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Original paper

Molecular identification of tapeworms from Konik Polski horses from Biebrza National Park

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ABSTRACT. Tapeworm infections in Konik Polski horses from Biebrza National Park were investigated in this study. Faecal samples were collected 10 times: in 2012 – 1 time, in 2013 – 4 times, in 2014 – 4 times and in 2015 – 1 time. In total, 162 faecal samples were collected and tested. Faecal egg counts (FECs) method was used in the study. Positive samples with cestode eggs were noted only twice – in October 2012 and December 2013 in two adult mares (9 and 11 years old). The determined prevalence was surprisingly low comparing to other studies, 4.3% in October 2013 and 28.5% in December 2013. Parasite genomic DNA was isolated from posterior proglottids of tapeworms found in horse faeces after deworming, PCR technique was applied in order to determine the species of these tapeworms. No specific products were obtained using primers specific to rDNA sequence, whereas, a 620 bp fragment encoding mitochondrial cytochrome oxidase I (COI) was amplified and sequenced. Nucleotide sequence analysis revealed a 100% homology to a corresponding fragment form *Anoplocephala perfoliata* tapeworm originating from a horse from the Czech Republic. To our knowledge this is the first report applying molecular techniques to tapeworm identification in Konik Polski horses.

Keywords: Konik Polski, *Anoplocephala*, PCR

Introduction

The Konik Polski (*Equus caballus*) is one of the two Polish breeds of primitive horses. The goal of keeping this breed is preservation of the original features, such as resistance, low requirements as to the keeping conditions, gentleness, intelligence and endurance. Approximately 8% of Konik Polski population is kept in free ranging, with small human interference. The caretakers intervened only in the case of threat of horse`s life or the health necessity. Konik Polski horses have been introduced to Biebrza National Park in 2004 to 200 ha enclosure, to maintain the biodiversity and prevent the encroachment of undesired vegetation. Biebrza National Park provide breeding habitats for rare and threatened water-marsh birds which needs open spaces for living or nesting. This habitat embraces of marshlands, forests and grasslands where oribatid mites (Acari, Oribatida), serving as the intermediate host for tapeworms, might be commonly found. During a pastural infection, anoplocephalosis poses a threat of widespread infection throughout the herd.

Horses are known to be hosts of several species of endoparasites. Because of the prevalence and pathogenicity the most important are nematodes and tapeworms [1,2]. *Anoplocephala perfoliata* is most pathogenic tapeworm species posing the greatest threat to the horse, incriminated as a significant cause of clinical disease (e.g., ileocaecal intussusception, caeco-caecal intussusception and/or caecal perforation), particularly in horses chronically infected with large numbers of worms.

So far the parasitological infections among Konik Polski horses were investigated in nature

reserves in Popielno and Roztocze National Park [3,4]. Those studies employed coproscopic methods and microscopic evaluation of morphological features of eggs or adults. Detection of parasite species using molecular biology methods is more reliable and accurate. Using a single multiplex PCR reaction allows to determine the species of *Anoplocephala* spp. tapeworm infecting horses [5].

The present study was an attempt to determine the species of *Anoplocephala* spp. tapeworm using molecular biology methods. To the best of the authors' knowledge, this is the first study employing molecular biology techniques to determine the species of tapeworms in free ranging Konik Polski horses in Poland.

Materials and Methods

The Konik Polski family groups living in Biebrza National Park in semiferal conditions differed in herd size and counts 7 to 10 adult members with offspring. The parasite load of 62 Konik Polski horses living in Biebrza National Park was monitored, using faecal egg counts (FECs) as routine monitoring of parasitological status of each horse [6]. Between October 2012 and September 2015, a total of 162 faecal samples from horses were collected 10 times: October 2012 – 10 horses, February 2013 – 16 horses, May 2013 – 16 horses, July 2013 – 15 horses, December 2013 – 23 horses, January 2014 – 22 horses, February 2014 – 16 horses, April 2014 – 10 horses, May 2014 – 16 horses, September 2015 – 18 horses. Feacal samples were collected before deworming from the ground straight after defecation, which enable to attribute the sample to defined horse identified by means of body positioning of whorls and markings, freezemarking or microchip identification (all Konik Polski horses are mouse-coloured). Faecal samples, approximately 200 g, were stored at 4°C until transported to the laboratory and analyzed with modified sedimentation-flotation method [7]. Faecal egg counts were carried out for each horse using a modified McMaster technique with a minimum detection limit of 100 EPG. Parasite eggs in faecal samples (4 g) were floated using a saturated NaCl solution. The sample were examined under a light microscope for parasite eggs. Anthelmintic treatment was performed routinely in Biebrza National Park once per year, without parasitic diagnostics being performed. Horses were dewormed in late November/December with various drugs containing mostly a combination of ivermectin/moxidectin and praziquantel.

In order to determine the species of *Anoplocephala* spp. tapeworm molecular analysis of genomic DNA was performed. Using Genomic Mini (A&A Biotechnology) parasite genomic DNA was isolated from posterior proglotids of tapeworms found in horse faeces after deworming or collected during necropsy. Examined tapeworms came from the following horses: Konik Polski from Biebrza National Park (BNP1), Arabian horse from Służewiec racing track (WRT1) and from a horse which died during transport from Russia and was necropsied at the Department of Pathology and Veterinary Diagnostics (RUS1). Multiplex PCR was conducted according to the method previously described by Bohorquez [5]. This reaction enables the discrimination between three species of tapeworms *A. perfoliata*, *A. magna* and *Paranoplocephala mamillana.* Another sets of primers: F 5' GCTATGAAATTTATTTGTAC 3', R 5' AAAACT TTATCAAGAACACA 3' and F 5' GGTCTGTGAT GCCCTTAGATGTCCG 3' and R 5' TCACGGTAC TTGTTCGCTATCGGACT 3' were designed in order to amplify a 620 bp long fragment of mitochondrial cytochrome oxidase I gene (COI) and a 2073 bp long fragment of ribosomal DNA (rDNA), respectively. COI and rDNA fragments were amplified using Phusion™ High-Fidelity DNA Polymerase (ThermoScientific) according to manufacturer's instructions. PCR products were then cloned into pJET 1.2 blunt vector using CloneJet PCR Cloning Kit (ThermoScientific). Recombinant vectors were sequenced using a commercial service (Genomed). Nucleotide sequences were analyzed using BLAST and Multalin version 5.4.1 [8,9].

Results

In the present study *Anoplocephala* spp. eggs were found only in 1.9% of tested samples – 2 konik`s mares (9 and 11 years old) and one foal, in two among 10 time points: October 2012 (mare and foal) and December 2013 (11 year old mare). In 2013, on the next day after deworming, proglottids of tapeworms were found in 11 year old mare faeces.

In order to determine the species of tapeworms found in Konik Polski horses a multiplex PCR analysis was performed according. This reaction gave no positive results on the template of ITS2

Table 1. Results of BLAST analysis of cytochrome oxidase I nucleotide sequence amplified on the template isolated from BNP1 tapeworm

GenBank accession no	BLAST score	Identity
JQ771109	*Anoplocephala* sp. A2, COI	98%
KR054960	*Anoplocephala perfoliata*, DNA mitochondrial	91%
AY568206	*Anoplocephala magna* isolate N53, COII	89%
AB099690	*Anoplocephala perfoliata*, COI	88%

DNA from BNP1 sample (Fig. 1A). On contrary, *A. perfoliata* specific products of 250 bp were amplified on the WRT1 template (Fig. 1B) and specific *A. magna* product of 382 bp was obtained on RUS1 DNA template (Fig. 1C).

Therefore we decided to amplify a fragment of mitochondrial COI using all three templates. Nucleotide sequences of fragments amplified on BNP1 and WRT1 templates differed in only one nucleotide and showed 100% (BNP1) and 99.84% (WRT1) identity to *Anoplocephala* spp. COI sequence (JQ771109) and 90% to a corresponding fragment of *A. perfoliata* mitochondrial genome (KR054960) (Fig. 2, Tab. 1). The COI fragment amplified from RUS1 template showed the identity of 90.65% to *A. perfoliata* COI sequence (AY568189), 89.53% to a corresponding fragment of *A. magna* mitochondrial genome (KU236385) and 87.1% to *A. perfoliata* mitochondrial genome (KR054960).

Because these results gave no precise information on investigated tapeworm species we amplified a fragment of rDNA containing partial 18S sequence, ITS-1, 5.8S, ITS-2 complete sequences and 28S partial sequence. Again, as in the case of multiplex PCR, a 2072 bp fragment was amplified only on the template isolated from WRT1 and RUS1 tapeworms. BLAST analysis of WRT1 rDNA fragment showed 94.18%, 94.08% and 94.00% identities to *A. perfoliata* fragments (AJ578151, AJ578152 and AJ578153, respectively). BLATS analysis of RUS1 rDNA nucleotide sequence showed 94.08%, 94.03% and 93.95% identity to *A. perfoliata* sequences (AJ578151,

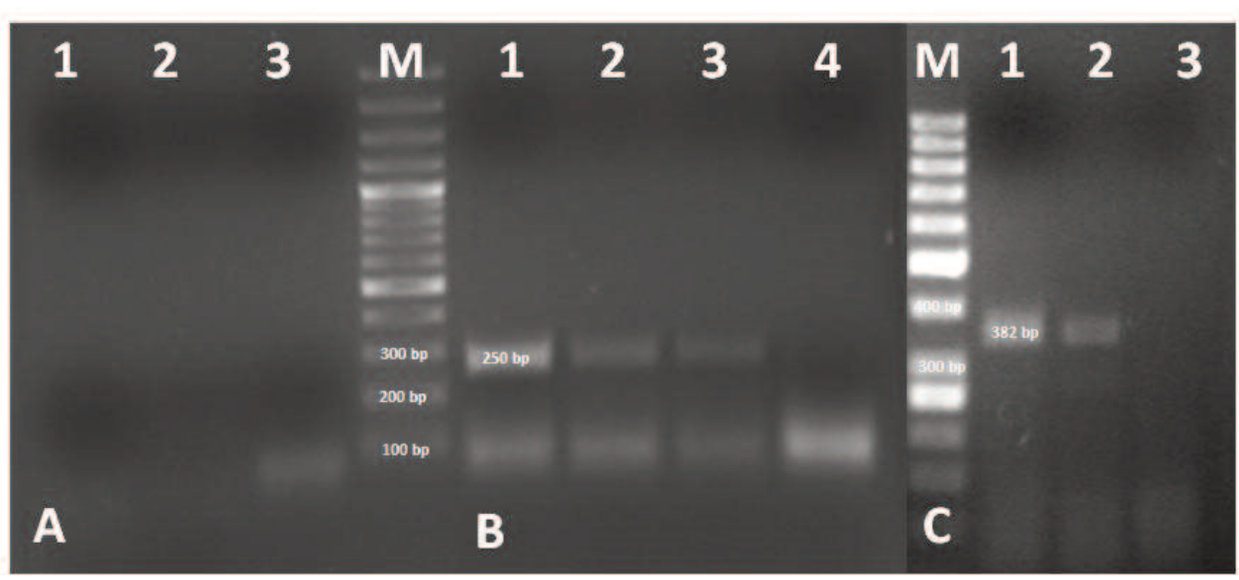

Figure 1. Multiplex PCR identification of Anoplocephalidae tapeworms [5]. Reactions were conducted on: A – BNP1 tapeworm DNA templates (1–2 – different DNA isolates, 3 – no template negative control, M – 100 bp molecular weight marker); B – *A. perfoliata* specific 250 bp products obtained on WRT1 template (1–3 – different isolates, 4 – no template negative control); C – *A. magna* specific 382 bp products obtained on RUS1 template (M – 50 bp molecular weight marker, 1–2 – different isolates, 3 – no template negative control)

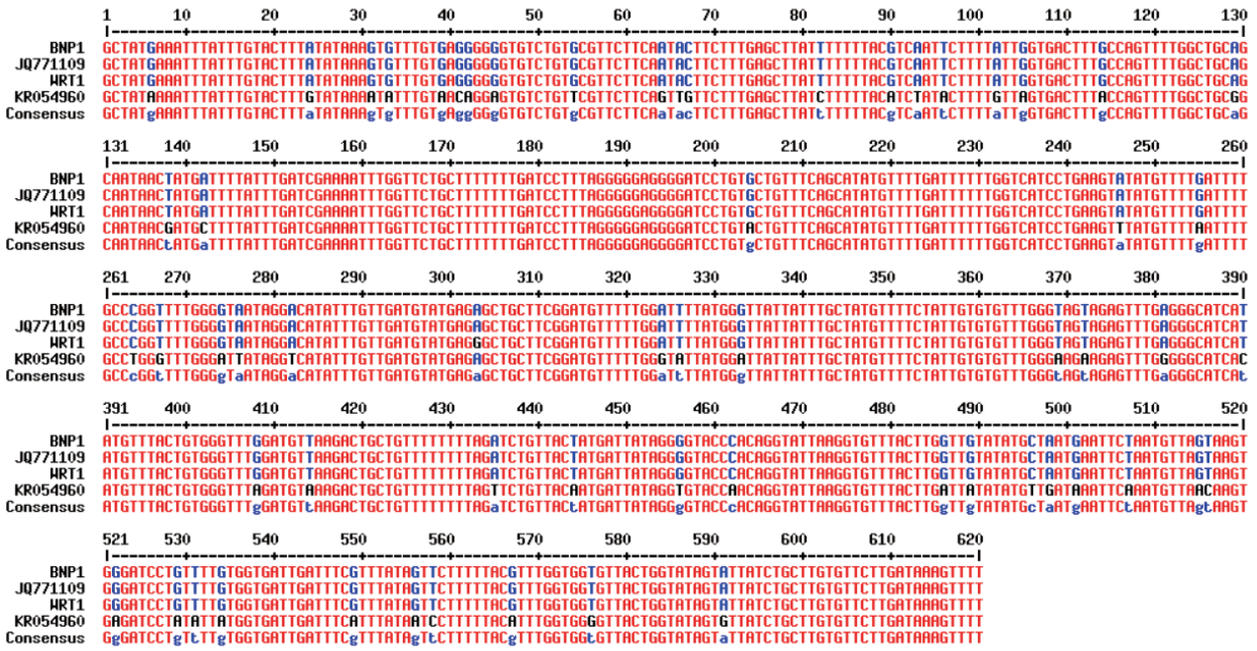

Figure 2. *Anoplocephala perfoliata* COI nucleotide sequence alignment. Alignment was performed using BNP1 and WRT1 amplified sequences as well as JQ771109 and KR054960 sequences from GenBank. Multialignment was performed using Multalin version 5.4.1 http://multalin.toulouse.inra.fr/multalin/

AJ578152 and AJ578153, respectively), 94.08%, 94.03% and 93.95% identity to *A. magna* rDNA (KF956074, KF956076, and KF956075, respectively) and 96.74% to *P. mamillana* (AJ578155). However, the analysis of phylogenetic trees constructed in BLAST showed that WRT1 tapeworm is the most closely related to *A. perfoliata* (Fig. 3) and RUS1 tapeworm to *Equinia mamillana* (syn. *P. mamillana*) (AJ578155).

As COI fragment amplified from BNP1 and WRT1 tapeworms were almost identical and rDNA analysis showed that WRT1 tapeworm belongs to *A. perfoliata* species, therefore, most probably BNP1 sample originates from *A. perfoliata* tapeworm.

COI nucleotide sequence amplified from BNP1 and WRT1 as well as rDNA sequences from WRT1 and RUS1 tapeworms were submitted to GenBank under the following accession numbers: MZ 576487, MZ 576488 and MZ 648229, MZ 648230.

Discussion

The first report about intestinal parasites infection in Konik Polski horses were conducted in sixties of the twentieth century, from over 50 years many different authors supplemented their results. A total of 38 species of helminths and five Diptera species were recorded from the Konik Polski horses [10]. In own study most often nematode eggs from the Strongylidae family were detected (100%). Mixed infection were reported (roundworms and small strongyles) only in young horses (2 female yearling and female-foal). Infection of *Oxyuris equi* in adult mare was reported only once. The achieved results do not differ with those obtained by other researchers [11–13]. Mixed infections were often remarked in the herd, eggs of Strongyloidae, roundworms, tapeworms and threadworm were found in the faecal samples. *Parascaris equorum* infections were observed mainly in foals and yearlings. This result is similar to the previous report of Kilani et al. [14]. In this study very low prevalence of tapeworm infection (1.9%) was observed. Infections with *A. perfoliata* and *P. equorum* were rarely reported also in the study of Hedberg-Alm et al. [15], and the data showed the same frequency for both parasites (1.4%, 1.0% cases, 1.7% controls). According to a study on coprological examination, it exhibits the highest diagnostic possibilities in late autumn and early winter [16]. In the previous study performed between October 2010 and September 2013, 284 faecal samples of horses originating from Lazio Region (Central Italy) were tested for the presence of *Anoplocephala* sp. eggs by a classic copro-microscopic technique [17]. *Anoplocephala* sp. eggs

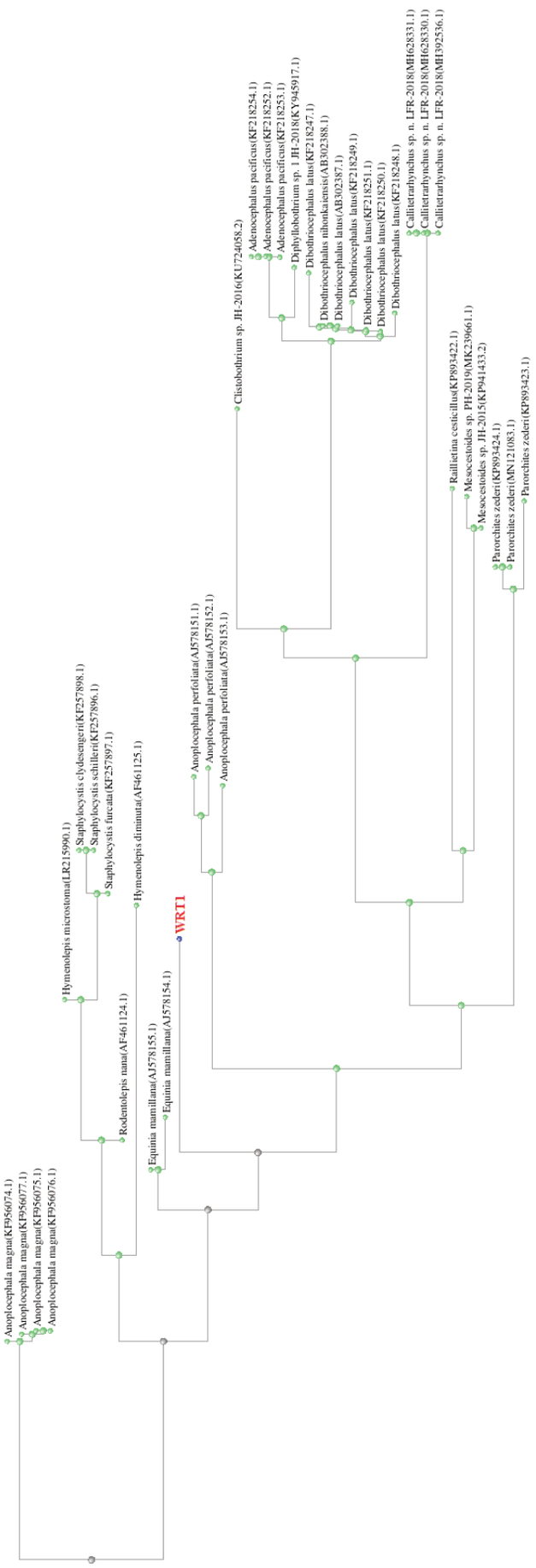

Figure 3. Phylogenetic analysis of WRT1 tapeworm rDNA sequence with corresponding sequences from GenBank (accession numbers are shown in brackets). The tree was constructed in BLAST

were found in 13.0% of tested horses. Higher values of prevalence were recorded in females (15.2%), younger animals (<6 months) (15.4%), and animals dependent on pasture for their diet (18.6%). In the study of Tomczuk et al. [18] the overall prevalence of *A. perfoliata* was 25.1% (21.4–29.0), and it was significantly affected by the age of horses. The highest prevalence was found in horses from 10 to 20 years old. Prevalence was not significantly affected by sex of horses, however more mares were infected with *A. perfoliata* than the rest of horses: mares = 28.8% (25.8–32.0); stallions = 12.0% (7.4–18.8); geldings = 23.7% (20.0–27.9). In the study of Hedberg-Alm et al. [15], a total of 15.7% (43/274) of the horses were positive for *A. perfoliata*. Seventeen horses (12.4%) were positive for *A. perfoliata* in the case group and 26 horses (19.0%) in the control group, with no significant difference between the two groups (P=0.14).

In the present study the prevalence of *A. perfoliata* was 4.8% (3 from 62 horses). A total of 20% (2/10) of the horses were positive for *A. perfoliata* in October 2012 and 4.3% (1/23) in December 2013. This result is quite surprising because the area of the Biebrza National Park (swamps, wetlands, meadows) seems to be a good habitat for oribatid mites, which serve as the pasture-related, intermediate hosts for Anoplocephalidae. During natural grazing, animals are especially prone to tapeworm infection. Tomczuk [19] showed that prevalence and intensity of the infection of *Anoplocephala perfoliata* tapeworms were the highest in horses that preferred wet pasture and low-lying soils in the vicinity of reservoirs. It can be associated with the presence of various species of mites which are intermediate hosts for *A. perfoliata* tapeworms [7,19,20]. The oribatid larvae die when humidity is low and appear in large numbers when the amount of moisture in the environment increases. Denegri and Alzuet [21] state that this environmental influence may be the key to controlling the intermediate host.

The results of other studies from around the world indicate that the most common species of tapeworm detected in horses is *A. perfoliata* [20,22–24]. In the earlier study of Konik Polski horses from the Popielno Reserve prevalence of tapeworm infection was 72.7%, while in the Roztocze National Park it was much lower and amounted to 37.5% [4]. Tapeworms were also rarely found in Przewalski's horses (14.3%) [12]. Despite the favorable environment for Oribatida development in Biebrza National Park the prevalence of tapeworm infection in Konik Polski horses was much lower compared with that reported in animals from Popielno nature reserves and Roztocze National Park.

During molecular analysis rDNA products were successfully amplified from WRT1 and RUS1 templates and COI fragments from all analyzed samples. Multialignment analysis showed that mitochondrial DNA fragments from tapeworms originating from Konik Polski (BNP1) and Arabian horse (WRT1) were almost identical and BLAST analysis showed that BNP1 sequence shares the highest homology of 100% to COI fragment from *Anoplocephala* sp. A2 (JQ771109). This sample originated from a horse from Czech Republic and was described by the authors of the study as *A. perfoliata* [25] (Tab. 1). This proves that the tapeworm collected from Konik Polski is *A. perfoliata*. Other *A. perfoliata* COI sequences available in GenBank (KR054960 and AY568189) which show lower homology (90% and 89.3%) to BNP1 and WRT1 originate from China [26] and Australia [27]. These differences probably result from intraspecies geographic diversity. Mitochondrial genomes were found useful in phyleogeographic analyses of other tapeworm species such as *Echinococcus granulosus* [28] and *Taenia hydatigena* [29].

Multiplex PCR as well as bioinformatic analysis of rDNA amplified from WRT1 sample proved that this tapeworm belongs to *A. perfoliata* species. This further confirms that BNP1 tapeworm also belongs to *A. perfoliata* as both samples have identical COI fragments.

On contrary, data obtained using RUS1 sample is inconsistent. Analysis of COI nucleotide sequence showed a similar homology both to *A. perfoliata* (90.65%) and *A. magna* (89.53%). Multiplex PCR according to Bohorquez [5] in which an ITS-2 fragment of rDNA is amplified showed that the sample belongs to *A. magna* species as a specific 382 bp fragment was amplified, whereas a phylogenetic distance tree constructed in BLAST program using a 2072 bp long rDNA fragment showed that the tapeworm was most closely related to *E. mamilliana*. These discrepancies may result from intraspecies geographic sequence diversity but also from scarcity of nucleotide sequences available in databases. Another cause may be the PCR technique itself, as the amplification of target DNA produces copies which may contain errors: editing errors that occur during DNA polymerase-catalyzed

enzymatic copying and errors due to DNA thermal damage [30]. This shows that molecular identification of tapeworm species is a complicated method, which is dependent on numerous factors.

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