First genetically verified occurrence of *Ligula pavlovskii* outside its native range and characteristics of its infection in *Neogobius fluviatilis*

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Abstract

In this study, we provide the first genetically verified distribution record of *Ligula pavlovskii*, a high-impact endoparasite of Ponto-Caspian gobies, beyond its native range. According to parasitological surveys, ligulosis was detected for the first time in monkey goby individuals collected from Lake Balaton in 2004, 34 years after the first record of monkey goby in the lake. During a Lake Balaton survey in 2018, we detected tapeworms in 44.4% of the sampled monkey gobies. This prevalence is about two to ten times higher than it was reported from within the native range of the fish hosts. The same survey revealed an uneven spatial distribution of the ligulosis in the monkey gobies inhabiting different shoreline sections of the lake. We assume that the occurrence and recent distribution data of this high-impact endoparasite may provide useful information for potential biocontrol measures of invasive Ponto-Caspian gobies in the future.

Keywords: Ponto-Caspian goby, Cestoda, distribution, shallow lake, parasite invasion, large lake

Introduction

Worldwide range expansion of Ponto-Caspian gobies (Pisces, Gobiidae) is a good example of recent human facilitated invasion of freshwater ecosystems. For example, habitat alterations along with human assisted spread of eggs, larvae and adult fish largely contributed to the invasion of European large rivers by several goby species (Kotta et al., 2016). Some species, like the tubenose goby (*Proterorhinus semilunaris*) and the round goby (*Neogobius melanostomus*), even got across the ocean and caused major changes in the structure and functioning of the Laurentian Great Lakes ecosystems (Kornis et al., 2012). Beside human assisted spread, a less known biotic factor, the lower parasite infection rate may also favour the mass invasion of gobies. Gobies in their non-native range are less parasitized and have less diverse parasite fauna compared to populations in their native range (Kvach et al., 2014; Kvach and Skóra, 2007; Kvach and Stepien, 2008; Molnár, 2006). These findings are in accordance with the “enemy release hypothesis”, that the temporary lack of natural enemies (predators, pathogens and parasites) can facilitate the invasion success of non-native species (Torchin et al., 2003).

Plerocercoid larvae of the genus *Ligula* (Cestoda, Diphyllobothriidae) have been recorded from the body cavity of numerous species of various fishes throughout the world (Bouzid et al., 2008; Lagrue et al., 2018). These *Ligula* species have been shown to be phylogenetically largely specific to the host fish species. For example, the tapeworm *Ligula pavlovskii* Dubinina 1959 occurs primarily in the Ponto-Caspian gobiids, and has been recorded from only a few fish species from the Ponto-Caspian region, specifically stellate tadpole-goby (*Benthophillus stellatus),* monkey goby (*Neogobius fluviatilis*), round goby, bighead goby (*Ponticola kessleri*), syrman goby (*Ponticola syrman*), marbled goby (*Pomatoschistus marmoratus*), and an unclear species from genus *Knipowitschia* (Yuryshynets et al., 2017). Although plerocercoids are large bodied parasites (Fig 1.), to our knowledge no study to date has reported their occurrence in non-native populations of Ponto-Caspian gobies (Kudrenko and Kvach, 2005; Kvach et al., 2015, 2014; Kvach and Stepien, 2008; Mierzejewska et al., 2014; Appendix A) except a single specimen from Ros river, near Kiev (Kvach and Ondračková, 2020; Zaichenko, 2015) that was found in a sample of 288 monkey goby individuals.

The first occurrence of monkey goby was reported from Lake Balaton in 1970 (Bíró, 1971). Since then the species became one of the most abundant non-native fish in the littoral zone of the lake (Czeglédi et al., 2019). During a fish survey conducted in the littoral zone of Lake Balaton in 2017, we found widespread tapeworm infection in monkey gobies. Knowledge of the distribution and infection rate of parasites facilitates the understanding of their effect on life-history and population dynamics of the host species (Claridge et al., 1985; May, 1983), which might modify the effect of invasive gobies on the recipient ecosystem.

Therefore the aims of our study in Lake Balaton were: (1) to identify the species of *Ligula* infecting the monkey goby and establishing its taxonomic position using molecular tools; (2) to determine the first occurrence of the parasite using unpublished historical data; and (3) to describe the recent distribution pattern, prevalence and intensity of ligulosis in the monkey goby stocks.

Methods

Study area

Lake Balaton is the largest shallow lake in Central Europe with a surface area of 596 km^2 and an average depth of 3.3 m. Due to the geographical and hydrological differences, the northern and southern shores have distinct characteristics: the water is deeper at the north coastline, and the mostly reed dominated habitats are closer to the natural state compared to the southern shores. The only outflow of the lake is Sió Canal, which connects the lake with Danube River (Fig. 2.). The water level of the lake is regulated with a sluice at the outflow, which also functions as a barrier for fish migrating upstream from the canal. The Danube serves as one of the most important invasion corridor of Ponto-Caspian species to Western Europe (Bij de Vaate et al., 2002).

Sample and data collections

Following the incidental finding of ligulosis of monkey goby individuals during a fish survey, we collated all available published and unpublished data between 1970 and 2016 potentially indicating the presence and describing the spatiotemporal dynamics of ligulosis in the monkey goby population of Lake Balaton. In addition, we collected monkey gobies at 17 sampling sites along the shoreline of the lake in autumn of 2018 (Fig 2, Table 1.) using an electrofishing gear. Collected fish were euthanized with clove oil and stored in freezer (-20°C). Before dissection standard length (SL) and total weight (W_f) of each fish was measured. Plerocercoid larvae were removed, counted and weighted (W_p). We also calculated the Parasite Index (PI) as follows: $PI = (W_p / W_f) \times 100$

Prevalence data (number of infected fish individuals per number of examined host), mean intensity of infection (mean number of parasites per infected fish individuals), and mean PI values of sampling sites were compared between the north and the south shorelines using Mann-Whitney U tests. Data analysis was performed using PAST software (Hammer et al., 2001). An alpha value of 0.05 was used to determine statistical significance of all tests.

Molecular analysis

Four of the removed tapeworms were randomly selected and preserved in 96% ethanol for taxonomic identification. DNA was extracted using a QIAGEN DNeasyTM tissue kit (animal tissue protocol; Qiagen, Hilden, Germany) and eluted in 200 μL AE buffer. The amplification of COI was executed using the primers PBI-cox1F_PCR, and PBI-cox1R_PCR, and the protocol published by Scholz et al. (Scholz et al., 2013). PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA (TAE) buffer gel, stained with 1% ethidium bromide. Purification was carried out with EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Canada). Purified PCR products of COI were sequenced with the primers PBI-cox1F_seq and PBI-cox1R_seq. ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing, and the sequences read using an ABI 3100 Genetic Analyser.

Phylogenetic analysis

Assembly of the sequenced fragments was done by MEGA X (Kumar et al., 2018) and ambiguous bases clarified using corresponding ABI chromatograms. Alignment of the gene

COI were conducted with the software CLUSTAL W (Thompson et al., 1994). The alignment was corrected manually using the alignment editor of the software MEGA X. Sequences were deposited in the GenBank under the accession numbers (MT209900-MT209903). DNA pairwise distances were calculated with the MEGA X software using the p-distance substitution model. Maximum likelihood (ML) analysis was performed. *Schistocephalus solidus* (KY552891) was chosen as outgroup species. The dataset was tested using MEGA X for the nucleotide substitution model of best fit, and the model shown by the Akaike Information Criterion (AIC) as the best-fitting one, was chosen for each partition. ML analysis was conducted in MEGA X under the GTR + G model. Bootstrap values were generated based on 1000 re-sampled datasets.

Results

First occurrence, recent prevalence data and description of the recent infection

Based on the collected unpublished data we found that, although investigations started soon after the first occurrence of the monkey goby in the lake, plerocercoid larvae were not recorded, and ligulosis was detected for the first time in monkey goby individuals of Lake Balaton in 2004 (Appendix A).

During the field survey, we detected tapeworms in 228 out of 514 dissected monkey gobies (44.4%). Mean intensity of infection was 2.0 (SD = 1.41, max: 9). Mean weight of tapeworm individuals was 0.54 g (SD = 0.38, max = 1.9 g) and mean PI of infected fish was 8.01 (SD = 3.68, max = 20.1, Table 1.). We found infected monkey goby individuals at all sampling sites, but prevalence showed high variation. The highest infection rate was detected at Site 10 (84.4%), while the lowest at Site 15 (7.4%) (Fig. 2., Table 1.). Results of Mann-Whitney U tests showed that monkey gobies collected at the northern shoreline of the lake can be characterised by significantly higher prevalence (U = 66, p = 0.003), higher intensity of infection (Mann-Whitney U test: p < 0.001; U = 70) and PI values (Mann-Whitney U test: p = 0.003; U = 66), compared to their conspecifics collected at the southern shore.

Molecular- and phylogenetic status

The partial mtCox1 gene of four samples was amplified and resulted in 497-564 bps long sequenced fragments. The alignment of the COI consisted of 521 bps, including 360 conservative and 161 variable (122 of them parsimony-informative) sites. The sequences of the four samples (GenBank Sequence IDs: MT209900-MT209903) were almost identical, the mean distance within the group was only 0.006. They showed the greatest similarity with the species *L. pavlovskii* (KY552876) with the pw distance ranging from 0.0 to 0.008 and the monophyly of this group was supported with high bootstrap value (94). Other *Ligula* species differed much more, *L. alternans* Rudolphi, 1810 (KY552873, NC039446) with 0.082-0.086, *L. intestinalis* Linneaus, 1758 (KY552874, KY552875, NC039445) with 0.106-0.116, *Ligula* sp. (EU241220) from algerian barb (*Luciobarbus callensis* Valenciennes, 1842) with 0.095-0.098 pairwise distance values. Moreover, *Ligula* species form a monophyletic clade (Fig. 3.), showing a considerable genetic distance from the *Diphyllobothrium* species.

Two *L. pavlovskii* specimens (reference numbers: 20889/1, 20890/1), and genomic DNA of the analysed four specimens (reference numbers: 20889/2, 20889/3, 20890/2, 20890/3) were deposited in the Parasitological Collection of Hungarian Natural History Museum.

Discussion

To our knowledge, this is the first study with molecular genetic evidence on occurrence of *L. pavlovskii* outside of its native range. This is particularly important in newly invaded areas because the great morphological uniformity among the plerocercoids of the species of the genus makes the use of molecular tools for their identification an essential element. Our study also yielded evidence on two to ten times higher prevalence of the parasite compared to data from its native range. Furthermore, we detected an uneven spatial distribution of its prevalence across habitats.

Within the native range of the species, relatively low infection rates have been reported. For example, a mean prevalence of 4.7% and 4.2% was found in the Budaksky Lagoon, Black Sea (Kvach, 2010, 2002), 11.9% in the North-western Black Sea (Kvach, 2005), and 7.7% and 3.1% in the area of Dniester Estuary (Kvach, 2010). In addition, no infected monkey goby was found in Dnieper Estuary (Kvach et al., 2014). The only available prevalence data of *L. pavlovskii* in a freshwater monkey goby population is higher than the above mentioned from marine and brackish environments: 22.8% was reported from Kakhovka reservoir in Ukraine (Yuryshynets et al., 2017). In this regard, the extremely high recent mean infection rate (44.4%) in Lake Balaton suggests that this parasite finds optimal environmental conditions in this lake (or generally in freshwater environments). This pattern might be related to the high density of the different host species, which are necessary to the development of the species of ligulids (Chubb, 1980). In brief, copepods get infected by the ingestion of free-swimming coracidia larva of the parasite, which matures into procercoid stage in the haemocoel cavity of the primary host. Infected copepods are eaten by gobies, where the procercoid larvae migrate from the alimentary tract to abdominal cavity of the fish host and transform into plerocercoid larvae, a stage that may last for a few years. The life cycle ends when the infected fish is eaten by a piscivorous bird. In the alimentary tract of the bird, the parasites reach their sexual maturity, lay eggs which leave the host with excreta within a few days (McDonald, 1969).

The zooplankton community of Lake Balaton is dominated by copepods with a great proportion of *Eudiaptomus gracilis* (Németh and G-Tóth, 2003). *Eudiaptomus gracilis* is the most favourable host for *L. intestinalis* (Loot et al., 2001), a closely related species to *L. pavlovskii*, suggesting the zooplankton community to be ideal for the development of the latter. Its fish host, the monkey goby is widespread in various habitat types of the entire lake (Czeglédi et al., 2019), and there are also numerous piscivorous waterbird species which commonly feed on fish from the lake (Kovács, 2017). According to these, the continuation of the parasite’s life cycle is guaranteed in Lake Balaton.

The mechanism of the delayed occurrence of the parasite in monkey gobies of the Lake Balaton remains unknown. We speculate that this may originate in the small founder population size of monkey goby in Lake Balaton. The monkey goby either reached the lake passively, stowing a vessel from the Danube, or by active movement, somehow surmounting the high wall of the sluice at the outflow of the lake (Bíró, 1971). In either case, the founder population supposedly was small, and its population had to attain a critical size after which it showed an explosive population growth. It is likely that the founder specimens were not infected by the parasite, and the parasite was introduced to the lake for example with delayed arrival of additional gobies or with piscivorous birds. In fact, the increasing numbers of the great crested grebe (*Podiceps cristatus*) and the great cormorant (*Phalacrocorax carbo*) from early 2000s (Kovács, 2017) may have contributed largely to the spread of the parasite.

The survey of the monkey goby population in 2018 revealed that although ligulosis was detected at all sites, the infection rates exhibit high variability between sampling sites. Moreover, a spatial pattern was found: individuals collected at the southern coast were less infected than those collected at the northern coast of the lake. This may be attributed to the morphology of the lake, which has fundamental effect on the distribution of the first intermediate and definitive hosts, therefore to the infection rates of monkey goby stocks as well. Zooplankton abundance is 1.5-3 times higher to the north from the central line than to the south from it (Németh and G-Tóth, 2003). However, the exact distribution pattern of great crested grebe is unknown at the lake, lakebed morphology and the habitat types around Lake Balaton suggest their higher density along the northern shore. Namely, grebes prefer to nest on the waterfront of the vegetation along the shore and most effectively feed in clear water by diving. Considerable amounts of reed-dominated habitats (~73%) are along the northern shore (Tóth, 2016), whereas the dominant habitat type of the southern shore is rip-rap. The water depth of the northern region is increasing more rapidly providing better conditions for diving closer to the nest. Therefore, the recorded pattern in prevalence, intensity of infection, and PI values seems to be affected by the abundances of first intermediate and definitive hosts. However, further studies are needed to confirm the stability of the observed spatial pattern and its ultimate reasons.

In conclusion, to our knowledge, this study is the first, which unequivocally proves the occurrence of *L. pavlovskii* outside of its native range using genetic analyses. We detected two to ten times higher prevalence in the invasive monkey goby population of Lake Balaton, than it was reported within the native range of the parasite. We assume that such a high level of infection may influence the life history of the monkey goby; a notion, which along with uneven spatial distribution of the prevalence, needs further investigation. We suppose that the occurrence and recent distribution data of this and other parasites may provide useful background information for the biocontrol of invasive Ponto-Caspian gobies in the future.

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Figure captions

Fig. 1: Location of *L. pavlovskii* plerocercoids in the body cavity of a monkey goby (a), and size of the parasite compared to the size of the host (b).

Fig 2: Recent prevalence of *L. pavlovskii* in Lake Balaton (a). Black parts of pie charts represent the ratio of infected fish individuals (for further details see Table 1.). The geographic position of Lake Balaton in Hungary and Hungary in Europe are indicated in the inserts B and C, respectively.

Fig. 3.: Maximum likelihood phylogeny tree derived from the analysis of the mtCOI sequence data. Posterior probabilities are assigned on nodes. Haplotypes revealed in this study are marked with Lig01-04 codes. GenBank accession numbers are indicated right after the species names.

Table 1: Codes, location, coordinates, number of individuals (n_{tot}), number of infected individuals (n_{inf}), prevalence (P), intensity (mean ± SD), and parasite index (PI; mean ± SD) of the 17 studied monkey goby samples collected from the shoreline of Lake Balaton in 2018.

Code	Shoreline	Coordinates	n_{tot}	n_{inf}	P (%)	Intensity	PI (%)
Site 01	North	N46.75353 E17.24760	26	7	26.9	1.71±0.95	1.28±2.4
Site 02	North	N46.75360 E17.31346	36	25	69.4	2.60±1.85	5.02±4.0
Site 03	North	N46.78366 E17.44474	31	15	48.4	1.57±0.65	3.36±3.9
Site 04	North	N46.82010 E17.59340	24	15	62.5	2.07±1.03	5.51±4.7
Site 05	North	N46.88238 E17.76592	40	30	75.0	2.07±1.34	5.72±4.6
Site 06	North	N46.89718 E17.86330	27	15	55.6	1.93±0.80	6.06±5.9
Site 07	North	N46.91408 E17.89288	30	14	46.7	1.93±1.07	5.74±7.0
Site 08	North	N46.95726 E17.91453	24	20	83.3	2.90±2.20	8.02±5.7
Site 09	North	N47.03380 E18.03174	10	3	30.0	3.33±1.53	3.87±6.5
Site 10	North	N47.02986 E18.10952	32	27	84.4	1.85±1.35	5.35±3.5
Site 11	South	N46.98797 E18.16235	43	17	39.5	1.59±0.80	3.34±4.5
Site 12	South	N46.91020 E18.04501	45	11	24.4	1.36±0.50	2.18±3.7
Site 13	South	N46.89081 E17.96831	36	13	36.1	1.62±0.77	2.35±3.7
Site 14	South	N46.82191 E17.79511	26	6	23.1	1.20±0.45	1.63±3.3
Site 15	South	N46.77494 E17.63691	27	2	7.4	1.00±0.00	0.33±1.3
Site 16	South	N46.71494 E17.47538	31	3	9.7	1.00±0.00	0.56±1.8
Site 17	South	N46.71516 E17.31576	26	5	19.2	1.00±0.00	1.01±2.1
			514	228	44.4	2.00±1.41	3.54±4.67

Fig 1.


a
1 cm
b
1 cm

Fig 2.

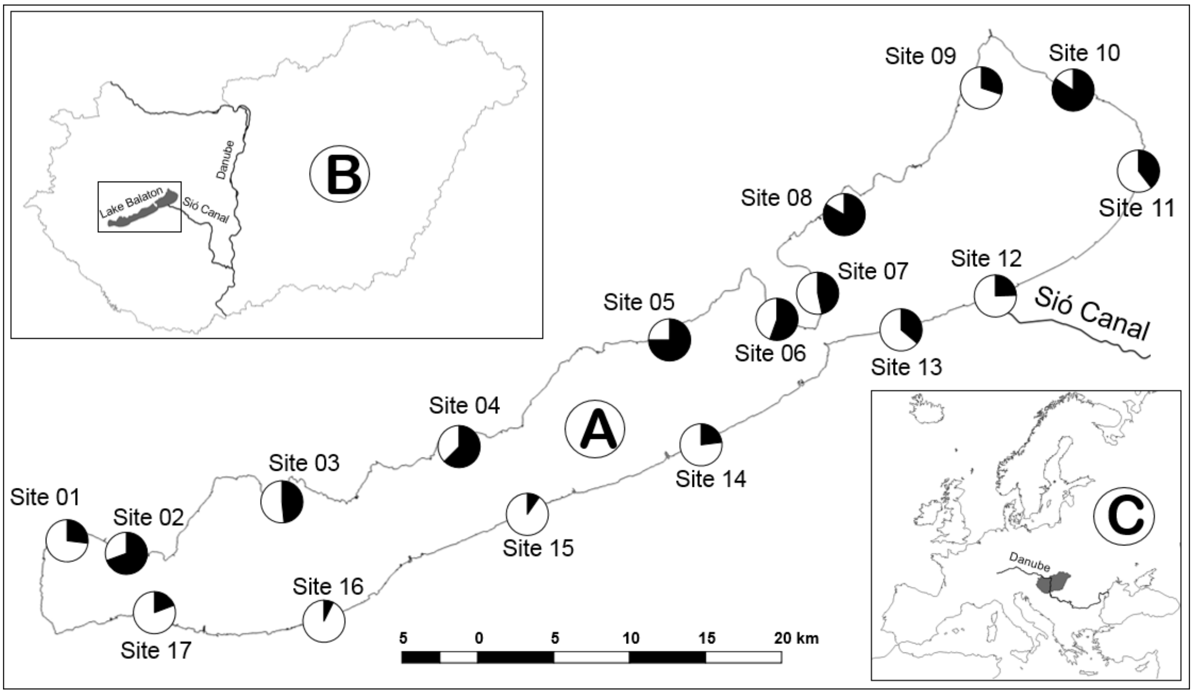
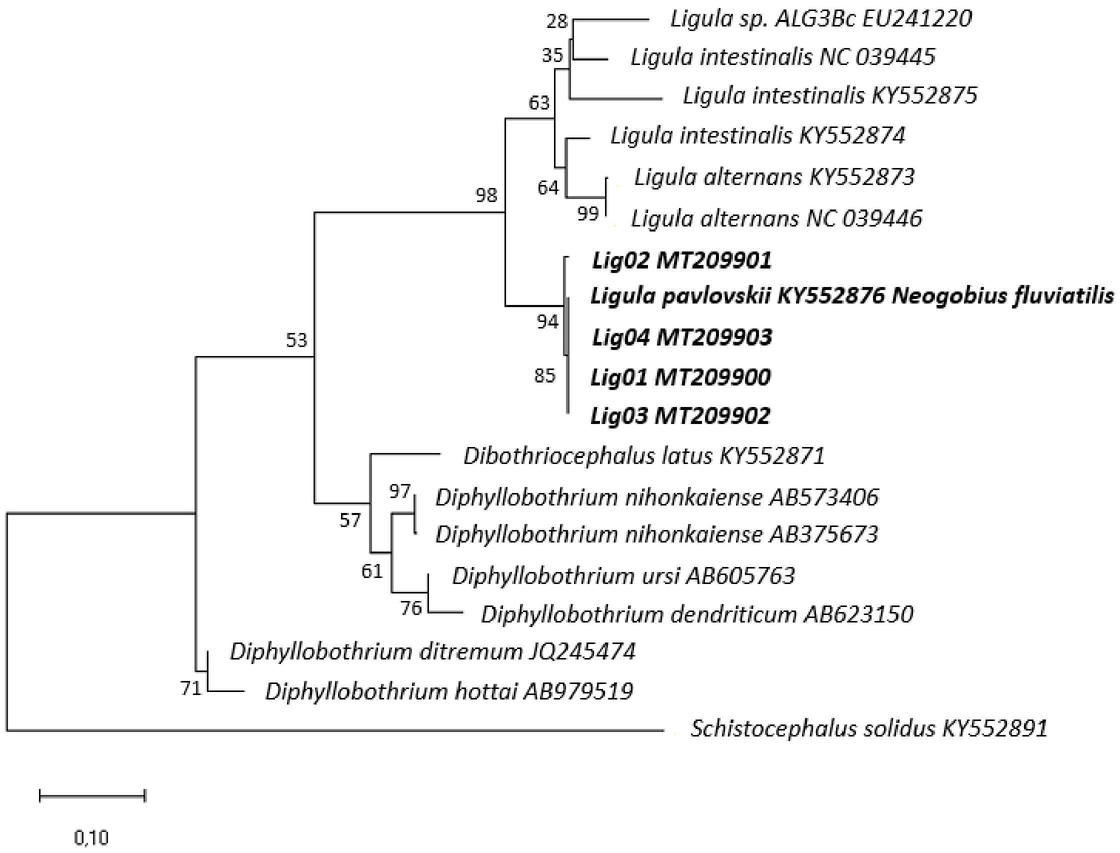

B
Danube
Lake Balaton
Sió Canal
Site 09
Site 10
Site 11
Site 08
Site 07
Site 12
Site 05
Site 06
Sió Canal
Site 13
Site 04
A
Site 14
Site 03
Site 01
Site 02
Site 15
Site 16
Site 17
5
0
5
10
15
20 km
C
Danube

Fig 3.


28
Ligula sp. ALG3Bc EU241220
35
Ligula intestinalis NC 039445
63
Ligula intestinalis KY552875
Ligula intestinalis KY552874
98
64
Ligula alternans KY552873
99
Ligula alternans NC 039446
Lig02 MT209901
Ligula pavlovskii KY552876 Neogobius fluviatilis
94
Lig04 MT209903
53
85
Lig01 MT209900
Lig03 MT209902
Dibothriocephalus latus KY552871
97
Diphyllobothrium nihonkaiense AB573406
57
Diphyllobothrium nihonkaiense AB375673
61
Diphyllobothrium ursi AB605763
76
Diphyllobothrium dendriticum AB623150
Diphyllobothrium ditremum JQ245474
71
Diphyllobothrium hottai AB979519
Schistocephalus solidus KY552891
0,10