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# Molecular validation of species determination of larval trematodes from freshwater snail hosts in Austria, with special emphasis on the genus *Trichobilharzia* Skrjabin & Zakharow, 1920

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**Abstract:** Digenean trematodes are parasites of vertebrates using obligatory molluscan intermediate hosts for their development. The infective larval stages (cercariae) of the "avian schistosomes" of the trematode family Schistosomatidae may cause dermatitis even in aberrant hosts, including humans. For Austria, larval findings have been reported repeatedly, but most records of schistosomes in Austria have not been identified to the species level. Thus, the species spectrum is not well documented yet. To facilitate the determination of larval trematodes, molecular genetic methods provide a valuable alternative to intricate morphological studies or time-consuming infection experiments to obtain adults. In this study, freshwater snails of the species *Lymnaea stagnalis*, *Planorbis planorbis* and *Planorbarius corneus* from locations in eastern Austria were screened for trematode infections with a focus on avian schistosomes. DNA-based protocols for validation of trematodes in general and of *Trichobilharzia* species in particular were developed. Besides the detection of several other trematodes, the occurrence of *Trichobilharzia szidati* in *Lymnaea stagnalis* and of *Bilharziella polonica* in *Planorbarius corneus* was confirmed. This is the first evidence of these species by DNA-based methods in Austria.

**Keywords:** Avian schistosomes, cercarial dermatitis, molecular genetic identifiction, *Trichobilharzia szidati*, *Bilharziella polonica*

**Zusammenfassung:** Digene Trematoden sind Parasiten von Wirbeltieren, die für ihren Entwicklungszyklus Mollusken als erste Zwischenwirte benötigen. Die Infektionslarven (Zerkarien) von „Vogelbilharzien", Trematoden aus der Familie Schistosomatidae, können im Fehlwirt Mensch Dermatitis verursachen. In Österreich wurden bisher Funde von Schistosomatiden-Zerkarien in den Überträgerschnecken aufgrund der schwierigen Bestimmung der Larven meist nicht verlässlich auf Artniveau bestimmt. Daher sind Artenspektrum und Verbreitung der Arten nur mangelhaft dokumentiert. Um die Bestimmung der Zerkarien zu erleichtern, bieten molekulargenetische Methoden eine interessante Alternative, die komplizierte morphologische Untersuchungen und zeitaufwändige Infektionsversuche zur Erlangung adulter Würmer für die Artbestimmung, ersetzen kann. In der vorliegenden Studie wurden Süßwasser-Schnecken der Arten *Lymnaea stagnalis*, *Planorbis planorbis* und *Planorbarius corneus* von Fundorten im Osten Österreichs auf das Vorkommen von Trematoden, mit Augenmerk auf „Vogelbilharzien", untersucht. Protokolle für den DNA-Nachweis von Trematoden im Allgemeinen und für Arten der Gattung *Trichobilharzia* im Speziellen wurden entwickelt. Neben dem Vorkommen unterschiedlicher Trematoden in den untersuchten Schnecken wurden die Arten *Trichobilharzia szidati* in *Lymnaea stagnalis* und *Bilharziella polonica* in *Planorbarius corneus* nachgewiesen. Dies ist der erste Nachweis dieser beiden Arten mit molekulargenetischen Methoden in Österreich.

**Schlüsselwörter:** Vogelbilharzien, Zerkariendermatitis, molekulargenetischer Nachweis, *Trichobilharzia szidati*, *Bilharziella polonica*

## Introduction

Digenean trematodes (= Digenea) are parasitic flatworms of animals (including humans). During their life cycles digeneans use two, three, four or more hosts, one being the definitive host, and the remaining being intermediate or paratenic hosts (Cheng 1986). Molluscs are serving as obligate first intermediate hosts, where the digeneans reproduce asexually (Kearn 1998). Infective stages, the so-called cercariae, are finally released by the mollusc and infest other hosts. Sexual reproduction takes place in the vertebrate final hosts. Many digenean trematode species are of medical and/ or veterinary importance. Particularly species of the family Schistosomatidae may cause severe diseases in birds and mammals, including humans. The family is characterized by a two-host-life cycle with a freely swimming miracidium, which invades the snail host where sporocysts and cercariae are produced. The ma-

ture cercariae are characterized by a bifurcated tail. They subsequently leave the snail host as free-living infectious stages that actively penetrate the skin of the definitive (final) hosts (Cheng 1986). "Bird schistosomes" or "avian schistosomes" belong to the family Schistosomatidae and live as adult endoparasites in the blood vessels of birds. Their cercariae may penetrate also aberrant hosts (including humans) and cause cercarial dermatitis (swimmer's itch), an inflammatory skin disease. Overviews of cercarial dermatitis outbreaks and schistosome species in Europe have been provided e.g. by Horák & Kolářová (1997), Soldánová et al. (2013), Horák et al. (2015).

Until now, there have been only few studies on the occurrence and distribution of these trematodes in Austria. First evidence of schistosomes causing dermatitis in humans were given by Graefe (1971) and Graefe et al. (1973). Further records and surveys were published by Dvořák et al. (1999), Auer & Aspöck (2002), Sattmann et al. (2004), Hörweg et al. (2006) and Reier et al. (2020). Cases of "swimmer's itch" caused by cercariae have been recorded from almost all Austrian federal states (Auer & Aspöck 2002, 2014). However, for Austria, not much information is available on the schistosome species spectrum (Reier et al. 2020). One reason might be that the larval stages in (or released by) intermediate hosts are tiny and often morphologically indistinct and partly not identifiable at the species level by microscopy. Reliable identification by life cycle experiments have confirmed the occurrence of *Trichobilharzia szidati* Neuhaus, 1952 in *Lymnaea stagnalis* (Linnaeus, 1758). Adults had been obtained from infected ducks and identified accordingly (Dvořák et al. 1999). A recent integrative morphological and molecular study of cercariae from *Radix auricularia* (Linnaeus, 1758) confirmed the first finding of *Trichobilharzia franki* Müller & Kimmig, 1994 in Austria (Reier et al. 2020).

The aim of the current study was to investigate particular aquatic snail species in the area of the river Leitha, in Lower Austria and Burgenland and to evaluate their current infection rates with digenean trematodes. Trematodes in focus of this study were – among others – species of the family Schistosomatidae. Snail species investigated were of the families Lymnaeidae and Planorbidae, as some of these species are known to frequently host schistosomes, particularly of the genera *Trichobilharzia* Skrjabin & Zakharow, 1920 and *Bilharziella* Looss, 1899 in Europe (Horák & Kolářová 1997, Soldánová et al. 2013, Horák et al. 2015). Since species delimitation based on morphological characters has proven to be delicate, molecular biological methods have been involved in this study to differentiate and identify species. The aim of this study was (1) to implement a protocol for DNA-based validation of trematodes in general, and (2) to identify *Trichobilharzia* from the intermediate host on species level. This manuscript is based on a master thesis of the first author (Gaub 2014) and presents parts of the data obtained.

## Material and methods

### Collection of the intermediate host snail

In total, 339 *Lymnaea stagnalis*, 63 *Planorbis planorbis* (Linnaeus, 1758) and 1 *Planorbarius corneus* (Linnaeus, 1758) were collected during 16 field trips from March to November 2012 in the floodplains of the river Leitha, between Götzendorf and Potzneusiedl, in Maria Ellend at the river Fischa, at Orth/Donau, and at St. Martin/Zicksee; at 31 locations in total (Fig. 1).

Depending on the weather conditions, every two weeks, 5 to 10 locations were sampled by 1 to 4 people. The field trips usually took 5 to 8 hours, spending 15 to 50 min on every single location. The different locations were characterized as river meanders, puddles or ponds.

The snails were picked up manually and were transported to the laboratory in containers with some water. In the laboratory, the snails (403 individuals in total) were placed at room temperature, each individual separately in a glass (250 ml) with water, close to a window for cercarial release experiments. Snails were kept and observed for at least 24 hours.

### Parasitological examinations

Cercariae released from snails (70 individuals) were classified by light-microscopy (Axioscope, Carl Zeiss, Austria) taxonomically to genus or family level, but in some cases only assigned to "cercarial types" (e.g. xiphidiocercariae) following the identification keys of Mikeš et al. (2001) and Faltýnková et al. (2007, 2008) and documented by microphotography (Nikon Eclipse E800 with attached Nikon DS-Fi2 camera, Optoteam Austria). In parallel, infected snails were dissected to obtain larval stages from the interior of the snails. For dissection, each snail was placed separately into a petri dish filled with water and then examined under a Wild/Leica M3Z stereomicroscope (Leica Mikrosysteme, Austria). The respective snail was fixed through the use of a forceps. In order to dissect the inner organs of the snail, the shell was broken and the soft body, especially the digestive organs, were cut into small pieces.

After morphological investigation, single released cercariae and the infected parts of the snails (mostly the midgut gland) were stored individually in 80 % EtOH in microtubes (1.5 to 2 ml) at -20 °C until use for molecular analyses.

### Molecular genetic analyses

**DNA isolation.** Total DNA was isolated from all prepared snail tissues as well as from selected cercariae, which were collected from all cercariae-releasing snails. For DNA extraction, 10 to 20 mg of the 70 midgut glands were cut into small pieces with a sterile razor blade and transferred individually into 1.5 ml microtubes. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany)

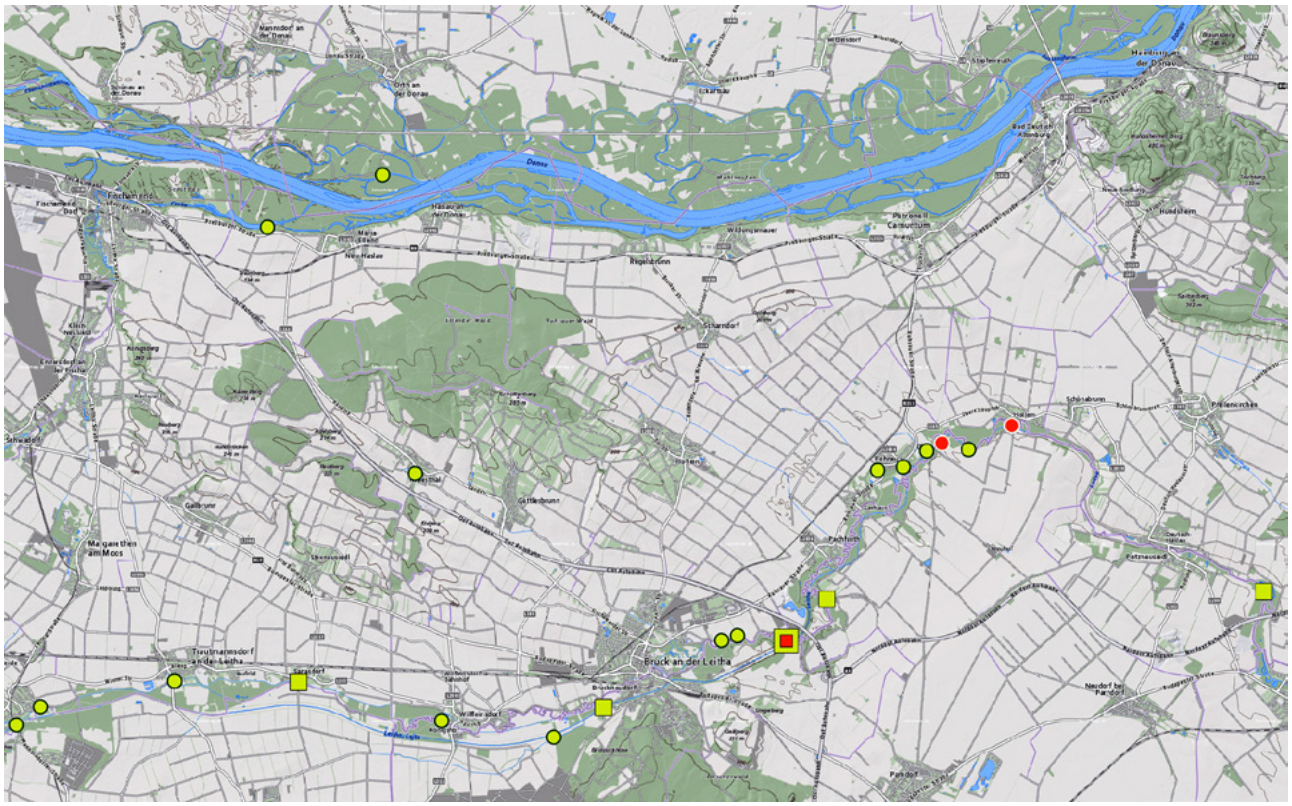

Fig. 1: Map of the sampled area. Sampling sites with infected snails mentioned in this study are marked with orange. Yellow dots indicate sites with other snail findings or with no infected snails. Squares are used for overlapping sampling sites. The location St. Martin/Zicksee lies outside (south) of the presented area. Map background © Geoland Basemap.

strictly following the protocol for tissue samples, with prior proteinase K digestion overnight. DNA extraction of the 70 cercariae was conducted as just described by digesting one entire cercaria per sample. The isolated DNA was stored at -20 °C until further use.

**PCRs**. As a proof of principle, infested tissues of all 70 snails releasing cercariae were screened by a universal trematode PCR targeting the nuclear *18S* rRNA gene (Haider et al. 2012). All of the 66 infested *L. stagnalis* were further screened by a PCR specific for the genus *Trichobilharzia*, which was designed for PCR-based detection of trichobilharzian infection in snails and amplifies the genomic region *ToSAU3A* (Korsunenko et al. 2010).

For species identification, primers were chosen, that amplify the region including internal transcribed spacer 1 (*ITS1*), the *5.8S* rRNA gene and the *ITS2*: its5Trem, which is complementary to the conserved region at the 3' end of the *18S* rRNA gene, and its4Trem, which is complementary to the conserved region at the 5' end of the *28S* rRNA gene (Dvořák et al. 2002). This PCR was run with all samples that were positive in the PCR specific for the genus *Trichobilharzia* and with all cercariae that morphologically had been identified as furcocercariae. Details for all primers used in this study are given in Table 1.

All PCR reactions were run in 0.2 ml soft PCR tubes in a nexus Mastercycler (Eppendorf, Hamburg, Germany). Each sample was tested in three different dilutions (1 µl, 3 µl, 6 µl of DNA per 50 µl reaction volume). The master mix contained of 5 µl PCR buffer (Solis BIODYNE), 5 µl MgCl2 (25 mM), 1 µl dNTP-Mix (20 mM of each, Solis BIODYNE), 11 µl distilled water, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 0.25 µl polymerase (Hot Fire DNA Polymerase I, 5 U/µl, Solis BIODYNE). All PCRs were run with a standard PCR programme (15 min at 95 °C followed by 30 cycles of 1 min denaturation, 1 min annealing, 3 min extension) applying an annealing temperature of 56 °C for the universal trematode-specific PCR and of 54 °C for the other two PCRs. Amplicons were visualized in 2 % agarose gels using GelRed (GeneOn GmbH, Germany).

**DNA sequencing**. All bands of the appropriate sizes were cut out and prepared for DNA sequencing. In brief, amplicons were purified from the gels with the Xact DNA Gel extraction Kit (genXpress, Austria) and sequenced with an automated ABI PRISM 310 Sequencer (PE Applied Biosystems, Langen, Germany). Sequences were obtained from both strands in two independent set-ups. Consensus sequences, excluding the primer regions, were used for multiple and pairwise alignments. Local alignments were performed with BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/) and EMBOSS (European Molecular Biology Open Software Suite, http://www.ebi.ac.uk/Tools/emboss/) to find the most similar reference

Table 1: Details of the PCR primers used in this study.

| Name | Sequence (5´- 3´) | Approximate amplicon length | Specificity | Reference |
|---|---|---|---|---|
| TremF | GGT TCC TTA GAT CGT ACA TGC | 430 bp | Trematodes | Haider et al. 2012 |
| TremR | GTA CTC ATT CGA TTA CGG AGC | | | |
| TR98F | CTC CGA CTG ATG ATG ACA AGA AGA | 400 bp | *Trichobilharzia* | Korsunenko et al. 2010 |
| TR98R | ATG AGT GGC GAA CGG TAT CCT | | | |
| its5Trem | GGA AGT AAA AGT CGT AAC AAG G | 1915 bp (*T. regenti*)<br>1330 bp (*T. szidati*) | Trematodes (amplicon length differs by species) | Dvořák et al. 2002 |
| its4Trem | TCC TCC GCT TAT TGA TAT GC | 1555 bp (*T. franki*) | | |

sequences. In order to be able to identify single nucleotide polymorphisms (SNPs), the reference groups and the sequences of the amplicons obtained with the respective primer pairs (TremF/TremR; TR98F/TR98R; its5Trem/its4Trem) were aligned using Clustal X and GeneDoc (Nicholas and Nicholas 1997).

Sequencing data obtained in this study was submitted to GenBank and is available under the following accession numbers (*T.s.* MW143564-65, *B.p.* MW144289).

## Results

### Microscopy

Of the altogether 403 snails investigated, 70 snails (i.e. 66 *L. stagnalis*, three *P. planorbis*, one *P. corneus*) released cercariae. Microscopical investigation of the cercariae released gave the following results, summarized in Table 2. Out of the 66 *L. stagnalis*, 45 individuals released xiphidiocercariae (cercariae with an oral stylet) (prevalence 13.3 %), six individuals released cercariae of the family Echinostomatidae (cercariae with an oral corona of hooks) (1.8 %) and seven individuals released cercariae of the family Notocotylidae (monostome cercariae) (2.1 %). Moreover, eight *L. stagnalis* were found to release furcocercariae (with a bifurcated tail). Out of these, two simultaneously released xiphidiocercariae. Six samples of furcocercariae were ocellate and were classified morphologically as *Trichobilharzia* sp. (1.8 %) (Fig. 2). Two of the furcocercariae were non-ocellate and were identified morphologically as diplostomids (0.6 %).


Fig. 2: Microscopic images of cercariae found. a: *Trichobilharzia* sp., b: double infection with xiphidiocercariae and *Trichobilharzia* sp.

Out of the three infected individuals of *P. planorbis*, two released xiphidiocercariae (3.2 %) and one released cercariae of the family Echinostomatidae (1.6 %).

The single individual of *P. corneus* was found to release schistosomatid furcocercariae, which we classified as *Bilharziella polonica* (Kowalewski, 1895).

### Molecular biology

The extracted tissues of all 70 snails that had been found to release cercariae were also positive in the PCR using the universal trematode primers (Haider et al. 2012). The 66 midgut gland samples of *L. stagnalis* were further screened by the PCR specific for *Trichobilharzia* spp. (Korsunenko et al. 2010) revealing nine positive samples, which is 3 more than detected by morphological investigation of the released cercariae.

Of the released cercariae, all nine furcocercariae were investigated by the PCR specific for the genus *Trichobilharzia* (Korsunenko et al. 2010). Altogether, six of the nine furcocercariae were positive for *Trichobilharzia* sp.

For species identification, all nine furcocercariae as well as the tissue from their host snails' midgut glands were further investigated with the primer pair its5Trem/its4Trem (Dvořák et al. 2002). Six of the nine investigated furcocercariae and seven out of the nine investigated midgut samples produced amplicons in this PCR. DNA sequencing of the amplicons obtained from the *L. stagnalis*-derived samples revealed *T. szidati* in all cases that gave reliable sequences (3). Two full length DNA sequences (GenBank Acc. MW143564-65) could be obtained, the hosting snails were both from the same location, a small pond in the

Table 2: Number of gastropods infected with digeneans of different types. Abbreviations: Xiph = xiphidiocercariae; Ech = echinostomatids; Not = notocotylids; Fur = furcocercariae; Dipl = diplostomids; Trich = *Trichobilharzia* sp.; Bil = *Bilharziella polonica*; prev.% = prevalence in %.

| Gastropods | studied (n) | infected (n)/ prev.% | Xiph/ prev.% | Ech/ prev.% | Not/ prev.% | Fur/ prev.% | Dipl/ prev.% | Trich/ prev.% | Bil |
|---|---|---|---|---|---|---|---|---|---|
| *Lymnaea stagnalis* | 339 | 66 /19.5 | 45/13.3 | 6/1.8 | 7/2.1 | 8/2.4 | 2/0.6 | 6/1.8 | - |
| *Planorbis planorbis* | 63 | 3/4.8 | 2/3.2 | 1/1.6 | - | - | - | - | - |
| *Planorbarius corneus* | 1 | 1/100 | - | - | - | 1 | - | - | 1 |
| **Total** | **403** | **70/17.4** | **47** | **7** | **7** | **9** | **2** | **6** | **1** |

floodplains of the river Leitha, that had the overall highest densities of *L. stagnalis*. The two sequences were both 1287 bp long, they were 100 % identical to one another and showed highest sequence similarity, namely 1 bp difference each to sequences of *T. szidati* from Czech Republic (GU233735) and Finland (FJ609409), respectively. One sample could only be identified as *Trichobilharzia* sp., because the obtained sequence was too short for species designation.

For the amplicon obtained from the *P. corneus*-derived sample, a 690 bp long fragment (GenBank Acc. MW144289) containing the *ITS1* region could be obtained. The sequence shows highest identity, namely 3 bp difference, to the sequence of a *B. polonica* isolate from Russia (MK264353).

In summary, 70 trematode infections were detected in 403 snails investigated, including also larval stages of *Trichobilharzia szidati,* an un-identified *Trichobilharzia sp.* and of *Bilharziella polonica*. Moreover, larval stages of the families Echinostomatidae and Notocotylidae and not further determined furcocercariae and xiphidiocercariae were found. All 3 *Trichobilharzia* samples that could be identified down to the species level were identified as *T. szidati*, with a prevalence in *L. stagnalis* of 0.89 % (3/339). A detailed list is given in Table 2.

## Discussion

A total of 339 *L. stagnalis*, 63 *P. planorbis* and one *P. corneus* were subjected to cercarial release experiments. 66 individuals of *L. stagnalis* (prevalence 19.47 %), three *P. planorbis* (4.76 %) and one *P. corneus* (100 %) expelled cercariae, whereby *L. stagnalis* was the host snail with the highest trematode diversity. The frequent findings of cercariae of the xiphidiocercariae-type were not identified further but much likely belong to the family Plagiorchidae. Two findings of furcocercariae were morphologically identified as representatives of the family Diplostomidae but could not be identified to the genus level. Furthermore, cercariae of the families Echinostomatidae, Notocotylidae and Schistosomatidae were recorded. Among the Schistosomatidae, *Bilharziella polonica* was identified morphologically and confirmed by DNA sequencing from the only *P. corneus* individual found during this study, whereas *Trichobilharzia szidati* was confirmed by molecular analyses from altogether three of the 339 *L. stagnalis* investigated in this study. Unfortunately, not all samples that were positive in the PCR specific for *Trichobilharzia* spp. also gave amplicons in the PCR allowing species differentiation within the genus *Trichobilharzia*. This might be due to the much higher amplicon length of the latter PCR (between 1330 and 1914 bp as compared to 400 bp), which significantly reduces sensitivity.

Overall, the infection rates of the current study are comparable to those of previous cercarial screenings from eastern Austria (Konecny et al. 1999, Dvorak et al. 1999). The total prevalence of trematodes in *L. stagnalis* in our study (19.47 %) is close to the average prevalence (23 %) of the combined data of 62 studies [(Kuris & Lafferty 1994) cit. in Loy & Haas 2001], but markedly higher than in Konecny et al. (1999) (10 %). A considerably higher prevalence of digenean trematodes (44.9 %) in *L. stagnalis* was reported from a pond system in southern Germany (Loy & Haas 2001). The higher infection rates may result from the high abundance and the diversity of vertebrate and invertebrate hosts in that area (Loy & Haas 2001).

It is known, that the lymnaeids represent the most frequent and widely distributed intermediate hosts for the genus *Trichobilharzia*. In the current study, a prevalence of 1.8 % for the genus *Trichobilharzia* and of 0.89 % for *T. szidati* was detected in *L. stagnalis*, which is similar to the prevalence for *T. szidati* reported by Dvořák et al. (1999), which was 1 %. Also, in the study by Loy & Haas (2001) the causative agents of cercarial dermatitis showed a constantly low prevalence (0.17 %) of "*T. ocellata"* (which is considered a synonym of *T. szidati*) in *L. stagnalis*. They assumed, that such a low prevalence is rather typical for areas where cercarial dermatitis occurs in humans (Loy and Haas 2001). Generally, in Europe, the prevalence of schistosomatids in snails seems to be rather low, while the infection rates in birds can be as high as 74.5 % [(Horák and Kolárová 2011; Horák et al. 2008) cit. in Soldánová et al. 2013]. Therefore, it has been suggested that human cercarial dermatitis is found most often along the major

avian migratory flyways (Kolárová 2007). In a study by Hörweg et al. (2006) they set up questionnaires to collect information on the waters and activities in the waters and details on the dermatitis itself. In their study, the duration of the exposure to water and the kind of activity had no statistically significant impact on the severity of disease, but it can be assumed, that longer stays in shallow water increase the probability of infection (Hörweg et al. 2006). In that study, they documented cases of cercarial dermatitis from May to August. In the current study, most infected snails with digenean trematodes, out of that two *Trichobilharzia szidati*, one *Trichobilharzia* sp. and one *B. polonica*, were found in June and August. August, in that year, was also the month with the lowest precipitation and the highest temperatures. It is known that higher temperatures enhance cercarial production [(Kendall and McCullough 1951) cit. in Mas-Coma et al. 2009]). It is still unknown, whether the risk for the mammalian hosts to be infected is higher for the genus *Trichobilharzia* or for the genus *Bilharziella*. In a study by Lichtenbergová & Horák (2012) it was postulated, that there is a higher health risk for mammals for infections with *T. regenti*, the nasal schistosome, than with visceral bird schistosomes (*T. franki*, *T. szidati*). In the current study, only *T. szidati* was found, but recently also *T. franki* was documented in Austria (Reier et al. 2020).

Altogether, a PCR-based approach enabled us to provide evidence for trematode infections in snails in general. Molecular genetic identification down to the species level was possible for *T. szidati* and *B. polonica*, corroborating that DNA sequencing is a valuable method for species delimitation, if good reference data are available. Trematode screenings are important to fill knowledge gaps. Only with reliable databases on parasite diversity and their distribution, epidemiological and ecological evaluations can be performed and reliable medical risk assessments become possible.

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