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Biodiversity assessment of *Eucyclops* Claus, 1893 (Copepoda: Crustacea) in the Baikal region using genetic methods

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Abstract

The genus *Eucyclops* is the most species-rich within the subfamily Eucyclopinae. Despite the significant research of *Eucyclops* in Russia, there are still vast areas including Siberia, the Baikal region, and the Far East with fragmentary study of this genus, particular with regard to molecular methods. In this work we have sequenced four molecular markers with different evolutionary rates and inheritance types (COI and 12S rRNA mtDNA, 18S rRNA and ITSn rDNA) to estimate the biodiversity of *Eucyclops* in the Baikal region. Five species *E. serrulatus*, *E. speratus*, *E. macruroides*, *E. arcanus*, and *E. macrurus baicalocorrepus* were identified through a combination of morphological and genetic methods in the Baikal region. The necessity for a taxonomic revision of endemic Baikal *E. macrurus baicalocorrepus* and *E. macruroides baicalensis* has been established. We assume that two forms of *E. macrurus baicalocorrepus* with short and long caudal rami are distinct endemic Baikal species. The genetic analysis of *E. serrulatus*, *E. speratus*, and *E. macruroides* revealed that the Baikal Cyclopoida, along with the Far Eastern and other Siberian representatives of the genus, form a distinct genetic lineage that differs from the majority of European representatives, with some exceptions.

Keywords

Cyclopoida, Copepoda, endemic, molecular phylogenetics, Siberia, speciation, systematics, taxonomy

Introduction

The estimation of biodiversity is a fundamental element in the processes of its conservation and the understanding of evolutionary mechanisms in the context of a changing climate, biological invasions and anthropogenic impact on functional changes in ecosystems (Lynch et al. 2023). It has been predicted that biodiversity among Copepoda will increase by 37–126% by 2100, reaching approximately 5000 species (Macêdo et. al. 2024). In the study of microcrustaceans such as Copepoda, especially closely related species, integrative approaches involving microscopy, morphometrics and genetic methods are crucial (Karanovic and Bláha 2019).

The genus *Eucyclops* Claus, 1893 is the most species-rich within the subfamily Eucyclopinae, with approximately 100 species (Alekseev and Defaye 2011; Alekseev 2019). This genus is comparable to the most species-rich genera among freshwater Cyclopoida: *Acanthocyclops* Kiefer, 1927 and *Diacyclops* Kiefer, 1927. Representatives of *Eucyclops* are ubiquitous, inhabiting permanent and temporary water bodies, including lakes, ponds, rivers, estuaries and groundwater. The majority of species are freshwater, although brackish-water species are also found. Similar to *Acanthocyclops* and *Diacyclops*, the genus *Eucyclops* is taxonomically complex due to the similarity in morphology among species, as well as the vast expanse of their ranges. Previously, *E. serrulatus* (Fischer, 1851) was considered to be cosmopolitan (Monchenko 1974). Today, this species, like *E. macrurus* (G.O. Sars, 1863), *E. macruroides* Lilljeborg, 1901 and *E. denticulatus* (Graeter, 1903), has a Palearctic distribution (Alekseev 2019), and all finds of *E. serrulatus* outside the Palearctic are most likely relatively recent introductions. As shown by integrative revisions, employing molecular genetic methods, these widely distributed copepod species may represent species complexes. For example, three morphotypes and two genetic lineages of *E. serrulatus* were identified in a type locality in St. Petersburg (Alekseev et al. 2006; Sukhikh and Alekseev 2015). Additionally, eight genetic lineages were identified among *E. serrulatus* inhabiting European water bodies (Hamrova et al. 2012). Specimens of the two most widespread genetic lineages have been found in a single water body. *Eurytemora affinis* (Poppe, 1880), previously considered a Holarctic species, has now been split into at least three species (Lee 1999; Sukhikh et al. 2023). Furthermore, several species of Harpacticoida have also demonstated complex intraspecific structure in genetic studies (Kochanova et al. 2022; Kochanova et al. 2024). The *Eucyclops* complex taxonomy has resulted in a constant increase of the described and redescribed species number (Alekseev et al. 2006; Mercado-Salas and Suárez-Morales 2014, 2020; Gaponova and Hołyńska 2019, 2022). The most recent genus taxonomic revision was conducted by V.R. Alekseev (2019). This revision resulted in the separation of two new genera *Isocyclops* Kiefer, 1957 and *Stygocyclops* Pleša, 1971 from the genus *Eucyclops*, which were previously considered subgenera. The remaining species were assigned to nine subgenera.

A fourteen-species *Eucyclops* fauna is known to occur in Russia, including the *E. serrulatus* species complex. Four Palaearctic species *E. macrurus*, *E. macruroides*, *E. denticulatus* and *E. speratus* (Lilljeborg, 1901) are found in the European part of Russia. *E. maritimus* (Alekseev et Monchenko, 1991), *E. persistens* (Monchenko, 1978), and *E. orthostylis* Lindberg, 1952 were recorded in the Ponto-Caspian basin. *E. delongi* Alekseev, 2019 and *E. roseus* Ishida, 1997 were recorded in Siberia and the Far East, while *E. roseus* was also recorded in Baikal and Crimea. *E. euacanthus* (Sars, 1909) and *Eucyclops cf. ohtakai* Ishida, 1997 were found in the Far East. *E. dumonti* Alekseev, 2000 and *E. arcanus* Alekseev, 1990 have been observed in Siberia (Alekseev 2019, 2023; Hołyńska et al. 2021; Novichkova and Chertoprud 2022). Four of these species have subspecies. These are *E. macrurus baicalocorrepus* Mazepova, 1955 and *E. macruroides baicalensis* Mazepova, 1978 from Lake Baikal, *E. arcanus arcticus* Alekseev, 2022 from Arctic bogs of Bolshezemelskaya tundra, *E. persistens persistens* from the mouths of rivers in the Caucasus and *E. persistens tauricus* from wells in the Crimea (Mazepova 1978; Gaponova and Hołyńska 2019; Alekseev 2022). The systematic position of *E. macrurus baicalocorrepus* is taxonomically difficult, as it was originally described as subspecies of *E. serrulatus*. However, V.I. Monchenko proposed that it should be classified as a subspecies of *E. macrurus* (Monchenko 1974; Alekseev and Defaye 2011). Also *E. roseus*, 2023 was transferred to the rank of subspecies *E. agiloides roseus* (Alekseev 2023).

The Baikal region is situated within the territory of Eastern Siberia, comprising the Irkutsk region and Zabaykal'skij Kraj, wich is confined to Lake Baikal. Lake Baikal is a rift, the deepest and most ancient lake on Earth. It is a significant center of speciation for numerous animal groups, including Cyclopoida (Timoshkin 2009). The genera *Diacyclops* and *Acanthocyclops*, among the Baikal cyclopids, are the most species-rich with a high degree of endemism. Four species of *Eucyclops* have been recorded in Baikal and the surround region: *E. macruroides*, *E. serrulatus*, *E. arcanus*, and *E. roseus*, which occur in Siberia and two endemic subspecies *E. macrurus baicalocorrepus* and *E. macruroides baicalensis* (Mazepova 1978; Alekseev 1990; Alekseev et al. 2019; Sheveleva et al. 2020; Alekseev and Chaban 2021). The species *E. serrulatus*, *E. arcanus*, and *E. macruroides baicalensis* inhabit the littoral zone of the lake at depths up to 45 m. *E. macrurus baicalocorrepus* is widespread in the littoral zone and occurs rarely at depths greater than 50 m. *E. macruroides baicalensis* Mazepova, 1978 has been recorded from 20 m to the maximum depths of Baikal (Mazepova 1978). Furhermore, a fifth species *E. dumonti* has been recorded in the water bodies of the Baikal region (Alekseev 2019).

Despite the significant research of *Eucyclops* in Russia, there are still vast areas including Siberia, the Baikal region, and the Far East with fragmentary study of this genus, particular with regard to molecular methods. Novikov et al. (2022) have conducted studies on representatives of *Eucyclops* from the Lena River delta in the Arctic, which have shown that the species composition of the genus is likely to be significantly expanded. Already at the beginning of the *Eucyclops* revision in this region, five potentially new species for science were identified.

In this study we have employed genetic methods to estimate the biodiversity of *Eucyclops* in the Baikal region.

Materials and methods

Sample collection

Samples were collected using a net and by scuba divers in 2009–2023 (Table 1). Copepods from the Baikal region, including Lake Baikal, water bodies on its shore, and water bodies from the Kuitun, Tulun, and Nizhneudinsk districts of the Irkutsk region, the vicinity of St. Petersburg, the Leningrad region, republic of Udmurtia, the Volgograd Reservoir, Sakhalin Island, and Kazakhstan were collected and subjected to analysis. The entire sample or only selected live copepods were fixed in 96% ethanol and stored at -20°C. Morphological analysis was performed using a Zeiss Imager A1 microscope (Zeiss, Germany), a MSP-1 (LOMO, Russia) and an Olympus CX 41 microscope (Olympus, Japan). Morphological features were measured using an Olympus CX 41 microscope (Olympus, Japan), a Levenhuk M 800 Plus digital camera and a LevenhukLite software (Levenhuk, Inc., USA). The mean error (m) was calculated according to the formula (1).

$$m = \frac{SD}{\sqrt{N-1}}, (1)$$

Where SD is the standard deviation and N is the number of observations.

For confocal laser scanning microscopy (CLSM), specimens were stained with Congo Red overnight and mounted on a slide in a drop of 50% glycerol following the procedure described by Michels and Büntzow (2010). The material was scanned using a Carl Zeiss LSM 710 laser confocal microscope (Zeiss, Germany); lens: Plan-Apochromat 20 ×/0.8 and 63×/1.40 Oil DIC M27; filters: 570–670 nm; lasers: 561 nm: 6.0%. Spinules groups on the antenna coxobases are numbered according to Alexeev et al. (2006).

Table 1. Locations and dates of sample collection

Specimens ID	Species	Sample location	Coordinates		Date
			N latitude	E longitude	
478 479	*E. macruroides* *E. macruroides*	lake, Novoje Devyatkino village, Leningrad region, Vsevolozhsk distrct, Russia	60.0564	30.4769	04.2020
746	*E. macruroides*	lake, Tankhoi settlement, republic of Buryatia, Russia	51.5621	105.1422	24.07.2021
BKE2	*E. macruroides*	lake, Bolshiye Koty, Irkutsk region, Russia	51.9174	105.0687	28.06.2023

Specimens ID	**Species**	**Sample location**	**Coordinates**		**Date**
			N latitude	**E longitude**	
T24	*E. arcanus*	temporary reservoir, Nizhneudinsk district, Irkutsk region, Russia	55.0198	98.9744	21.07.2021
F15-5	*E. arcanus*	lake, Onguren settlement, Olkhonsky district, Irkutsk region, Russia	55.0198	98.9744	02.06.2021
23-54	*E. arcanus*	lake, Okha, Okha district, Sakhalin region, Russia	53.6487	142.9762	28.07.2022
23-58	*E. arcanus*	temporary reservoir, Poronaysk, Poronaysk district, Sakhalin region, Russia	49.2285	143.0951	22.07.2022
727	*E. arcanus*	lake, Tankhoi settlement, republic of Buryatia, Russia	51.5621	105.1422	24.07.2021
729	*E. arcanus*				
720	*E. speratus*	Volgograd reservoir, Volzhanka settlement, Sredneakhtubinsk district, Volgograd region, Russia	48.9984	44.8315	02.09.2020
23-27	*E. speratus*	Orlovsky Pond, St. Petersburg, Leningrad region, Russia	59.8669	29.9377	25.06.2009
T13	*E. speratus*	river, Aleksandrovka village, Tulun district, Irkutsk region, Russia	55.3905	100.8231	25.07.2021
K1-6	*E. speratus*	lake, Kuitun, Kuitun District, Irkutsk Region, Russia	54.3472	101.5286	06.2021
23-50	*E. speratus*	river, Nogliki, Nogliki District, Sakhalin region, Russia	51.7981	143.1250	25.07.2022
23-51	*E. speratus*				
23-52	*E. speratus*				
406	*E. speratus*	Orlovsky Pond, St. Petersburg Leningrad region, Russia	59.9468	30.3765	04.2014
468	*E. cf. serrulatus*				
409	*E. serrulatus*				
472	*E. serrulatus*	lake, Karkalay village, Uvinsky district, republic of Udmurtia, Russia	57.6718	52.1284	05.2017
457	*E. serrulatus*	pond of the Tauride Garden, St. Petersburg, Leningrad region, Russia	59.9468	30.3767	04.2014

Specimens ID	**Species**	**Sample location**	**Coordinates**		**Date**
			N latitude	**E longitude**	
23-63	*E. serrulatus*	Irtysh river flood, Ust-Kamenogorsk, East Kazakhstan region, Kazakhstan	49.9337	82.6041	05.10.2023
TC1	*E. serrulatus*	lake, Bolshiye Koty settlement, Irkutsk district, Irkutsk region, Russia	51.8883	105.0369	21.06.2023
TC2	*E. serrulatus*				
BKE1	*E. serrulatus*	lake, Bolshiye Koty settlement, Irkutsk district, Irkutsk region, Russia	51.9174	105.0687	28.06.2023
Og1	*E. serrulatus*	lake, Onguren settlement, Olkhonsky district, Irkutsk region, Russia	53.6553	107.6653	10.06.2023
F15-3	*E. serrulatus*				02.06.2021
F15-8	*E. serrulatus*				
T15-1	*E. serrulatus*	temporary reservoir, Uk settlement, Nizhneudinsk district, Irkutsk region, Russia	55.0198	98.9744	21.07.2021
F10-1	*E. macrurus baicalocorrepus*, form 3	Lake Baikal, Bolshiye Koty settlement, Irkutsk district, Irkutsk region, Russia	51.8985	105.1525	31.05.2021
F10-2	*E. macrurus baicalocorrepus*, form 3				
F10-6	*E. macrurus baicalocorrepus*, form 3				
F10-7	*E. macrurus baicalocorrepus*, form 3				
F192-5	*E. macrurus baicalocorrepus*, form 1	Lake Baikal, Listvyanka settlement, Irkutsk district, Irkutsk region, Russia	51.8681	104.8296	27.04.2023
F192-6	*E. macrurus baicalocorrepus*, form 1				
F192-7	*E. macrurus baicalocorrepus*, form1				

DNA extraction, PCR, and sequencing

DNA was extracted by two methods. In the first case, we used whole specimen or cephalothorax and the ExtractDNA Blood DNA extraction kit according to instructions (Evrogen, Russia). In the second case, the biological material (egg sac or cyclopids antennula) was incubated in a mixture of 2x Encyclo buffer for PCR (Evrogen, Russia) and 0.1 mg/ml Proteinase K for 1-3 hours at 56°C. The enzyme was then inactivated by heating for 5 min at 96°C. The resulting mixture containing the DNA was used as a matrix in PCR in 10-fold dilution and stored at -20°C. PCR was performed using universal primers (Table 2) in a 10-20 μl mixture of 1x Encyclo buffer (Evrogen, Russia), 3.5 mM magnesium, 0.5 μM of each primer, 0.2 mM of each dNTP, 0.5 units of Encyclo DNA polymerase (Evrogen, Russia) and 1-2 μl of DNA-containing solution in a 100TM thermocycler (Bio Rad, USA).

The PCR thermoprofile for all genes included a step of heating to 94°C for 4 min; 35–40 cycles consisting of the following steps: matrix melting at 94°C for 15 s, primer annealing at specific temperature (Table 2) for 20 s, DNA synthesis at 72°C for 1 min; elongation step at 72°C for 4 min. The amplicons were electrophoretically separated in a 0.6% agarose gel in 0.5x Tris-acetate buffer. A piece of gel containing the target DNA fragment was excised, followed by freezing at -20°C. The tube with gel then centrifuged for 10 min at 10,000 rpm, after which the resulting solution was used in sequencing as a matrix. Direct DNA sequencing was conducted using the ABI PRISM BigDye Terminator sequencing kit v. 3.1 in 8-capillary genetic analysers ABI 3500 (Thermo Fisher Scientific, USA) and Nanofor 05 (Syntol, Russia).

Table 2. Features of PCR primers

Molecular marker	Primer	Primer direction	Primer sequence (5'-3')	Annealing temperutre, °C	Reference
COI	LCO-1490	Forvard	GGTCAACAAATCATAAAGATATTGG	48–50	Folmer et al. 1994
	HCO-2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		
	cop-COI-2189R	Reverse	GGGTGACCAAAAAATCARAA	48	Bucklin et al. 2010
12S	L13337-12S	Forvard	YCTACTWTGYTACGACTTATCTC	57–60	Machida et al. 2002
	H13845-12S	Reverse	GTGCCAGCAGCTGCGTTA		
ITSn	ITS-5	Forvard	GGAAGTAAAAGTCGTAACAAGG	57	White et al. 1990
	ITS-4	Reverse	TCCTCCGCTTATTGATATGC		
18S	18sI	Forvard	AACTYAAAGGAATTGACGG	50	Spears et al. 1992
	18s329	Reverse	TAATGATCCTTCCGCAGGT		

Molecular phylogenetic analysis

The study included 38 specimens of *Eucyclops*, for which 66 sequences of one to four molecular markers were obtained. All sequences were deposited in GenBank and their NCBI accession numbers are provided in Table 3: for 18SrRNA PQ164692-PQ164696, for nITS PQ165826-PQ165851, PQ319846-PQ319849 for 12SrRNA-PQ218851.1-PQ218871.1, for COI PQ216000-PQ216007. The sequences were aligned using MegaX (Kumar et al. 2018) and CLC Sequence Viewer 8.0.0(URL: https://resources.qiagenbioinformatics.com/manuals/clcsequenceviewer/current/index.php?manual=Introduction_CLC_Sequence_Viewer.html, accessed on August 20, 2024). The model-corrected genetic distances and p-distances were calculated using MegaX. The saturation of nucleotide substitutions was evaluated using the DAMBE (Xia et al. 2003). Nucleotide substitution model selection based on the Bayesian information criterion and phylogenetic tree construction by maximum likelihood (ML) method were performed using IQ-TREE2 software (Minh et al. 2020). Bootstrap algorithm was employed to evaluate the branching node support, with 1000 replications. *Eucyclops* and other cyclopid species sequences available in the GenBank were included in the analyses as outgroups. The GenBank accession numbers are provided on the phylogenetic trees. The trees were visualised and edited using the Interactive Tree Of Life (iTOL) version 6.8.1 (URL: https://itol.embl.de, accessed 07 May 2024) (Letunic, Bork, 2021) and Inkscape 1.3.2.

Table 3. NCBI accession numbers of sequences obtained in the study

Specimens ID	Species	12S rRNA	COI	ITSn	18S rRNA
78	*E. macruroides*	PQ218856.1	–	PQ165833.1	–
479	*E. macruroides*	–	–	PQ165834.1	–
746	*E. macruroides*	PQ218857.1	–	PQ165837.1	PQ164692.1
BKE2	*E. macruroides*	PQ218859.1	–	PQ165839.1	–
T24	*E. arcanus*	PQ218871.1	PQ216006.1	PQ165850.1	–
F15-5	*E. arcanus*	PQ218865.1	PQ216004.1	–	–
23-54	*E. arcanus*	–	PQ216001.1	PQ165830.1	–
23-58	*E. arcanus*	PQ218852.1	–	PQ165831.1	–
727	*E. arcanus*	–	–	PQ165835.1	–
729	*E. arcanus*	–	PQ216003.1	PQ165836.1	–
720	*E. speratus*	–	PQ216002.1	–	–
23-27	*E. speratus*	–	–	PQ165826.1	–
T13	*E. speratus*	–	PQ216005.1	–	–
K1-6	*E. speratus*	–	–	PQ165847.1	PQ164695.1
23-50	*E. speratus*	PQ218851.1	PQ216000.1	PQ165827.1	–

Specimens ID	**Species**	**12S rRNA**	**COI**	**ITSn**	**18S rRNA**
23-51	*E. speratus*	–	–	PQ165828.1	–
23-52	*E. speratus*	–	–	PQ165829.1	–
406	*E. speratus*	–	–	PQ165832.1	–
468	*E. cf. serrulatus*	PQ218855.1	–	PQ319848.1	–
409	*E. serrulatus*	PQ218854.1	PQ344269	PQ319846.1	–
23-63	*E. serrulatus*	PQ218853.1	–	–	–
TC1	*E. serrulatus*	–	–	PQ165851.1	PQ164696.1
TC2	*E. serrulatus*	–	PQ216007.1	–	–
BKE1	*E. serrulatus*	PQ218858.1	–	PQ165838.1	–
Og1	*E. serrulatus*	PQ218870.1	–	PQ165848.1	–
F15-3	*E. serrulatus*	PQ218864.1	–	–	–
457	*E. serrulatus*	–	–	PQ319847.1	–
472	*E. serrulatus*	PQ349277.1	–	PQ319849.1	–
F15-8	*E. serrulatus*	PQ218866.1	–	PQ165844.1	–
T15-1	*E. serrulatus*	–	–	PQ165849.1	–
F10-1	*E. macrurus baicalocorrepus*, form 3	PQ218860.1	–	PQ165840.1	–
F10-2	*E. macrurus baicalocorrepus*, form 3	–	–	PQ165841.1	–
F10-4	*E. macrurus baicalocorrepus*, form 3	PQ218861.1	–	–	–
F10-6	*E. macrurus baicalocorrepus*, form 3	PQ218862.1	–	PQ165842.1	–
F10-7	*E. macrurus baicalocorrepus*, form 3	PQ218863.1	–	PQ165843.1	PQ164693.1
F192-5	*E. macrurus baicalocorrepus*, form 1	PQ218867.1	–	PQ165845.1	PQ164694.1
F192-6	*E. macrurus baicalocorrepus*, form 1	PQ218868.1	–	PQ165846.1	–
F192-7	*E. macrurus baicalocorrepus*, form 1	PQ218869.1	–	–	–

Results

The 12S rRNA sequences were obtained for 22 specimens of *Eucyclops* (182-435 bp). Of these, 15 specimens were collected in Lake Baikal and in water bodies on its shore, and one specimen was collected in the Irkutsk region. We identified *E. serrulatus*, *E. speratus*, *E. macruroides*, *E. arcanus*, and two forms of *E. macrurus baicalocorrepus* inhabiting Lake Baikal using a combination microscopic examination and molecular genetic analysis.

The morphological differences between specimens of *E. m. baicalocorrepus* form 1 (F192-5 to F192-7) and *E. m. baicalocorrepus* form 3 (F10-1, F10-6, F10-7) for which molecular data were obtained include shorter caudal rami (Lf/Wf), smaller proportion of the length and width of the third endopodite segment of the fourth pair of swimming legs (LenpP4/WenpP4), longer inner apical spine relative to the outer apical spine of the same segment (IAS/EAS), greater proportion of inner apical spine and length of the third endopodite segment of the fourth pair of swimming legs (IAS/LenpP4) (Table 4), larger spinules of the caudal rami lateral row, longer row of spinules near to the lateral seta (Fig. 1A, B), additional row of small spinules on the caudal side of the coxopodite of the fourth pair of swimming legs (P4) (Fig. 1D) and one row of large spinuless in group 18 on the antenna coxobase on the caudal side instead of two rows of small spinules.

Table 4. Morphometric parameters of *E. m. baicalocorrepus* and *E. m. baicalensis*

Parameter	***E. m. baicalocorrepus*, form 1***	***E. m. baicalocorrepus*, form 3***	***E. m. baicalocorrepus*****	***E. m. baicalensis*****
		M±m		
Lf/Wf	3.79±0.117	6.84±0.232	3.4-8.2	7.2-9
LenpP4/WenpP4	2.04±0.008	2.20±0.055	2-2.5	2
IAS/EAS	1.39±0.012	1.16±0.027	1.3-1.5	The inner apical spine is slightly longer than the outer one
IAS/LenpP4	1.49±0.103	1.23±0.021	–	–

Note: *– our measurements; **– data of G.F. Mazepova (1978).

A saturation analysis of nucleotide substitutions in the 12S dataset using fully resolved sites showed Iss = 0.3073, significantly less than Iss.c = 0.6539 for the symmetric topology and less than Iss.c = 0.7267 for the asymmetric topology. The sequences for the symmetric topology have a small saturation of nucleotide substitutions, rendering them suitable for phylogenetic analysis. The intraspecific p-distances are 15.3% among *E. macruroides*, 14.5% among *E. serrulatus*, and 0.2% among *E. arcanus*. The interspecific p-distances ranged from 4.1 to 33.7%. *E. arcanus* is the most distant from all others species (30.1-33.7%). *E. speratus* and V genetic line of *E. serrulatus* according to Hamrova et al. (2012) are the closest (4.1%). The p-distance between the Baikalian *E. m. baicalocorrepus* forms 1 and 3 is 14.7%. One haplotype was selected from the population for inclusion in the 12S phylogenetic tree data set for each species. The *E. macrurus baicalocorrepus* forms 1 and 3 from Lake Baikal are sister taxons (Fig. 2). Specimens of *E. macruroides* from the eastern and western sides of the Baikal coast have the same haplotype, and form a cluster with *E. macruroides* from the vicinity of St. Petersburg and the VII and VIII genetic lineages of *E. cf. serrulatus* as defined by Hamrova et al. (2012). However, Baikalian *E. macruroides* are genetically isolated from *E. macruroides* from the vicinity of St. Petersburg. Specimens of *E. serrulatus* from water bodies on the shores of Baikal, Udmurtia and Kazakhstan belong to a single genetic lineage. This lineage was identified as the I lineage of *E. cf. serrulatus* by Hamrova et al. (2012), and is genetically close to *E. cf. serrulatus* from Great Britain. A single pond in St. Petersburg (the type locality for the species) contains individuals of *E. serrulatus* that are presented by not two, as previously thought (Sukhikh and Alekseev 2015), but three genetically distant lineages. These belong to lineages I, III and possibly a new, closest VI lineage of *E. cf. serrulatus* according to Hamrova et al. (2012) (specimen 468). The specimen 468 was identified as *E. cf. serrultus* based on morphology and together with the specimen from United Kindom forms a separate cluster VI.

Representatives of *E. arcanus* from distant populations in the Baikal region and the Far East are genetically close. Three individuals of *E. arcanus* have two haplotypes, one of which is represented in both the Irkutsk region and on Sakhalin Island.

The COI sequences were obtained from nine specimens of *Eucyclops* (219-645 bp), five of which were collected in the Baikal region. The species *E. serrulatus*, *E. speratus*, *E. arcanus*, and *E. macruroides* were identified by the integral method. The COI dataset, which included GenBank sequences, was saturated at the third codon position. The value of Iss = 0.6104 for symmetric trees is slightly less than Iss.c = 0.7407, while for asymmetric trees it is slightly greater than Iss.c = 0.4949. A minor effect is observed at the first and second codon positions (Iss = 0.4430 is significantly smaller than Iss.c = 0.7003). The intraspecies genetic model-corrected distances (K2P+G), calculated on the basis of the1st and 2nd codon positions ranged from 0 to 3.3%. The most genetically diverse species were *E. serrulatus* (3.6%), *E. arcanus* (2.7%), and *E. speratus* (2%). The interspecies, genetic model-corrected distances considering the 1st and 2nd codon positions ranged from 3.1 to 10.1%. The most closely related species are *E. taiwanensis* and *E. speratus* (3.1%). The p-distance values were close to the model-corrected distances, ranging from 0-3.2% within species and 2.9-8.7% between species. The phylogenetic tree was constructed using only the 1st and 2nd codon positions (Fig. 3). The nucleotide sequences of *E. speratus* and *E. taiwanensis* from Taiwan form a distinct cluster. Specimens of *E. speratus* from the Baikal and Far Eastern populations are genetically close and separated from a pair of other genetically similar specimens from the Volgograd reservoir and the Orlovsky pond in St. Petersburg.

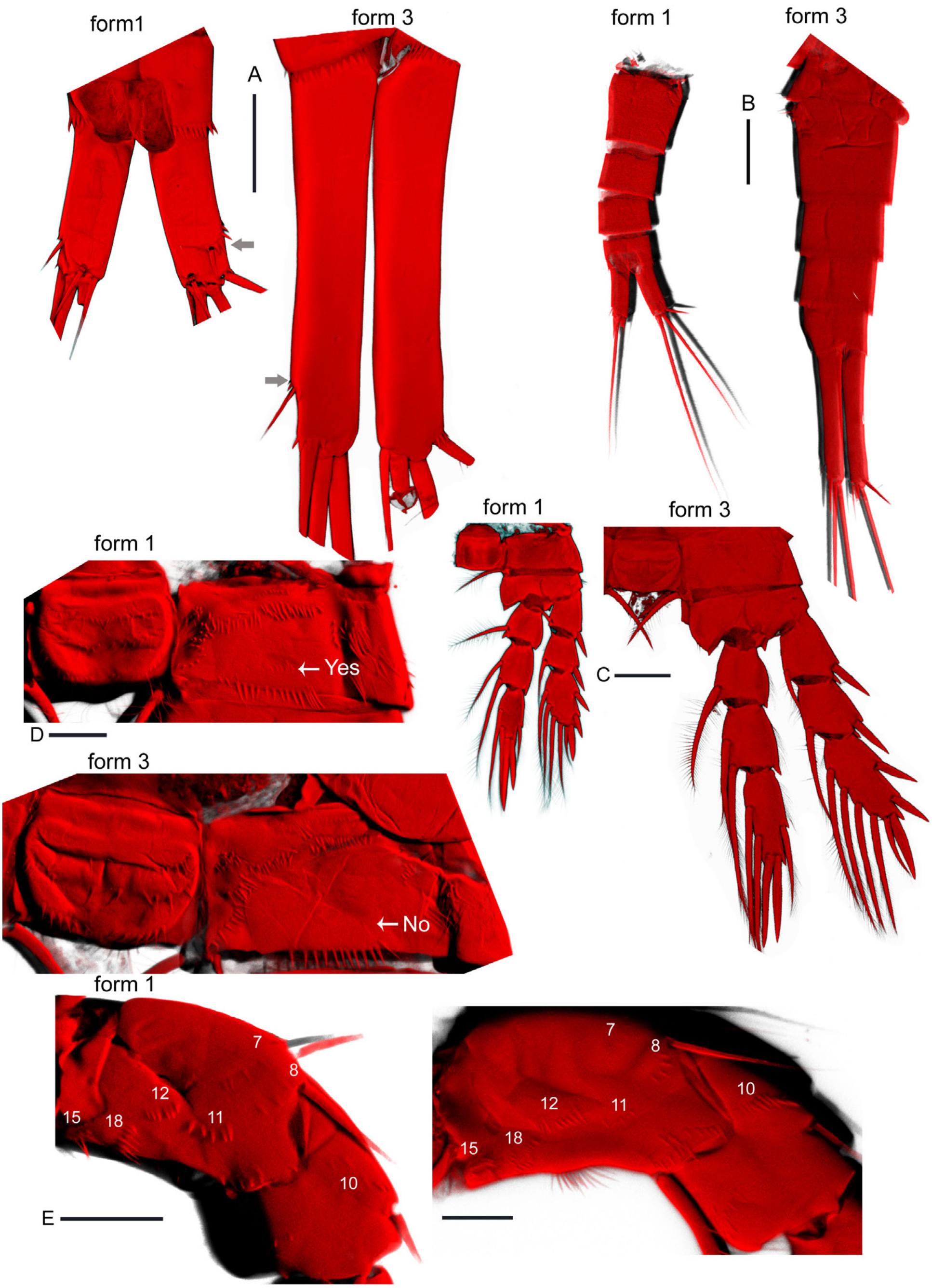

Figure 1. CLSM microphotographs of *E. m. baicalocorrepus* forms 1, 3. A-caudal rami; arrows indicate lateral row of spinules. B-abdomen. C-4th pair of swimming legs (P4), caudal. D-intercoxal sclerite and coxa of P4, caudal. E-antenna coxobase, caudal. Scale: A, C-50 μm; B-100 μm; D, E-20 μm.

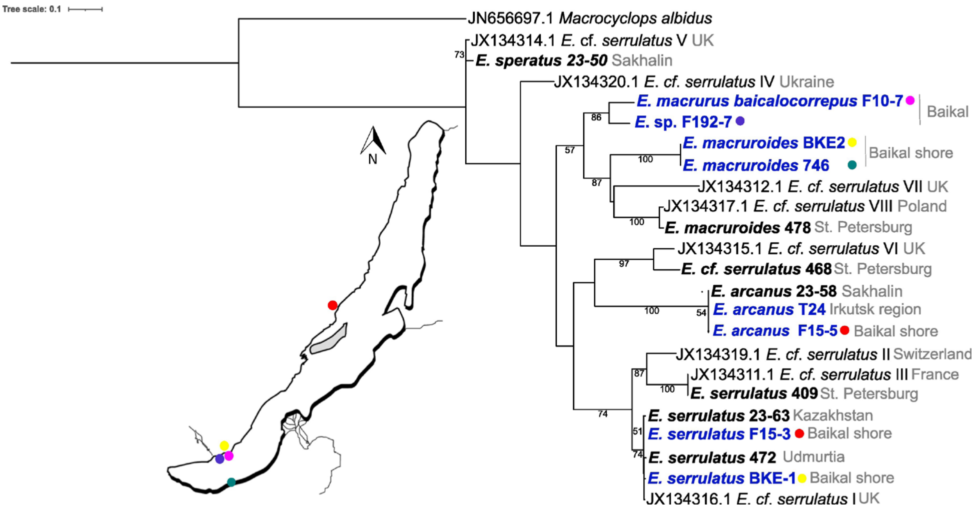

Figure 2. Phylogenetic tree based on 12S rRNA (TPM2u+F+I+G4) and sampling map. The number in the node is the bootstrap support value. Sequences obtained in this study are marked in bold. Sequences of specimens from the Baikal region are marked in blue. Sequences from GenBank are preceded by their accession numbers. Roman numerals indicate genetic lineages.

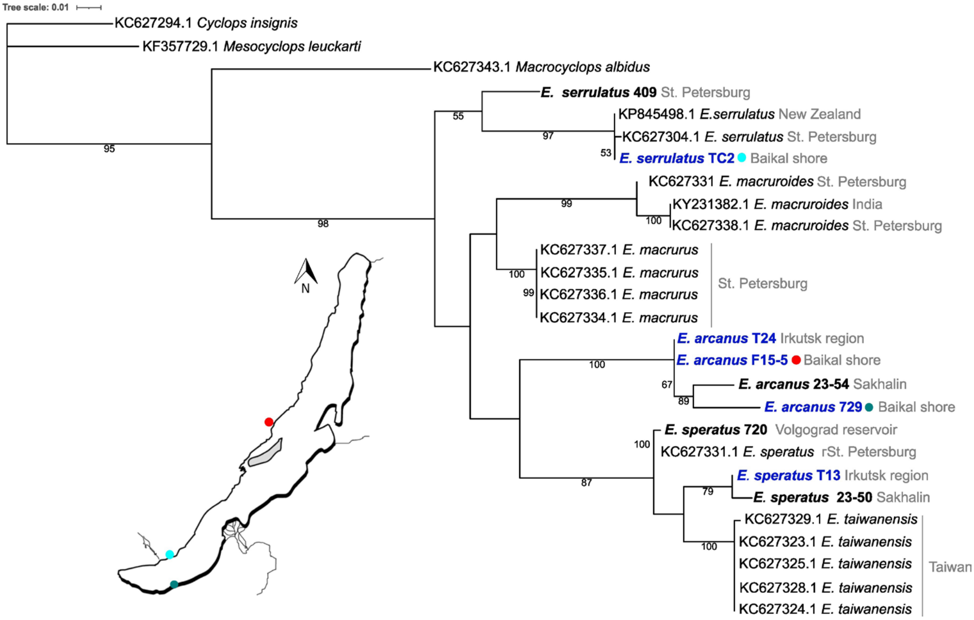

Figure 3. Phylogenetic tree based on the 1st, 2nd codon positions of COI (TN+F+I+G4) and sampling map. The number in the node is the bootstrap support value. Obtained sequences are marked in bold. Sequences of individuals from the Baikal region are marked in blue. Sequences from GenBank are preceded by their access numbers.

The CO1 *E. arcanus* sequences from the Baikal region and Sakhalin Island form a separate cluster, comprising three genetic lineages. The first lineage is represented by a specimen from Sakhalin Island, the second by a specimen from the eastern coast of Lake Baikal, and the third by specimens from the western side of the Baikal coast and the Irkutsk region. The Baikal region representative of *E. serrulatus* is genetically close to a specimen from New Zealand and a representative of clade II (eastern lineage) of *E. serrulatus* from St. Petersburg (Sukhikh and Alekseev 2015; Kochanova et al. 2021). The sequences of *E. serrulatus* collected in the same pond in the vicinity of St. Petersburg exhibit a significant degree of genetic divergence. Specimen 409, as observed in the 12S tree, represent a descrete lineage from the eastern clade. The sequences of *E. macruroides* form a separate cluster with two genetic lineages found in the vicinity of St. Petersburg. One of the lineages includs also *Eucyclops* from India.

The ITSn sequences, comprising the first and second transcribed spacers of rDNA and the 5.8S rRNA gene, were determined for 30 individuals belonging to five species: *E. speratus*, *E. macrurus baicalocorrepus* (forms 1 and 3), *E. arcanus*, *E. serrulatus*, and *E. macruroides* (206-681 bp). The intraspecific model-corrected (JC+G) genetic distances ranged from 0 to 10.3%. The maximum values were observed in the distances among *E. macruroides* (10.3%), *E. serrulatus* (2.5%), and *E. speratus* (2.1%). The interspecific model-corrected genetic distances ranged from 3.9 to 25.1%. *E. macrurus baicalocorrepus* form 1 and *E. macrurus baicalocorrepus* form 3 from Baikal genetically are the closest (3.9%), while and the greatest divergence was observed between *E. arcanus* and other species (22.1–25.1%). The intra- and interspecific p-distances were similar to the model-corrected distances and were 0-8.6% and 3.7-18.6%, respectively. The mitochondrial genes and nuclear ITSn tree topologies are consistent in general (Fig. 4).

The ITSn sequences of *E. speratus* form a separate cluster, in which sequences of the Far Eastern and Baikal populations are genetically isolated from those of the St. Petersburg population. The two forms of *E. macrurus baicalocorrepus* are closely related and sister to *E. macruroides*, which is genetically subdivided into two lineages from geographically distant populations: the Baikal region and St. Petersburg. The individuals of *E. arcanus* from the Baikal region and the Far Eastern populations are genetically close according to this fragment and form a distinct cluster. The *E. serrulatus* specimens from the four water bodies in the Baikal region belong to the same genetic lineage and are closest to *E. serrulatus* from St. Petersburg and Udmurtia. The representatives of *E. serrulatus* from St. Petersburg form three lineages, with one being the most distant and represented by a specimen of *E. cf. serrulatus* (468).

An 18S rRNA gene fragment (326 to 565 bp) was obtained from five specimens sourced from the Baikal region. The interspecific genetic model-corrected distances ranged from 0 to 1.7%, while p-distances ranged from 0 to 1.9%. The maximum values were observed between *E. agilis* and other species. The phylogenetic tree, based on 18S rRNA, with a high degree of support reveals two clusters. *E. agilis* from the USA, and *E. ensifer* from Brazil are sister species. *E. macruroides* from a water body on the shore of Lake Baikal, the VII and VIII genetic lineages of *E. cf. serrulatus*, as delineated by Hamrova et al. (2012) from Europe, form another cluster (Fig. 5). *E. macruroides* from the Baikal region and St. Petersburg reservoir (GenBank data) form distant genetic lines and the reason for this is unknown.

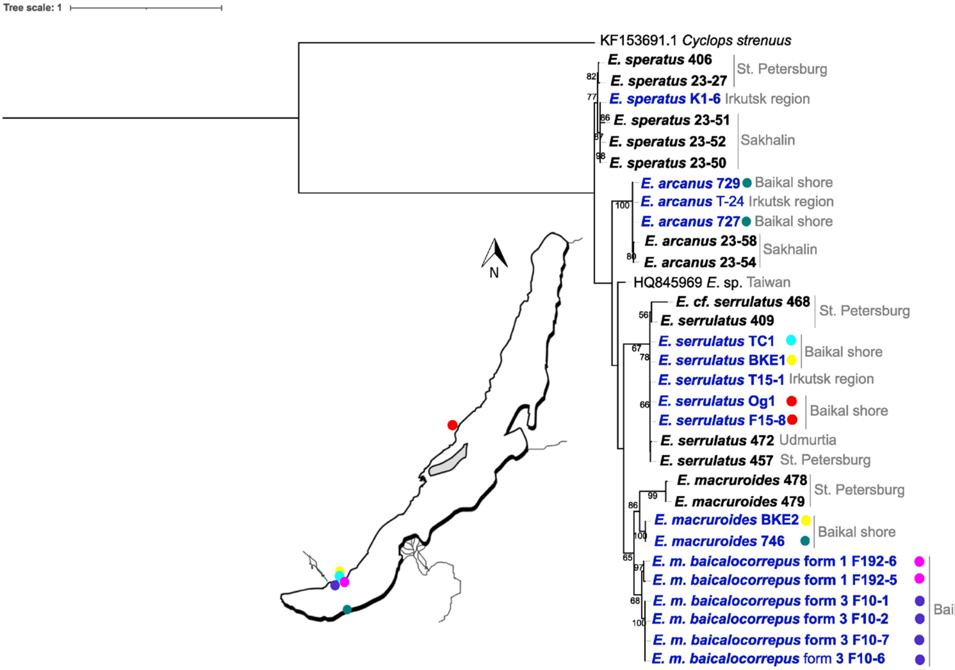

Figure 4. Phylogenetic tree based on ITSn rDNA (TNe+G4) and sampling map. The number in the node is the bootstrap support value. Obtained sequences are marked in bold. Sequences of specimens from the Baikal region are marked in blue. Sequences from GenBank are preceded by their access numbers.

Discussion

In the present study, four molecular markers with different evolutionary rates and inheritance types were sequenced: COI and 12S rRNA mtDNA, 18S rRNA and ITSn rDNA. The study permitted a considerable expansion of the genetic database of *Eucyclops* species, as well as estimation of its diversity and phylogeny within the Baikal region. Of the four molecular markers, the ITSn rDNA fragment, comprising two internal transcribed spacers and the 5.8S rRNA gene, was amplified with the highest efficiency using universal primers. For the COI data set, a saturation effect of nucleotide substitutions at the third codon position was detected, which appears to be characteristic phenomen among Copepoda. This phenomen has been shown in the genus *Diacyclops*, which is species-rich among freshwater Cyclopoida and the widespread species *Eurytemora affinis* (Lee 1999; Mayor et al. 2010; Novikov et al.

2024). For another mtDNA fragment, 12S rRNA, our analysis revealed extremely high interspecific genetic distances. In the case of *E. arcanus* and the other *Eucyclops* species, p-distances were approximately 30-35%. The high genetic diversity among *Eucyclops* may be related to both an ancient evolutionary age and an accelerated rate of mtDNA evolution. In light of the considerable taxonomic diversity observed in copepods, Boxshall and Jaume (2000), suggested that they represent the earliest colonisers of freshwater habitats, with an evolutionary history extending back to the Palaeozoic era. This hypothesis is confirmed by the fossil record of crustaceans. Harpacticoids of the extant family Canthocamptidae, were found in a bitumen clast of a late Carboniferous age (ca. 303 Ma) from a glacial diamictite deposit in eastern Oman (Selden et al. 2010). Conversely, Copepoda fossils are rare and therefore there are no estimates of specific evolutionary rates. The evolutionary dating of Copepoda uses estimates of the overall mtDNA evolutionary rate for Crustacea, which ranges from 0.9 to 2.6% per MY (Miracle et al. 2013; Marrone et al. 2013; Yong et al. 2014; Cornils and Held 2014). The latest estimates of evolutionary rates for aquatic invertebrates, including arthropods, indicate that the level of nucleotide substitutions of mtDNA can be significantly higher than the commonly used evolutionary rates for Copepoda (Calvo et al. 2015; Loeza-Quintana et al. 2019). Kochanova et al. (2024) observed an unexpectedly high level of mtDNA genetic diversity in *Harpacticella inopinata* Sars, 1908 from Lake Baikal, which they attributed to a possible accelerated evolutionary rate. A similar hypothesis was proposed by Sukhikh et al. (2023) in their study of the *E. affinis* group.

The integrative method employed in our study enabled the identification of five *Eucyclops* species from the Baikal region: *E. speratus*, *E. serrulatus*, *E. macruroides*, *E. arcanus*, and *E. macrurus baicalocorrepus*. *E. speratus* was detected for the first time in the area. All other species had previously been recorded in the area by microscopy methods (Mazepova 1978; Alekseev 1990; Sheveleva et al. 2020; Alekseev 2022). Interestingly that only *E. macrurus baicalocorrepus*, which presents two forms by Mazepova (1955), was found in Lake Baikal. The remaining species were found in the various water bodies of the Baikal region. The available data indicate that, the fauna of the Baikal region is similar to that of Siberia, reflecting their common evolutionary history. However, the fauna of Lake Baikal is the most specific, it is although influenced by the Siberian fauna. The lake is notable for its high endemic species richness. We did not find the same species in Lake Baikal and the Baikal region. The sole exception is the species *E. macruroides*, which was found by us on the Baikal coast and is quite different from its European relatives. *E. macruroides* with the subspecies *E. macruroides baicalensis* was also described from Lake Baikal. The subspecies differs from *E. macruroides* in morphology, including in the shape and armament of the caudal rami, the structure of P5, and so forth. Moreover, the drawings provided for this taxon (Mazepova 1978) are more correspond to *E. macrurus* and the long-furcal form of *E. m. baicalocorrepus* that was previously described by the same author. The specimens of *E. m. baicalocorrepus* with long caudal rami, designated as form 3, differ from *E. m. baicalensis* by the cylindrical shape of the caudal rami without thickening downwards and converge with it towards the side of the outer terminal seta of the caudal rami. The structure of P5 makes it challenging to distinguish between these two taxa, as both have similar ratios of the inner spine to the length of the segment: 1.1-1.6 for *E. m. baicalensis* and 1.0-1.8 for *E. m. baicalocorrepus*.

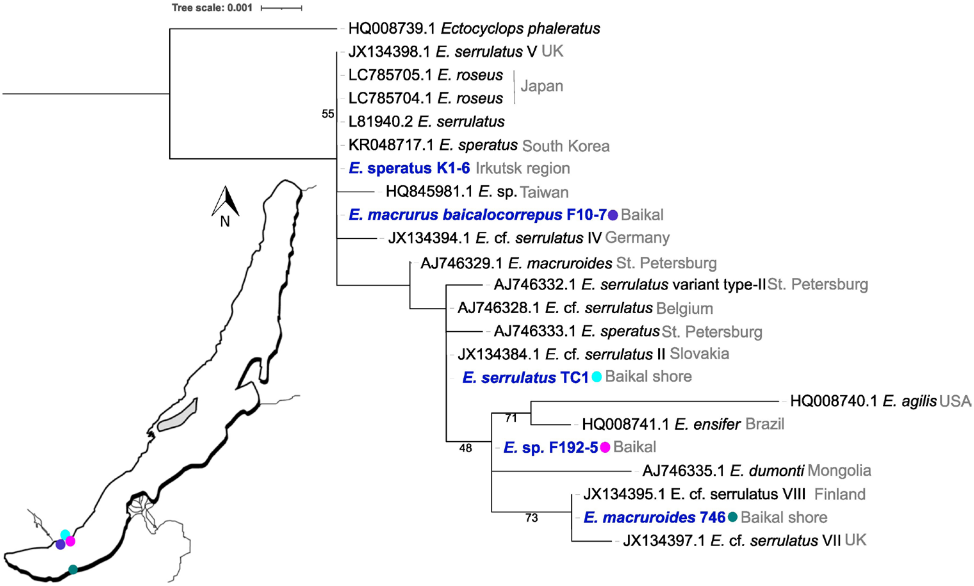

Figure 5. Phylogenetic tree based on 18S rRNA (JC+I) and sampling map. The number in the node is the bootstrap support value. Obtained sequences are marked in bold. Sequences of individuals from the Baikal region are marked in blue. Sequences from GenBank are preceded by their access numbers.

The taxonomic status of *E. macrurus baicalocorrepus*, initially described as *E. serrulatus baicalocorrepus* (Mazepova, 1955), requires further verification using genetic methods and the inclusion of *E. macrurus* from the type locality in the analysis. The results of morphological analysis (Monchenko, 1974) and our genetic study of the two forms demonstrate that *E. macrurus baicalocorrepus* is genetically distinct from any genetic lineage of *E. serrulatus*, not confirming its original description as *E. serrulatus baicalocorrepus*. It is evident that the considerable range of morphological variability (length of caudal rami from 3.4 to 8.2 and serra from a few spinules to the entire length of caudal rami, etc.) described for this subspecies cannot be attributed to a single taxon. It seems probably that we are dealing with several species or at least subspecies at once, which require accurate taxonomic revision and redescription. Similar species complexes were revealed for three endemic Baikal species of *Diacyclops* (Mayor et al. 2024). In this study, at least two of the three forms of *E. m. baicalocorrepus* identified by the caudal rami index were genotyped. The two forms of *E. m. baicalocorrepus* form a separate clade based on mitochondrial and nuclear DNA fragments. At the same time, the p-distances between two forms are 3.7% for ITSn and 14.7% for 12S rRNA, which corresponds to the species level among Copepoda (Zagoskin et al. 2014; Krajicek et al. 2016; Sukhikh et al. 2023). The forms of *E. m. baicalocorrepus* differ both in quantitative (index of caudal rami, indices of the third endopodite of the fourth pair of swimming legs) and qualitative characters (ornamentation of the P4 coxa and antenna coxobase). The level of genetic and morphological differences observed between the forms corresponds to the species level. Baikal is the deepest and most ancient lake on Earth, and is one of the most significant centers for hydrobiont speciation. The fauna of Baikal Cyclopoida includes 46 species and subspecies, of which 64% are endemic (Sheveleva et al. 2012). It is probable that both forms of *E. macrurus baicalocorrepus* diverged in the recent past in Baikal and are endemic. Genetic and morphological comparative analysis of the type material as well as other population of the species will allow to solve this question.

The tree topologies obtained by mitochondrial and nuclear markers are generally in agreement. Forms 1 and 3 of *E. macrurus baicalocorrepus* formed a monophyletic group with *E. macruroides*, indicating their common ancestral form. Nevertheless, the bootstrap support for this node is relatively low (57 and 65%), and it is conceivable that constructing a single tree based on several genetic fragments could enhance the reliability of this node. However, in the present study, we did not employ this method, as the data on four genetic fragments were obtained for different individuals due to varying PCR efficiency. The close relationship between *E. dumonti* and *E. macruroides* was previously demonstrated on the basis of the initial molecular genetic studies of *Eucyclops* using 18S rRNA (Alekseev et al. 2006). The latest revision of the genus places *E. arcanus* and *E. dumonti* in the subgenus *Speratocyclops*, *E. macruroides* in the subgenus *Denticyclops*, and *E. macrurus* in the subgenus *Macrurocyclops* (Alekseev 2019). However, the molecular phylogeny data do not fully agree with this systematics for this group of species.

In our analysis, *E. macruroides* is represented by a number of sister genetic lines. The isolation of the lines from the Baikal region and St. Petersburg was confirmed by ITSn and 12S rRNA analysis. Furthermore, two lines from the vicinity of St. Petersburg were isolated according to COI. The intraspecific genetic heterogeneity is shown for *E. speratus* by 12S rRNA and ITSn fragments. Similarly, to *E. macruroides* genetic lines from the Baikal region/Far East and St. Petersburg are distinguished from one another. Probably these lineages may represent new species, the status of which requires further study. All *E. serrulatus* specimens from the Baikal region belong to a single genetic lineage. This lineage has been identified as one of two in the study conducted by Sukhikh and Alekseev (2015) and as one of eight lineages within the species according to Hamrova et al. (2012). Specimens of this lineage are distributed across a vast area, including Siberia, Central Asia, and even New Zealand, where the species is apparently introduced. In the vicinity of St. Petersburg, three genetically distinct lineages were found in one pond, wich is the type locality of *E. serrulatus*. These lineages are consistent in 12S rRNA with clades 1–3 published by Hamrova et al. (2012). Three more clades out of eight under the name *E. cf. serrulatus* in the cited work reliably belong to other species (12S rRNA tree).

Conclusion

Five species of *Eucyclops* were identified through a combination of morphological and genetic methods in the Baikal region: *E. serrulatus*, *E. speratus*, *E. macruroides*, *E. arcanus*, and *E. macrurus baicalocorrepus*. *E. speratus* is included in the list of Copepoda fauna of the region for the first time. The necessity for a taxonomic revision of the species *E. macrurus baicalocorrepus* and *E. macruroides baicalensis*, which are endemic to Baikal, has been established. The results of the analysis of the description, in conjunction with the findings of the morphological and genetic studies, indicate that at least two species are under the common subspecies name *E. macrurus baicalocorrepus*. Additionally *E. macruroides baicalensis* is actually *E. macrurus* and most likely corresponds to one of the form of *E. m. baicalocorrepus*. Each analysed species was genotyped by four mitochondrial and nuclear genes: 12S rRNA, COI, ITSn, and 18S rRNA. The genetic analysis of *E. serrulatus*, *E. speratus*, and *E. macruroides* revealed that the near Baikal Cyclopoida, along with the Far Eastern and other Siberian representatives of the genus, form a distinct genetic lineage that differs from the majority of European representatives, with some exceptions.

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