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GENOTYPING OF BLACK SEA TREMATODES OF THE FAMILY OPECOELIDAE BY MITOCHONDRIAL MARKERS

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Opecoelidae Ozaki, 1925 (Trematoda: Opecoeloidea) is the biggest trematode family in the Black Sea in terms of species and genera number. Maritae of the most common Black Sea Opecoelidae trematodes are well described morphologically; nevertheless, information on their genomes structure is sketchy, and data on mitochondrial genomes are absent. The aim was to study the structure of mitochondrial genome fragments of Black Sea trematode species: *Cainocreadium flesi* Korniychuk & Gaevskaya, 2000, *Gaevskajatrema perezi* (Mathias, 1926) Gibson & Bray, 1982, and *Helicometra fasciata* (Rudolphi, 1819) Odhner, 1902. Sequences were made for CO1 (the cytochrome c oxidase subunit I) and 16S mitochondrial genes. To amplify CO1 gene fragment of *Cainocreadium* and *Helicometra* trematodes, primers were developed. Phylogenetic relationships within the analyzed part of the Opecoelidae family were reconstructed on the basis of our data and the corresponding GenBank data by the Maximum Likelihood algorithm, implemented in MEGA X program. To root the phylogenetic trees, the corresponding sequences of the closely related trematode *Brachycladium goliath* (Brachycladioidea: Brachycladiidae) were used. For the first time, nucleotide sequences of CO1 and 16S mitochondrial genes fragments of Black Sea trematodes *C. flesi*, *G. perezi*, and *H. fasciata* from different definitive fish hosts were identified and deposited in GenBank. In case of *C. flesi*, no host-specific lines were found in the structure of CO1 mitochondrial gene fragment, but high CO1 nucleotide diversity was noted. Black Sea *H. fasciata*, parasitizing peacock wrasse, *Symphodus tinca*, were revealed to be a host-specific CO1 haplogroup; its taxonomic status requires further clarification, and ecological and genetic studies of the putative *H. fasciata* species complex from different water areas are needed. No host-specific genetic lines were found when analyzing the sequences of *H. fasciata* 16S rRNA mitochondrial gene fragment. No significant differences in 16S fragment were registered between *G. perezi* trematodes from different Black Sea definitive hosts; however, the intraspecific 16S nucleotide diversity was rather high.

Keywords: Black Sea, Trematoda, Opecoelidae, *Cainocreadium*, *Gaevskajatrema*, *Helicometra*, mitochondrial genes, CO1, 16S rRNA

Opecoelidae Ozaki, 1925 (Trematoda: Opecoeloidea) is the leading Black Sea trematode family in terms of genera and species number (Gaevskaya & Kornyychuk, 2003). Over the past approximately 50 years, it has experienced a progressive decrease in species and genera representation in this water basin (Dmitrieva et al., 2018), and its reasons are not completely clear. To date, maritae of the most widespread Opecoelidae species in the Black Sea (*Cainocreadium flesi* Korniychuk & Gaevskaya, 2000, *Gaevskajatrema perezi* (Mathias, 1926) Gibson & Bray, 1982, and *Helicometra fasciata* (Rudolphi, 1819) Odhner, 1902) are detailed only morphologically, by light microscopy

methods (Gaevskaya & Solonchenko, 1989 ; Korniychuk, 2009 ; Kornyychuk & Gaevskaya, 2000 ; Opredelitel' parazitov pozvonochnykh…, 1975). The available data on the genomes structure of Opecoelidae species (and generally trematodes), known from the Black Sea, are mainly represented by nucleotide sequences of nuclear DNA (18S rDNA, 28S rDNA, ITS1, and ITS2) (Katokhin & Kornyychuk, 2018 ; Andres et al., 2014 ; Born-Torrijos et al., 2012 ; Bray et al., 2016 ; Jousson & Bartoli, 2001). Few data on mitochondrial genes structure of Opecoelidae genera of the Black Sea were obtained only from the Pacific material (Donald et al., 2004 ; Donald & Spencer, 2016 ; González et al., 2013 ; Lagrue, 2016 ; López et al., 2015 ; Martin et al., 2019 ; Yano & Urabe, 2017); they are of little use for confirming species identification of Black Sea trematodes by molecular genetic methods.

The aim of the study is to obtain the first data on the structure of mitochondrial genome fragments of trematode species, being the most common ones in the Black Sea in the current period (*C. flesi*, *G. perezi*, and *H. fasciata*), for further clarification of their taxonomic status.

MATERIAL AND METHODS

Maritae of Opecoelidae trematodes (*Cainocreadium flesi*, *Gaevskajatrema perezi*, and *Helicometra fasciata*) were obtained from Black Sea fish, caught in Sevastopol and Batiliman water areas, as well as in Karadag nature reserve water area (Table 1). The species were identified by generally accepted morphological criteria (Gaevskaya & Solonchenko, 1989 ; Korniychuk, 2009 ; Kornyychuk & Gaevskaya, 2000 ; Opredelitel' parazitov pozvonochnykh…, 1975).

For molecular genetic analysis, trematode mitochondrial genes fragments, encoding 16S rRNA and the cytochrome c oxidase subunit I (CO1), were selected. Total DNA was isolated by the CTAB method (Wilke et al., 2006). To amplify 257-nucleotide 16S rRNA fragment, the primers OMP38 5'-AGACGGAAAGACCCCGAG-3' and OMP04 5'-CTCACGCCGGTCTTAACT-3' were used, as well as the thermal profile of the polymerase chain reaction as follows: denaturation at +94 °C for 3 minutes, 40 cycles (denaturation at +94 °C for 20 seconds; primer annealing at +52 °C for 30 seconds; synthesis at +72 °C for 25 seconds). These primers were developed and used for the reconstruction of opisthorchiid trematodes mitogenomes (Shekhovtsov et al., 2010), and they are also suitable for Opecoelidae species genotyping. Amplification was performed under standard reaction conditions (see http://molbiol.ru/protocol/12_01.html).

To amplify 276-nucleotide CO1 gene fragment, we used the primers PlagiHenC1F 5'-GTTGTTTGGGCTCATCATATGTTTA-3' and OpeCo1uniR2 5'-AGCCACCACAAACCAAGTATCATG-3', as well as the thermal profile of the polymerase chain reaction, described above, but with primer annealing at +54 °C. Since CO1 sequences for *Cainocreadium* trematodes were not known before our study, we used the primer, which was proposed earlier for Prosthogonimidae trematodes (Heneberg et al., 2015), as the basis for the development of the PlagiHenC1F primer. After the alignment with the sequences from GenBank (NCBI), this primer was modified to be suitable for Opecoelidae species genotyping. When developing the OpeCo1uniR2 primer, we used the primer, which was developed and used for opisthorchiid genotyping, as the basis; it was similarly modified to work with Opecoelidae trematodes DNA (Fig. 1).

The amplicons were sequenced by the Sanger method. The analysis of the sequencing reaction products after purification by isopropanol precipitation was carried out in the sequencing core facility in the Institute of Molecular and Cellular Biology of Siberian Branch of RAS on a capillary sequencer ABI 3730xl Genetic Analyzer (Applied Biosystems).

Table 1. Trematoda (Opecoelidae) maritae samples, analyzed in the study

Sample identifier	Trematode species	Fish host species	Sampling area	No. in GenBank	
				16S	CO1
009Cai-PfSKr05-21	*Cainocreadium flesi*	*Platichthys flesus*	Sevastopol	MT472528	MT472167
010Cai-PfSKr05-22				MT472529	MT472168
022Cai-PfSKr05-23				MT472530	MT472169
001Cai-GmSKr05-21		*Gaidropsarus mediterraneus*	Sevastopol	MT472531	–
002Cai-GmSKr05-22				MT472532	–
003Cai-GmSKr05-23				MT472533	MT472170
004Cai-GmSKr05-24				MT472534	–
011Cai-GmKKr18-21			Karadag	MT472535	MT472171
012Cai-GmKKr18-22				MT472536	–
013Cai-GmKKr18-23				MT472537	MT472172
014Cai-GmKKr18-24				MT472538	MT472173
018Cai-GmKKr18-26				MT472539	–
019Cai-GmKKr18-27				MT472540	–
005Hel-SpSKr05-21	*Helicometra fasciata*	*Salaria pavo*	Sevastopol	–	MT472174
006Hel-SpSKr05-22				MT472542	MT472175
015Hel-GmBKr17-22		*Gaidropsarus mediterraneus*	Batiliman	MT472543	MT472176
042Hel-GmKKr18-21			Karadag	MT472548	–
043Hel-GmKKr18-22				MT472549	–
047Hel-GmKKr18-23				MT472551	MT472177
048Hel-GmKKr18-24				MT472552	–
036Hel-AsKKr18-21		*Aidablennius sphynx*	Sevastopol	MT472545	–
037Hel-AsKKr18-22				MT472546	–
040Hel-AsKKr18-25				MT472547	–
049Hel-SpKKr06-21		*Scorpaena porcus*	Karadag	MT472553	MT472178
045Hel-SrBKr02-21		*Symphodus roissali*	Sevastopol	MT472550	–
056Hel-StSKr19-22		*Symphodus tinca*	Sevastopol	MT472554	MT472179
057Hel-StSKr19-23				MT472556	–
041Gae-SsSKr19-21	*Gaevskajatrema perezi*	*Symphodus ocellatus*	Sevastopol	MT472557	–
051Gae-SoSKr19-22				MT472558	–
052Gae-StSKr19-23		*Symphodus tinca*	Sevastopol	MT472559	–
053Gae-StSKr19-24				MT472556	–
–	*Helicometra fasciata*	*Labrisomus philippii*	Pacific Ocean, Chile coast	–	KJ996004 [20]
–	*Helicometra fasciata*	*Paralabrax humeralis*		–	KJ996005; KJ996006 [20]
–	*Brachycladium goliath*	*Balaenoptera acutorostrata*	Atlantic Ocean	KR703278	KR703278 [17]

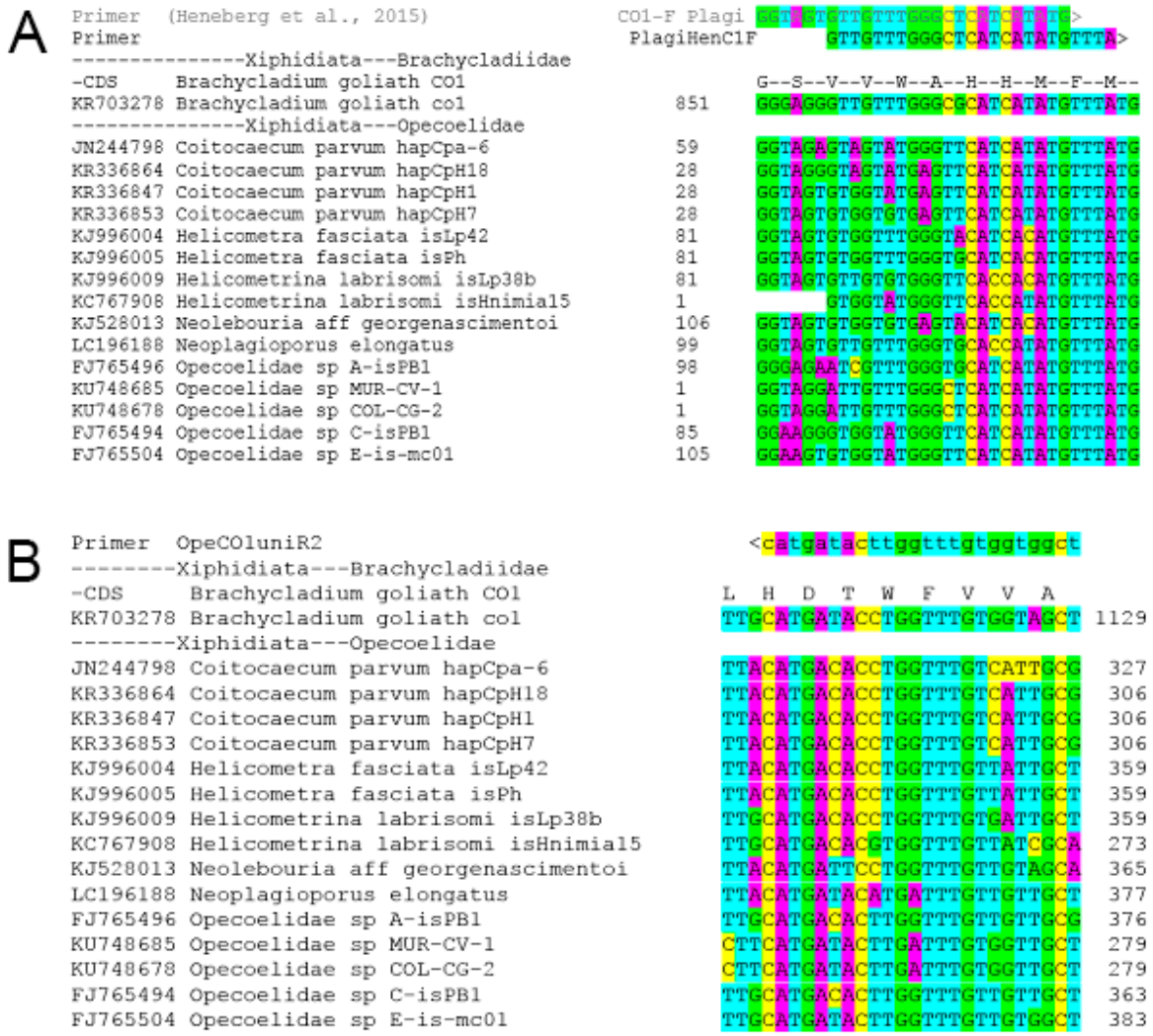

Fig. 1. Alignment of CO1 gene fragments of Opecoelidae trematodes and *Brachycladium goliath*; position of PlagiHenC1F (A) and OpeCo1uniR2 (B) primers

The sequences obtained were aligned by the Clustal W algorithm; genetic distance calculation and multiple alignment analysis were carried out by the MEGA X program (Kumar, 2018).

Nucleotide sequences of CO1 and 16S mitochondrial gene fragments of Black Sea trematodes, identified in this work, were deposited in GenBank (see Table 1). For phylogenetic analysis, the corresponding 16S and CO1 sequences of Opecoelidae from GenBank were used (Table 1). For *Gaevskajatrema perezi*, CO1 fragment failed to amplify; the possible reason of the failure is excessive polymorphism in template fragments, targeted by primers. Phylogenetic relationships within the analyzed part of the Opecoelidae family were reconstructed on the basis of our data and the corresponding GenBank data by the Maximum Likelihood (ML) algorithm and the HKY nucleotide substitution model, recommended by the Model Test subprogram (the MEGA X program). To root the phylogenetic trees, the corresponding sequences of the closely related trematode *Brachycladium goliath* (van Beneden, 1858) Fraija-Fernández, Aznar, Raga, Gibson & Fernández, 2014 (Brachycladioidea: Brachycladiidae) were used.

Haplotypes were identified and analyzed by the DnaSP 5.10 software (Librado & Rozas, 2009). Haplotype networks were built by the Network 10 program (http://www.fluxus-engineering.com/sharenet.htm) (Bandelt et al., 1999).

RESULTS AND DISCUSSION

The analysis of the genetic distances between Black Sea Opecoelidae trematodes from different genera according to the sequences of 16S and CO1 mitochondrial genes fragments revealed significant quantitative differences between them, with a much lower intraspecific variability of the corresponding trematode genome fragments (Table 2).

Table 2. Estimates of genetic distances (number of base substitutions per site) intra- (in brackets, bold) and between studied Black Sea Opecoelidae trematodes for CO1 (above diagonal) and 16S mitochondrial genes fragments (below diagonal)

CO1 / 16S	*Cainocreadium flesi* (**0.2175**)	*Helicometra fasciata* (**0.0423**)
Cainocreadium flesi (**0.0031**)		0.3108
Helicometra fasciata (**0.0109**)	0.0719	
Gaevskajatrema perezi (**0,0127**)	0.0688	0.0616

The position of Black Sea Opecoelidae trematode species on the phylogenetic tree, based on 16S rRNA fragment under study (Fig. 2), adequately reflected the assignment of trematode species to genera.

***Cainocreadium flesi* Korniychuk & Gaevskaya, 2000.** As a rule, 16S mitochondrial gene is used to distinguish trematode taxa above the species level (most reliably to distinguish families) (Blasco-Costa et al., 2016). On the intraspecific level, any significant differences in its nucleotide sequences are unlikely. However, in *Cainocreadium* trematodes, six 16S haplotypes were identified, and the transitions between them are clearly illustrated in Fig. 3. The samples analyzed from the European flounder *P. flesus* are represented by three haplotypes (No. 4–6), differing by one nucleotide substitution and/or insertion-deletion. All analyzed *Cainocreadium* maritae samples from shore rockling *G. mediterraneus* are represented by three other haplotypes (No. 1–3), differing by one nucleotide substitution. The most common haplotype is No. 1: it was identified from *Cainocreadium* specimens, sampled in different years, in different areas, and from different fish host specimens. The host specificity of 16S gene haplogroups indicates a certain multidirectionality of microevolutionary processes in relation to Black Sea *Cainocreadium* maritae, parasitizing shore rocklings and European flounders.

The analysis of similarity and difference of *C. flesi* trematodes from various hosts by the structure of CO1 mitochondrial gene fragment (Fig. 4) revealed no host-specific clusters, which is consistent with the previously obtained data on the absence of genetic variability of these trematodes from shore rockling and European flounder by ITS1 nucleotide sequences (Katokhin & Kornyychuk, 2018). Moreover, it apparently may indicate belonging of Black Sea *Cainocreadium* maritae from different definitive fish hosts, having, nevertheless, obvious morphological differences (Kornyychuk, 2008), to one and the same species: *C. flesi*. Meanwhile, in *C. flesi* samples analyzed, a very high CO1 haplotypic diversity was observed (Fig. 4): each of them belonged to a separate haplotype; 14 nonsynonymous substitutions (0.21 %) and 28 synonymous ones (0.42 %) were found.

The analysis by the Network 10 program revealed as follows: CO1 haplotypes of Black Sea *Cainocreadium* are divided into two groups (Hap 1÷4 and Hap 5÷7), separated by 42 mutational events; within the haplogroups, the differences do not exceed 5 mutational steps (Fig. 5). Meanwhile, neither

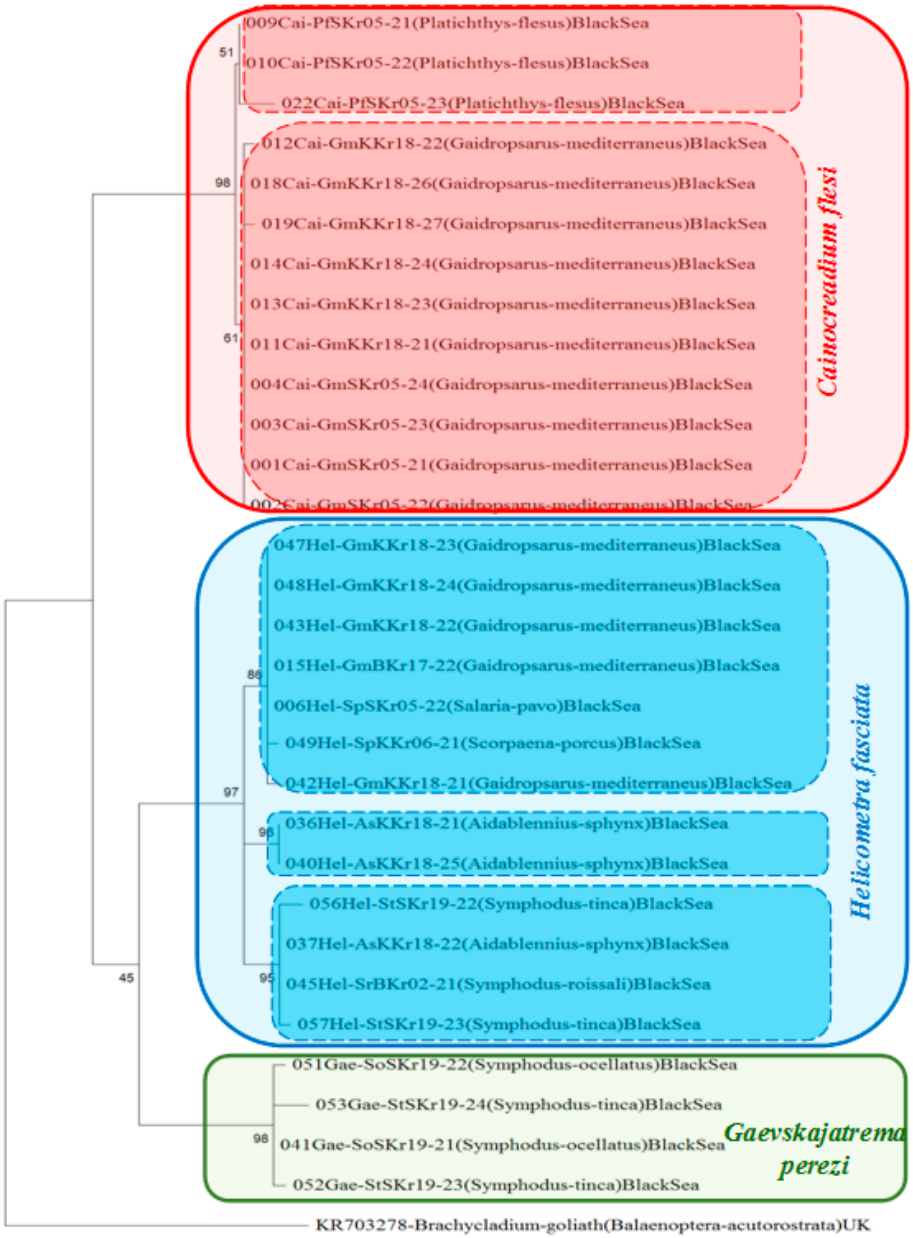

Fig. 2. ML phylogram of Black Sea Opecoelidae trematodes, based on 16S rRNA mitochondrial gene fragment

host nor spatial specificity of CO1 haplogroups was noted (Fig. 4): each of them includes trematodes from both shore rockling and European flounder, caught in two Black Sea water areas, being almost 200 km apart along the coast.

Cainocreadium entered the Black Sea apparently from the Mediterranean Sea and got completely new definitive and intermediate hosts (Kornyychuk, 2008). A new species, *C. flesi*, was formed from trematode ancestral form (Kornyychuk & Gaevskaya, 2000); the adaptation to exploiting new Black Sea hosts is clearly ongoing.

***Helicometra fasciata* (Rudolphi, 1819) Odhner, 1902.** Based on 16S RNA structure, Black Sea *H. fasciata* maritae (see Fig. 2) were divided into three clusters, with none of them reflecting narrow specificity to the definitive host: trematodes from *A. sphynx* both formed a separate cluster and were present in another cluster (from different fish hosts).

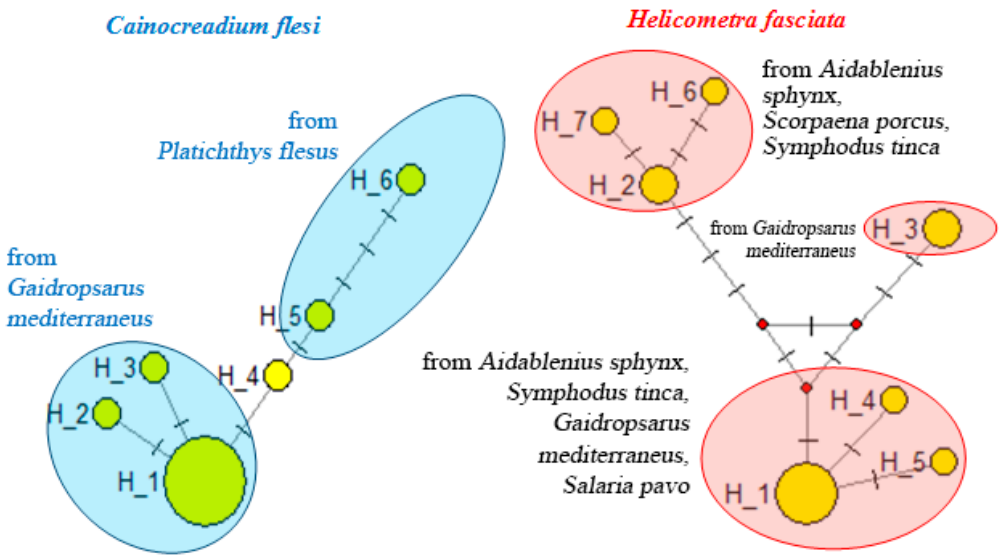

Fig. 3. Haplotype networks of 16S of two Black Sea trematodes

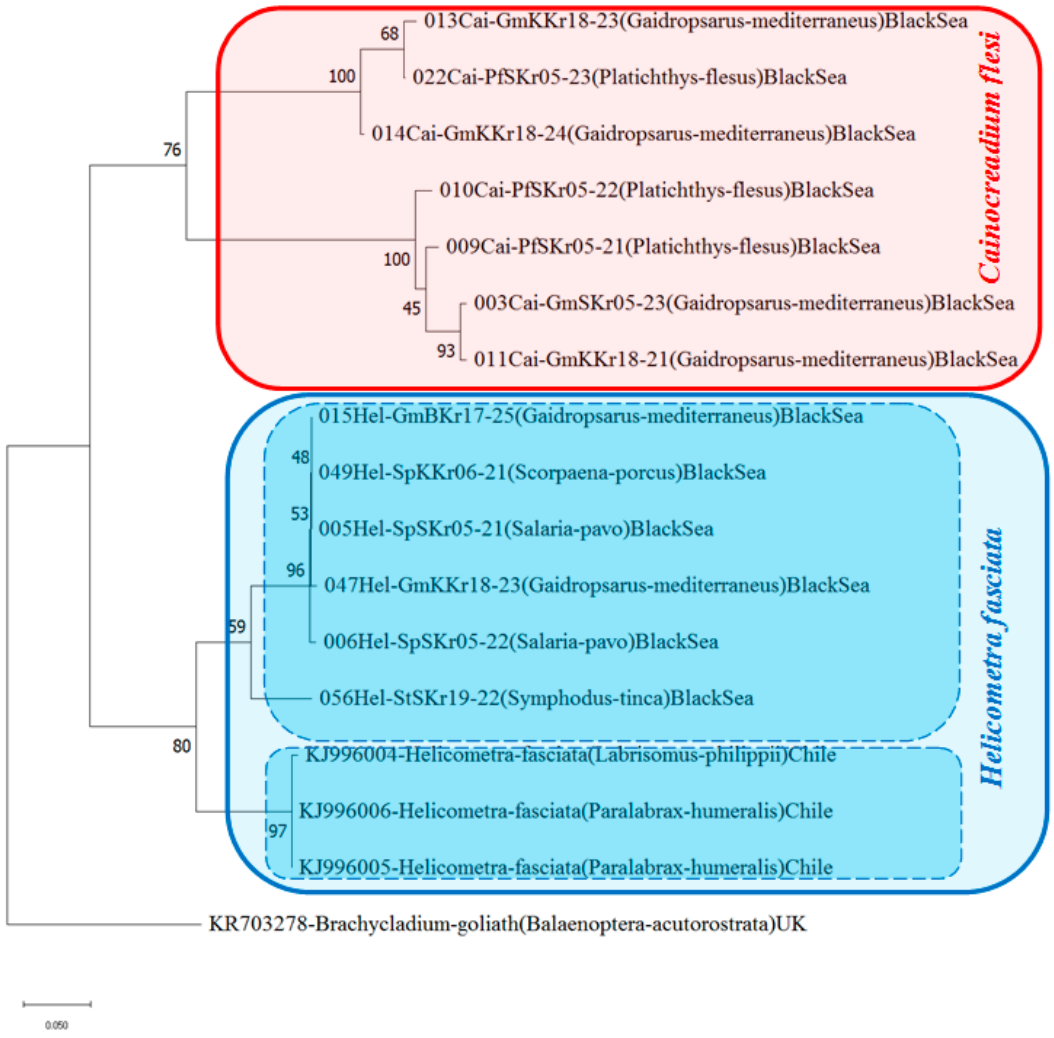

Fig. 4. ML phylogram of Black Sea Opecoelidae trematodes, based on mitochondrial gene fragment, encoding the cytochrome c oxidase subunit I (CO1)

Three groups of *H. fasciata* 16S haplotypes were identified. Hap 4÷7 are confined to Sevastopol region only; Hap 2 and 3 were found in Karadag only; Hap 1 was registered in maritae from fish from geographically significantly distant water areas (Sevastopol, Batiliman, and Karadag; more than 200 km in a straight line between the extreme points). Meanwhile, none of fish hosts is able to move long distances (Svetovidov, 1964).

Black Sea *H. fasciata* maritae studied, parasitizing peacock wrasse, *Symphodus tinca*, significantly differ in CO1 structure from *H. fasciata* maritae from the other fish hosts studied, which are grouped into another cluster on the tree (Fig. 4).

We compared our data on CO1 structure of Black Sea *H. fasciata* with the corresponding GenBank data (see Table 1) on *H. fasciata* trematodes off Chile coast. It turned out that *Helicometra* from this geographically distant area and specimens, parasitizing Black Sea fish from other families, are clustered in different groups.

CO1 haplotypes of *H. fasciata* samples studied were subdivided into three haplogroups (Fig. 5). The haplotypes of *H. fasciata* from the majority of Black Sea fish studied are closely related (Hap 1÷3; average intragroup genetic distance is 0.032). They differ significantly from haplotypes of *H. fasciata* from *S. tinca* (Hap 4; average genetic distance to above described complex of *H. fasciata* is 0.088).

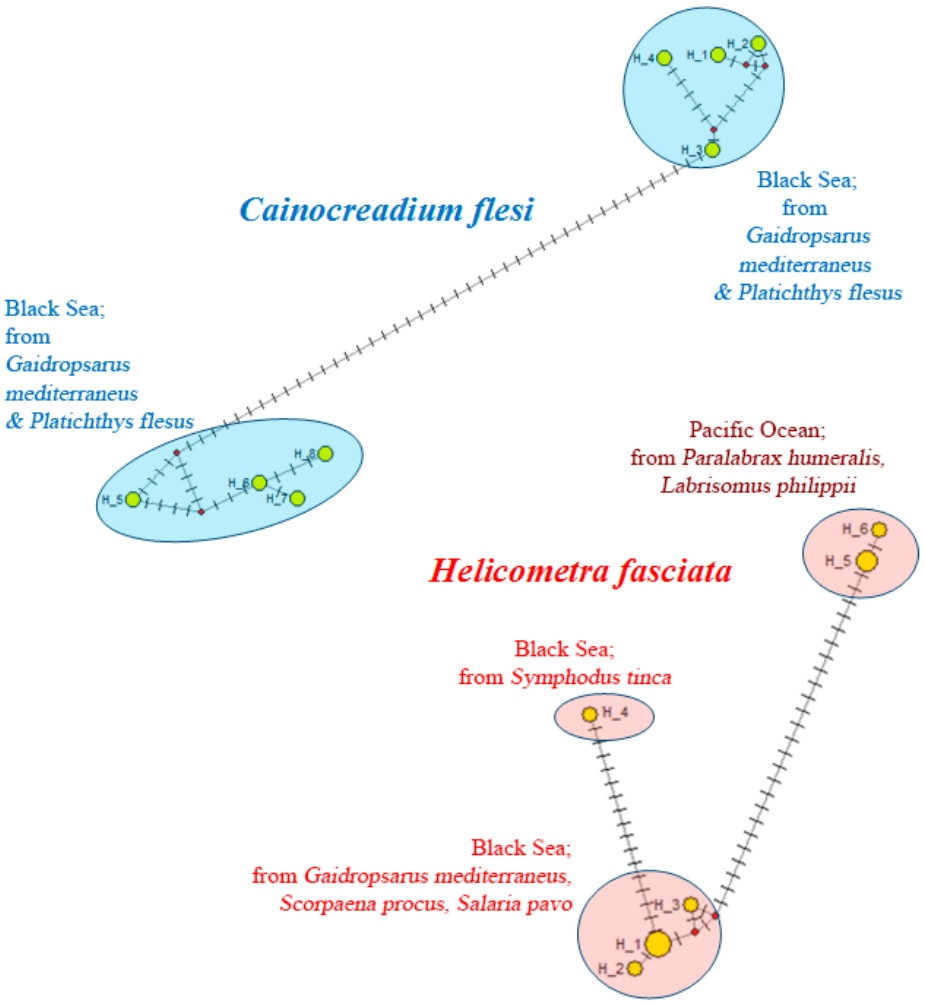

Fig. 5. Haplotype networks of CO1 of Black Sea trematodes

The most distant *H. fasciata* haplogroup (CO1 Hap 5÷6) included Pacific representatives of the species: average genetic distance is 0.154 (range 0.146÷0.158); the distance from Black Sea *H. fasciata* from *S. tinca* is 0.148, from other Black Sea *H. fasciata* – 0.155.

The values of genetic distances between two CO1 haplogroups of Black Sea *H. fasciata* and between each of these groups and Pacific *H. fasciata* are in the range of interspecific CO1 differences for trematodes, established by several researchers (León-Règagnon, 2010 ; Pérez-Ponce et al., 2016 ; Rosser et al., 2017 ; Vilas et al., 2005). These data, as well as previously established morphological peculiarity of Black Sea *H. fasciata* maritae from *S. tinca* (Korniychuk, 2000), allow suggesting as follows: all three CO1 haplogroups of *H. fasciata*, mentioned above, may be taxonomically unequal. Verification of this hypothesis requires a comprehensive study of the putative *H. fasciata* species complex: obtaining more complete information on CO1 haplotypic composition of host maritae groups, studying morphological features of *H. fasciata* of different CO1 haplogroups, and assessing presence of transitional morphotypes.

***Gaevskajatrema perezi* (Mathias, 1926) Gibson & Bray, 1982.** *G. perezi* trematodes are specific only to Labridae fish and are common in these hosts in the Black Sea (Korniychuk, 2001). Studied maritae of this species from two species of Black Sea wrasses (ocellated wrasse and peacock wrasse) appeared to have the close structure of 16S fragment (Fig. 2). It will be interesting to check this conclusion in future, using other markers.

In general, the use of two mitochondrial markers for answering the question of the status of Opecoelidae species seems to be promising. It is clear that the involvement in the analysis of new samples from different hosts and water areas will provide a lot of unique data on species diversity and intrapopulation genetic structure of these trematodes.

The data obtained on the structure of fragments of mitochondrial genes, encoding 16S rRNA and the cytochrome c oxidase subunit I (CO1), of Black Sea trematodes studied were deposited in GenBank (see Table 1) and integrated into the website of parasitic organisms subcollection of IBSS collection of the World Ocean hydrobionts (http://marineparasites.org/taxa/?dna-sequences).

Conclusions:

1. For the first time, the primers PlagiHenC1F and OpeCo1uniR2 were developed to amplify CO1 gene fragments of *Cainocreadium* and *Helicometra* trematodes.
2. For the first time, nucleotide sequences of mitochondrial gene fragments of Black Sea trematodes were identified: 16S gene of *Cainocreadium flesi* Kornyychuk & Gaevskaya, 2000, *Gaevskajatrema perezi* (Mathias, 1926) Gibson & Bray, 1982, and *Helicometra fasciata* (Rudolphi, 182) Odhner, 1902, as well as CO1 gene of *C. flesi* and *H. fasciata* from different definitive fish hosts.
3. In *C. flesi* trematodes, no host-specific groups in the structure of 16S and CO1 mitochondrial genes fragments were found; however, high CO1 nucleotide diversity was revealed.
4. In Black Sea *H. fasciata* maritae from *Symphodus tinca*, CO1 haplotype was identified, being significantly distant from CO1 haplogroup of *H. fasciata* from other fish hosts studied; the taxonomic status of maritae from *S. tinca* requires clarification. When analyzing the sequences of 16S rRNA mitochondrial gene fragment, host-specific genetic lines of *Helicometra* were not found.
5. CO1 haplogroup of Pacific *H. fasciata* is significantly distant from the haplogroups of Black Sea ones, and this requires clarification of the taxonomic status of Pacific *H. fasciata* as well.
6. No differences were found in the structure of studied 16S gene fragment in Black Sea *G. perezi* from different definitive hosts.

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ГЕНОТИПИРОВАНИЕ ЧЕРНОМОРСКИХ ТРЕМАТОД СЕМЕЙСТВА OPECOELIDAE ПО МИТОХОНДРИАЛЬНЫМ МАРКЕРАМ

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Opecoelidae Ozaki, 1925 (Trematoda: Opecoeloidea) — ведущее по числу видов и родов семейство трематод в Чёрном море. Мариты наиболее распространённых видов черноморских опецелидных трематод подробно описаны морфологически, однако сведения о структуре их геномов отрывочны, а данные о митохондриальных геномах отсутствуют полностью. Цель исследования — получить первые сведения о строении участков митохондриального генома представителей наиболее распространённых в Чёрном море в современный период родов трематод семейства Opecoelidae для последующего уточнения их таксономического статуса. Филогенетические отношения внутри анализируемой части этого семейства реконструированы на основе данных, полученных нами, и соответствующих данных из GenBank с помощью алгоритма Maximum Likelihood и модели нуклеотидных замен HKY. Для укоренения филогенетического дерева использованы соответствующие последовательности *Brachycladium goliath* (Brachycladioidea: Brachycladiidae). Поскольку последовательности CO1 — стандартного и наиболее популярного митохондриального маркера — для исследуемых родов опецелид до сих пор не были известны, нами на основе известных соответствующих последовательностей Xiphidiata разработаны праймеры для амплификации фрагмента CO1, чтобы впервые провести соответствующий филогенетический анализ. Впервые определены и депонированы в GenBank нуклеотидные последовательности фрагментов митохондриальных генов CO1 и 16S черноморских трематод *Cainocreadium flesi* Korniychuk & Gaevskaya, 2000, *Gaevskajatrema perezi* (Mathias, 1926)

Gibson & Bray, 1982 и *Helicometra fasciata* (Rudolphi, 1819) Odhner, 1902 от разных видов дефинитивных хозяев — рыб. У *C. flesi* не выявлено специфичных к окончательным хозяевам — рыбам линий по структуре фрагмента митохондриального гена CO1, однако отмечено высокое CO1-нуклеотидное разнообразие. У марит черноморских *H. fasciata* определена приуроченная к зеленушкам-руленам *Symphodus tinca* CO1-гаплогруппа, статус которой требует дальнейшего выяснения; необходимы экологические и генетические исследования предполагаемого видового комплекса *H. fasciata* из разных акваторий. При анализе последовательностей фрагмента митохондриального гена 16S рРНК гостальных генетических линий у *H. fasciata* выделить не удалось. У черноморских *G. perezi* не обнаружено значительных различий по фрагменту 16S между трематодами из окончательных хозяев разных видов, однако внутривидовое 16S-нуклеотидное разнообразие оказалось высоким.

Ключевые слова: Чёрное море, Trematoda, Opecoelidae, *Cainocreadium*, *Gaevskajatrema*, *Helicometra*, митохондриальные гены, CO1, 16S рРНК