Phylogenetic relationships of Paradiclybothrium pacificum and Diclybothrium armatum (Monogenoidea: Diclybothriidae) inferred from 18S rDNA sequence data
Phylogenetic relationships of *Paradiclybothrium pacificum* and *Diclybothrium armatum* (Monogenoidea: Diclybothriidae) inferred from 18S rDNA sequence data

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**Abstract**

The Diclybothriidae (Monogenoidea: Oligonchoinea) includes specific parasites of fishes assigned to the ancient order *Acipenseriformes*. Phylogeny of the Diclybothriidae is still unclear despite several systematic studies based on morphological characters. Together with the closely related Hexabothriidae represented by parasites of sharks and ray-fishes, the position of Diclybothriidae in different taxonomical systems has been matter of discussion. Here, we present the first molecular data on Diclybothriidae. The SSU rRNA gene was used to investigate the phylogenetic position of *Paradiclybothrium pacificum* and *Diclybothrium armatum* among the other Oligonchoinea. Complete nucleotide sequences of *P. pacificum* and *D. armatum* demonstrated high identity (98.53%) with no intraspecific sequence variability. Specimens of *D. armatum* were obtained from different hosts (*Acipenser schrenckii* and *Huso dauricus*); however, variation by host was not detected. The sequence divergence and phylogenetic trees data show that Diclybothriidae and Hexabothriidae are more closely related to each other than with other representatives of Oligonchoinea.

**Keywords:** 18S rDNA; *Acipenseriformes*; *Monogenoidea*; *Diclybothriidae*; Parasites; Phylogeny

**1. Introduction**

Species of Diclybothriidae Price, 1936 (Monogenoidea) are specific gill parasites of the acipenseriform fishes. Sturgeons (*Acipenseridae*) and paddlefishes (*Polyodontidae*) are members of the order *Acipenseriformes*, a group of approximately 27 extant species distributed throughout North America and Eurasia [1,2]. At present, four valid diclybothrid species are known: *Diclybothrium armatum* Leuckart, 1835 from sturgeons in Eurasia, including Amur River basin; *D. hamulatum* (Simer, 1929) from North American paddlefish *Polyodon spathula* (Walbaum); *Diclybothrium atriatum* Choudhury et Dick, 1996 from North American lake sturgeon *Acipenser fulvescens* Rafinesque and shortnose sturgeon *Acipenser brevirostrum* Le Sueur; *Paradiclybothrium pacificum* Bychowsky et Gussev, 1950 from the Sakhalin sturgeon *Acipenser mikadoi* Hilgerndorf of the Tatar Strait and Sea of Okhotsk; from the kaluga *Huso dauricus* (Georgii) in the lower Amur River; and the green sturgeon *Acipenser medirostris* Ayres from the North American Pacific [3–15].

The number of characters (morphology, anatomy and ontogenesis) points to high divergence between Diclybothriidae and other Monogenoidea. For example, studies on anatomy have revealed the presence of paired multichannel sperm ducts in Diclybothriidae [5,10]. Paired sperm ducts, while common in Turbellaria with multiple testes, are unusual among the Monogenoidea [10]. Due to such peculiarities, the position of Diclybothriidae together with the closely related Hexabothriidae was inconsistent between various systems, based on morphological characteristics. These differences are reflected in the five most popular systems proposed for Monogenoidea: (1) Odhner–Price [see Ref. [6]]; (2) Bychowsky [6]; (3) Yamaguti [16]; (4) Boeger and Kritsky [17], and (5) Lebedev [18].

The system developed by Odhner–Price was most widely used until the 1950s. This system divides the order Monogenea (Trematoda) into two suborders: Monopisthocotylea (vagina present, genito-intestinal canal usually absent) and Polypisthocotylea (vagina, when present, single or double, with ventral, lateral or dorsal pore or pores, genito-intestinal canal generally present). The latter includes Polystomatidae and Hexabothriidae, the latter of which contains Hexabothriinae and Diclybothriinae established by Price [4,19]. These monogeneans were placed in one superfamilial due to the presence of haptor with three pairs of suckers. In 1950, Bychowsky and Gussev [5] revised the diclybothrid monogeneans and proposed *Paradiclybothrium* Bychowsky et Gussev, 1950 with a single representative *P. pacificum*. They also revised and elevated the previously proposed Diclybothriidae and conducted the analysis of its position among other monogeneans. These findings later were described in a new system of monogenean worms [6]. This system was based on larval development and the ontogenesis of hooks in different groups of Monogenea. As a result of his work, the order Monogenea was elevated to class and its name was changed to Monogenoidea. This class was then divided into the
subclasses Polychoinea and Oligochinea with Diclybothriidae and Hexabothriidae belonging to the Dicybothriidea Bychowsky, 1957. The system of Yamaguti [16], generally speaking, is a modified Odhner–Price system. Yamaguti restored the previous names and states of Monopisthocotylea and Polyopisthocotylea and recognized Dicybothriidea as a separate taxon, but placed it together with Hexabothriidea and Polystomatidae into Polystomatoida. The last two systems, Boeger and Kritsky [17] and Lebedev [18], are based on the system of Bychowsky [6] with some improvements. In part, Monogenoidea was restored as a class and divided into three subclasses: Polychoinea, Polystomatoida and Oligochinea with Dicybothriidea, which contains Dicybothriidae and Hexabothriidae. Later, Boeger and Kritsky [20] proposed Heteronchoinea to include the sister taxon of the Monogenoidea (i.e., the polystomes and oligochineans). As a result, Polystomatoida and Oligochinea became infrasuborders of the Heteronchoinea.

Currently, the phylogenetic relationships within Monogenoidea are still unclear. In analyses based on morphology, Monogenoidea has been considered as a monophyletic group [21,22], but the results of the molecular analysis based on 28S rDNA sequences contradicted this monophyly [23]. Later, the monophyly of Monogenoidea was claimed the molecular analysis based on 28S rDNA sequences contradicted this monophyly [23]. Later, the monophyly of Monogenoidea was claimed the molecular analysis based on 28S rDNA sequences contradicted this monophyly [23]. Later, the monophyly of Monogenoidea was claimed the molecular analysis based on 28S rDNA sequences contradicted this monophyly [23]. Later, the monophyly of Monogenoidea was claimed the molecular analysis based on 28S rDNA sequences contradicted this monophyly [23].

Regardless of the number of works on the molecular characterization and phylogeny of Monogenoidea, such data on Diclybothriidae and Hexabothriidae are absent. Here, we present the first molecular data on 18S rDNA sequences and evaluated the internal phylogenetic relationships of *P. pacificum* and *D. armatum* within the Dicybothriidea and its position among the other Oligochinea.

### 2. Materials and methods

#### 2.1. Samples

Monogenoidean specimens were collected from the gills of *H. dauricus* (103 specimens) and *Acipenser schrenckii* Brandt (116 specimens) captured in the Amur River near Nikolaevsk-na-Amur (53° 06’ N, 140° 4’ E) and at several sites of Amur Liman (Table 1) in 2009–2011 during parasitological investigations. Most worms were analyzed using glycerol-gelatin slides. Some specimens were flattened under slight pressure, fixed in 70% ethanol, stained with alum carmine and after dehydrating and clearing were mounted in Canada balsam. Morphological studies were conducted using an Axioscope optical microscope (Carl Zeiss Microimaging GmbH, Germany). In total, two species *D. armatum* and *P. pacificum*, morphologically identical to those described earlier [5,10], were revealed.

For the phylogenetic analysis, 10 specimens of *D. armatum* from two Amur sturgeons and one kaluga as well as three specimens of *P. pacificum* from kaluga were taken. Worms were identified under slight pressure between two glass slides, fixed in 96% ethanol and stored at −20 °C until DNA extraction.

### 2.2. Extraction of total genomic DNA, PCR, and sequencing

Total genomic DNA was extracted from posterior part of individual worms using the Hot–SHOT technique [37]. The anterior part was stored in 96% ethanol at −20 °C.

The complete sequences of 18S rDNA were amplified using the following primer pairs: forward 5′-CAATGTCGGATTCTGATGATTAC-3′ and reverse 5′-CTGATCCTTGAGTCTCCATAC-3′ [38]. PCR reaction was performed in a 25 μl reaction mixture containing 10 μl of each primer, 12.5 μl of PCR Master Mix (Thermo Scientific, Vilnius, Lithuania), and 4 μl of extracted DNA. Amplification started with an initial denaturation step at 96 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 4 min with a final extension at 72 °C for 10 min. Products were separated by 1.0% agarose gel electrophoresis and visualized with ethidium bromide staining using an ultraviolet transilluminator. Sequencing of the amplified gene was performed on ABI 3130 automated sequencer using the following internal primers: 570 (5′-GCTATTTGGACTGTGAATTAC-3′), 1137 (5′-GTGCCCTTCCGCTCAAT-3′), 892C (5′-GTCAAGCTGAATCTTCTT-3′), 1286C (5′-GTGGATCGATGGCCTTCTTA-3′), and 1/F (5′-CAACGCCGTCGTCG-3′) [39].

### 2.3. Sequence data analysis and construction of phylogenetic trees

Nucleotide sequences of 18S rDNA were assembled and aligned using the MEGA 5.10 [40] software package. Sequences were deposited in GenBank under the following accession numbers: KP796242–KP796244 for *P. pacificum*, and KP796245–KP796254 for *D. armatum*, respectively.

The phylogenetic relationships were inferred by the following methods: Neighbor-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference using MEGA 5.10 and Mr. Bayes 3.1.2 [41], respectively, from our data and the nucleotide sequences of 18S rDNA from subclasses Oligochinea and Polystomatoida obtained from the NCBI GenBank database. We also used 18S rDNA sequences from Gyrodactylidae (Monogenea: Polychoinea) and Echinostomatidae (Digenea) as outgroup taxa. The resulting sequence alignment included 32 representatives of Monogeneoidea and four outgroup species. After alignment sequences were edited, the ends of each sequence were trimmed to match the shortest sequence in the alignment. Distance matrices for the 18S rDNA were constructed with the absolute pairwise character difference. Pairwise comparisons of absolute sequence divergence for all taxa were calculated with gaps treated as missing data. The appropriate model of nucleotide substitutions was calculated using Modeltest 3.06 [42] with an AIC informational criterion [43]. The GTR model with proportion of invariable sites 0.35 and gamma value 0.39 was chosen as the best fit model. The phylogenetic relationship significance was estimated using a bootstrap analysis [44] with 1000 replications for NJ trees and 100 replications for MP and ML trees. For BI, the standard deviation of split frequencies was used to assess if the number of generations completed was sufficient; the chain was sampled every 1000 generations and the dataset was run for one million generations. Burn-in was determined empirically by examination of the log likelihood values of the chains. NJ, MP and ML phylogenetic trees were constructed using MEGA 5.10, and the BI

### Table 1. Hosts and sample sites.

<table>
<thead>
<tr>
<th>Date/Most</th>
<th>N</th>
<th>AC, cm</th>
<th><em>D. armatum</em></th>
<th>P (%)</th>
<th>MI (limit)</th>
<th><em>P. pacificum</em></th>
<th>P (%)</th>
<th>MI (limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amur River near Nikolaevsk-na-Amure</strong></td>
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<tr>
<td><em>H. dauricus</em></td>
<td>23</td>
<td>64–216</td>
<td>13.0</td>
<td>3.33</td>
<td>(1–5)</td>
<td>21.7</td>
<td>1.4</td>
<td>(1–3)</td>
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<tr>
<td>A. schrenckii</td>
<td>16</td>
<td>41–136</td>
<td>81.3</td>
<td>48.7</td>
<td>(5–165)</td>
<td>0</td>
<td>0</td>
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<td>September–October 2009</td>
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<tr>
<td><em>H. dauricus</em></td>
<td>19</td>
<td>56–160</td>
<td>100</td>
<td>29</td>
<td>(4–136)</td>
<td>0</td>
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<tr>
<td>A. schrenckii</td>
<td>23</td>
<td>32–132</td>
<td>91.3</td>
<td>27.8</td>
<td>(1–106)</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td><strong>Amur Liman</strong></td>
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<tr>
<td>June–July 2011</td>
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<tr>
<td><em>H. dauricus</em></td>
<td>51</td>
<td>80–133</td>
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<td>7.8</td>
<td>3.8</td>
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<td>1.5</td>
<td>(1–2)</td>
<td>0</td>
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</table>

N – number of examined fishes; AC – fish length range; P – prevalence; MI – mean intensity.
tree was visualized using FigTree 1.4.2. In this study, we used the taxonomical macrosystem of Monogenoidea described by Lebedev [18].

3. Results

3.1. Parasite occurrence

The parasite species, their hosts, size, and number of analyzed fishes are listed in Table 1. In freshwater *D. armatum* was found both in *A. schrenckii* and *H. dauricus*, whereas in Amur Liman, this species was revealed only in Amur sturgeon. *P. pacificum* was found only in *H. dauricus*. In fresh water only the large specimens of kaluga (AC = 170–216 cm) were infected.

3.2. Sequence characteristics

The complete sequences of 18S rRNA gene from *P. pacificum* and *D. armatum* were 2007 and 2006 base pairs long, and their guanine–cytosine (GC) contents were 49.9 and 49.3%, respectively. No intra- or host-specific variation was found. The sequences were closely related between two species with the similarity rate of 98.53%. The main differences between sequences were in 23 transitions (16 C→T, 7 G→A), six transversions (2 A→C, 2 A→T and 2 C→G) and one single base pair-long indel. Most nucleotide substitutions were located in the first half of the SSU rRNA sequence (Fig. 1).

3.3. Comparative DNA analysis

The aligned sequences contain 2076 characters (including gaps), 603 of which were variable and 473 were parsimony-informative. The GC content was similar in all monogenoidean species and ranged from 46 to 52%.

The genetic divergence between *P. pacificum* and *D. armatum* was 1.55%. Intrafamily divergence in Oligonchoinea ranged from 2.21% in Allodiscocotylidae Tripathi, 1959 to 5.75% in Diclydophoridae Cerfontaine, 1895. The genetic distances (Table 2) point to closer relationships between Diclybothriidae and Hexabothriidae (d = 9.24%), than with all the other Oligonchoinea. Unfortunately, the lack of molecular data did not allow us to examine the range of divergence within Diclybothriidae. Nevertheless, the divergence value between Diclybothriidae and Hexabothriidae was within the interfamily values of the Oligonchoinea (d = 3.41–12.11%). The genetic differences in Polystomatoidea ranged from 0.53% to 11.60%. The lowest divergence value was found between *Polystoma marmorati* and *Polystoma integerrimum*, while the highest was between *Wetapolystoma almae* and *Concinocotyla australiensis*. The genetic difference between Oligonchoinea and Polystomatoidea was 11.11%.

3.4. Phylogenetic analysis

All methods employed for inferring the phylogenetic relationships of *P. pacificum* and *D. armatum* showed similar results, and divided all the sequences into two large clusters with high bootstrap support (Fig. 2). The first cluster, in its turn, included two subclusters: the first one consists of members of Diclybothriidae (Diclybothriidae and Hexabothriidae); and the second gathered all other species of Oligonchoinea. The second clade included representatives of Polystomatidae with strong phylogenetic support.

4. Discussion

As was mentioned above (see Introduction section), before 1950, *Diclybothrium* was a member of Hexabothriidae, which belonged to Polystomatoida [4,19]. Due to morphological differences between *Diclybothrium* and the other genera of Hexabothriidae, Price [19] placed this genus into Dicylbothriinae. Later, during their studies on larval and adult specimens, Bychowsky and Gussev [5] revised the position of dicylbothriids among the other Monogenoidea. This issue was subsequently discussed by Bychowsky [6]. According to Bychowsky and Gussev [5,8] the combination of Hexabothriidae and Polystomatidae is rather artificial because it based on either originally initial/primary (i.e. pleismorphies) or obviously convergent characters. These characters cannot be accounted for in making these groups phylogenetically close. Polystomatidae was attributed to Polonchoinea. The differences in several characters, such as haptor structure, the anterior region, eggs, and hosts between dicylbothriids, including *Paradiclybothrium* and other members of Hexabothriidae allow Bychowsky and Gussev to establish the separate Dicylbothriidae. Morphological divergence between Diclybothriidae and Hexabothriidae points to the ancient divergence of the ancestral species of these families. Nevertheless, they were both placed in a new order Dicylbothriidea. Morphological characters also indicated that Dicylbothriidae and Hexabothriidae are more closely related with each other than with the remaining Oligonchoinea, and closer to Oligonchoinea than to Polonchoinea [6]. This is reflected in other systems of Monogenoidea [17,18].

The results of the present study are in agreement with previous morphological [5,6,17,18,20] and molecular [26,45] works on Monogenoidea. The genetic divergence between dicylbothriids and the *Pseuhexabothrium* as well as between Dicylbothriidea and the remaining representatives of Oligonchoinea appears to be higher than interfamily differences in Oligonchoinea without Dicylbothriidea. The difference between Dicylbothriidea and Oligonchoinea was close to that between Oligonchoinea and Polystomatoidea. The phylogenetic reconstructions confirm the data on genetic divergence. Representatives of Dicylbothriidea and Hexabothriidea formed distinct branches and gathered in their own subclust with *Pseudohexabothrium taeniurae* as the sister-group. The remaining Oligonchoinea formed the other subclust.

Here, we have presented the molecular data on the Dicylbothriidea. The analysis of sequence divergence and the phylogenetic trees data points to close relationships between Dicylbothriidea and Hexabothriidea, and distance between Dicylbothriidea, the other Oligonchoinea and Polystomatoidea. High differences between Dicylbothriidea and Oligonchoinea are indirectly confirmed hypothesis of Boeger and Kritsky [20] on introduction of Heteronchoinea. It is possible that the additional molecular data for closely relative taxa Hexabothriidae and Chimaerocotylidae [18,20] could clarify the phylogenetic relationships of Dicylbothriidea in Oligonchoinea.

<table>
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<tr>
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<td>12.11</td>
<td>8.46</td>
<td>7.74</td>
<td>7.86</td>
<td>7.72</td>
<td>7.72</td>
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Fig. 1. Nucleotide differences between *P. pacificum* and *D. armatum* complete 18S rDNA sequences.
**Fig. 2.** Consensus trees topology inferred from 18S rDNA sequences. Sequences obtained in this study marked with bold. Results of phylogenetic support are shown as follows: Nj/Mp/Ml/Bi.

**Conflict of interest statement**

The authors declare that they have no competing conflict of interest.

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**References**


