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THE FIRST RECORD OF DIMEROSACCUS ONCORHYNCHI (TREMATODA: OPECOELIDAE) IN FISHES FROM RIVERS OF PRIMORSKY TERRITORY, RUSSIA, WITH A DISCUSSION ON ITS TAXONOMIC POSITION USING MORPHOLOGICAL AND MOLECULAR DATA

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The opecoelid trematode *Dimerosaccus oncorhynchi* (Eguchi, 1931) Shimazu, 1980, hitherto known only from the Japanese archipelago, has been found in three species of freshwater salmonid fish (*Oncorhynchus masou*, *Brachymystax tumensis*, *Salvelinus curilus*) from rivers of Primorsky Territory, the Sea of Japan basin, Russia. This is the first record of the parasite in the continental part of Asia. Fishes *B. tumensis* and *S. curilus* are new hosts for *D. oncorhynchi.* The ecological notes, morphological description and drawings of the found trematodes clarifying the morphology of the male reproductive system are given. In the present study phylogenetic relationships of the species *D. oncorhynchi* with the related taxa were revealed using partial sequences of the 28S rDNA. Obtained results indicate the validity of *D. oncorhynchi* as the member of the subfamily Opecoelinae, which was closely related to the genus *Opecoeloides*, a representative of this subfamily.

Key words: *Dimerosaccus oncorhynchi*, Opecoelidae, morphology, salmonid fish, Primorsky Territory of Russia, 28S rDNA.

ОБНАРУЖЕНИЕ DIMEROSACCUS ONCORHYNCHI (TREMATODA: OPECOELIDAE) У РЫБ ИЗ РЕК ПРИМОРСКОГО КРАЯ РОССИИ С ОБСУЖДЕНИЕМ СИСТЕМАТИЧЕСКОГО ПОЛОЖЕНИЯ ЭТОГО ВИДА ПО МОРФОЛОГИЧЕСКИМ И МОЛЕКУЛЯРНЫМ ДАННЫМ

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Трематода *Dimerosaccus oncorhynchi* (Eguchi, 1931) Shimazu, 1980 впервые обнаружена у лососевых рыб (*Oncorhynchus masou*, *Brachymystax tumensis*, *Salvelinus curilus*) рек Приморского края. Ранее этот вид был зарегистрирован только у рыб Японского архипелага. Ленок *B. tumensis* и южная мальма *S. curilus* впервые отмечены в качестве хозяев *D. oncorhynchi*. В работе приведены данные по экологии, морфологическое описание, рисунки найденных трематод, уточнено строение мужской половой системы. Филогенетические взаимоотношения *D. oncorhynchi* обсуждаются с использованием частичной последовательности 28S рДНК. *Dimerosaccus oncorhynchi* кластеризуется с представителями рода *Opecoeloides*, что подтверждает принадлежность этого вида к подсем. Opecoelinae.

Ключевые слова: *Dimerosaccus oncorhynchi*, Opecoelidae, морфология, лососевые рыбы, Приморский край, 28S рДНК.

Dimerosaccus oncorhynchi (Eguchi, 1931) Shimazu, 1980 is the type species and only representative of the opecoelid genus *Dimerosaccus* Shimazu, 1980 (see Cribb, 2005). This parasite has been recorded only in the Japanese archipelago — the Islands of Honshu, Hokkaido and Shikoku (Shimazu, 2003, 2008, etc.). This species possesses a rich synonymy; it was described as *Allocreadium oncorhynchi* Eguchi, 1931, *Plagioporus oncorhynchi* (Eguchi, 1931) Peters, 1957 and *P. honshuensis* Moravec et Nagasawa, 1998 (see Shimazu, 2000). This is certainly the opecoelid species, but its subfamily belonging remains controversial (see Shimazu, 1988, 2000; Moravec, Nagasawa 1998; Cribb 2005).

D. oncorhynchi was recorded for the fish genera *Liobagrus* Hilgendorf, 1878 (Amblycipitidae), *Cottus* Linneus, 1758 (Cottidae), *Rhinogobius* Gill, 1859 and *Tridentiger* Gill, 1859 (Gobiidae), but more frequently for salmonids (Salmonidae s. str.): the masu *Oncorhynchus masou* (Brevoort, 1856) (= *O. rhodurus*), the whitespotted char *Salvelinus leucomaenis* (Pallas, 1814) and *S. l. pluvius* (Hilgendorf, 1876) (Shimazu, 1988, 2000, 2008, et al.). A single case (Shimazu, 2000) of parasitism of this trematode was described in the intestine of the Japanese clawed salamander, *Onychodactylus japonicus* (Houttuyn, 1782).

This species was found by us in three salmonid species, namely *O. masou*, *Salvelinus curilus* (Pallas, 1814) and *Brachymystax tumensis* Mori, 1930 from rivers of Primorsky Territory in Russia, and was previously mentioned in the summary (Shedko et al., 2010). This is the first report on the presence of *D. on-*

corhynchi in the mainland Asia and in Russia. *B. tumensis* and *S. curilus* were recorded as new hosts of the parasite.

The goal of the present publication is the documentation of the presence of *D. oncorhynchi* in a new territory and discussion of systematic position of this species using morphological data combined with the data on 28S rDNA partial sequences.

MATERIALS AND METHODS

Collecting of hosts and parasites

The material was collected from three salmonid fish species (103 specimens were examined) caught in six continental rivers of the Sea of Japan basin, Primorsky Territory, Russia (table 1). The majority of trematode species were flattened under slight pressure, fixed with 70 % ethanol, and stained with alum carmine. Several living trematode specimens were killed and fixed with acetic acid carmine without crushing (Sudarikov, Shigin 1965). After dehydrating and clearing, flukes stained by both methods were mounted in Canada balsam. Several parts of the structure of the reproductive system were examined in both living specimens and on slides prepared from organs dissected from the body of the parasite fixed with acetic acid carmine.

Morphological methods

Ninety specimens of mature worms taken from three fish species were used for the description of *D. oncorhynchi*. Measurements of 77 whole-mounted specimens fixed according to the first method were performed. Specimens with the presence of the advanced vitellarium and at least one egg were considered as matures. Similarly to Shimazu (1988), the forebody length was measured according to Yamaguti's (1971) scheme, i. e. from the anterior extremity of the body to the mid-level of the ventral sucker.

The length of the membranous sac was measured along a straight line between its anterior and posterior edges, without curves organ. The space between the ventral sucker and the ovary was measured from the posterior edge of suckers to the front edge of the ovary.

Sample parameters included M (mean value), ± standard deviation (σ). All measurements are given in millimeters (mm). Morphological studies were conducted using an Axio Skope optical microscope. Drawings were made using a Karl Zeiss drawing tube. Measurements and microphotographs were performed using an AxioCam HR CCD-camera and AXIOVISION 4.6.3 software (Carl Zeiss MicroImaging GmbH, Germany) in the Microscopy Center for Collective Use of Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (IBSS FEB RAS).

Some of the material on *D. oncorhynchi* is held in the collections of Zoological Museum (ZM) of IBSS FEB RAS (34 specimens of four glass slides; # 1371—1374) and of Helminthological Museum of Parasitology Centre of the Institute of Ecological and Evolutionary Problems RAS, Moscow, Russia (10 specimens of one glass slide; # 3981).

Table 1. Occurrence of trematode *Dimerosaccus oncorhynchi* in fishes from continental water bodies of Sea of Japan basin, Primorsky Territory of Russia

Water body	Host	Date	Fork length, mm	Prevalence	Intensity, ind.	Mature/immature trematodes ratio
Kedrovaya R. 43° 06′ N, 131° 32′ E	*Salvelinus curilus*	2.IV.2001	125	1/1*	22	14/8
		2.VII.2004	80—105	5/9	1—2	4/3
		2.XI.2010	56—98	5/7	1—13	24/8
	Oncorhynchus masou	2.IV.2001	130	1/1	2	2/0
		13.VII.2001	104	1/1	2	2/0
		5.V.2002	95—105	5/6	1—6	7/5
		14.V.2002	40—50	1/3	1	1/0
		2.VII.2004	130—135	1/2	1	1/0
		2.XI.2010	56—165	9/19	1—18	46/13
Klyuch Glubokyi R. (Shkotovka R. basin) 43° 20′ N, 132° 51′ E	*S. curilus*	17.VIII.2003	110—155	10/12	2—28	39/52
		21.VII.2010	60—10	3/3	6—12	7/13
		24.X.2010	50—130	9/10	2—38	91/15
Steklyanukha R. (Shkotovka R. basin) 43°21′ N, 132°27′ E	*O. masou*	19.XI.2010	65—90	2/4	1	2/0
Klyuch R. 43°18′ N, 132°15′ E	*O. masou*	22.X.2000	100—130	1/9	1	1/0
		22.IV.2001	110	1/1	2	2/0
Ikryanka R. (Partizanskaya R. basin) 43°16′ N, 133°22′ E	*O. masou*	20.IX.2003	78—135	2/6	1—3	4/0
	Brachymystax tumensis	20.IX.2003	170—282	1/4	2	2/0
Sukhoy Klyuch R. (Kievka R. basin) 43°02′ N, 133°42′ E	*O. masou*	8.X.2007	103—132	1/5	2	1/1

Note. * — before the slash the number of infected fish, after — number of examined.

Molecular methods

Mature trematodes of *D. oncorhynchi* (n = 17) were obtained from the intestine of definitive host species (table 2) and then fixed with 96 % ethanol. DNA extraction was performed from whole worms using silica based DNA purification procedure described earlier (Boom et al., 1990; Melzak et al., 1996).

The polymerase chain reaction (PCR) was employed to amplify of the nuclear 28S rDNA using the following primers: DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′), described in Tkach et al. (2003). Initial PCR reaction was carried out in a total volume of 20 µl. Each reaction contained 0.25 mM of each primer pair, combined with 1 µl of water solution of DNA, 10× Taq buffer, 1.25 mM dNTP, 1.5 mM magnesium, and 1 unit of Taq polymerase. Amplification of a 1200-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) with 3 min denaturation hold at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 52 °C, 2 min at 72 (C and 7 min extension hold at 72. PCR contamination control was performed by including negative controls alongside positive controls, using both primers. PCR products were initially directly sequenced using ABI Big Dye Terminator v.3.1 Cycle Sequencing kit (as instructed by manufacturer) and internal sequencing primers: 300F, ECD2, 900F, 1200R (Tkach et al., 2003). Reading of the cycle PCR products was performed with ABI 3130 genetic analyzer on the basis of IBSS FEB RAS.

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software and aligned using MEGA 5.0 (Tamura et al., 2011) alignment explorer with default options. Regions that could not be aligned unambiguously were excluded from the analyses. A number of variable, parsimony-informative sites, nucleotide composition and substitution analysis performed using MEGA 5.0. Genetic divergence was estimated using genetic p-distance (d) values, which were calculated including all substitution types. Phylogenetic analysis of nucleotide sequences was undertaken, using Bayesian algorithm with GTR+G evolutionary model. This model showed the best fit to the data using jModeltest v.2.1.5 software (Darriba et al., 2012). Bayesian inference was obtained using MrBayes v. 3.1.2 software (Huelsenbeck, Ronquist, 2001). The resulting networks were rooted with the outgroup taxa. Bayesian inference was employed using the following nucleotide substitution parameters: lset nst = 6, rates = gamma, ncat = 4 and basefreq = empirical, that correspond to a general time reversible (GTR)

Table 2. List of specimens of *Dimerosaccus oncorhynchi* collected from the of Primorsky Territory, Russia, incorporated in sequence analysis and their respective host species, genBank accession number for the 28s

Sample Number/Number of specimens	Host species	Location	Gene Bank accession numbers
1417/6	*Salvelinus curilus*	Klyuch Glubokyi River	FR870246 — FR870251
1461/1	*Oncorhynchus masou*	Steklyanukha River	FR870252
1423/6	*O. masou*	Kedrovaya River	FR870253 — FR870258
1427/3	*Salvelinus curilus*	» »	FR870259 — FR870261
1438/1	*S. curilus*	» »	FR870262

model including estimates of gamma distributed among-site rate variation. Markov chain algorithm was performed with 1 000 000 generations and 2 parallel runs and burnin 150 000 generations. Phylogenetic relationships of the genus *Dimerosaccus* were inferred from our data and the nucleotide sequences of 28S rDNA of other specimens from NCBI GenBank database, representing different genera of the family Opecoelidae: *Bentholebouria_colubrosa* Andres, Pulis et Overstreet, 2014 (J001207) (Andres et al., 2014), *Biospeedotrema biospeedoi* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014 (KF733986) (Bray et al., 2014), *B. jolliveti* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014 (KF733985) (Bray et al., 2014), *Buticulotrema thermichthysi* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014 (KF733984) (Bray et al., 2014), *Cainocreadium labracis* (Dujardin, 1845) (JQ694144) (Born-Torrijos et al., 2012), *C. lintoni* (Siddiqi et Cable, 1960) (KJ001208) (Andres et al., 2014), *Gaevskajatrema halosauropsi* Bray et Campbell, 1997 (AY222207) (Olson et al., 2003), *G. perezi* (Mathias, 1926) (AF184255) (Tkach et al., 2001), *Hamacreadium mutabile* Linton 1910 (KJ001209) (Andres et al., 2014), *Macvicaria macassarensis* (Yamaguti, 1952) (AY222208) (Olson et al., 2003), *M. mormyri* (Stossich, 1885) (AF184256) (Tkach et al., 2001), *M. obovata* (Molin, 1859) (JQ694146) (Born-Torrijos et al., 2012), *Neolebouria lanceolata* (Price, 1934) (KJ001210) (Andres et al., 2014), *Opecoeloides furcatus* (Bremser in Rudolphi, 1819) (AF151937) (Tkach et al., 2000), *O. fimbriatus* (KJ001211) (Andres et al., 2014), *Peracreadium idoneum* (Nicoll, 1909) (AY222209) (Olson et al., 2003), *Plagiocirrus loboides* Curran, Overstreet et Tkach, 2007 (EF523477) (Curran et al., 2007), *Podocotyloides brevis* Andres et Overstreet, 2013 (KJ001212) (Andres et al., 2014), *Pseudopycnadena tendu* Bray et Justine, 2007 (FJ788506) (Bray et al., 2009). Also we used 28S rDNA sequences of the trematodes from the families Allocreadiidae and Paragonimidae as an outgroup taxa: *Crepidostomum cornutum* (Osborn, 1903) (EF032695) (Curran et al., 2006), *Bunodera luciopercae luciopercae* (Muller, 1776) (GU462124) (Petkeviciute et al., 2010), *Allocreadium lobatum* Wallin, 1909 (EF032693) (Curran et al., 2006) and *Paragonimus kellicotti* Ward, 1908 (HQ900670) (Curtis, Fischer, and Weil, 2013, direct submission), *P. westermani* (Kerbert, 1878) (AY116874) (Olson et al., 2003).

RESULTS

Ecological characteristics

Definitive hosts: *Oncorhynchus masou*, *Salvelinus curilus*, and *Brachymystax tumensis* (Salmonidae).

Site of infection: stomach, pyloric caeca, intestine.

Localities: Primorsky Territory of Russia, Sea of Japan basin — Kedrovaya R., Klyuch R., river basins of Partizanskaya (Ikryanka R.), Shkotovka (Klyuch Glubokyi R., Steklyanukha R.) and Kievka (Sukhoy Klyuch R.).

Intensity: range 1—38.

Prevalence: in fish samples, for $n > 10$ — from 47.4 to 90.0 %.

The ratio of mature and immature trematodes in samples is given in table 1.

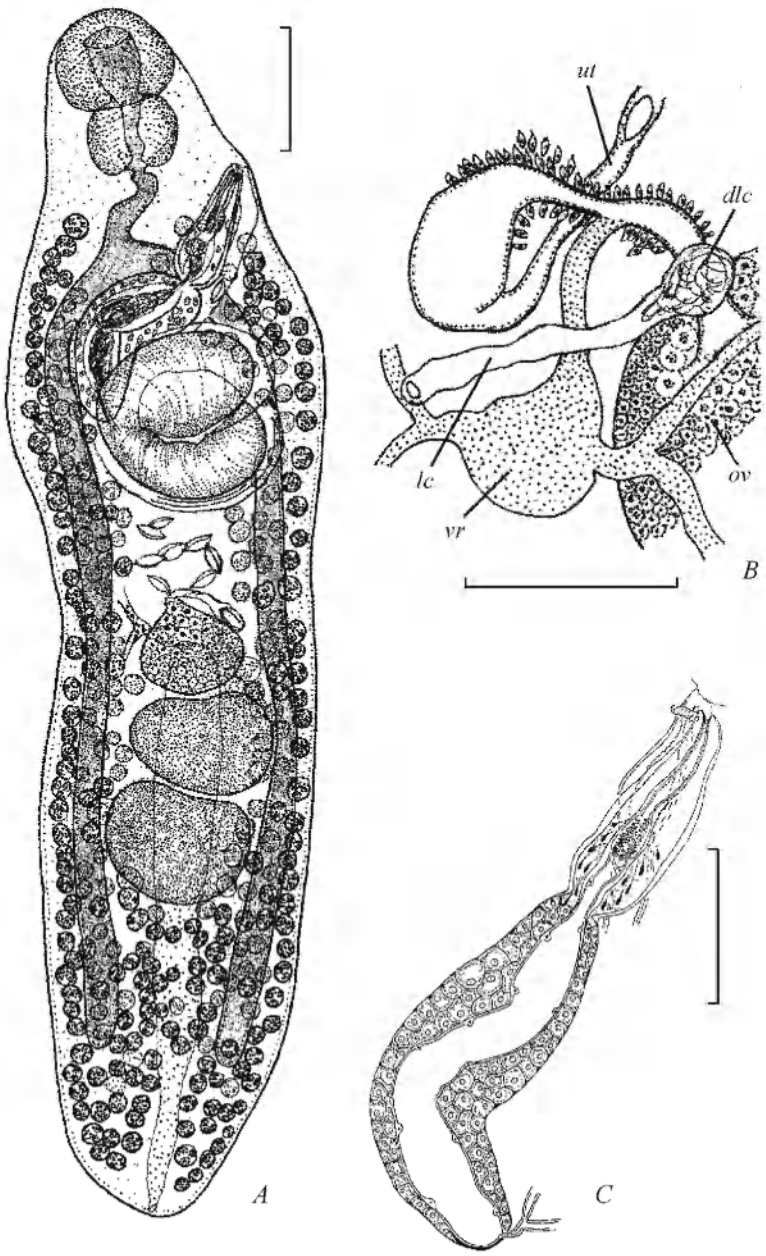

Fig. 3. *Dimerosaccus oncorhynchi* from *Salvelinus curilus* of Klyuch Glubokyi River.
A — entire body, ventral view; *B* — ovarian complex, dorsal view; *C* — terminal genitalia, ventral view. *lc* — Laurer's canal, *dlc* — expansion of the proximal part of Laurer's canal, *ov* — ovarium, *vr* — vitelline reservoir, *ut* — uterus. Bars: *A* — 0.2 mm; *B*, *C* — 0.1 mm.

Morphological description (figs 1—3).

The body is elongate, often distinctly widened laterally at the level of the ventral sucker (fig. 1, 3, *A*). The ventral part of the acetabular area of the body forms a conical muscular protrusion which carries the ventral sucker. Tegument

Fig. 1. Photograph of live gravid specimens of *Dimerosaccus oncorhynchi* from *Salvelinus curilus* of Klyuch Glubokyi River, flattened under slight pressure, in ventral view.
Bar — 0.5 mm.

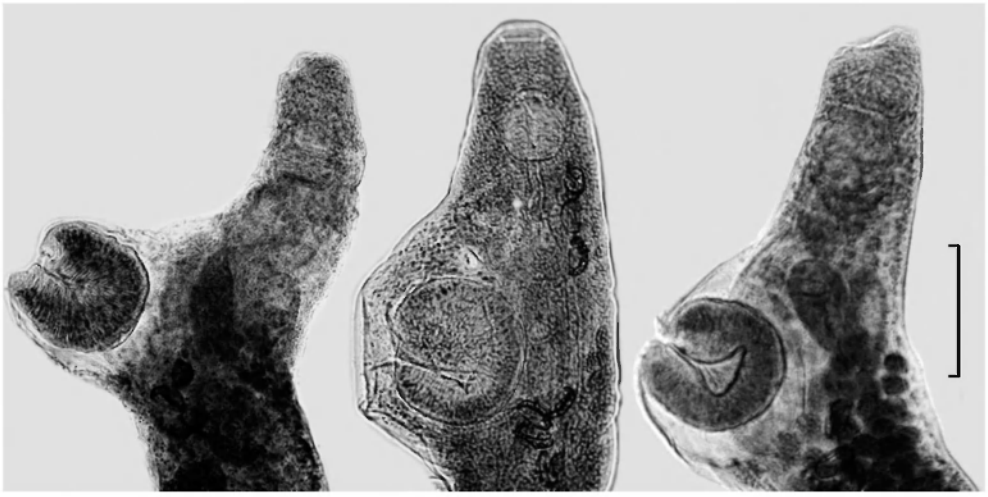

Fig. 2. Anterior part of body of gravid specimens of *Dimerosaccus oncorhynchi* from *Oncorhynchus masou* of Kedrova River, fixed with acetic acid carmine without crushing in lateral view.
Bar — 0.1 mm.

Table 3 Diversity of *Dimerosaccus oncorhynchi* on the extension of vitellarium

Variants of anterior border of vitellarium	Water body, host species and number of trematodes, %							Total (all rivers and hosts)
	Klyuch R.	Kedrovaya R.		Shkotovka R. basin		Partizanskaya R. basin		
	Oncorhynchus masou	*O. masou*	*Salvelinus curilus*	*S. curilus*	*O. masou*	*O. masou*	*Brachymystax tumensis*	
I — to posterior margin of ventral sucker	1	2 (8.0 %)	2 (16.7 %)	0	0	0	0	5 (6.9 %)
II — to middle of ventral sucker	0	8 (32.0 %)	5 (41.7 %)	0	0	1	0	14 (19.4 %)
III — to anterior margin of ventral sucker	1	7 (28.0 %)	4 (33.3 %)	2 (7.7 %)	0	2	0	16 (22.2 %)
IV — between anterior margin of ventral sucker and oesophagus bifurcation	0	8 (32.0 %)	1 (8.3 %)	12 (46.2 %)	1	1	2	25 (34.7 %)
V — anteriorly bifurcal level	0	0	0	12 (46.2 %)	0	0	0	12 (16.7 %)
Number of case	2	25	12	26	1	4	2	72

Table 4 Measurements of mature specimens of *Dimerosaccus oncorhynchi* (length and width in mm) according to original and literature data

Characters	Original data			Shimazu (1980, 2008); Shimazu, Awakura (1993)	Shimazu (2008); Shimazu, Urabe (2005)*; Moravec, Nagasawa (1998)
Hosts	*Salvelinus curilus* $N_{trematodes} = 41$	*Oncorhynchus masou* $N_{trematodes} = 34$	*Brachymystax tumensis* $N_{trematodes} = 2$	Salmonidae	Amblycipitidae and Gobiidae
	min—max (M ± σ)	min—max (M ± σ)	min—max	min—max	min—max
Body: length (L)	1.06—2.36 (1.68 ± 0.36)	0.70—2.77 (1.97 ± 0.45)	1.80—2.10	1.77—4.18	0.95—3.89
Body: width (W)	0.28—0.65 (0.43 ± 0.10)	0.31—0.65 (0.48 ± 0.09)	0.51—0.60	0.58—1.12	0.42—1.20
Oral sucker: L	0.11—0.18 (0.15 ± 0.02)	0.11—0.19 (0.15 ± 0.02)	0.17—0.19	0.17—0.46	0.12—0.20

Oral sucker: W	0.12—0.21 (0.16 ± 0.02)	0.11—0.20 (0.15 ± 0.02)	0.15—0.17	0.19—0.56		0.12—0.25	
Forebody: L	0.37—0.79 (0.54 ± 0.11)	0.19—0.84 (0.61 ± 0.14)	0.63—0.71	0.80—1.12		0.56—0.96	
Forebody: % of body-length	25.58—40.88 (32.59 ± 3.59)	25.46—36.67 (31.21 ± 2.8)	33.81—34.72	25.00—37.00		16.00—45.00	
Ventral sucker: L	0.15—0.31 (0.23 ± 0.04)	0.18—0.32 (0.24 ± 0.04)	0.22—0.25	0.25—0.72		0.19—0.35	
Ventral sucker: W	0.16—0.37 (0.25 ± 0.06)	0.18—0.32 (0.26 ± 0.04)	0.25—0.30	0.30—0.80		0.23—0.43	
Ratio: oral sucker (W) to ventral sucker (W)	1.23—2.17 (1.58 ± 0.24)	1.27—2.50 (1.70 ± 0.27)	1.67—1.77	1.40—2.00		1.10—1.87	
Pharynx: L	0.08—0.15 (0.12 ± 0.02)	0.08—0.16 (0.12 ± 0.02)	0.09—0.14	0.12—0.32		0.10—0.15	
Pharynx: W	0.08—0.15 (0.12 ± 0.02)	0.08—0.15 (0.11 ± 0.02)	0.11—0.13	0.12—0.45		0.09—0.16	
Ratio: oral sucker (L) to pharynx (L)	1.00—1.55 (1.25 ± 0.13)	0.93—1.55 (1.29 ± 0.13)	1.21—2.11	—		1.73—2.09**	
Oesophagus: L	0.01—0.15 (0.09 ± 0.03)	0.03—0.20 (0.13 ± 0.04)	0.12—0.14	0.17—0.72		0.01—0.27	
Post-testicular field: % of body-length	20.61—36.03 (27.26 ± 3.59)	17.14—37.21 (28.58 ± 3.84)	24.76—30.00	—		—	
Anterior testis: L	0.07—0.25 (0.15 ± 0.04)	0.08—0.23 (0.16 ± 0.04)	0.15—0.15	0.18—0.25		0.06—0.19	0.15—0.42
Anterior testis: W	0.07—0.32 (0.19 ± 0.05)	0.09—0.31 (0.20 ± 0.05)	0.21—0.24	0.34—0.44	0.80—0.88***	0.20—0.45	×0.22—0.43***
Posterior testis: L	0.07—0.23 (0.17 ± 0.04)	0.09—0.30 (0.19 ± 0.04)	0.18—0.20	0.25—0.31		0.09—0.20	
Posterior testis: W	0.07—0.29 (0.20 ± 0.05)	0.09—0.31 (0.20 ± 0.05)	0.22—0.24	0.34—0.44		0.1—0.44	
Cirrus-sac: L	0.07—0.23 (0.16 ± 0.03)	0.12—0.25 (0.18 ± 0.04)	0.18—0.22	0.11—0.48		0.07—0.27	

Continuation of table 4

Characters	Original data			Shimazu (1980, 2008); Shimazu, Awakura (1993)	Shimazu (2008); Shimazu, Urabe (2005)*; Moravec, Nagasawa (1998)
Hosts	*Salvelinus curilus* $N_{trematodes} - 41$	*Oncorhynchus masou* $N_{trematodes} - 34$	*Brachymystax tumensis* $N_{trematodes} - 2$	Salmonidae	Amblycipitidae and Gobiidae
	min—max (M ± σ)	min—max (M ± σ)	min—max	min—max	min—max
Cirrus-sac: W	0.05—0.09 (0.06 ± 0.01)	0.05—0.08 (0.06 ± 0.01)	0.06—0.07	0.08—0.24	0.05—0.11
Membranous sac: L	0.09—0.40 (0.21 ± 0.08)	0.12—0.32 (0.22 ± 0.05)	0.25—0.35	0.40—1.28	0.22—0.69
Membranous sac: W	0.05—0.12 (0.10 ± 0.02)	0.04—0.14 (0.09 ± 0.02)	0.10—0.10	0.19—0.40	0.07—0.25
Field between ventral sucker and ovary: L	0.04—0.33 (0.16 ± 0.08)	0.06—0.34 (0.21 ± 0.08)	0.10—0.30	—	—
Ovary: L	0.06—0.20 (0.12 ± 0.03)	0.06—0.18 (0.12 ± 0.03)	0.13—0.13	0.11—0.64	0.05—0.25
Ovary: W	0.08—0.27 (0.15 ± 0.04)	0.08—0.22 (0.17 ± 0.38)	0.15—0.15	0.15—0.67	0.16—0.38
Number of eggs	1—30 (12 ± 7)	1—77 (18 ± 7)	29—38	5—11	—
Eggs: L	0.042—0.069 (0.058 ± 0.004)	0.049—0.072 (0.063 ± 0.005)	0.051—0.062	0.018—0.065	0.049—0.066
Eggs: W	0.025—0.037 (0.028 ± 0.003)	0.025—0.040 (0.032 ± 0.003)	0.025—0.032	0.016—0.038	0.021—0.040

N o t e. * — measurements from Shimazu, Urabe (2005) multiplied by 0.8 (according to personal comment Dr. Shimazu: «All our own measurements were erroneously given. To obtain correct ones, multiply them by 0.8»); ** — ratio of sucker sizes (Moravec, Nagasawa, 1998); *** — diameter or length and width of testes without specifying exactly what — anterior or posterior (on: Shimazu (1980), Shimazu, Awakura (1993), Shimazu, Urabe (2005), respectively).

spines are visible only in this region of the body. The oral sucker is oval, subterminal, considerably smaller than the ventral sucker. The prepharynx is shorter than the pharynx. The pharynx is slightly smaller than the oral sucker. The oesophagus is bifurcating fairly anterior to the ventral sucker. The caeca ends blindly at a short distance from the posterior end of the body, 0.05—0.28 (0.12). The ventral sucker is situated near the junction of the anterior and middle one-thirds of the body, without appendages at the aperture (fig. 1, 3). Depending on the functional state, it is immersed deep into flying its ventral projection of the body or pushed out of it (fig. 2). Testes are transversely oval, less frequently almost spherical, or unevenly lobed, tandem, situated in the middle third of the body. The cirrus-sac is short and thick-walled (thickness constituting about 0.007—0.010), containing the cirrus, pars prostatica, and short tubular internal seminal vesicle. The ejaculatory duct opens into a very short genital atrium, its width constitutes about 0.047, it is situated sinistro-submarginally or sinistro-submedially, near the posterior end of the pharynx. Testes are transversely oval, less frequently they are almost spherical or unevenly lobed, tandem, situated in the middle third of the body. External seminal vesicle is convoluted or elongated, surrounded by gland-cells enclosed in a membranous sac (fig. 3, *C*). The posterior border of the external seminal vesicle is situated in a space between the posterior and anterior margins of the ventral sucker. Vasa efferentia coalescing near external seminal vesicle. The ovary is transversely oval or triangular, median, situated just anterior to anterior testis. The uterus is preovarian, intercecal; the metraterm is shorter than the cirrus-sac, ending as a sphincter. The vitellarium is follicular; follicles are large, anteriorly separate, circumcaecal; posteriorly entering post-testicular region confluent. The anterior extent of the vitellarium is situated in a space between the posterior margin of the ventral sucker and the last third of the oesophagus, pointed out five options for the location of the upper limit (table 3). The ootype complex is preovarian. The Laurer's canal is rather long, inflated in the proximal part, usually containing sperm (fig. 3, *B*). Canalicular seminal receptacle is absent. Eggs are oval, operculated, without filaments, not embryonated. The excretory vesicle is I-shaped, reaching as far as the ovary. Metric characteristic of the maritae are given in table 4.

Alignments and phylogenetic analysis

In the present study data on the genetic divergence and phylogenetic relationships of the *D. oncorhynchi* species from the Russian Far East were obtained, using 28S rDNA partial sequences. Amplicons were about 1300 bp in length and the sequences, using for data processing after alignment were 1208 bp, including 6 variable, parsimony-informative sites. Genetic p-distances between far eastern *D. oncorhynchi* and different genera of the family Opecoelidae ranged from 9.3 % (*D. oncorhynchi* / *Opecoeloides furcatus*) to 11.5 % (*D. oncorhynchi* / *Pseudopycnadena tendu*). *Dimerosaccus onchorhynchi* was closely related to the genus *Opecoeloides* Odhner, 1928, a member of the subfamily Opecoelinae.

The phylogenetic analysis performed with the use of Bayesian methods, recognized *D. oncorhynchi* as a distinct species and revealed two clusters within *D. oncorhynchi*, corresponding to different geographical locations (fig. 4). The

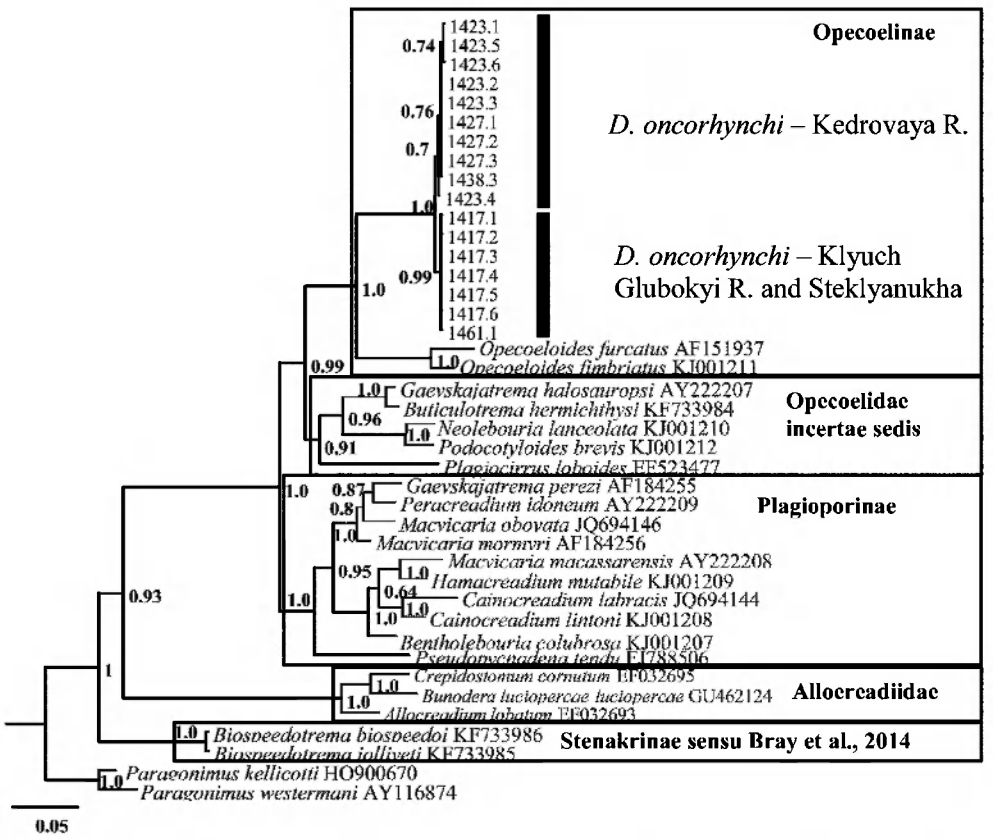

Fig. 4 — Bayesian phylogram of family Opecoeliodae employing a GTR + G substitution model for partial 28S rDNA sequence dataset.

Nodal numbers is a posterior probabilities.

first one was formed by specimens of *D. oncorhynchi* from the Kedrovaya River and the second one included specimens from the Klyuch Glubokyi and Steklyanukha Rivers and was supported by high bootstrap values. Divergence between 28S rDNA sequences of *D. oncorhynchi* from different locations was 0.4 %. 28S rDNA sequences of these specimens were distinguished by six substitutions, four of them were fixed. In general, there were four different variants of 28S sequences in *D. oncorhynchi*, three of them being revealed in trematodes from the Kedrovaya River from different host species. However, specimens of *D. oncorhynchi* obtained from different hosts from other two locations possessed their own common sequence variant. Thus, the genetic differentiation of *D. oncorhynchi*, obtained from our data, does not depend on host species.

DISCUSSION

The mean values of dimensional attributes *D. oncorhynchi* of the Primorsky Territory of Russia fit into the size range specified for this species in the literature (table 4). We have not found specimens whose length would exceed 3 mm,

i. e. was comparable with the large specimens from the collection of Shimazu (table 4). Sampling was performed in the same season as in Japan. The small size of trematodes from Russia can be probably explained by the shorter warm period in this territory.

The examined trematodes are mainly similar to *D. oncorhynchi* described early. However, we have some comments on the morphology of this species.

The acetabulum of the studied specimens is markedly expanded forwards (fig. 2). The ventral sucker occupies the terminal part of the ventral muscular protrusion body that is capable to move forward and to shut tightly with the complete isolation of the ventral sucker bag from the environment (fig. 1). At the same time, *D. oncorhynchi* described early has the typical sessile sucker (Shimazu, 1988, 2000; Shimazu, Urabe, 2005). However, according to personal message of T. Shimazu, he had «often observed protrudent or even stalked ventral sucker in fresh and hot formalin-fixed specimens of this species».

According to Shimazu (1980, 1988) and Cribb (2005) there is a small cirrus-sac and the long tubular external seminal vesicle, which surrounds gland-cells in *D. oncorhynchi*. The external seminal vesicle together with the gland-cells is enclosed in a membranous sac. The distal part of the male duct exhibited by *D. oncorhynchi* bears some superficial resemblance to that one in some Lepidapedidae, especially in the genera *Lepidapedon* Stafford, 1904 and *Paralepidapedon* Shimazu et Shimura, 1984 (Bray, 2005; Bray, Cribb, 2012). According to Manter (1954) and Shimazu, Shimura (1984) the membranous sac of *Lepidapedon* spp. and *Paralepidapedon* spp. may be interpreted as a continuation of the wall of the cirrus-sac. In later publications, Shimazu (Shimazu, 2000, 2008; Shimazu, Awakura, 1993; Shimazu, Urabe, 2005) describes the morphology of the cirrus-sac of *D. oncorhynchi* within this concept, considering the tubular seminal vesicle (external seminal vesicle in Shimazu, 1980) in a complex with the gland-cells and the membranous sac of this species as a proximal part of the bipartite cirrus-sac. Distal part of the male duct of *D. oncorhynchi* was described using the terminology of Shimazu (1980, 1988) and Cribb (2005).

We have registered a variation of posterior border of external seminal vesicle (fig. 1), that was previously noted by Shimazu, Urabe (2005). Of the 67 individuals with a clearly visible external seminal vesicle, in 25 cases its posterior boundary was located at the anterior edge of ventral sucker, in 18, mainly in the middle level of the ventral sucker, in the remaining 24 cases it was extended posteriorly beyond the ventral sucker. In our opinion, this may be due to the method of fixation or the physiological state of the individual (the degree of fullness of the seminal vesicle by genital products).

Shimazu (1988) described two morphological forms of *D. oncorhynchi*, differing by location of the anterior end of the vitellarium: the «Honshu-form» and the «Hokkaido-form» named according to the collecting site (island). In the first form, the vitellarium does not extend anteriorly to the bifurcal level, but in the latter form, its anterior part extends as far as the median level of the oesophagus (Shimazu, 1988; Shimazu, Awakura, 1993). Later, both forms were found in the same water body from Honshu Island in the Takami River (Shimazu, Urabe, 2005) and the Ide River (Shimazu, 2007). At the same time, it was noted that in the waters of the Cikoku Island the «Hokkaido-form» dominated in gobiida, whereas «Honshu-form» prevailed in masu (Shimazu, 2008). The difference in

the anterior limit of the vitellarium was believed to be slight and of no taxonomic value (Shimazu, 1988, 2007, 2008; Shimazu, Urabe, 2005).

In our studies, we observed variations in the anterior border of the vitellarium. It was noted that five different variants (table 3): variants I—IV corresponded to the «Honshu-form», V — to the «Hokkaido-form». In total, variant IV was dominant, whereas variant I occurred very rare. For trematodes from *S. curilus*, all five variants of the anterior border of the vitellarium were marked; variants IV and V were the most frequent ones (34.2 and 31.6 %, respectively) (fig. 1). Trematodes from *O. masou* belonged only to the «Honshu form» (variants I—IV); most commonly (34.4 %) the vitellarium extended to the anterior margin of the ventral sucker (variant III). All five variants were never found simultaneously found in any of the studied water bodies. The highest number of them (four) was noted in the Kedrovaya River, and all of them belonged to the «Honshu form». The simultaneous presence of Hokkaido and Honshu forms was found in specimens from the Klyuch Glubokyi River in *S. curilus*.

Together with the variability in the position of the anterior border of the vitellarium, variations in 28S rDNA fragment in *D. oncorhynchi* from different locations were also observed. It was found that specimens of this species from geographically distant Kedrovaya and Klyuch Glubokyi Rivers (Amursky Bay and Ussuri Bay of respectively) had 0.4 % of 28S rDNA sequences differentiation. Moreover, diversity of 28S sequences from the south-western Kedrovaya River was higher (three sequence variants) than in the south-eastern Steklyanukha River, where only a single sequence variant was found. It is of a considerable interest because many studies pointed to the high conservation of 28S rDNA within digenean species (Maurelli et al., 2007; Otranto et al., 2007; Atopkin, Shedko, 2014; Petkeviciute et al., 2010; Atopkin, 2011).

Divergence of 28S rDNA sequences of *D. oncorhynchi* is strongly associated with the geographical distribution of this trematode or its hosts. Moreover, we have found that this differentiation does not depend on definitive host species. So we can suggest that the origin of the genetic variation of *D. oncorhynchi* agrees with the distribution of the first intermediate host species — mollusks. The agreement of phylogeography of parasites and mollusks was noted in some studies (Hasegawa, 1999; Blair et al., 2001; Attwood et al. 2004; Webster et al., 2007). Any changes in mollusks populations could be reflected as the genetic variation of its parasite, *D. oncorhynchi*. Nevertheless, there are some difficulties hampering the resolution of this problem. The life cycle of *D. oncorhynchi* is yet unknown. Further studies are needed to find its first and second intermediate host species. Investigation of population genetic structure of these species will allow us performing a comparative genetic analysis of *D. oncorhynchi* and mollusks to examine a hypothesis about agreement of host-parasite relationships for these species.

All samplings of trematodes, collected in different years in the period from April to November, contained both mature and immature individuals with underdeveloped organs of the reproductive system, while the number of mature individuals of *D. oncorhynchi* was higher than the number of immature ones (table 1). In different seasons, the ratio between these individuals was different: 2:1 (n = 39 specimens) in April—May, 1.6:1 (n = 228) in June-September and 1.4:1 (n = 200) in October—November. Apparently, the infestation of fishes by these trematodes occurs during their active feeding period (from spring to autumn). Infestation site of the immature trematodes is the pyloric caeca, of matures, the

intestines. In addition, two immature and single mature trematodes were found in the stomach of three specimens of the fish, as it was observed in previous studies (Shimazu, Awakura, 1993).

We observed *D. oncorhynchi* in freshwater fish: *B. tumensis*, juvenile and dwarf males of *O. masou*, residential forms of *S. curilus*. Undoubtedly, this species is of a freshwater origin, which agrees with data of Shimazu, Awakura (1993).

Only a single species of freshwater opecoelid, *Plagioporus imanensis* Belouss, Skrjabin et Koval, 1958, was found in salmonid fishes in Russian continental rivers of Sea of Japan basin. This parasite has been listed for *Oncorhynchus masou* from Razdolnaya River (Ermolenko, Butorina, 1988) and for *Salvelinus curilus* (*S. malma*, according to the author) from the Kedrovaya River (Ermolenko, 1992). In both cases, only a single specimen of this fluke was found.

The type host of the trematode *P. imanensis* is the minnow *Rhynchocyprinus* sp. (according to the authors, *Phoxinus lagowskii oxycephalus* (Sauvage et Dabry de Thiersant, 1874)), Cyprinidae, and the type locality is the Bol'shaya Ussurka River, Ussuri River basin, and Primorsky Territory of the Russian Far East (Belous, 1952; Skrjabin, Koval, 1958).

One of the authors of this publication (M. B. Shedko) studied a specimen of «*P. imanensis*», which was found by A. V. Ermolenko in *S. curilus* (stored in ZM IBSS FEB RAS). This parasite was established as belonging to the species *Crepidostomum metoecus* (Braun, 1900). The material on *P. imanensis* from *O. masou* was not preserved. Reviewing the circumstances (including our discovery of a single species from Opecoelidae in salmonids of the Kedrovaya River) give us all reason to doubt the adequacy of findings of this species of salmonids in continental waters of the Sea of Japan basin. Thus, *D. oncorhynchi* is the only species of freshwater opecoelid, in fact recorded in the salmonids from these waters.

Up to date, this parasite was occurred only in the rivers of Primorsky Territory, to the south of 43° 35′ N. In this region, we had examined more than 450 specimens of freshwater fish from different families, including salmonid fishes, from 14 rivers (including basins of the Razdolnaya Artemovka Rivers), but the parasite was found only in six of them (table 1). In spite of the fact that we had examined more than 300 specimens of the salmonid and grayling fish (*Thymallus* spp.) from the Ussuri River basin (Primorsky Territory) and the continental rivers of the Japan Sea basin, located to the north of 43° 35′ N, as well as salmonids in rivers of the southern Sakhalin Island (Belaya, Bakhura, and Lyutoga Rivers.), *D. oncorhynchi* was not detected.

According to Shimazu, Awakura (1993), adult *D. oncorhynchi* were also found in *O. masou* caught in sea, and may be capable of surviving in the sea for at least five to nine months. These authors discuss the possibility of using this species as a potential natural tag for ecological studies on *O. masou* in the sea. However, the discovery of *D. oncorhynchi* in the Russian territory should be taken into account in studies of this kind.

The taxonomic position of *D. oncorhynchi* is discussed repeatedly in literature. According to Shimazu (1980, 1988), this parasite belongs to the subfamily Opecoelinae Ozaki, 1925. However, since 1993 this species was recognized as a member of the subfamily Plagioporinae Manter, 1947 (Shimazu, Awakura, 1993; Moravec, Nagasawa, 1998; Shimazu, 2000). This point of view, based on

the concept of family Opecoelidae, was suggested by Gibson, Bray (1982) and Gibson (1996). The reason for the taxonomic revision of *D. oncorhynchi* was based on a new interpretation of the reproductive system morphology: the presence of the cirrus sac, which contains whole seminal vesicle, and the presence a little widening in the proximal part of the Laurer's canal, which can be considered as rudiment canalicular seminal receptacle (Shimazu, Awakura, 1993; Shimazu, 2000). Cribb (2005) believes that the canalicular seminal receptacle in *D. oncorhynchi* is absent and the distal part of the male duct is represented by a small cirrus sac with the adjacent external seminal vesicle enclosed entirely by a membranous sac. On this basis, the author considered this species as the member of Opecoelinae.

To clarify the systematic position of the genus *Dimerosaccus*, we used molecular data. It should be taken into account that presented in NCBI GenBank database data on different genera and subfamilies of Opecoelidae mostly belong to the not type-taxa. Therefore, the taxonomical interpretations obtained on their basis are preliminary.

All available molecular data of opecoelids, including *Dimerosaccus*, were grouped into four clusters. The first cluster included *D. oncorhynchi*, which was closely related to the genus *Opecoeloides* Odhner, 1928, a member of the subfamily Opecoelinae (see Cribb, 2005) that confirmed a point of Cribb about membership of *Dimerosaccus* to this subfamily. First cluster was a sister to a second one, which includes the species *Gaevskajatrema halosauropsi*, *Buticulotrema hermichthysi*, *Neolebouria lanceolata*, *Podocotyloides brevis* and *Plagiocirrus loboides*. According to Cribb (2005), genera, which include these species belong to the subfamily Plagioporinae with the exception of genus *Buticulotrema*, a member of the subfamily *Opecoelinae*. These two clusters formed a separate group, which was related to a large group (cluster 3), wholly consisting of representatives of the subfamily Plagioporinae, according to the system of Cribb (2005). The fourth cluster includes two species of the genus *Biospeedotrema* Bray, Waeschenbach, Dyal, Littlewood et Morand, 2014, which are members of the subfamily Stenakrinae, according to Bray et al. (2014). Our results indicate high differences between the second and the third clusters and the highest differences between *Biospeedotrema* (cluster 4) and other opecoelids that was noted earlier (Bray et al., 2014). Using Allocreadiidae as the outgroup taxa provided a question about systematic position of the genus *Biospeedotrema*, which was separated from Opecoeliidae (fig. 4).

The species *Gaevskajatrema perezi* (the type species of genus *Gaevskajatrema* Gibson et Bray, 1982) and *G. halosauropsi* were included into the clusters 3 and 2, respectively. *G. halosauropsi* is closely related to *Buticulotrema hermichthysi*, a member of the subfamily Opecoelinae. For this reason *G. halosauropsi* certainly should be assigned to another genus. Earlier, a similar conclusion was made by Andres et al. (2014). Subfamiliar membership of the species *Neolebouria lanceolata* and *Podocotyloides brevis* also needs to be adjusted. In addition, our analysis confirms the point of view of Curran et al. (2007) and Bray et al. (2014) that *Plagiocirrus loboides* does not belong to the subfamily Plagioporinae. Until obtaining of further molecular genetic data on other genera of the subfamiles Opecoelinae and Plagioporinae, we include *G. halosauropsi*, *Buticulotrema hermichthysi*, *N. lanceolata*, *Podocotyloides brevis* and *P. loboides* in the group Opecoelidae incertae sedis.

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