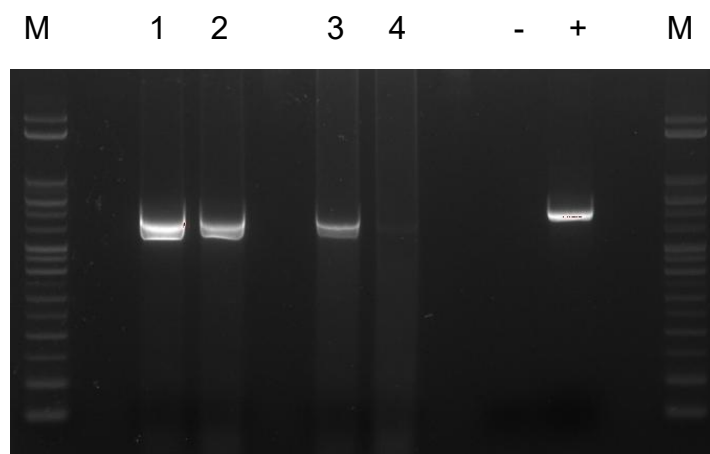


Figure 16. Agarose gel electrophoresis following PCR using LCO1490/COR722b primer pair. (M) Molecular size marker DirectLoad™ Step Ladder, 50bp; (1-2) DNA from sample A in a concentration of (1) 1 and (2) 0.5; (3-4) DNA from sample B in a concentration of (3) 1 and (4) 0.5; (-) negative control; (+) positive control.

All amplicons were purified and DNA sequences were obtained from both strands for sequence comparison.

3.2.2. Sequencing

3.2.2.1. Sequencing of *Fascioloides magna*

Out of 1,794 samples, one tested positive for *F. magna* resulting in a prevalence of 0.056%. The consensus sequence is shown in figure 17. Sequencing of the positive sample using Cox1F and Cox1R primers showed 99% sequence similarity to sequences from *F. magna* sampled in the Czech Republic (GenBank numbers: GU599870.1 and EF594997.1) (see Table 4).

3.2.2.2. Sequencing of trematodes

After performing PCR using the general TremF and TremR primers, the number of positive samples decreased from 154 to a total of 110. Out of these, five samples were chosen at random to be sequenced using universal trematode primers with the aim of determining the exact trematode species. Most notably, two of these samples (D217_1 and D217_3) were found to be infected with the human pathogen *E. revolutum*. Both originated from specimens identified as *R. labiata*, sampled at the same sampling site in Germany but were collected at different habitats. When comparing them to reference sequences from the GenBank, both samples showed the highest sequence similarities of 99% to samples obtained from *E. revolutum* isolated from freshwater snails in Iran (GenBank number: OM956144.1) and Vietnam (GenBank number: MZ436915.1). Sample 1 had an overall similarity of 338/343 base pairs, while sample 2 showed an overlapping of 334/338 base pairs. The other three samples were shown to be infected with other parasites: Sample D 205_1 was identified as *R. labiata* and was collected in Germany. It showed the highest similarity of 98% to an *Opisthioglyphe ranae* (GenBank number: MZ787567.1) sequence obtained from a freshwater snail in Iran with an overlapping of 359/367 base pairs. In contrast, sample D 233_4a and D 233_4b were identified as *G. truncatula* and were collected in the same area of one German sampling site. Both samples showed a sequence similarity of

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97% to a sample allocated to *Orchipedum tracheicola* (GenBank number: AJ287551.1). However, while sample D 233_4a showed a convergence of 333/345 base pairs, the latter had a higher similarity of 335/345 base pairs.

3.2.2.3. Sequencing of snail species

Of the two bands obtained in the snail-specific PCR, one resulted in a sequence of 622 base pairs and showed 99% sequence similarity to three sequences isolated from *R. labiata* in Germany (GenBank numbers: LT623598.1 (621/622 bp); HG932226.1 (619/622 bp); LT623599.1 (617/622 bp)).

RESULTS

3.2.3. Consensus sequence of *Fascioloides magna*

```

      *           20           *           40           *
1r : ASMRI GGTCA TGGGGT TATAAT GATTTTTT TTTCTG ATGCC TGTGTTG : 50
2r : -MSGI GGTCA TGGGGT TATAAT GATTTTTT TTTCTG ATGCC TGTGTTG : 49
1f : -----
2f : -----G : 1
      g

      60           *           80           *           100
1r : ATAGGGGGG GTTGGTAATTATTTATTGCC TTTGTTATTAGG TATACCAGA : 100
2r : ATAGGGGGG GTTGGTAATTATTTATTGCC TTTGTTATTAGG TATACCAGA : 99
1f : -----GGTAATTATTTATTGCC TTTGTTATTAGG TATACCAGA : 38
2f : ATAGGGGGG GTTGGTAATTATTTATTGCC TTTGTTATTAGG TATACCAGA : 51
      ataggggggtttGGTAATTATTTATTGCC TTTGTTATTAGG TATACCAGA

      *           120           *           140           *
1r : TTTAAAATT SCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCC TT : 150
2r : TTTAAAATT GCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCC TT : 149
1f : TTTAAAATT GCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCC TT : 88
2f : TTTAAAATT GCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCC TT : 101
      TTTAAAATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCC TT

      160           *           180           *           200
1r : CTTGIGTATGTTTGTCCTTAGTTTGATAGGGGGGCTGGGGGTAGGTTGG : 200
2r : CTTGIGTATGTTTGTCCTTAGTTTGATAGGGGGGCTGGGGGTAGGTTGG : 199
1f : CTTGIGTATGTTTGTCCTTAGTTTGATAGGGGGGCTGGGGGTAGGTTGG : 138
2f : CTTGIGTATGTTTGTCCTTAGTTTGATAGGGGGGCTGGGGGTAGGTTGG : 151
      CTTGTGTATGTTTGTCCTTAGTTTGATAGGGGGGCTGGGGGTAGGTTGG

      *           220           *           240           *
1r : ACTTTTTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTA : 250
2r : ACTTTTTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTA : 249
1f : ACTTTTTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTA : 188
2f : ACTTTTTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTA : 201
      ACTTTTTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTA

      260           *           280           *           300
1r : TTTTTTGATGTTTCTCTTCACTTAGCCGGTATTTCTAGGGTTTGGGTT : 300
2r : TTTTTTGATGTTTCTCTTCACTTAGCCGGTATTTCTAGGGTTTGGGTT : 299
1f : TTTTTTGATGTTTCTCTTCACTTAGCCGGTATTTCTAGGGTTTGGGTT : 238
2f : TTTTTTGATGTTTCTCTTCACTTAGCCGGTATTTCTAGGGTTTGGGTT : 251
      TTTTTTGATGTTTCTCTTCACTTAGCCGGTATTTCTAGGGTTTGGGTT

      *           320           *           340           *
1r : CTATTAAATTTATATGTA CTATCTTGGAGGTTATG TSGATGAGGGGGTA : 350
2r : CTATTAAATTTATATGTA CTATCTTGGAGGTTATG TGGATGAGGGGGTA : 349
1f : CTATTAAATTTATATGTA CTATCTTGGAGGTTATG TGGATGAGGGGGTA : 288
2f : CTATTAAATTTATATGTA CTATCTTGGAGGTTATG TGGATGAGGGGGTA : 301
      CTATTAAATTTATATGTA CTATCTTGGAGGTTATG TGGATGAGGGGGTA

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RESULTS

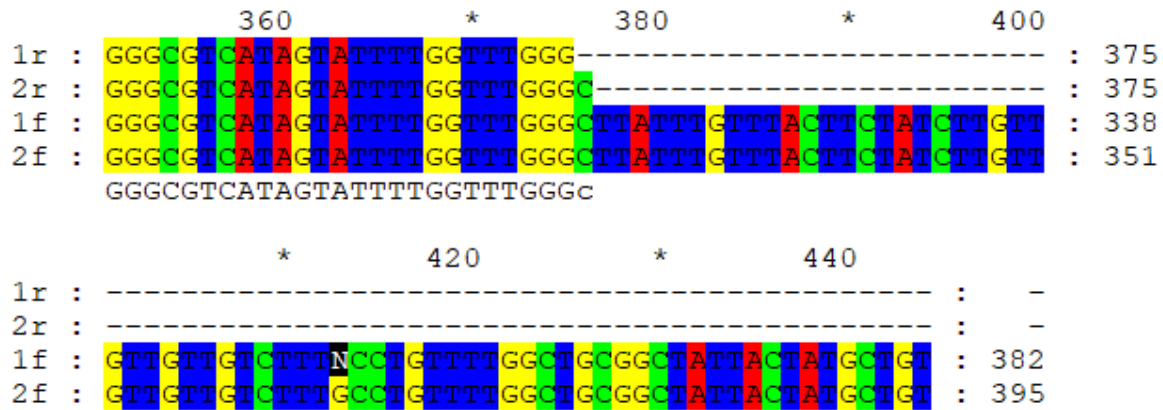


Figure 17. Sequence alignment of all sequences (row 1-4) obtained from the *F. magna* amplicon from *R. labiata* isolate from the Czech Republic (CZ 336_3) by sanger sequencing and the resulting consensus sequence (below row 4).

3.2.4. Comparison of *Fascioloides magna* haplotypes

An alignment of the newly obtained sequence from sample CZ 336_3 with the reference sequences from GenBank (Table 3) was performed. The resulting alignment is shown in figure 18. The sequence obtained from sample CZ 336_3 showed highest sequence similarity of 99% (438/439 bp) to two isolates of *F. magna* from the Czech Republic (GenBank number: GU599870.1 and EF534997.1). The first sequence was obtained from red deer in the Bohemian Forest while the latter was obtained from deer in Mirosov, a town in the West of the Czech Republic. Next followed a similarity of 436/439 base pairs (GenBank number: GU599868.1) and 432/439 base pairs (GenBank number: GU599864.1). Both sequences were obtained by directly taking adult flukes from the livers of infected red deer in the Bohemian Forest. Sequencing also revealed a sequence similarity of 97% (428/439 bp) to *F. magna* obtained in red deer in La Madria in Italy (GenBank number: GU599860.1) and a similarity of 427/439 base pairs to a Canadian sequence collected from wapiti in Alberta (GenBank number: GU599871.1).

Most notably, the one distinction that could be made between the newly obtained sequence from the Czech Republic and all the reference sequences, was position 43 where a point mutation led to the substitution of A with a G.

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This mutation was only found in the new sequence. Other substitutions can be identified throughout the alignment. However, only the substitutions in base pairs 63, 147, 291, and 390 show clear distinction between sequences obtained from the Czech Republic and ones from Italy and Canada. While sequences from the Czech Republic all have a T at position 63 and 147, the Italian and Canadian sequences have a C at position 63 and an A at position 147. For position 291 and 390 the sequences obtained from the Czech Republic have a G and a C whereas the Italian and Canadian both have an A and a T.

Table 3. Sequence similarity between the new sequence and reference sequences for the most closely related haplotypes from the GenBank.

sequence ID	Cox1 haplotype	sequence similarity	region
GU599870.1	CO1-Ha5	438/439 bp	Czech Republic
EF534997.1	-	438/439 bp	Czech Republic
GU599868.1	CO1-Ha4	436/439 bp	Czech Republic
GU599864.1	CO1-Ha3	432/439 bp	Czech Republic
GU599860.1	CO1-Ha1	428/439 bp	Italy
GU599871.1	CO1- Ha6	427/439 bp	Canada

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3.2.5. Sequence alignment of *Fascioloides magna*

```

          *          20          *          40          *
F.magna      : GGTTCATGGGGTTATAATGATTTTTTTTNTTTCTGATGCCTGTTGTTGATAGG : 50
GU599870.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
GU599868.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
EF534997.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
GU599864.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
GU599860.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
GU599871.1   : GGTTCATGGGGTTATAATGATTTTTTTTCTTTCTGATGCCTGTTGTTGATAGG : 50
Cox1fwd      : GGTTCATGGGGTTATAATGA----- : 19
Cox1rev      : ----- : -

```

```

          60          *          80          *          100
F.magna      : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
GU599870.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
GU599868.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
EF534997.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
GU599864.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
GU599860.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
GU599871.1   : GGGGTTTGGTAATTGATTTATTGCCTTTGTTATTAGGTATACCAGATTTAA : 100
Cox1fwd      : ----- : -
Cox1rev      : ----- : -

```

```

          *          120          *          140          *
F.magna      : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
GU599870.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
GU599868.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
EF534997.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
GU599864.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
GU599860.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
GU599871.1   : AATTGCCTCGTTTGAATGCGCTTAGTGCTTGGTTGTTGTTGCCTTCTTGT : 150
Cox1fwd      : ----- : -
Cox1rev      : ----- : -

```

```

          160          *          180          *          200
F.magna      : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
GU599870.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
GU599868.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
EF534997.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
GU599864.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
GU599860.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
GU599871.1   : GTATGTTTGTCTCCTTAGTTTGATAGGGGGGTCTGGGGTAGGTTGGACTTT : 200
Cox1fwd      : ----- : -
Cox1rev      : ----- : -

```

```

          *          220          *          240          *
F.magna      : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
GU599870.1   : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
GU599868.1   : TTATCCTCCTCTTTCTAGGGCTAGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
EF534997.1   : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
GU599864.1   : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
GU599860.1   : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
GU599871.1   : TTATCCTCCTCTTTCTAGGGCTGGTTATTCTGGTTGGGGTGTGTTGATTTTT : 250
Cox1fwd      : ----- : -
Cox1rev      : ----- : -

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RESULTS

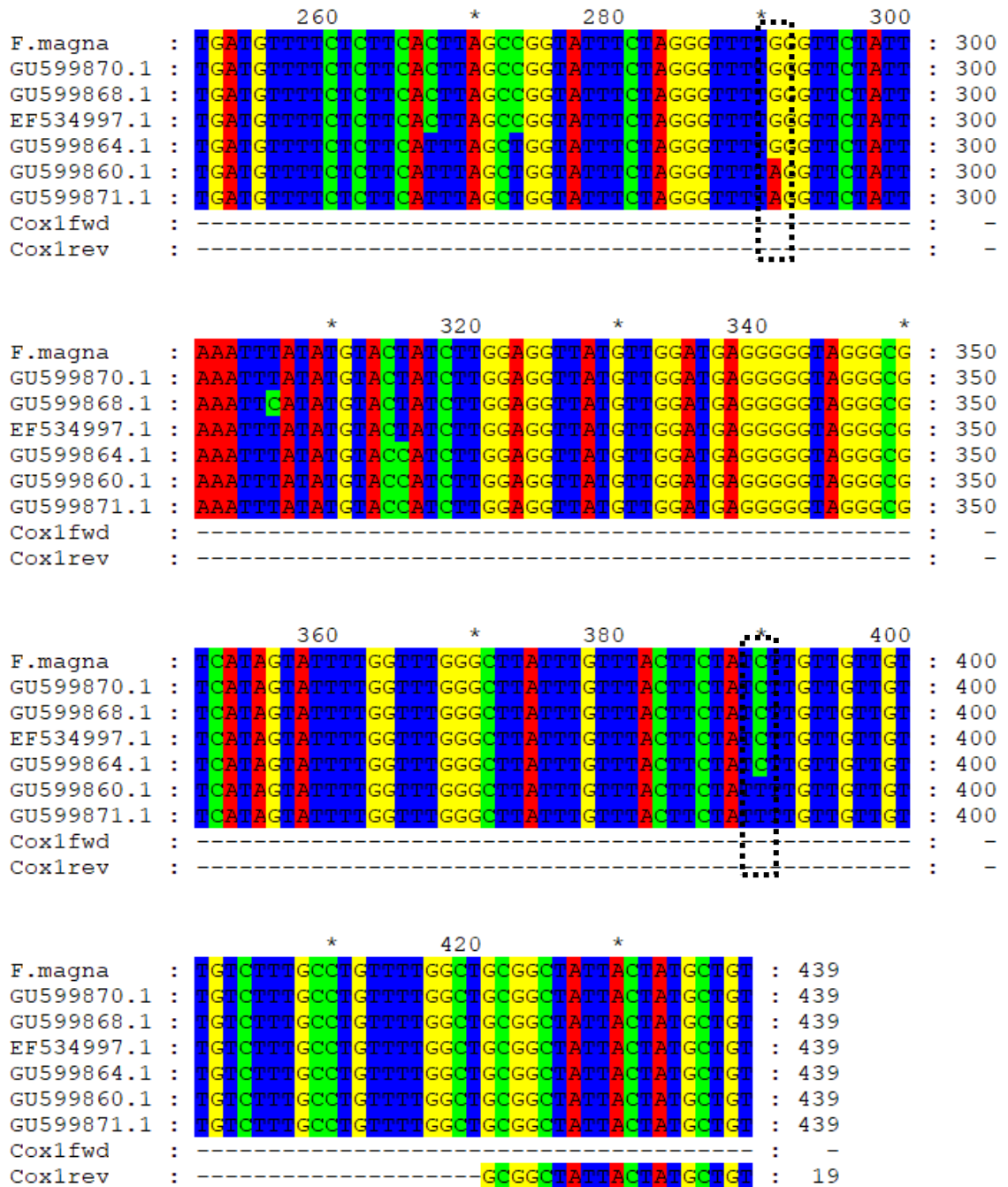


Figure 18. Sequence alignment of the newly obtained sequence of *F. magna* isolated from the *R. labiata* sample (CZ 336_3) from the Czech Republic (row 1) with reference sequences from the GenBank (row 2-7) and forward and reverse primer (row 8-9).

4. DISCUSSION

In this study, one sample collected in the Czech Republic tested positive for *Fascioloides magna* resulting in a prevalence of 0.056% of the liver fluke in all samples examined. The prevalence of *F. magna* in samples collected in the Czech Republic was 1.616% while the total infection rate with trematodes was 8.58%. Moreover, the human pathogen *Echinostoma revolutum* was detected in two specimens sampled in the Bavarian National Park and the prevalence of trematode infections in all samples examined was determined to be 6.02%. Sequencing analysis of the positive sample revealed that *F. magna* was infecting a *Radix labiata* and comparison of the obtained sequence with reference sequences from GenBank revealed an affiliation to the Cox1 haplotype 5.

4.1. Prevalence of trematodes in snails

4.1.1. Prevalence of trematodes in freshwater snails

In this study, out of 1,794 snails collected, 154 samples were found to be infected by morphological examination via light microscopy after dissection under the microscope, relating to an infection rate of 8.58%. Out of these, 110 samples were confirmed to be infected with trematodes by PCR, resulting in an infection rate of 6.02%. This number is consistent with prevalences presented in studies examining *Galba truncatula* only. Haider et al. (2012) collected a total of 3,444 *G. truncatula* starting in April of 2008 and resuming sampling in October of the same year. The sampling was done in Austria and resulted in a prevalence of trematodes of 2.41%. This finding was supported by Hörweg et al. (2011) who also examined snails in Austria and stated that out of 10,059 collected *G. truncatula*, 2.43% were infected with at least one trematode. A similar prevalence of trematode infections of 2.93% was given for *G. truncatula* populations in France (Rondelaud et al., 2016). Compared to this, the prevalences of trematode infections in freshwater snails are higher. One of the older studies performed on this topic stems from Faltýnková (2005) and focused on trematode infections in freshwater snails collected in the Czech Republic resulting in a prevalence of trematodes of 33.9%. This

frequency of infections is substantiated by a second study by Faltýnková et al. (2007). In that study the focus was on the snails in Central European countries including the Czech Republic, Germany and Austria. The monitoring was initiated in areas with close proximity to major rivers in 1998 and resumed in 2005 and resulted in the collecting of 3,628 freshwater snails. Examination led to the following numbers: The highest prevalence of trematode infections in freshwater snails between the three countries was found in in the Czech Republic (37.9%), followed by a prevalence of 15.3% in Germany and 10.8% in Austria. The total prevalence of trematode infections in all samples collected was 26.3%. Selbach et al. (2020) investigated freshwater snails in Germany collecting specimens from 2012 to 2013 and found a prevalence of trematode infections of 19.6%. The trematode families with the highest prevalences were Diplostomatidae, Cyclocoelidae, Strigeidae, and Echinostomatidae. Duan et al. (2021) investigated the subject in Denmark by collecting 5,657 freshwater snails in 2019. A prevalence of trematodes of 12.6% was obtained highlighting the diversity in different countries. Interestingly, a paper published in Canada showed the closest match to the prevalence found in this study: McPhail et al. (2025) collected a total of 20,557 freshwater snails over the course of 3 years. Morphological examinations showed that 1,786 samples were infected, resulting in a prevalence of 8.69%. Molecular methods such as PCR using Cox1 and Nad1 primers identified 72 different species of trematodes belonging to the families Diplostomidae, Strigeidae, and Schistosomatidae to name a few.

The differences between the infection rates in *G. truncatula* and *R. labiata* and the prevalences found in freshwater snails in general could be explained by various factors. One explanation could be the difference in shell size of the species collected. While this study focused on *G. truncatula* and *R. labiata*, other studies mentioned predominantly finding *Lymnaea stagnalis* (Faltýnková, 2005; Faltýnková et al., 2007). Similar to the first two species, *L. stagnalis* is a freshwater snail that acts as intermediate host for trematodes (Kuroda and Abe, 2020). However, where *G. truncatula* and *R. labiata* stay small with the latter growing marginally bigger and reaching a maximum size of 20 mm (Yildirim et al., 2006), *L. stagnalis* can grow up to 50 mm (Kuroda

and Abe, 2020). This bigger shell size was shown to result in heavier infections (Brown et al., 1988; Graham, 2003) and could explain the presented difference in prevalences. Another explanation could be the natural fluctuation of trematode prevalences in snail populations from year to year. This phenomenon was described by Brown et al. (1988) who examined the infection rates of freshwater snail populations in three ponds in Indiana. Over the span of 4 years, it was found that the average prevalence of trematodes in snails across all ponds changed from 19.7% in 1982 to 12.2% in 1983, 22.2% in 1984, and 15.4% in 1985 highlighting the dynamic nature of trematode communities in snail populations.

Data on the prevalence of trematode infections in definitive hosts such as red or roe deer is not available. A comparison of the infection rates between the intermediate and definitive hosts was therefore not possible.

4.1.2. Prevalence of *Fascioloides magna* in freshwater snails

One snail was confirmed to be infected with *F. magna*, resulting in a prevalence of 0.056%. The infection rate of all snails collected in the Czech Republic, however was 1.616%. These results are comparable to numbers obtained in various studies. Kasny et al. (2012) investigated snails of the genera *G. truncatula* and *Radix* spp. in several locations of the Czech Republic and obtained the following results: Out of 1,100 *G. truncatula* individuals, 26.7% were infected with *F. magna*, while all 1,800 snails belonging to the genus *Radix* spp. tested negative. This resulted in a total prevalence of 0.084% of *F. magna* in the collected snails. A marginally higher number was documented two years later by Leontovyč et al. (2014). That study focused on the West of the Czech Republic and showed that 0.18% of the collected *Radix* spp. was infected with *F. magna*. However, out of 50 *G. truncatula*, 64% tested positive resulting in a total prevalence of 2.72% in all snails collected. A comparison of both studies showed overlapping areas in the West, indicating the permanent presence of the liver fluke in that area. Hörweg et al. (2011) investigated *G. truncatula* in the surroundings of the Danube in the East of

Austria starting in 2004. Over the course of one year, 10,000 snails were collected and a prevalence of 0.03% of *F. magna* in *G. truncatula* was determined. Three years later, Haider et al. (2012) focused on a smaller section of the previously examined area and found that the prevalence in *G. truncatula* had increased to 0.23%. This could imply that the region in the second study was more suitable for the snail species. Smith et al. (2024) examined the environments in which *G. truncatula* was previously detected and found that the species prefers shallow and slow-running waterbodies with temperatures between 10°C to 25°C. Conditions at the location examined by Haider et al. (2012) could be more favorable for the snails than in the bigger area examined by Hörweg et al. (2011), resulting in a higher abundance of the snail and therefore higher numbers of flukes as well. Another possibility for this increase of the *F. magna* prevalence in Austria could be that the fluke is adapting to the European conditions and its prevalence is therefore increasing over time. This seems especially likely as more and more countries are reporting infections in their deer populations for the first time (Halász et al., 2023; Popovici et al., 2024; Žele Vengušt et al., 2025).

In comparison, the prevalence of *F. magna* in definitive host populations is significantly higher. One of the older studies on the subject was done by Novobilský et al. (2007) who examined the red and fallow deer populations of 20 regions in the Czech Republic. Over the span of two years, 52 livers and 30 fecal samples were collected resulting in prevalences of *F. magna* ranging from 4 to 95%. Ursprung and Prosl (2011) investigated the red deer population in Austria by collecting both livers and fecal samples of red deer and found a prevalence of *F. magna* of 46.7%. Compared to this, the infection rate in Germany appears to be even lower, as shown by Sommer et al. (2022). Starting in 2019 and resuming the investigation in 2020, a total of 700 livers of game animals was collected resulting in a prevalence of 5.9% of *F. magna* in specimens examined. The different numbers between the three countries also further support the theory of an introduction into Europe through the Czech Republic (Králová-Hromadová et al., 2011). This is especially true as the higher infection rate in deer in the Czech Republic point to a longer history of

the fluke in it and could indicate that it had more time to adapt to the new environment and therefore grow in numbers.

4.1.3. Prevalence of *Echinostoma revolutum*

In this study, two samples collected in Germany tested positive for *E. revolutum*, a species that belongs to the family of Echinostomatidae and is known to infect animals and humans alike (Toledo and Esteban, 2015). Similar to *F. magna*, *E. revolutum* depends on intermediate hosts such as mollusks to complete its life cycle (Chai and Jung, 2022). It infests the intestines and bile ducts of various animals such as birds and rodents (Parija & Chaudhury, 2024), causing internal bleeding and digestive problems (Chai, 2009). The species was originally discovered and described in 1798 in Germany, where adult flukes were first found in the large intestines of wild ducks (Kanev, 1994 in Chai et al., 2020). The first human infection with *E. revolutum* however, was documented in 1929 in Taiwan (Anazawa, 1929 in Chai et al., 2020), followed by cases in other parts of China (Mao, 1991 in Chai et al., 2020) and Thailand (Radomyos et al., 1994 in Chai et al., 2020). Despite its global distribution (Chai and Jung, 2022), infections with *E. revolutum* are most commonly found in Asia (Toledo and Esteban, 2015). This could be linked to the differences in infection rates of intermediate host snails. Selbach et al. (2020) collected freshwater snails in Germany and determined a prevalence of *E. revolutum* of 2.2%. A comparable result of 2.7% was obtained by Faltýnková (2005) when investigating 2,802 freshwater snails in the Czech Republic. In contrast, numbers in Asia are higher. Li et al. (2022) examined 8,238 freshwater snails in China specifically for their *E. revolutum* infection rate and found a prevalence of 13%. This significant difference to the numbers from European countries highlights the impact that *E. revolutum* has in Asia. This is further supported by the only study done on humans. In 2007, stool samples from 471 children from different schools in Cambodia were tested. The prevalences ranged from 7.5% to 22.4% (Sohn et al., 2011). However, despite these differences in infection rates, it seems likely that the cause of the higher prevalences in Asia comes from eating habits. This seems especially true as *E. revolutum* is known to be transmitted through the consumption of raw

intermediate hosts (Hu et al., 2024). A practice that is common in various areas in Asia (Chai and Jung, 2022) leading to more opportunities to contract an infection (Toledo et al., 2011).

In addition to identifying samples infected with *E. revolutum*, two samples collected in Germany tested positive for *Orchipedum tracheicola* which infests the trachea of water birds (Webster, 1959 in Palandačić et al., 2024). Another sample collected at a different sampling site in Germany however, tested positive for *Opisthioglyphe ranae* which is usually found in the intestines of frogs and uses snails such as *L. stagnalis* as intermediate host (Tepe and Yilan, 2021).

4.2. *Radix labiata* as host for *Fascioloides magna*

One sample in this study tested positive for *F. magna*. Coincidentally, the snail species of the infected specimen was determined to be *R. labiata*, an unexpected result, as *G. truncatula* is considered to be the more common intermediate host (Králová-Hromadová et al., 2011) in Europe. Consequently, most studies on the topic focus entirely on *G. truncatula* and do not consider *Radix* spp. as a host for *F. magna* resulting in a lack in data. This is especially surprising as there are studies showcasing the suitability of the snail as such. Cshivcsik et al. (2023) noted that *R. labiata* might have a lower efficacy in the production of cercaria, a statement that is also supported by Leontovyč et al. (2014). However, *R. labiata* also shows a higher abundance (Cshivcsik et al., 2023) and thrives in conditions that are suboptimal for *G. truncatula* such as areas with acidic soil (Leontovyč et al., 2014). These properties could enable a further spread of the fluke, especially into areas previously not accessible due to limitations of *G. truncatula*.

The ability of *Radix* spp. to not only get infected with *F. magna* but also to provide an environment in which the development of *F. magna* can be completed was proven by 2006 (Faltýnková et al., 2006). The paper did not only provide experimental results but also discussed the first discovery of an infected *R. labiata* in nature. This susceptibility of the genus *Radix* to *F. magna*

infections was also tested by Huňová et al. (2012), who collected snails in the Czech Republic to then perform infection experiments. The snails were infected with *F. magna* by incubating them with *F. magna* eggs isolated from the livers of infested final hosts, achieving a prevalence of *F. magna* of 5%. A number that is considerably higher than the prevalence of 0.18% provided by Leontovyč et al. (2014), who collected 1,155 snails of the genus *Radix* spp. in the Czech Republic. Out of these, two samples tested positive for *F. magna*, introducing the first paper to provide tangible numbers for snails collected in nature. This finding is also comparable to the prevalence of 0.09% presented in this study and highlights the importance of *R. labiata* in the distribution of *F. magna* in Europe.

4.3. Identification of the *Fascioloides magna* haplotype

4.3.1. Comparison of the obtained sequence to CO1-Ha5

One individual redia of *F. magna* was isolated from a snail from the Sumava National Park and examined for its Cox1 haplotype. The sequence was compared to reference sequences of *F. magna* haplotypes from GenBank, revealing the highest sequence similarity of 438/439 base pairs with a sequence obtained from adult *F. magna* flukes from the livers of red deer in the west of the Czech Republic (GenBank number: GU599870.1) (Radvánský et al., 2011) and belonging to Cox1 haplotype 5 (CO1-Ha5). The same sequence similarity was found with a strain obtained from adult liver flukes from red deer in the Czech Republic (GenBank number: EF534997.1). Although not assigned to any haplotype, its sequence similarity to GU599870.1 and its localization in the west of the country suggest an affiliation to Cox1 haplotype 5 (CO1-Ha5). This high similarity between the newly obtained sequence in this study and Cox1 haplotype 5 suggests that the *F. magna* population, of which an isolate was obtained in this study was not recently introduced, but rather derives from a population already endemic in the Czech Republic. However, determining its exact origin proves difficult as several areas in the country are known to be endemic for *F. magna*. Erhardová-Kotrlá (1971) (cited in Novobilský et al., 2007) was the first to determine four areas

located in the west of the Czech Republic in the surrounding area of Prague as endemic for *F. magna*. Decades later, Novobilský et al. (2007) found more endemic regions west of the already established ones after collecting fresh faeces and examining livers of fallow and red deer. The results indicated a spreading of *F. magna* toward the German border. A theory that was later supported by Radvánský et al. (2011), who examined the livers of red deer in the Bohemian Forest and found even closer movement toward the border. This theory seems verified by the findings in this study that place the fluke found in the Czech Republic in close proximity to the German border. In summary, the results demonstrate an affiliation of the isolated *F. magna* to Cox1 haplotype 5 (CO1-Ha5), and therefore fit well to previous findings of *F. magna* in the central west area of the Czech Republic.

4.3.2. Comparison of the obtained sequence to haplotypes in Italy and Canada

Compared to the sequence similarities of the newly obtained sequence to haplotypes from Central Europe, the similarities to haplotypes from Italy and Canada are lower. Sequencing showed 97% (428/439 bp) sequence similarity to a sequence obtained from *F. magna* sampled in La Mandria in Italy (GenBank number: GU599860.1) belonging to Cox1 haplotype 1 which can be found in Italy and parts of North America. This result is supported by Králová-Hromadová et al. (2011) and Juhásová et al. (2016), who found no close genetic relationship between the haplotypes in Italy and the ones in Central Europe. This clear differentiation between the populations of these locations supports the theory proposed by Králová-Hromadová et al. (2011) that the populations of these two hot spots in Europe originated from different areas in North America and that they did not come into contact with each other. This assumption is further backed by the following result: The sequence similarity to a sequence sampled in Canada and belonging to Cox1 haplotype 6 was even lower with a similarity of 427/439 base pairs (GenBank number: GU599871.1). This suggests a closer relationship between the populations in Canada and Italy, indicating that the Italian populations might have originated

in Canada. It also shows that the introduction to the Czech Republic most probably occurred directly from Northern America and not from Italy. Altogether, these results highlight the distant relationship between the haplotypes in the Czech Republic and ones found in Italy and Canada, and provides more verification to the theory of several introductions of *F. magna* to Europe as mentioned by Bazsalovicsová et al. (2015).

4.4. Epidemiological importance of the findings

In this study, snails infected with flukes of both, veterinary and medical importance were identified. *F. magna* belongs to the first group and is known to drastically impact the health of game animals (Konjević et al., 2021) and livestock (Foreyt, 1990) while not causing infections in humans. It is already established in Europe and was recently identified for the first time in new countries such as Serbia, Romania, and Slovenia (Popovici et al., 2024; Trailović et al., 2016; Žele Vengušt et al., 2025), indicating a constant spread of the fluke in Europe. Related species with medical importance are *F. hepatica* and *F. gigantica* which have been proven to cause fasciolosis in humans. With the highest prevalences found in countries such as Bolivia, China or Spain (Andrews et al., 2021) it is estimated that up to 17 million people might be infected worldwide (Mas-Coma et al., 2019). It should be also noted, that Boray and Enigk (1964) (cited in Andrews et al., 2021) observed a correlation between the surrounding temperature and the survival rate of metacercariae. They experimentally proved that infectivity of *F. hepatica* metacercariae is lost at -20°C which in connection with global temperatures rising (Hansen et al., 2023) and the resulting milder winters could lead to higher infection rates in humans worldwide.

The rising temperatures (Hansen et al., 2023) could also have a big impact on *E. revolutum* which is known to infect both animals and humans. It has a global distribution with prevalences being highest in Asia, while the infection rate in the European population are low (Toledo & Esteban, 2015). This difference in prevalences does not stem from a lack of intermediate hosts or parasites in Europe as both are abundant on the continent (Georgieva et al., 2014), but is

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likely linked to differences in eating habits (Toledo et al., 2011). In this case, higher temperatures could result in faster development of *E. revolutum* (Marcogliese, 2001). This could lead to higher prevalences of the fluke in the environment which in turn could increase infection rates of *E. revolutum* in the population of already endemic areas.

5. CONCLUSION

To conclude, this study focused on the liver fluke *Fascioloides magna* in the Bayerischer Wald and Sumava National Parks in Germany and the Czech Republic and confirmed its presence in the area. Overall, out of all 1,794 samples (1,098 *Radix* spp. and 696 *Galba truncatula*) examined, one specimen of *Radix labiata* from the Czech Republic tested positive for the fluke resulting in a prevalence of 0.056%. The infection rate of the samples with at least one trematode infection was 6.05% and the presence of the zoonotic human pathogen *Echinostoma revolutum* in the National Parks was confirmed as well. Interestingly, the sample infected with *F. magna* was identified as *R. labiata*, providing one of the few pieces of evidence that this snail species can act as natural intermediate host for the fluke. Finally, a fragment of the CO1 gene of the *F. magna* isolate was sequenced and comparison to reference sequences from the GenBank showed the highest sequence similarity (99.74%) to two Cox1 haplotype 5 (CO1-Ha5) sequences obtained from red deer in the Bohemian Forest and Mirosov in the Czech Republic. This suggests that this lineage of *F. magna* was not recently introduced into the country but belongs to the population already established in the West of the Czech Republic. This also supports the theory that the liver fluke is still adapting to the environment in Europe and will continue to spread further.

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