Original Article

The first detection of coccidia (Conoidasida: Eimeriidae) DNA in Godlewski's sculpin *Abyssocottus* (*Limnocottus*) *godlewskii* (Dybowski, 1874)

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ABSTRACT. For the first time, fragments of the *cox1* gene of a representative of the family Eimeriidae were obtained by high-throughput sequencing in the digestive tract of Godlewski's sculpin *Abyssocottus* (*Limnocottus*) *godlewskii* (Dybowski, 1874). The nucleotide sequences of the coccidia, which accounted for less than 0.01% of the total data set, belonged to a single genotype and were significantly different from all previously known. Phylogenetic reconstruction based on the translated amino acid sequences reliably revealed the basal location of branches belonging to representatives of the family Eimeriidae among fishes. The question of the genus of the detected organism remains unresolved due to the limited nucleotide data for representatives of the genera *Eimeria*, *Calyptospora*, and *Goussia* from fish.

Keywords: Eimeriidae, *Abyssocottus* (*Limnocottus*) *godlewskii* (Dybowski, 1874), *cox1* gene, Lake Baikal

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1. Introduction

The analysis of fish parasites is an essential part of studies on their ecology. The advantage of the molecular genetic approach using high-throughput sequencing technologies is the ability to analyze and identify relatively short fragments of foreign DNA from the contents of the digestive tract, organs, and tissues of fish. These methods are efficient due to their high resolution and ability to identify a wide range of species (Harms-Tuohy et al., 2016; Jakubavičiūtė et al., 2017; Yoon et al., 2017). Despite a number of drawbacks, including inaccurate species identification due to the limited genetic data in publicly available databases (Siddall et al., 2012; Kvist, 2013) and the detection of organisms from the digestive tract of food using DNA (Sakaguchi et al., 2017), metabarcoding can serve as a complementary approach to traditional methods for studying fish parasite fauna (Ogedengbe et al., 2011; Villsen et al., 2022; Denikina et al., 2023a; b).

All members of the protozoan type Sporozoa or Apicomplexa of the Alveolata group are unicellular obligate parasites of multicellular animals and are also considered to be among the most successful parasites in the world (Morrison, 2009). It is estimated that more than 6,000 described species represent only 0.1% of the total diversity of the group (Morrison, 2009). Representatives of the genera *Cryptosporidium*, *Plasmodium, Toxoplasma*, and *Babesia* are causative agents of human and animal diseases. Coccidia cause significant damage to agricultural production (Conoidasida: Eimeriidae). Despite their widespread distribution and economic importance, research on the evolutionary relationships within this group is still in its infancy (Arisue and Hashimoto, 2015; Xavier et al., 2018). The taxonomy of coccidia is still evolving, with many genera being paraphyletic. This raises questions about the value of strict morphological and ecological traits for their classification (Ogedengbe et al., 2018; Xavier et al., 2018). Representatives of the family Eimeriidae are less well studied in aquatic animals than in terrestrial animals. Nevertheless, even the limited sequence data available for the small subunit ribosomal RNA (ssrRNA) enable to suggest that these are the base groups within the families (Jirků et al., 2009; Xavier et al., 2018; Denikina et al., 2023b). The NCBI database currently contains mtDNA *cox1* gene sequences for the following fish species: redlip blenny *Ophioblennius macclurei* (Silvester, 1915), white perch *Morone americana* (Gmelin, 1789), and belica *Leucaspius delineatus* (Heckel, 1843).

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Godlewski's sculpin *Abyssocottus (Limnocottus) godlewskii* (Dybowski, 1874) is an endemic species of lake sculpins that inhabits depths ranging from 100 to 900 m (Bogdanov, 2023). Difficulties in studying of the ecology and parasite fauna of deepwater species arise from the limited number of fish samples due to the labor-intensive capture process. A study of the food spectrum of Godlewski's sculpin using next-generation sequencing techniques has resulted in coccidia sequences. The aim of the work was to determine the phylogenetic position of a representative of the family Eimeriidae from the digestive tract of Godlewski's sculpin.

2. Materials and methods

The samples were collected in September 2019 from the R/V "G.Y. Vereshchagin" in the area around the Chivyrkuisky Bay of Lake Baikal (53°59.674'N, 109°09.086'E) at depths of 790 to 820 m. The fish species were identified according to the latest revisions (Bogdanov, 2017; 2023). Five individuals of Godlewski's sculpin with weights ranging from 8.7 to 28.5 g and total lengths from 95 to 149 mm were used for the analysis.

In vitro, the contents of the entire digestive tract (250-700 μl) of each individual were diluted with an equal volume of mQ water, ground and mixed thoroughly. Total DNA was extracted using the DNA-sorb-AM kit (Russia) according to the manufacturer`s instructions. An approximately 350 bp fragment of the *cox1* gene was amplified for each sample in 30 cycles with reducing the annealing temperature by 0.3°C from the initial 55°C, using MiSeq primers: COIintF 5´tcgtcggcagcgtcagatgtgtataagagacagGGWACWGGWTGAACWGTWTAYCCYCC and dgHCO2198 5´gtctcgtgggctcggagatgtgtataagagacagTAIACYTCIGGRTGICCRAARAAYCA (Leray et al., 2013). All amplicons from the digestive tract were pooled and used to prepare the sample for sequencing.

A library was constructed from the purified amplicon pool using the Nextera XT kit (Illumina, Hayward, California, USA). The nucleotide sequences were determined using Illumina NextSeq. The registration number of the data obtained in the international NCBI database is PRJNA1086215.

All original reads were trimmed for quality using the program Trimmomatic V 0.39 (Bolger et al., 2014) with options: average read quality 20, minimum read length 140. The original reads were assembled into contigs corresponding to the full-length amplification products using the program metaSPAdes (Nurk et al., 2017) with k-mer lengths of 21, 33, 55, 77, 99, and 121. The chosen k-mer lengths allowed the aggregation to be brought into single contigs containing only reads specific to the original *cox1* fragments of the DNA mixture of different metagenomic sample species.

The complete sequence set of the *cox1* marker from the International Barcode of Life Database (iBOL) (https://ibol.org/) was used as a reference database for the taxonomic analysis. The DNA sequences of the amplicon assembly were compared to a reference database using the local BLASTn application (Altschul et al., 1990). The results of the BLAST analysis were converted into a table of taxonomic representation in the DNA of the host digestive tract contents. The primary processing of the obtained nucleotide sequences of representatives of the family Eimeriidae and the corresponding data in the NCBI database (Table 1) was performed with the editor BioEdit and aligned with the program ClustalW. The sequence is registered in NCBI under the number PP552829. Phylogenetic analysis, including model selection for estimating evolutionary divergence and reconstructing evolutionary history, was performed using the program MEGA7 (Kumar et al., 2016). The evolutionary divergence between the sequence groups was estimated with the maximum likelihood method using the Tamura-Nei model (TrN DNA evolutionary model) (Tamura and Nei, 1993).

Phylogenetic reconstruction of evolutionary history based on amino acid sequences was performed with the maximum likelihood method using the Le-Gascuel model with gamma correction for differences in rates of substitution accumulation at different sites (LG + G protein evolutionary model) (Nei and Kumar, 2000; Le and Gascuel, 2008). A non-parametric booster (1000 replicates) was used to test the validity of the phylogenetic tree topology.

3. Results and discussion

As a result of analyzing data from metagenomic DNA sequencing of the Godlewski's sculpin digestive tract contents, sequences from representatives of the family Eimeriidae with a relative representation of $<0.01\%$ were detected. The sequences obtained belonged to the only haplotype significantly different from all known sequences of the *cox1* gene of coccidia, including *G. bayae* and Eimeriidae derived from the belica, and showed the highest degree of homology (86.71%) with the nucleotide sequences of *Cyclospora cayetanensis* (Ortega, Gilman & Sterling, 1994).

Fish coccidia are relatively understudied, and very little nucleotide data is available for them. In addition to the sequences of the *cox1* mtDNA gene from the common sunbleak, which were previously obtained in a similar experiment (Denikina et al., 2023b), only two sequences of representatives of the family Eimeriidae from fish are currently available in the NCBI database. The sequences of *G. bayae* from the gall bladder of the white perch (Matsche et al., 2019) and a sequence from the blood of the redlip blenny were also obtained. However, the latter, referred to as *Coccidia* sp. (NCBI: OR822199.1), actually belongs to a clade of a new widespread group of fish parasites of the Apicomplexa type, sister to the order Corallicolida and called "ichthyocolids" by the authors (Bonacolta et al., 2024). Based on the above, these data were not included in the phylogenetic analysis. The phylogenetic tree was constructed using data from representatives of the family Eimeriidae of vertebrates; the sequence of the *Toxoplasma gondii* mtDNA *cox1* gene was represented as an outgroup (Nicolle & Manceaux, 1908) (Table 1, Fig. 1).

Table 1. The *cox1* gene nucleotide sequence numbers from the NCBI database used in the analysis.

Host	No.No. NCBI; Species
Mammalia: Placentalia	MN260359; MN260361; MN260362; MN260363; MN260364; MN316534; MN316535; *Cyclospora cayetanensis* Ortega, Gilman & Sterling, 1994
	KP025693; *Eimeria flavescens* Marotel & Guilhon, 1941
	KT203398; *Eimeria mephitidis* Andrews 1928
	JQ993698; *Eimeria piriformis* Kotlan & Pospesch, 1934
	HM771687; KX495130; OL770312; *Eimeria zuernii* (Rivolta, 1878) Martin, 1909
	MN077082; *Toxoplasma gondii* (Nicolle & Manceaux, 1908)
Mammalia: Marsupialia	MK202809; *Eimeria gaimardi* Barker, O'Callaghan, and Beveridge, 1988
	MK202808; *Eimeria mundayi* Barker, O'Callaghan, and Beveridge, 1988
	MK202807; *Eimeria potoroi* Barker, O'Callaghan, and Beveridge, 1988
	JN192136; *Eimeria trichosuri* O'Callaghan & O'Donoghue, 2001
	MK202806; *Eimeria woyliei* Northover et al., 2019
Reptilia	KF859856; *Caryospora bigenetica* Wacha and Christiensen, 1982
	KR108297; MW720599; *Isospora amphiboluri* Cannon, 1967
	MW720599; *Isospora lunulatae* Yang, Brice, Berto & Zahedid, 2021
Aves	EF158855; *Eimeria acervulina* Tyzzer, 1929
	MH758793; *Eimeria anseris* (Kotlan, 1932)
	HM771675; *Eimeria brunetti* Levine, 1942
	JQ659301; KX094945; *Eimeria praecox* Johnson, 1930
	MF497440; *Eimeria tenella* (Railliet & Lucet, 1891) Fantham, 1909
	KC346355; *Isospora gryphoni* Olson, Gissing, Barta & Middleton, 1998
	KT224377; *Isospora manorinae* Yang, Brice, Jian & Ryan 2016
	NC_065382; *Isospora picoflavae* Rejman, Hak-Kovacs & Barta, 2021
	ON584773; *Isospora serini* (Aragao, 1933)
	KX276860; *Isospora serinuse* Yang, Brice, Elliot & Ryan 2015
Amphibia	KT184381; *Lankesterella minima* (Chaussat, 1850) Nöller, 1912
Actinopteri	PP590353; PP590354; PP590355; PP590356; Eimeriidae
	MH792860; *Goussia bayae* Matsche, Adams & Blazer, 2019

It is important to note that for all currently available sequences of the family Eimeriidae from fish, the closest homologs are those of parasites from homeothermic animals and birds: *G. bayae* is homologous to *Choleoeimeria taggarti* (Amery-Gale et al., 2018) Kruth, Michel, Amery-Gale & Barta, 2020 (79.33%, NCBI: MK813349) from the yellow-footed antechinus *Antechinus flavipes flavipes* (Waterhouse, 1838). Representatives of the family Eimeriidae from the belica are most closely related to *Eimeria praecox* (Johnson, 1938) (82.95%, NCBI: KX094945) from the red junglefowl *Gallus gallus* (Linnaeus, 1758); *Isospora serini* (Aragao, 1933) (84.62%, NCBI: ON584773) and *Isospora serinuse* (Yang, Brice, Elliot & Ryan, 2015) (82.37%; NCBI: KX276860) from the common canary *Serinus canaria* (Linnaeus, 1758). A comparative analysis of the nucleotide sequences revealed a high degree of similarity between representatives of the family Eimeriidae from Godlewski's sculpin and parasites of marsupials (Table 2).

Analysis of phylogenetic relationships based on the *cox1* mtDNA nucleotide sequences proved to be uninformative; the tree was unresolved with low support. However, representatives of the family Eimeriidae of fish have formed basal branches. The phylogenetic reconstruction based on translated amino acid sequences (Fig. 1) demonstrates that representatives of the family Eimeriidae from fishes are reliably located at the base of the tree. The hypothesis that fish coccidia were the source of all known coccidia lineages in other vertebrates (Rosenthal et al., 2016; Xavier et al., 2018; Matsche et al., 2019; Denikina et al., 2023b) was indirectly confirmed.

It has been previously suggested that the *cox1* gene fragment has sufficient phylogenetic potential to contribute to the resolution of the apparent paraphyly within coccidia (Ogedengbe et al., 2011). The results obtained do not allow us to definitely confirm this hypothesis, as data on *cox1* mtDNA sequences of representatives of the genera *Eimeria, Calyptospora,* and *Goussia* from fish are currently insufficient. For the above reasons, it is premature to determine to which genus the detected representative of the family Eimeriidae belongs.

Metagenomic studies (metabarcoding) of eukaryotes from marine and terrestrial ecosystems have shown the high diversity and dominance of Apicomplexa representatives (Mahé et al., 2017; Lentendu et al., 2018), which are parasites of invertebrates and vertebrates, and have complex life cycles that differ significantly between groups (Votýpka et al., 2016; Rueckert et al., 2019). The family Eimeriidae is the most diverse taxon of protozoa. The main characteristic of its representatives is the formation of environmentally stable oocysts, that are released with the host's feces. The general morphology of the oocysts, as well as the number of sporocysts and sporozoites are commonly used to identify individual genera. However, the results of recent phylogenetic studies correlate poorly with current taxonomy. They have also shown that several diagnostic traits thought to be unique and are also found in representatives of several genetically distant genera (Votýpka et al., 2016). It is now known that members of the genera *Eimeria, Goussia* and *Calyptospora* are most commonly found in various species of marine and freshwater fish (Xavier et al., 2018).

Previously, five species of coccidia were identified in fish from Lake Baikal (Shulman and Zaika, 1964; Zaika, 1965; Pronina, 1990), and only one was observed in representatives of the Cottidae family:

1. *Goussia carpelli* (Leger et Stankovitch, 1921) (Syn.: *Eimeria carpelli* (Leger et Stankovitch, 1921); *E. cyprini* (Plehn, 1924); *Goussia carpelli* sensu (Dykova et Lom, 1983). The parasite is localized in the intestinal and gall bladder walls of the bighead sculpin *Batrachocottus baicalensis* (Dybowski, 1874), the sandy sculpin *Leocottus kesslerii* (Dybowski, 1874), the broadsnout sculpin *Abyssocottus (Cyphocottus) eurystomus* (Taliev, 1955), and the siberian river minnow *Phoxinus rivularis* (Pallas, 1773).

2. *Goussia leucisci* (Schulman et Zaika, 1964) Lom, Desser, Dykova, 1989 (Syn.: *Eimeria leucisci* (Schulman et Zaika, 1964); *E. freemani* (Molnar et Fernando, 1974); *Goussia freemani* (Molnar et Fernando, 1974)). The parasite is localized in the kidneys and in the walls of the gall bladder of the Siberian dace *Leuciscus baicalensis* (Dybowski, 1874).

3. *Eimeria esoci* Schulman et Zaika, 1964. The parasite is localized in the intestinal and gall bladder walls of the northern pike *Esox lucius* (Linnaeus, 1758).

4. *Eimeria percae* (Riviere, 1914) (Syn.: *Coccidium percae* Riviere, 1914; *Eimeria percae* Reichenow, 1921; *E. rivieri* Yakimoff, 1929). The parasite is localized in the intestinal walls and kidneys of the European perch *Perca fluviatilis* (Linnaeus, 1758).

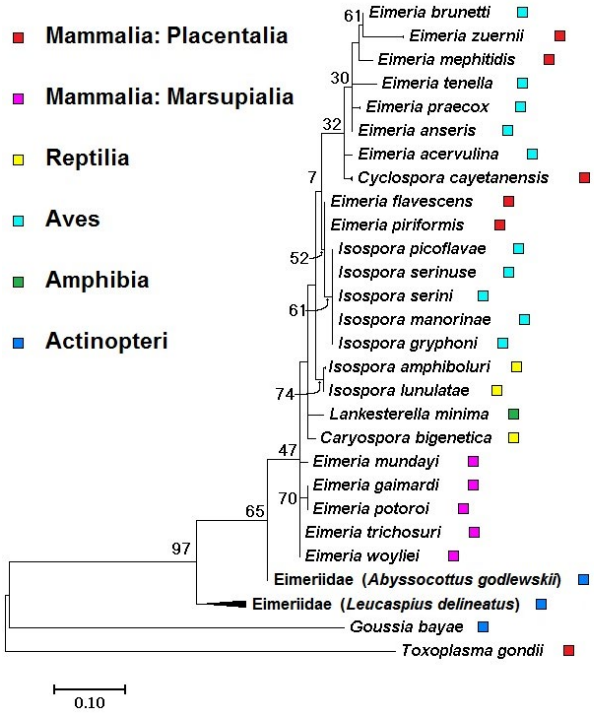

Fig.1. A phylogenetic tree of representatives of the family Eimeriidae constructed using the maximum likelihood method based on translated amino acid sequences of the mtDNA *cox1* gene fragments. *T. gondii* as an outgroup

5. *Eimeria* sp. The parasite is localized in the intestinal walls of the Baikal omul *Coregonus migratorius* (Georgi, 1775).

One species, *G. carpelli*, has previously been recorded in representatives of the family Cottidae, including coastal species of the bigheaded and sand sculpins, as well as in the deep-water species, the broadsnout sculpin. For the parasitic protozoa Apicomplexa, which are transmitted and spread by oral-fecal means, the resistance of the oocysts to environmental factors is of great importance (Clopton et al., 2016). Due to these properties, they can be detected in a variety of environmental samples, including paleontological samples (Rueckert et al., 2011; Côté and Le Bailly, 2018; Le Bailly et al., 2019; Singer et al., 2020; Beltrame et al.,

Table 2. The estimation of evolutionary divergence between sequence groups. The standard errors are given above the diagonal

	1	2	3	4	5	6	7
1. Eimeriidae (*Abyssocottus godlewskii*)		0.029	0.062	0.019	0.020	0.022	0.076
2. Eimeriidae (*Leucaspius delineatus*)	0.119		0.047	0.026	0.024	0.021	0.069
3. *Goussia bayae*	0.385	0.271		0.053	0.049	0.050	0.065
4. Mammalia: Marsupialia	0.061	0.105	0.315		0.010	0.012	0.077
5. Reptilia + Amphibia	0.070	0.102	0.293	0.020		0.004	0.068
6. Mammalia: Placentalia + Aves	0.087	0.089	0.303	0.034	0.010		0.069
7. *Toxoplasma gondii*	0.486	0.450	0.423	0.487	0.433	0.443	

2022). Oocysts, including those of the genera *Eimeria* and *Goussia*, may be present in the external environment, including bottom sediments (Siński and Behnke, 2004). In coccidia of aquatic animals, young oocysts are usually released with the feces that are not sporulated and are not infectious, as their development is terminated only in the external environment, where the formation of sporocysts with sporozoites occurs (Votýpka et al., 2016). Two modes of transmission are observed in the life cycle of coccidia in fish: direct with fecal contamination and indirect, which includes invertebrates (Steinhagen and Korting, 1988; Davis and Ball, 1993). It can therefore be assumed that the DNA of a representative of the family Eimeriidae could enter the digestive tract of Godlewski's sculpin with equal probability in two ways: directly from the external environment and/or indirectly via its food objects.

Sequences derived from representatives of the family Eimeriidae accounted for <0.01% of all metagenomic DNA sequencing data from the contents of the digestive tract of fish. However, we cannot currently confirm whether the parasite we detected is specific to the Godlewski's sculpin. *G. carpelli,* which is found in members of the family Cottidae, is considered a specific parasite of the common carp *Cyprinus carpio* (Linnaeus, 1758) (Molnár et al., 2005). However, other fish species on its host list have their own separate coccidia species (Sokolov and Moshu, 2014). In this context, a comprehensive morphological and molecular genetic study of these parasites is required, with particular attention to the widespread *G. carpelli* from different systematic fish groups.

4. Conclusion

When analyzing the metagenomic DNA sequencing data of the Godlewski's sculpin digestive tract contents with a relative representation of <0.01%, sequences from representatives of the family Eimeriidae were detected for the first time. The sequences obtained belonged to the only haplotype that was reliably different from all previously known. In contrast to the analysis of the nucleotide sequences of the *cox1* mtDNA, the phylogenetic reconstruction based on translated amino acid sequences reliably demonstrated the basal location of the branches of representatives of the family Eimeriidae in fish. The question of the genus of the detected organism remains unresolved due to the limited nucleotide data for representatives of the genera *Eimeria, Calyptospora,* and *Goussia* in fish. The results obtained indicate the need for targeted and complex studies, including molecular genetic studies, of the fauna of parasitic protozoa in fish.

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Conflict of Interest

The authors declare no conflicts of interest.

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