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Never ending diversity: two new species of the genus *Allocreadium* (Digenea: Allocreadiidae) including new keys to the genus

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Research Paper

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Abstract

Two new species of the genus *Allocreadium* were isolated from the intestines of the Lake minnow *Rhynchocypris percnura* caught in the backwater of the Komissarovka River in the South of the Russian Far East. The morphology of *A. anastasii* n. sp. corresponds to that of *Allocreadium* sp. from Lake Khar (Mongolia) and *Allocreadium* sp. Belous, 1952 from the Primorsky region of Russia except for the preacetabular anterior border of the vitelline follicles in *A. anastasii* n. sp. from the Komissarovka River vs. at anterior half of ventral sucker in *Allocreadium* sp. Genetic analysis revealed the identity of *A. anastasii* n. sp. to *Allocreadium* sp. 1 from the Nezhinka River and Lake Khar. *Allocreadium macrolecithum* n. sp. was differentiated from Palaearctic *Allocreadium* spp. by having the following features: respectively large vitelline follicles extending from posterior extremity to anterior margin of the ventral sucker; relatively short caeca reaching the border of middle and posterior thirds of hindbody; and small testes in the middle of hindbody. Interspecific genetic p-distances between *Allocreadium* spp. were 0.16–7.23% in 28S gene and 18.62–31.54% in *Cox1* mtDNA gene. In the phylogenetic tree reconstructed with Maximum parsimony and Bayesian Inference methods, *A. anastasii* n. sp. and *A. macrolecithum* n. sp. were nested into different species groups of the genus *Allocreadium* – sister to *A. khankaiense* and *A. bursense*, respectively. Modified dichotomous keys were prepared for 31 Palaearctic species of *Allocreadium* including *A. crassum*, *A. dogieli*, *A. papilligerum*, *A. bursense*, *A. anastasii* n. sp., and *A. macrolecithum* n. sp.

Introduction

The genus *Allocreadium* Looss, 1900 represents cypriniform fish-associated trematodes comprising significant species richness in relation to other allocreadiids – 107 valid species (WoRMS 2024). At present, the systematics of *Allocreadium* is based on both morphological (mainly adult stages) and genetic data. Thus, for the last four years, four new species of *Allocreadium* were described from the Primorsky region of Russia, South Africa, and North Western Turkey (Vainutis 2020; Dos Santos *et al.* 2021; Vainutis *et al.* 2023; Aydogdu *et al.* 2023). It was revealed that 10 *Allocreadium* species representing different lineages maintain the general morphological characters of the type species *A. isoporum* (Aydogdu *et al.* 2023; Vainutis *et al.* 2023). Some species sharing great morphological similarity and infecting the same fish hosts in close geographic regions are still not genetically confirmed (e.g., over 30 species from India). Based on the WoRMS database, only 13% (14 species) of *Allocreadium* spp. have been confirmed with application of molecular genetic data: *A. transversale* (Rudolphi, 1802) Odhner, 1901; *A. isoporum* (Looss, 1894) Looss, 1900; *A. lobatum* Wallin, 1909; *A. gotoi* (Hasegawa & Ozaki, 1926) Shimazu, 1988; *A. crassum* (Wesenberg-Lund, 1934) Vainutis, Voronova, Urabe & Kazarin, 2023; *A. schizothoracis* Pande, 1938; *A. dogieli* Koval, 1950; *A. neotenicum* Peters, 1957; *A. hemibarbi* Roitman, 1963; *A. papilligerum* (Rees, 1968) Moravec, 1984; *A. khankaiense* Vainutis, 2020; *A. apokryfi* Dos Santos, Gilbert, Avenant-Oldewage & Dumbo, 2021; *A. pseudoisoporum* Vainutis, Voronova, Urabe & Kazarin, 2023; *A. bursense* Aydogdu, Vainutis, Voronova & Aydogdu, 2023 (Vainutis 2020; Dos Santos *et al.* 2021; Vainutis *et al.* 2023; Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Sokolov *et al.* 2023; Solórzano-García *et al.* 2024).

This study was aimed at resolving the taxonomic status and phylogenetic relationships of two *Allocreadium* species found in the backwaters of the Komissarovka River (Primorsky region, Russian Far East). Description of the new species and recent confirmation of the species *A. transversale*, *A. dogieli*, *A. papilligerum*, and *A. bursense*, with genetic data (Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Sokolov *et al.* 2023), resulted in modification of dichotomous keys including 31 Palearctic *Allocreadium* species to date.

Material and methods

Material collection and morphological analyses

Twelve adult specimens of *Allocreadium* spp. were isolated from the intestines of two Lake minnows *Rhynchocypris percnura* caught in two backwaters of the Komissarovka River, Khankaysky district, Primorsky region, Russia (44°57′56.4″N 131°44′37.3″E). Collected worms were identified to genus level according to morphological characteristics under a light microscope, rinsed in saline, killed in hot distilled water, and stored in 70% ethanol for morphological analysis and 96% ethanol for molecular genetic studies.

Six specimens of *Allocreadium* selected for the morphological study were stained in alum carmine, dehydrated in a graded ethanol series (75%, 85%, 95%, ~100% absolute ethanol), cleared in clove oil, and mounted in Canada balsam. Measurements of the entire body and hindbody were provided in millimetres (mm), and measurements of other structures were in micrometres (μm). The holotypes and paratypes of both species were deposited to the parasitological collection of the Water Bioresources and Aquaculture department of the Far Eastern State Technical Fisheries University (FESTFU), Vladivostok, Russia. Dichotomous keys (Vainutis *et al.* 2023) were modified by including six species of which two were described as new in this study and four from previous works (Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Sokolov *et al.* 2023): *A. bursense*, *A. transversale*, *A. dogieli*, and *A. papilligerum*.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from four specimens using alkaline lysis method HotShot (Truett 2006). A fragment of the 28S rRNA gene was amplified using forward primer U178 (5′-GCA CCC GCT GAA YTT AAG-3′) and reverse primer L1642 (5′-CCA GCG CCA TCC ATT TTC A-3′) (Lockyer *et al.* 2003). A fragment of *Cox1* mtDNA of *A. anastasii* was amplified using forward primer JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (Bowles *et al.* 1992) and reverse primer CO1-R-trema (5′-CAA CAA ATC ATG ATG CAA AAG G-3′) (Miura *et al.* 2005). For the analysis of genetic divergence, short fragments of three specimens of *Allocreadium hemibarbi* (ASP6 from Vainutis 2020) were amplified with forward primer JB3 and reverse primer JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) (Bowles *et al.* 1992). The PCR mixture contained 2X DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 0.5 μL forward and reverse primers and 5 μL templates in total volume of 20 μL. The amplification protocol for 28S rDNA was performed under the following conditions: 2 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 a 7 min extension hold at 72°C. The amplification protocol for *Cox1* mtDNA: 1 min denaturation hold at 94°C, 30 cycles of 15 s at 94°C, 30 s at 50°C, 2 min at 72°C, and a 7 min extension hold at 72°C. Each PCR reaction included a negative and positive control, using both primers to detect possible contamination. PCR products were directly sequenced using the Bright Dye Terminator Cycle Sequencing kit (Nimagen, The Netherlands) as instructed by the manufacturer. Internal sequencing primers implemented for the 28S gene fragment were as follows: 3S (5'-CGG TGG ATC ACT CGG CTC GTG-3') (Bowles *et al.* 1995), 1200F (5'-CCC GAA AGA TGG TGA ACT ATG C-3'), 1200R (5'-GGG CAT CAC AGA CCT G-3'), 900F (5'-CCG TCT TGA AAC ACG GAC CAA G-3') (Lockyer *et al.* 2003). The PCR products were read with an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA) at the NSCMB FEB RAS.

Alignment and phylogenetic analyses

Four partial 28S rDNA and two partial *Cox1* mtDNA sequences obtained in this study were assembled with MEGA X software and aligned using ClustalW (Kumar *et al.* 2018). New sequences of *Cox1* gene of *Allocreadium hemibarbi* were deposited to GenBank with accession numbers OR945219–OR945221. Sequences used for the phylogenetic reconstruction are represented in Table 1. Genetic p-distances were estimated using the Tamura-Nei model with 1,000 bootstrap replicates in the MEGA X software; p-value was lower than 0.05. The species *Acrolichanus auriculatus* (NCBI Accession numbers MN524579, MN750364) was chosen as outgroup taxon. Following previous studies (Aydogdu *et al.* 2023; Vainutis *et al.* 2023), unidentified species from Ukraine and China were designated as *Allocreadium* sp. 2 and *Allocreadium* sp. 3, respectively. Phylogenetic analyses were carried out with Bayesian Inference (BI) algorithm (Huelsenbeck *et al.* 2001) with the GTR+I+G model selected in jModeltest v. 2.1.5 software as the best (Darriba *et al.* 2012). The MCMC algorithm was performed using two independent runs and 500,000 generations (the average standard deviation of split frequencies was less than 0.01); 25% of generations were discarded as burn-in in MrBayes v. 3.1.2 software (Huelsenbeck *et al.* 2001). An additional phylogenetic tree for the same sampling was reconstructed using the Maximum Parsimony (MP) method in the MEGA X software, with 1,000 bootstrap replicates. In this study, different species groups of *Allocreadium* on the phylogenetic trees were indicated with the Latin letters (from *A* to *B*) following the previous phylogenetic works (Aydogdu *et al.* 2023; Vainutis *et al.* 2023).

Results

Taxonomy

Phylum: Platyhelminthes Gegenbaur, 1859
Class: Trematoda Rudolphi, 1808
Subclass: Digenea Carus, 1863
Order: Plagiorchiida La Rue, 1957
Suborder: Xiphidiata Olson, Cribb, Tkach, Bray & Littlewood, 2003
Superfamily: Gorgoderoidea Looss, 1901
Family: Allocreadiidae (Looss, 1902) Stossich, 1903
Genus: *Allocreadium* Looss, 1900
Allocreadium anastasii n. sp. (Figure 1)
urn:lsid:zoobank.org:act:EDC56D5A-C212-40D2-9DBB-E1D514C771D9

Material examined: Nine adult specimens were isolated from the intestine of one individual of *Rhynchocypris percnura* (Pallas, 1814).

Morphological description. Based on five specimens. Relatively small trematodes, body elongate, fusiform in contracted state, length 1.456–2.122 (1.774). Tegument smooth, unarmed. Widest part of body posterior to ventral sucker in utero-ovarian region in relaxed worms, 553–886 (703), or at level of anterior third of anterior testis when body contracted. Forebody narrowing at anterior end, short, 355–591 (426), 20.85–27.85% (23.92%) of body length; hindbody 0.868–1.278 (1.118), 59.62–65.65% (62.31%) of body length. Oral sucker subterminal, subrounded (n=3) or transversely oval (n=2), 199–254 ×214–250 (233×237), 11.26–15.73% (13.28%) of body length; perioral muscular papillae absent. Tegument protrusion absent at anterior margin of body. Small pigment eyespots present, in contracted worms at level of

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Table 1. Information on the species, host, geographic origin, and the genetic data (partial 28S rRNA and *Cox1* mtDNA sequences) for the species used in the phylogenetic reconstructions

Parasite species	Host	Locality	Sequence data accn.		Reference
			28S rRNA	*Cox1* mtDNA	
Family Allocreadiidae					
Allocreadium					
Allocreadium macrolecithum n. sp.	*Rhynchocypris percnura*	Russia, Primorsky region, Khankaysky district, backwater of Komissarovka River	OR066228–OR066229	–	This study
Allocreadium anastasii n. sp.	*Rhynchocypris percnura*	Russia, Primorsky region, Khankaysky district, backwater of Komissarovka River	OR066230–OR066231	OR945222–OR945223	This study
	Phoxinus phoxinus	Russia, Nadezhdinsky district, tributary of the River Nezhinka (Razdolnaya River basin)	MK211209–MK211210	MK818870–MK818871	Vainutis 2020; Vainutis *et al.* 2021
	Oreoleuciscus potanini	Mongolia, Great Lakes' Hollow, Lake Khar	OQ427384	–	Sokolov *et al.* 2023
Allocreadium bursense	*Oxynoemacheilus angorae*	Turkey, Bursa, the Nilüfer Stream	OK045521–OK045523	OQ249866–OQ249868	Aydogdu *et al.* 2023
Allocreadium pseudoisoporum	*Carassius gibelio*	Russia, Primorsky region, Yakovlevsky district, Arsenyevka River (near Yablonovka village)	MK258685–MK258687	OM914849–OM914851	Vainutis *et al.* 2023
Allocreadium khankaiense	*Rhynchocypris lagowskii*	Russia, Khankaisky district, Komissarovka River	–	MW729428–MW729429	Vainutis *et al.* 2021
		Russia, Chuguevsky district, Pavlovka River (near Pavlovka village)	MZ448170–MZ448171	–	Vainutis *et al.* 2023
Allocreadium hemibarbi	*Hemibarbus labeo*	Russia, Khankaisky district, Komissarovka River	MK211220–MK211221	OR945219–OR945219	Vainutis 2020; this study
Allocreadium transversale	*Cobitis taenia*	Lithuania, Curonian Lagoon	OQ359128–OQ359129	–	Petkevičiūtė *et al.* 2023
Allocreadium papilligerum	*Salmo trutta*	Russia, Karelia, River Syskyänjoki	OQ427386	–	Sokolov *et al.* 2023
Allocreadium apokryfi	*Labeobarbus aeneus*	South Africa, Vaal River	MW907591–MW907595	–	Dos Santos *et al.* 2021
Allocreadium isoporum	*Alburnus alburnus*	Russia, Karelia, Lake Oster	GU462125–GU462126	–	Petkevičiūtė *et al.* 2010
Allocreadium isoporum	*Barbatula barbatula*	Russia, River Il'd, upper Volga River basin	MH143102	–	Petkevičiūtė *et al.* 2018
Allocreadium crassum	*Pisidium amnicum*	Finland, Siilaisenpuro River	JF261142–JF261143	–	Petkevičiūtė *et al.* 2012
Allocreadium dogieli	*Blicca bjoerkna*	Russia, Karelia, Lake Pertozero	OQ427387	–	Sokolov *et al.* 2023
Allocreadium lobatum	*Semotilus corporalis*	Maine, USA	EF032693	–	Curran *et al.* 2006
Allocreadium lobatum	*Luxilus cornutus*	USA, Wisconsin, West Twin River	–	OR987847	Solórzano-García *et al.* 2024
Allocreadium neotenicum	*Hydroporus rufifrons*	United Kingdom, Cumbria, Lake District	JX977132	–	Bray *et al.* 2012
Allocreadium neotenicum	*Pisidium casertanum*	Russia, Crimea, River Burulcha	MH143103	–	Petkevičiūtė *et al.* 2018
Allocreadium neotenicum	*Pisidium casertanum*	Norway, Lake Takvatn	MH143104	–	Petkevičiūtė *et al.* 2018
Allocreadium gotoi	*Misgurnus anguillicaudatus*	Japan, Nagano, Iiyama, Midori	LC215274	LC215273	Shimazu *et al.* 2017
Allocreadium schizothoracis	*Tariqilabeo latius*	India	OP584922	–	Rajput *et al.* unpublished
Allocreadium sp.	*Sphaerium corneum*	Ukraine, River Teterev	GU462121	–	Petkevičiūtė *et al.* 2010

(*Continued*)

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Table 1. (*Continued*)

Parasite species	Host	Locality	Sequence data accn.		Reference
			28S rRNA	*Cox1* mtDNA	
Allocreadium sp.	*Schizothorax parvus*	China	MN969626	–	Li, Fan 2020
	Schizothorax yunnanensis		MN969627	–	
Crepidostomum					
Crepidostomum metoecus	*Barbatula toni*	Russia, Primorsky region, Artyomovsky district, Artyomovka River	MT196355	–	Vainutis *et al.* 2021
Crepidostomum oschmarini	*Pisidium casertanum*	Lithuania, River Nedzinge	MH159994	–	Petkevičiūtė *et al.* 2018
Crepidostomum chaenogobii	*Gammarus* sp.	Russia, Sakhalin Island, Sakhalin region, Tyoply Klyuch brook (Belaya River tributary)	MK818589	–	Vainutis *et al.* 2021
Stephanophiala					
Stephanophiala farionis	*Salvelinus leucomaenis*	Russia, Sakhalin region, Belaya River (near the Sokol village)	MW368678	–	Vainutis *et al.* 2021
Stephanophiala pseudofarionis	*Salvelinus alpinus*	United Kingdom, Scotlant, Loch Rannoch	OP580487	–	Rochat *et al.* 2022
Bunodera					
Bunodera luciopercae	*Pisidium amnicum*	Lithuania, dammed up River Nemunas near Kaunas	GU647219	–	Petkevičiūtė *et al.* 2010
Bunodera acerinae	*Pisidium amnicum*	Russia, River Tvertsa, upper Volga River basin	GU462112	–	Petkevičiūtė *et al.* 2010
Acrolichanus					
Acrolichanus sp.	*Acipenser schrenkii*	Russia, Amur region, Amur Estuary	MN524579	–	Atopkin *et al.* 2020
Acrolichanus auriculatus	*Acipenser fulvescens*	USA, Wisconsin, Lake Winnebago	MN750364	–	Atopkin *et al.* 2020

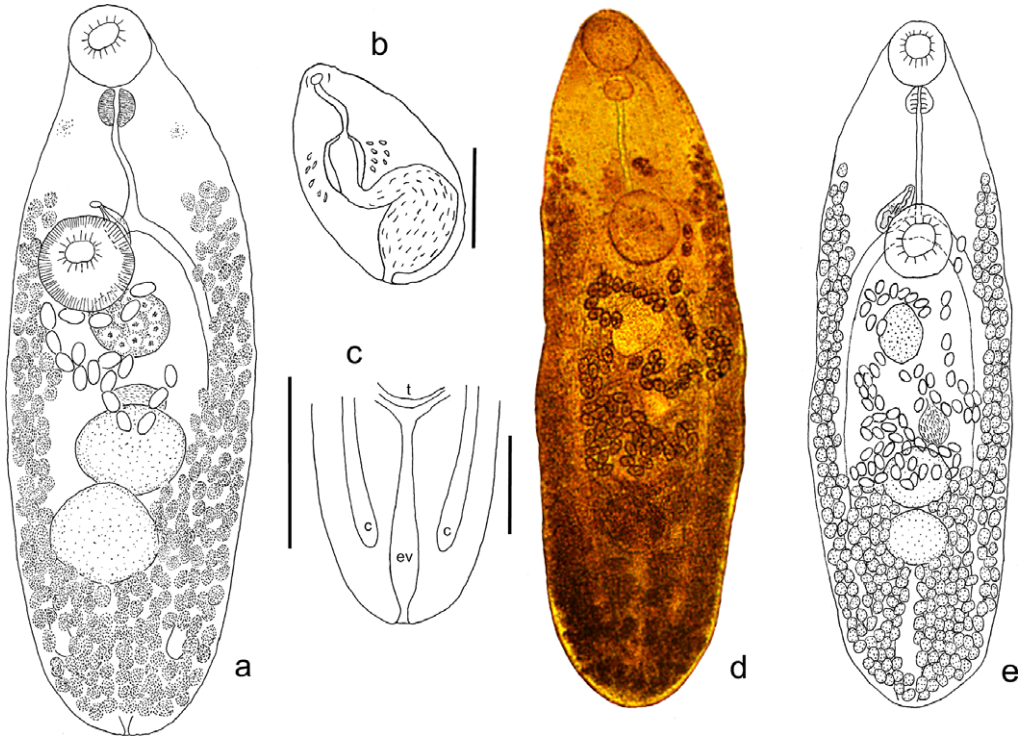

Figure 1. *Allocreadium anastasii* n. sp., registration No. T1: (a) holotype No. T1-1 from the backwater of the Komissarovka River, entire worm, ventral view, scale bar = 500 μm; (b) schematic drawing of the cirrus pouch, scale bar = 50 μm; (c) schematic drawing of posttesticular space with caeca and excretory vesicle, scale bar = 200 μm; abbreviations: t – testis, c – caeca, ev – excretory vesicle; (d) Microphotograph of *A. anastasii* n. sp. from the Nezhinka River, based on the material used for DNA extraction in the work of Vainutis (2020); (e) sketch drawing of *A. anastasii* n. sp. from the Nezhinka River.

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posterior margin of oral sucker, in relaxed worms at level of posterior margin of pharynx; eyespots 5.6–19×5–14(12×10) (n=4). Prepharynx not observed. Pharynx transversely oval 95–137×114–144 (114×134), 5.33–7.59% (6.5%) of body length. Oesophagus relatively long, 154–278 (199), 9.39–13.1% (11.14%) of body length, bifurcating dorsally to anterior third or middle of ventral sucker. Caeca reaching posterior third of posttesticular space, at 179–217 (201) from posterior end of body. Ventral sucker on border of anterior and middle thirds of body, subrounded (n=3) or transversely oval (n=2), larger than oral sucker, 230–288×221–280 (259×260), 13.29–15.97% (14.67%) of body length. Distance between oral and ventral suckers 121–356 (192). Suckers length ratio 1:1–1.18 (1:1.11); suckers width ratio 1:0.98–1.2 (1:1.1). Testes tandem or suboblique in middle third of hindbody, subrounded, transversely oval or irregular, tightly adjoining each other, anterior testis 179–489×195–404 (275×317), 10.01–27.11% (15.49%) of body length; posterior testis 185–632×220–523 (317×315), 12.7–35.03% (17.72%) of body length. Distance between anterior margin of anterior testis and posterior end of body 761–1023 (865); length of posttesticular space 364–471 (421). Cirrus pouch saccate, thin-walled, suboval, 118–168×73–85 (150×78), anterior to ventral sucker or its proximal part at anterior third of ventral sucker. Cirrus pouch containing seminal vesicle, short pars prostatica and short cirrus (119). Seminal vesicle tubular, curved crescently, locating in posterior half of cirrus pouch. Genital atrium containing male (mgp) and female (fgp) genital pores preacetabular, medium, 81×105 (n=1); mgp 23–27×21–29 (25×25); fgp 26–43×24–39 (33×29). Distance anterior to genital atrium 298–448 (343). Ovary subrounded or oval, 141–210×140–253 (183×223), 9.63–10.92% (10.35%) of body length, pretesticular, its anterior third dorsal to posterior third of ventral sucker; distance between ovary and anterior testis 147–190 (169). Seminal receptacle saccate, 85–193×73–219 (134×113), 5.83–9.09% (7.4%) of body length; posterior to ovary, biased posteriorly to anterior testis. Laurer's canal not observed. Lateral fields of vitellarium extending ventrally and dorsally, overlapping caeca; its anterior border anterior to ventral sucker at middle of esophagus or at level of anterior margin of ventral sucker at 344–462 (379), posterior border at 60–88 (74) from posterior end of body. Vitelline follicles oval, subrounded or irregular, relatively small, 66–203×45–154 (109×86). Uterus between ventral sucker and posterior testis reaching posterior third of anterior testis; in contracted worms lower part of uterus at anterior third of posterior testis. Eggs 23–82, oval, 63–102×40–52 (82×46). Excretory vesicle tubular, reaching posterior margin of posterior testis or immediately posterior to it, length 279–492 (374); width at posterior part – 26–64 (50), anterior part – 39–89 (69). Excretory pore terminal.

Synonyms. Allocreadium sp. Belouss, 1952; *Allocreadium* sp. 1 (Vainutis, 2020); *Allocreadium* sp. (Sokolov *et al.* 2023).

Etymology. The species name *anastasii* was given after the remarkable scientist Anastasia Voronova (Leading researcher at Pacific branch of Russian Federal Research Institute of Fisheries and Oceanography (TINRO)) in gratitude for the guidance and support on my scientific path.

Type host and locality. Lake minnow *Rhynchocypris percnura* (Pallas, 1814) caught from a backwater of Komissarovka River, Khankaysky district, Primorsky region, Russia (44°57′56.4″N 131°44′37.3″E).

Other hosts. Phoxinus lagowskii oxycephalus (=*Rhynchocypris oxycephala* (Sauvage & Dabry de Thiersant, 1874)) in Belouss (1952); *Oreoleuciscus potanini* (Kessler, 1879) in Sokolov et al. (2023).

Other localities. Tributary of the Nezhinka River, Razdolnaya River basin, Nadezhdinsky district, Russia (43°25′57.1″N 131° 46′21.8″E) (Vainutis, 2020); Lake Khar, Great Lakes' Hollow, Mongolia (48° 19′ N; 93° 08′ E) (Sokolov *et al.* 2023).

Type material. Holotype (No. T1-1) and four paratypes (No. T1-2–5) were deposited to the helminthological collection of the Far Eastern State Technical Fisheries University, Vladivostok, Russia. Deposition date: 15 November 2023.

Molecular genetic data. Complete region ITS1-5.8S-ITS2 rRNA – MW480031–MW480032 (this study), partial 5.8S-ITS2 region OQ427388 (Sokolov *et al.* 2023); 28S rRNA gene – OR066230–OR066231 (this study), MK211209–MK211210 (Vainutis 2020), OQ427384 (Sokolov *et al.* 2023); *Cox1* mtDNA gene – MK818870–MK818871 (Vainutis *et al.* 2021), OR945222 – OR945223 (this study).

Diagnosis. Allocreadium anastasii n. sp. has morphological characters of both *Allocreadium* sp. of Belouss (1952) from the Primorsky region and *Allocreadium* sp. of Sokolov *et al.* (2023) from Mongolia. Their common features are as follows: anterior half of pharynx dorsal to posterior margin of oral sucker; intestinal bifurcation dorsal to anterior half of ventral sucker; proximal part of cirrus pouch dorsal to anterior margin of ventral sucker; anterior margin of ovary dorsal to ventral sucker; lower part of uterus overlapping anterior third or half of anterior testis or anterior third of posterior testis ventrally; excretory vesicle reaching anterior quarter of posttesticular space, immediately posterior to posterior testis. *Allocreadium anastasii* n. sp. from the Primorsky region of Russia has several morphological features distinguishing it from Mongolia's *A. anastasii*: anterior border of vitellarium preacetabular vs. at anterior half of ventral sucker; testes tandem or suboblique, subrounded, transversely oval or irregular and tightly adjoining each other vs. tandem, rounded and separated; excretory vesicle reaching posterior margin of posterior testis or immediately posterior to it vs. reaching anterior quarter of post-testicular space.

Among other Far Eastern *Allocreadium* species, *A. gobii* is the most similar to *A. anastasii* n. sp. by the following features: proximal part of cirrus pouch dorsal to ventral sucker reaching its midlevel; genital pore anterior to intestinal bifurcation; intestinal bifurcation dorsal to ventral sucker; anterior border of vitelline fields in forebody. But *A. anastasii* n. sp. differs from *A. gobii* by the following features: uterus posteriorly reaching anterior third of anterior testis vs. uterus in region between ventral sucker and anterior testis; anterior border of vitellarium at anterior half of ventral sucker (Mongolia) or anterior to it at level of genital pore (Primorsky region, Russia) vs. anterior border of vitellarium at posterior margin of pharynx.

Allocreadium anastasii n. sp. differs from relative species *A. khankaiense* by numerous morphological characters such as anterior border of vitellarium in forebody or at anterior third of ventral sucker vs. at level of anterior or posterior half of ventral sucker; proximal part of cirrus pouch reaching midlevel of ventral sucker vs. posterior margin of ventral sucker; uterus posteriorly reaching anterior third or posterior margin of anterior testis vs. strictly pretesticular or overlapping anterior margin of anterior testis.

Allocreadium macrolecithum n. sp. (Figure 2)

urn:lsid:zoobank.org:act:5F70A3B5-BDB7-4CDF-A7BC-C-41AB7FB004E.

Material examined. Three adult specimens were isolated from the intestine of one individual of *Rhynchocypris percnura.*

Morphological description. Based on one specimen. Relatively small trematodes, body elongate-ellipsoid, length 2.451. Tegument

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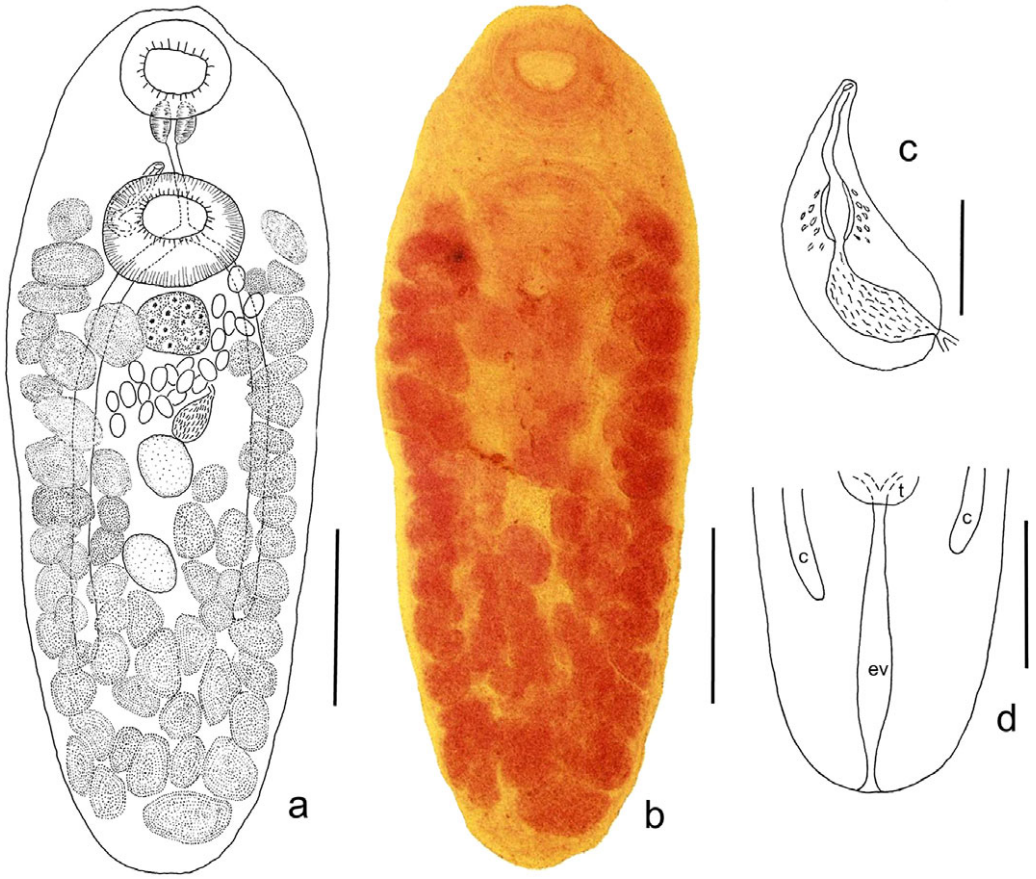

Figure 2. *Allocreadium macrolecithum* n. sp., registration No. T2: (a) holotype No. T2-1 from the backwater of the Komissarovka River, entire worm, ventral view, scale bar = 500 μm; (b) microphothograph of the entire worm, scale bar = 500 μm; (c) schematic drawing of the cirrus pouch, scale bar = 100 μm; (d) schematic drawing of posttesticular space with caeca and excretory vesicle; abbreviations: t – testis, c – caeca, ev – excretory vesicle, scale bar = 400 μm.

smooth, unarmed. Widest part of body in utero-ovarian region, 917, posterior to ventral sucker. Forebody short, 485; hindbody 1.664, slightly narrowing to posterior end. Oral sucker subterminal, round, 309×326; perioral muscular papillae absent. Bipartite tegument protrusion present at anterior margin of body. Eyespots absent. Prepharynx not observed. Pharynx subrounded 171×163. Oesophagus short, 225, bifurcating at level of middle third of ventral sucker. Caeca reaching middle third of hindbody. Ventral sucker on border of anterior and second fourths of body, oval, larger than oral sucker, 334×413. Distance between oral and ventral suckers 157. Testes suboblique allocating in tandem in mid-line of body and in anterior half of hindbody, anterior testis subrounded, 196×175, length 7.99% of body length; posterior testis oval, 193×136, length 7.87% of body length. Distance between anterior and posterior testes 117; between ventral sucker and anterior testis 366; between posterior testis and posterior end of body 81. Cirrus pouch saccate, thin-walled, 245×141, dextro-dorsal to ventral sucker, its proximal part at middle third of ventral sucker. Cirrus pouch containing seminal vesicle, prostatic part and short cirrus. Seminal vesicle tubular, 116×39, with slightly widened posterior end, locating in posterior third of cirrus pouch, where at mid-length curving at right angle. Prostatic part 73×42. Genital pore preacetabular, dextro-submedian. Ovary subrounded, 223×304, pretesticular, in midsagittal line; distance between ovary and anterior testis 172. Seminal receptacle small, club-shaped, 138×89, postero-dorsal and antero-dorsal to ovary and anterior testis respectively. Laurer's canal not observed. Vitellarium extending in lateral fields of body ventrally and dorsally, overlapping caeca; its anterior border at anterior third of ventral sucker, posterior border at posterior end of body. Vitelline follicles relatively large, 95–268×76–168, nuclei of vitellocytes oval or subrounded 23–26×19–23, containing nucleolus nearly four times smaller than nuclei in diameter. Follicles not reaching posterior end of body at 99. Uterus pretesticular, immediately posterior to ventral sucker. Eggs 21, relatively large, 78–96×59–69. Excretory vesicle tubular, reaching posterior margin of posterior testis. Excretory pore terminal.

Etymology. The species name *macrolecithum* is given due to enormously large vitelline follicles in respect to those of other *Allocreadium* species.

Type host and locality. Rhynchocypris percnura (Pallas, 1814) caught from a backwater of Komissarovka River, Khankaysky district, Primorsky region, Russia (44°57′56.4″N 131°44′37.3″E).

Type material. Holotype (No. T2-1) was deposited to the helminthological collection of the Far Eastern State Technical Fisheries University, Vladivostok, Russia. Deposition date: the 15th of November 2023.

Molecular genetic data. 28S rRNA gene – OR066228–OR066229.

Diagnosis. Allocreadium macrolecithum n. sp. is very similar to *Allocreadium transversale* in following morphological characters: ventral sucker is significantly larger than the oral sucker; testes

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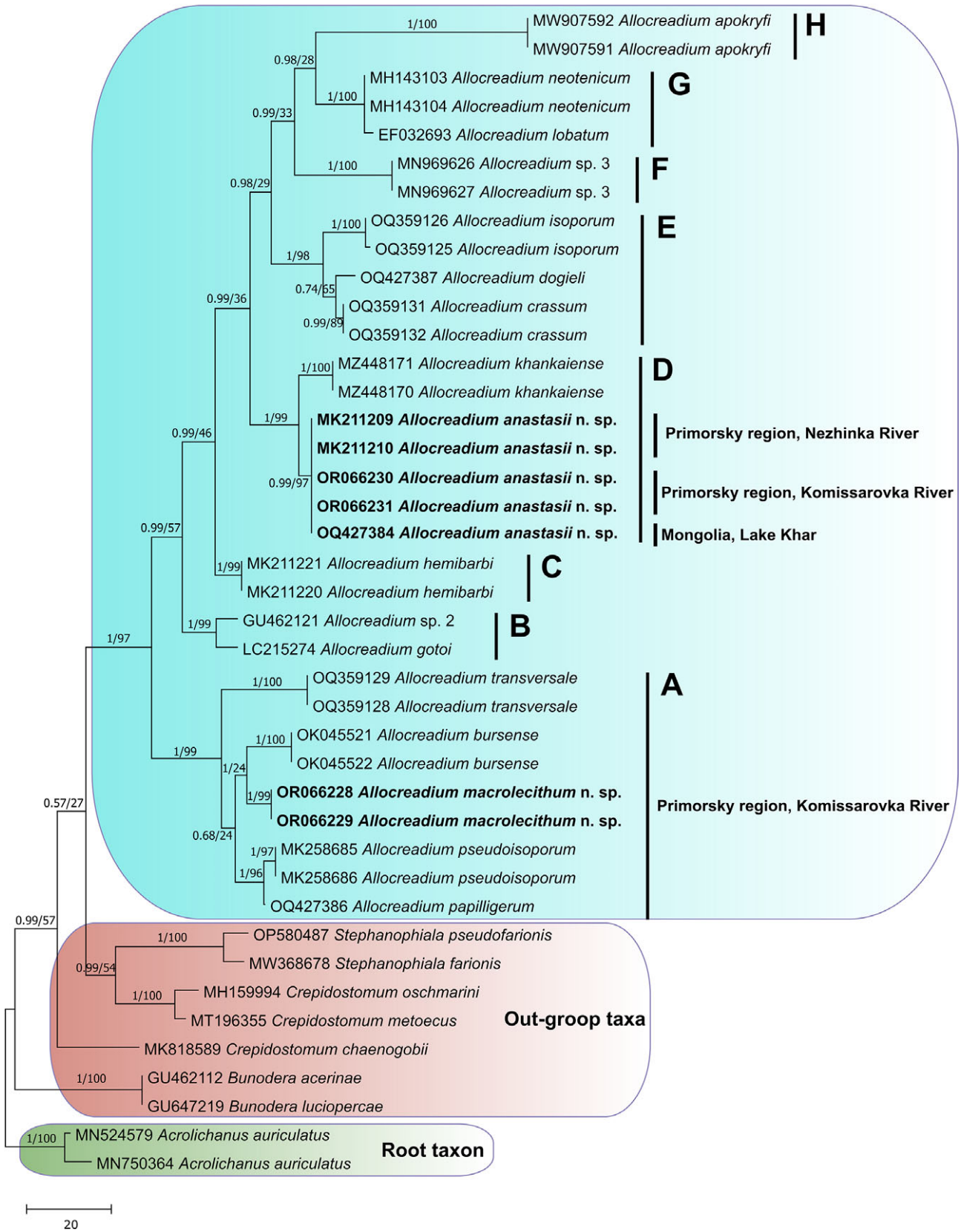

Figure 3. Phylogenetic tree of five allocreadiid genera. Emphasis was made on the evaluation of phylogenetic relationships of 15 *Allocreadium* species (blue rectangle). The new species are in bold. Reconstruction made with the Bayesian Inference (BI) and Maximum Parsimony (MP) methods based on the 1269 bp of 28S rRNA gene fragment. Numbers on the branches are posterior probabilities of BI and % of MP.

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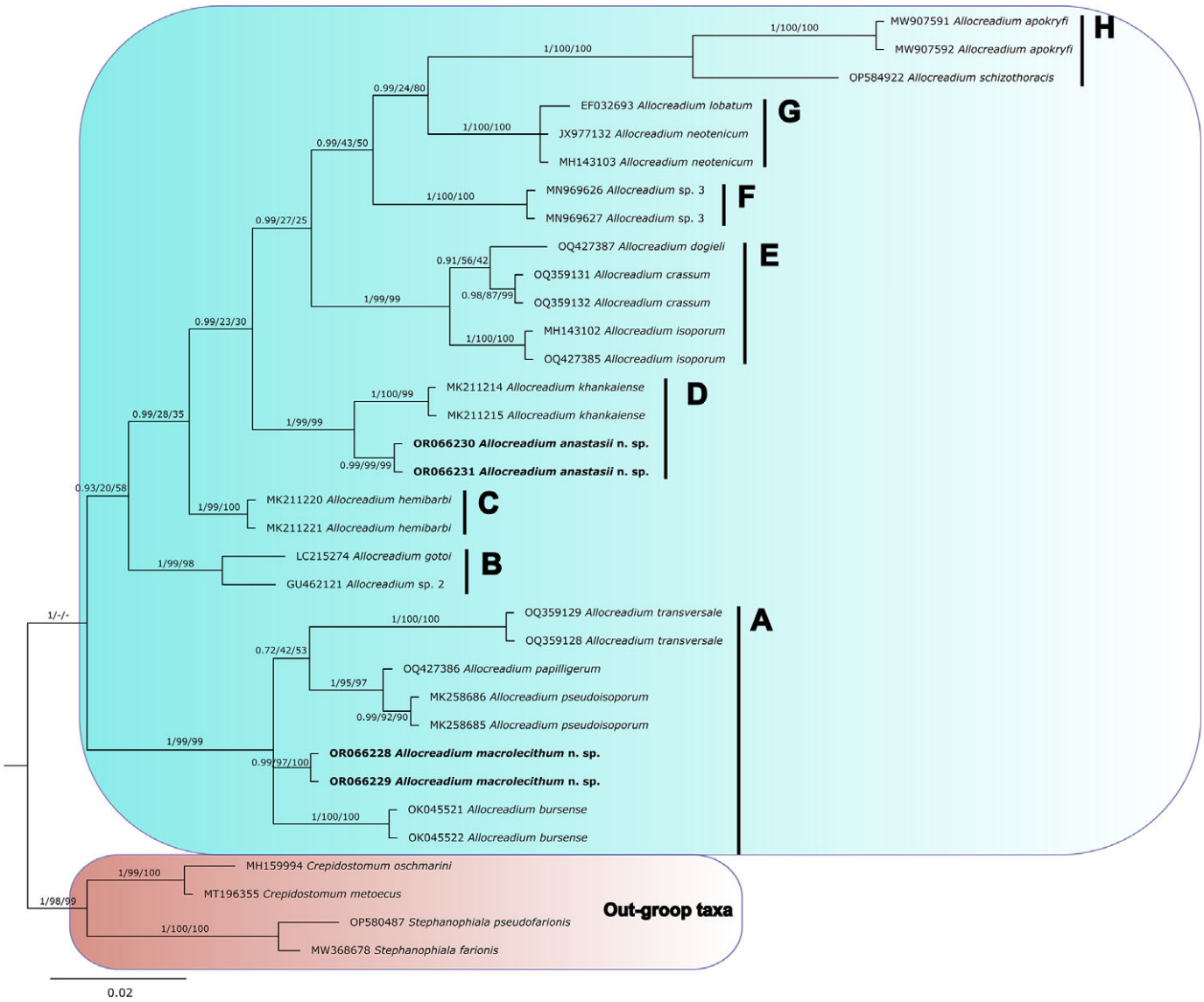

Figure 4. Phylogenetic tree of three allocreadiid genera representing phylogenetic relationships of 16 *Allocreadium* species (blue rectangle). Four sequences of two new species are in bold. Reconstruction made with the BI, MP, and Maximum Likelihood (ML) methods based on the D2 domain of 28S rRNA gene fragment (499 bp). Numbers on the branches are posterior probabilities of BI and % of MP and ML.

small, oval, suboblique with a little distance between them; large vitelline follicles reaching the anterior margin of the ventral sucker (Bauer 1948; Koval 1957; Roitman 1963; Petkevičiūtė *et al.* 2023). *Allocreadium macrolecithum* differs from *Allocreadium transversale* by the following characters: bipartite tegument protrusion present at anterior end of body vs. protrusions absent; anterior border of vitelline fields at level of ventral sucker vs. in forebody; genital pore anterior to intestinal bifurcation vs. posterior. Vitelline follicles of *A. macrolecithum* are comparable with that of *A. dogieli* in Sokolov *et al.* (2023). The authors did not provide the measurements of follicles but noted the size of the follicles is almost equal to that of gonads. In *A. macrolecithum*, length of follicles (95–268) and width (76–168) are a little larger than those of *A. dogieli* gonads: anterior testis 74–124×62–106, posterior testis 62–149×62–92, and ovary 92–183×94–127.

Allocreadium anastasii n. sp. and *A. macrolecithum* n. sp. differ from each other by the following features: testes relatively large – anterior testis 10.01–27.11% and posterior testis 12.7–35.03% of body length vs. 7.99% and 7.87% of body length, respectively; anterior border of vitelline fields in forebody vs. at level of ventral sucker; vitelline follicles oval or subrounded, small, with maximum length 203 and width 154 vs. irregular, large in relation to body dimensions (268×168); uterus overlapping anterior half of anterior testis vs. pretesticular; excretory vesicle posterior to testes vs. reaching posterior testis.

Molecular genetic analysis

Genetic analysis of the 28S rRNA gene

Partial sequences of the 28S rRNA gene of two *A. anastasii* n. sp. (1734 bp) and two *A. macrolecithum* n. sp. (1686 bp) individuals were identical within each species. Pairwise genetic distances between 15 species of *Allocreadium* and unidentified *Allocreadium* sp. 2 and *Allocreadium* sp. 3, were 0.16 – 7.23% (Supplementary Table 1). Genetic distance between *A. anastasii* n. sp. and *A. macrolecithum* n. sp. was 4.51%. *Allocreadium anastasii* n. sp. was similar to

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Table 2. Modified dichotomous keys to 31 Palearctic species of *Allocreadium* Looss, 1900, based on morphology of the adult worms

Couplets	Character states	Species
1a	Vitelline fields in hindbody.	2
1b	Anterior border of vitelline fields in acetabular region or in forebody.	3
2a	Ventral sucker larger or nearly equal to oral sucker.	4
2b	Ventral sucker smaller than oral sucker.	*A. hemibarbi* Roitman, 1963
3a	Anterior border of vitelline fields in acetabular region.	5
3b	Anterior border of vitelline fields in forebody.	6
4a	Ventral sucker nearly equal to oral sucker.	7
4b	Ventral sucker larger than oral sucker.	8
5a	Excretory vesicle reaching posterior or anterior testis.	9
5b	Excretory vesicle not reaching posterior testis.	10
6a	Genital pore anterior to intestinal bifurcation.	11
6b	Genital pore at level of or posterior to intestinal bifurcation.	12
7a	Body length not exceeding 1.7 mm. Proximal part of cirrus pouch reaching the posterior margin of ventral sucker.	*A. pseudoisoporum* Vainutis, Voronova, Urabe & Kazarin, 2023
7b	Body length more than 1.9 mm. Proximal part of cirrus pouch slightly extending beyond anterior margin of ventral sucker or at middle third of it.	13
8a	Excretory vesicle reaching posterior testis.	14
8b	Excretory vesicle not reaching posterior testis.	15
9a	Excretory vesicle reaching anterior testis.	*A. bursense* Aydogdu, Vaınutıs, Voronova & Aydogdu, 2023
9b	Excretory vesicle reaching posterior testis.	16
10a	Ovary oval or three-lobed.	*A. japonicum* Ozaki, 1926
10b	Ovary oval, rounded, comma-shaped, or irregular shape.	17
11a	Intestinal bifurcation dorsal to anterior margin of ovary.	*A. pseudaspii* (Achmerov, 1960) Bychovskaya-Pavlovskaya, 1962 Synonym: *A. elongatum* (Achmerov, 1960) Bychovskaya-Pavlovskaya, 1962 (preoccupied name)
11b	Intestinal bifurcation dorsal or anterior to ventral sucker.	18
12a	Genital pore at level of intestinal bifurcation.	19
13a	Body length more than 2 mm reaching 5 mm.	*A. isoporum* (Looss, 1894) Looss, 1902
13b	Body length 1.89–1.99 mm.	*A. crassum* (Wesenberg-Lund, 1934) Vainutis, Voronova, Urabe & Kazarin, 2023 Synonym: *Cercariaeum crassum* Wesenberg-Lund, 1934 (cercarial stage)
14a	Cirrus pouch preacetabular. Anterior border of vitelline follicles at level of posterior margin of ventral sucker.	*A. montanus* Sidorov & Butenko, 1966
14b	Cirrus pouch preacetabular. Anterior border of vitelline follicles at level of ovary.	*A. brevivitellatum* Shimazu, 1992
15a	Testes rounded or ellipsoid. Seminal vesicle bipartite. Uterine loops reaching posterior testis.	*A. tribolodontis* Shimazu & Hashimoto, 1999
15b	Testes of irregular shape, deeply indented. Seminal vesicle unipartite. Uterine loops pretesticular.	*A. hasu Ozaki, 1926*
16a	Length of anterior testis 7.99% of body length, posterior testis 7.87% of body length. Vitelline follicles large, some equal or larger than testes.	*A. macrolecithum* n. sp.
16b	Both testes length occupying more than 12% of body length. Vitelline follicles large or average-sized, smaller than testes.	21
17a	Body spindle-shaped with distinctly truncated anterior end. Anterior testis rounded to oval or subtriangular, entire; posterior testis subrhomboid to almost crescent-shaped or irregular, entire to indented in outline.	*A. dogieli* Koval, 1950
17b	Body elongate. Testes of irregular shape, slightly indented.	22

(*Continued*)

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Table 2. (*Continued*)

Couplets	Character states	Species
18a	Intestinal bifurcation at level of ventral sucker.	23
18b	Intestinal bifurcation anterior to ventral sucker.	*A. tosai Shimazu, 1988*
19a	Cirrus pouch preacetabular. Anterior border of vitelline fields at level of pharynx.	*A. shinanoense* Shimazu, 2003
19b	Proximal part of cirrus pouch posterior to anterior margin of ventral sucker. Anterior border of vitelline fields posterior to pharynx.	24
20a	Small auricular outgrowths on anterior margin of oral sucker. Anterior border of vitelline follicles at level of intestinal bifurcation.	*A. erythroculteris* (Achmerov, 1960) Bychovskaya-Pavlovskaya, 1962 Junior synonym: *A. maculati* Achmerov, 1963
20b	Perioral outgrowths absent. Anterior border of vitelline follicles at level of anterior margin of ventral sucker.	25
21a	Uterus strictly pretesticular or posterior uterine loops covering anterior margin of anterior testis. Proximal part of cirrus pouch reaching posterior margin of ventral sucker.	*A. khankaiense* Vainutis, 2020
21b	Uterus between ventral sucker and posterior testis. Proximal part of cirrus pouch reaching anterior margin of ventral sucker.	*A. carparum* Odening, 1959
22a	Cirrus pouch anterior to ventral sucker. Body length 2.56–2.90 mm.	*A. aburahaya* Shimazu, 2003
22b	Cirrus pouch extending to posterior border of ventral sucker. Body length 4.71–4.73 mm.	*A. tamoroko* Shimazu and Urabe, 2013
23a	Proximal part of cirrus pouch dorsal to ventral sucker.	26
23b	Cirrus pouch preacetabular.	27
24a	Proximal part of cirrus pouch posterior to ventral sucker. Anterior border of vitelline fields between pharynx and intestinal bifurcation.	28
24b	Proximal part of cirrus pouch at midlevel of ventral sucker. Anterior border of vitelline fields at level of anterior margin of ventral sucker.	*A. danjiangense* Gao, Wang, Xi, Yao, Nie, 2008
25a	Proximal part of cirrus pouch reaching midlevel of ventral sucker.	*A. transversale* (Rudolphi, 1802)
25b	Cirrus pouch preacetabular.	*A. gotoi* (Hasegawa & Ozaki, 1926) Shimazu, 1988
26a	Proximal part of cirrus pouch reaching midlevel of ventral sucker.	29
26b	Proximal part of cirrus pouch slightly beyond anterior margin of ventral sucker.	*A. papilligerum* (Rees, 1968) Moravec, 1984
27a	Anterior border of vitelline fields at half distance between suckers.	*A. baueri* Spassky et Roitman, 1960
27b	Anterior border of vitelline follicles at level of posterior margin of oral sucker.	*A. markewitschi* Koval, 1949
28a	Body relatively small (length 2.4–3.3 mm), fusiform.	*A. qianweiense* Zhang, Yang, 1994
28b	Body large, elongate-oval.	30
29a	Uterus in region between ventral sucker and anterior testis. Anterior border of vitellarium at posterior margin of pharynx.	*A. gobii* Roitman, 1963
29b	Uterus posteriorly reaching anterior third of anterior testis. Anterior border of vitellarium at anterior half of ventral sucker (Mongolia) or anterior to it at level of genital pore (Primorsky region, Russia).	*A. anastasii* n. sp.
30a	Testes large (length 0.4–0.6 mm), irregular shape, entire.	*A. hypophthalmichthydis* (Achmerov, 1960) Bychovskaya-Pavlovskaya, 1962
30b	Testes relatively large (length 0.56–0.64 mm), irregular shape, slightly indented.	*A. conicum* Wang, Jiang, 1985

A. khankaiense (0.89%) and differed from the other *Allocreadium* spp. on 2.13–6.37%. *Allocreadium macrolecithum* n. sp. was similar to the species *A. papilligerum* (1.21%), *A. bursense* (1.29%), and *A. pseudoisoporum* (1.45%), and distant from other *Allocreadium* spp. on 2.21–6.28%.

Both BI and MP phylogenetic trees based on the partial 28S rDNA sequences displayed the same branch topology (Figure 3). Allocreadiid species were divided into two clades of which the earliest branching taxon was *Acrolichanus* chosen as root and the second consisted of outgroup taxa (subclades I–III) and *Allocreadium* (subclade IV). Subclade I included two species of *Bunodera.* Subclade II comprised single species *Crepidostomum chaenogobii.* Subclade III was subdivided into two groups: first, two species of *Crepidostomum*, and second, two species of *Stephanophiala.* Subclade IV was formed with 15 species of *Allocreadium* and branched into eight separate groups. Group *A* belonged to *A. pseudoisoporum*, *A. bursense*, *A. papilligerum*, *A. transversale*, and *A. macrolecithum* n. sp. Group *B* included *A. gotoi* from Japan and *Allocreadium* sp. 2. Group *C* was presented with the single species *A. hemibarbi.* Group *D* included *A. khankaiense* and *A. anastasii* n. sp. from the Komissarovka and Nezhinka Rivers (Primorsky region, Russia). Group *E* included European species

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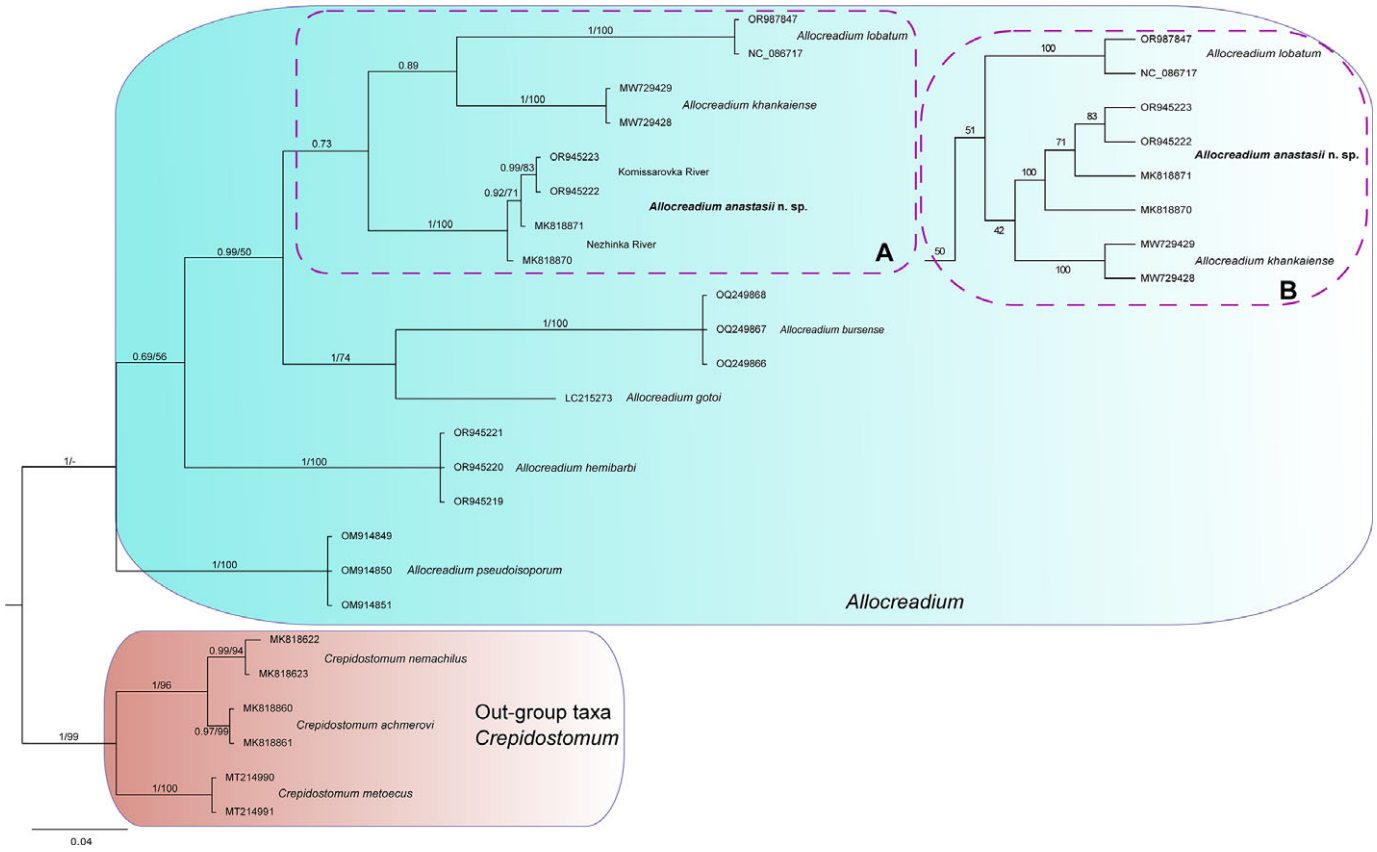

Figure 5. Phylogenetic tree reconstructed for seven *Allocreadium* species based on the 381 bp of *Cox1* mtDNA gene fragment. The new species *Allocreadium anastasii* is in bold. Reconstructions were performed with the BI and MP methods. Numbers on the branches are posterior probabilities of BI and % of MP. (A) Variant of resolution of fourth subclade in BI tree; (B) variant of fourth subclade in MP tree.

A. isoporum, *A. crassum*, and *A. dogieli.* Group *F* consisted of the single species *Allocreadium* sp. 3 from China, which is sister to the terminal node containing group *G* (*A. neotenicum* and *A. lobatum*) and group *H* (*A. apokryfi*).

Phylogenetic tree including *Allocreadium schizothoracis* was reconstructed using the shorter 28S fragment (Figure 4). Original alignment was trimmed to the shortest sequence based on the length of only sequence of *A. schizothoracis* available in GenBank (499 bp). In general, BI, MP, and ML reconstructions shared the same topology, but statistical support in nodes between species groups was highest in the BI tree and lower and unreliable in MP and ML. The genera *Crepidostomum* and *Stephanophiala* were chosen as outgroup taxa representing the first major clade and rooting the tree. African group *H* with *Allocreadium apokryfi* included *A. schizothoracis* with strongest support obtained by three methods (1.0/100/100).

Genetic analysis of the Cox1 mtDNA gene

Two identical sequences (767 bp length) were obtained for *Allocreadium anastasii* n. sp. The *Cox1* fragment of *A. macrolecithum* n. sp. was not sequenced because of both pairs of primers (JB3 and CO1-R-trema, JB3 and JB4.5) were not specific to this species. Due to the short sequences of *A. hemibarbi*, the final length of analyzable fragment of the *Cox1* gene was 381 bp for the whole sampling. Intraspecific genetic distances of two populations of *A. anastasii* (Nezhinka and Komissarovka Rivers) were 0.54–1.07%. The interspecific genetic distances distinguishing *A. anastasii* n. sp. from *A. pseudoisoporum*, *A. bursense*, *A. gotoi*, *A. khankaiense*, *A. hemibarbi*, and *A. lobatum* were in the range 18.36–30.22%. In general, the range of interspecific genetic distances of seven *Allocreadium* species was the following: 18.36–39.78% (Supplementary Table 2).

Phylogenetic trees based on the *Cox1* fragment comprising ten allocreadiid species of two genera were reconstructed with BI and MP methods. The resulting tree had a different topology than that of the 28S tree. The consensus tree (Figure 5) was subdivided into two clades of which the earliest branching clade was formed by outgroup taxa (three species of *Crepidostomum*) chosen as the root-group. The second clade represented the ingroup of *Allocreadium* spp. and consisted of four subclades: *A. pseudoisoporum* occupied the first basal subclade; the second subclade included *A. hemibarbi*; the third subclade comprised *A. gotoi* and *A. bursense*, and was sister to the fourth subclade combining *A. anastasii* n. sp., *A. khankaiense*, and *A. lobatum.*

Discussion

Current research supplemented several works devoted to the study of the type genus of the family Allocreadiidae mainly in the Russian Far East: description of three new species (Vainutis 2020; Vainutis *et al.* 2023; Aydogdu *et al.* 2023), genotyping of *A. khankaiense* and *Allocreadium* sp. 1 (=*A. anastasii* n. sp.) (Vainutis *et al.* 2021), and assumptions on co-evolution with cyprinid hosts (Bogatov and Vainutis 2022). The description of two new species increased the diversity of the genus *Allocreadium* in the Russian Far East, and particularly in the south of the Primorsky region. Morphological

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and genetic data obtained originally and from published materials (Vainutis 2020; Sokolov *et al.* 2023) clarified the taxonomic status of *Allocreadium* sp. 1 from the Primorsky region of Russia and Mongolia.

Considering the genetic distances estimated with 28S rRNA and *Cox1* mtDNA genes and genetic data obtained in last several studies (Vainutis 2020; Dos Santos *et al.* 2021; Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Sokolov *et al.* 2023; Vainutis *et al.* 2023), *Allocreadium anastasii* n. sp. and *A. macrolecithum* n. sp. are distinct species. Both species belong to already known species groups of *Allocreadium* – *A. macrolecithum* n. sp. to group *A* and *A. anastasii* n. sp. to Asian group *D*.

Among other species groups, group *A* is the earliest branching clade strongly supported with BI (Bogatov and Vainutis 2022; Aydoğdu *et al.* 2023; Sokolov *et al.* 2023; Vainutis *et al.* 2023), Maximum Likelihood (Vainutis *et al.* 2023; Petkevičiūtė *et al.* 2023), Neighbor-Joining (Aydoğdu *et al.* 2023), and Maximum Parsimony methods (Aydoğdu *et al.* 2023). It was revealed that group *A*, containing only *A. pseudoisoporum* in Vainutis *et al.* (2023) (*Allocreadium* sp. 2 in Bogatov and Vainutis (2022)), has wider species composition including *A. pseudoisoporum*, *A. bursensis*, *A. papilligerum*, and *A. transversale* (Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Sokolov *et al.* 2023). Likewise, the group *A* is characterized by diverse diagnostic morphological features (Table 2) and geographic distribution – Eastern and Western Asia and Eastern and Northern Europe. *Allocreadium macrolecithum* n. sp. supplemented this group (Figure 3) forming a sister relationship with *A. bursense* and diverging from all representatives of group *A* on 1.21–2.21% based on the 28S rRNA gene. These values are characteristic for interspecific divergence of *Allocreadium* particularly (Aydogdu *et al.* 2023; Vainutis *et al.* 2023) and trematodes in general. The phylogenetic tree based on the *Cox1* gene revealed alternative but unreliable topology. *Allocreadium pseudoisoporum* occupied basal subclade, but other representatives were rearranged (Figure 5). Of them, the fourth subclade had two variants depending on the methods: BI variant formed a separate branch for *A. anastasii* and showed a sister relationship of *A. khankaiense* and *A. lobatum*; the MP variant showed a sister relationship of *A. anastasii* and *A. khankaiense* and generated a separate branch for *A. lobatum* – in both cases, resolution had low statistical support. The selected fragment of the *Cox1* gene is too short for trustworthy phylogenetic analysis; therefore, by reducing the number of informative sites, the impact of parallel mutations and reversions directly increases. Specifically, *A. gotoi* and *A. bursense* have more common conservative sites (312 of 381, 81.9%) than *A. bursense* and *A. pseudoisoporum* (301 of 381, 79%), although the close relationship between *bursense* and *pseudoisoporum* has been demonstrated (Aydogdu *et al.* 2023; Sokolov *et al.* 2023). Discrepancy occurred when comparing *A. anastasii* and *A. khankaiense* (325 of 381, 85.3%), and *A. khankaiense* and *A. lobatum* (315 of 381, 82.7%) – although *A. anastasii* and *A. khankaiense* are more similar, it did not affect their sister relationship in the BI tree. Thus, due to the higher rates of evolution of *Cox1*, the use of short fragments of this gene up to 700 bp, especially at the species level within the same genus, can lead to the generation of deliberately false phylogenies.

Allocreadium anastasii n. sp. is more widely distributed than its sister species, *A. khankaiense*; the former is found in the rivers Nezhinka (Razdolnaya River basin) and Komissarovka (Khanka Lake basin) (this study) of Southern Primorye of Russia and Lake Khar of Western Mongolia (Vainutis 2020; Sokolov *et al.* 2023), whereas *A. khankaiense* occurs in the rivers of Southern Primorye – Komissarovka, Pavlovka (Ussuri River basin) and Artyomovka (Muravyinaya Bay basin) (Vainutis 2020; Vainutis *et al.* 2021; Vainutis *et al.* 2023). The wider distribution of *A. anastasii* possibly affected its morphological variability that is an extension of the anterior border of vitellarium, spatial arrangement, shape, and interlocation of testes, and extension of excretory vesicle.

According to the values of genetic distances, Aydogdu *et al.* (2023) revealed that Indian species *A. schizothoracis* is closely related to group *A*, including five *Allocreadium* species. In this study, the short 28S sequence of *A. schizothoracis* does not provide opportunity to reconstruct a robust phylogeny with strong nodal support of ML and MP methods, except for the BI method, which revealed strong support and the same topology obtained based on the longer sequences. On the tree reconstructed with the BI method using the ITS2 rDNA region, Sokolov *et al.* (2023) revealed the sister position of *A. schizothoracis* in relation to the branch containing *A. transversale* and *A. papilligerum.* Considering this phylogeny, *A. schizothoracis* should be nested within the basal group or between groups *A* and *B.* In addition, newly added *A. macrolecithum* n. sp. is more related to *A. schizothoracis* (11.18%) than other species of group *A* (11.75%, 12.01%, 12.11%, 14.09%). It was shown earlier that among *Allocreadium* spp., *A. apokryfi* is most closely related to *A. schizothoracis* (6.05%) (Aydogdu *et al.* 2023). On the original tree (Figure 4) based on the second variable domain of the 28S rRNA gene (499 bp), *A. schizothoracis* was nested within the group *H* containing *A. apokryfi.* This group was strongly supported with BI, MP, and MP (1/100/100) methods, but bootstrap values of MP and ML in ancestral nodes of species groups was lower than 75%. The Indian species *A. schizothoracis* is sister to the South African *A. apokryfi.* This particularly confirms the initial assumption of Aydogdu *et al.* (2023) concerning the speciation of *A. apokryfi* and *A. schizothoracis* from the common South East Asian lineage. Since that, African group *H* should be considered Ethiopian-Oriental based on the names of the biogeographic realms which *A. apokryfi* and *A. schizothoracis* belong to – Ethiopia and Oriental realm, respectively.

Allocreadium apokryfi is not closely related to group A because of larger number of accumulated mutation steps. Among terminal groups *G* and *H*, *Allocreadium apokryfi* is the first confirmed of nine African species (Dos Santos *et al.* 2021). Genetic distances revealed relative equidistance of *A. apokryfi* in relation to other *Allocreadium* spp. – 5.03–7.23%. This could potentially explain the inconsistencies in resolution of *apokryfi* on the phylogenetic trees: (1) location between basal group *A* and *A. gotoi* (*B*) (Dos Santos *et al.* 2021; Sokolov *et al.* 2023), (2) sister in relation to group *G* (*A. neotenicum* and *A. lobatum*) (Figure 3; Aydogdu *et al.* 2023; Petkevičiūtė *et al.* 2023; Vainutis *et al.* 2023). First topology is controversial because of insufficient statistical support of the BI method: 0.8 (Dos Santos *et al.* 2021) and 0.62 (Sokolov *et al.* 2023).

Conclusion

The description of two new species, *A. anastasii* n. sp. and *A. macrolecithum* n. sp., revealed a higher diversity of *Allocreadium* spp. in Russian Far East, and particularly in the Southern Primorsky region of Russia than previously known. The list of *Allocreadium* species known from the basin of the Komissarovka River (Primorsky region, Russia) has been increased to four species: *A. hemibarbi*, *A. khankaiense*, *A. anastasii* n. sp., and *A. macrolecithum* n. sp. In total, ten species of *Allocreadium* inhabit

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inland waters of the Russian Far East. New species *A. anastasii* has the wider distribution in relation to its relative *A. khankaiense*: in the rivers Nezhinka (Razdolnaya River basin) (Vainutis 2020) and Komissarovka (Khanka Lake basin) (this study) of Southern Primorye of Russia and Lake Khar of Western Mongolia (Sokolov *et al.* 2023). Two new species, *Allocreadium anastasii* n. sp. and *A. macrolecithum* n. sp., share general morphological features characteristic for the most of 31 Palaearctic *Allocreadium* spp.: body elongate, anterior border of vitelline fields in acetabular region or in forebody (23 sp.), ventral sucker larger than oral (27 sp.), tandem testes (31 species), cirrus pouch pre- or dorso-acetabular, ovary pretesticular (31 species). On the phylogenetic tree reconstructed with the 28S rRNA gene using Bayesian Inference and Maximum Parsimony methods, the new species *A. macrolecithum* n. sp. and *A. anastasii* n. sp. belong to already known species groups of *Allocreadium*: basal group *A* and Asian group *D*, respectively.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000440.

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