

Origin and taxonomic position of Far Eastern island populations *Eurytemora caspica tethysiana* subsp. nov

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Abstract

The article describes a new subspecies *Eurytemora caspica thetysiana* subsp. nova.

that, according to its genetic and morphological features, is close to the recently described species *Eurytemora caspica caspica* from the Caspian Sea. According to our hypothesis, both of these taxon are the relics of the fauna of the Tethys Sea in the last phase of existence (Paratethys).

The described form occupies an intermediate position between *Eurytemora caspica caspica* and *Eurytemora carolleae* by its morphological characteristics. The time of divergence of the described subspecies and the original forms according to molecular clocks is in good agreement with the geological events associated with the evolution of the Tethys Sea (8–20 MYA), which indicates a very slow rate of evolution among *Eurytemora*. The morphological differences of our subspecies from the nominative Caspian subspecies are: the shapes of the genital double-somite and P5 distal segment in females. In males these differences are in the shapes of left rudimentary P5 exopod and distal segment of the right rudimentary P5, in ratio L abdomen/ L caudal rami.

At the same time, both forms belong to the *affinis* group of species, which includes also *Eurytemora carolleae* and *Eurytemora affinis*. The new taxon is described and the problem of the *Eurytemora* species' evolution in the *affinis* group is discussed in the article.

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Introduction

Eurytemora affinis (Poppe, 1880) was described at the end of the last century from the Elbe and subsequently its distribution was considered to be circumpolar. Later, in 1999 it was suspected as being a complex of cryptic species (Lee 1999; Lee and Frost 2002), inhabiting fresh- and brackish water of the Holarctic basin, but this species does not tolerate normal oceanic salinity (Rylov 1922).

This species complex is assumed to be native to the Ponto–Caspian region (Kipp et al. 2023) and reported from the North American Atlantic coast, including the Gulf of Mexico, the North American Pacific coast, the western European coast, and parts of Asia (Mills et al. 1993; Torke 2001; Dussart and Defaye 2002; Lee and Frost 2002). In Europe and North American Atlantic areas, these copepods are mainly known from coastal brackish water environments, but also from large continental lakes like Lake Ladoga in Europe and the Great Lakes in North America (Rylov 1922; Engel 1962; Faber and Jermolajev 1966; Mills et al. 1993). However, the suggested epicentre of diversity for the genus *Eurytemora* lies along coastal Alaska, where several species are endemic (Heron and Damkaer 1976; Dodson et al. 2010).

Global phylogeography of the *Eurytemora affinis* group of the species was first studied by Lee (2000) and revealed a number of clade and subclade highly remote from each other using mitochondrial 16SrRNA and CO1 genes. Analysis of nuclear genes nITS showed high congruence with mitochondrial ones (Sukhikh et al. 2020). At the same time the study of the more conservative nuclear gene 18SrRNA was uninformative within the *affinis* group and all clades were practically identical according to this search (Sukhikh et al. 2020). The only exception was Asian clade, which has one nucleotide substitution in the 18SrRNA part of the gene compared to *E. affinis* and *E. carolleae*. A similar picture of 18SrRNA analysis was shown for many other Copepoda species and a conclusion was made about the unsuitability for closely related species phylogeography (Hamrová et al. 2012; Sukhikh and Alekseev 2015; Sukhikh et al. 2016; Kochanova et al. 2021). Today this group of species is represented by three species: North American *Eurytemora carolleae*, European *Eurytemora affinis* and Caspian *Eurytemora caspica caspica*, the first two of these are still represented by a few remote mitochondrial clades.

The paper deals with resolving the question about the taxonomical position of Russian Far East and Japan populations. Until now, Asian *Eurytemora* were attributed as *Eurytemora affinis*. Like many other populations of *Eurytemora* aff. *affinis* crustaceans from Japan were widely studied mainly from an ecological perspective. The Sakhalin population is even less studied; there are just a few works on species distribution and some population dynamics (Ueno 1935, 1935a, Borutzky and Bogoslovskii 1964; Usova et al. 1980; Samatov et al. 2002; Kafanov et al. 2003, Zavarzin 2003, 2004, 2005, 2011, 2021; Zavarzin & Safronov 2001; Zavarzin et al. 2022; Labay et al. 2013, 2016). The question about the correct taxonomic positions of the Japan and Far East *Eurytemora* populations was set up in previous papers (Makino 2017; Sukhikh et al. 2020), but has not yet been solved.

Comparative morphological studies are almost absent in these regions. Genetic studies of mitochondrial 16SrRNA and CO1 genes revealed two lineages on Hokkaido Island.: from Lakes Ohnuma, Akanko and Baratoka, and the River Ishikari, according to Lee (2000). The populations from Lakes Ohnuma and Akanko were thought to have been introduced from the island of Honshu in Japan (Ban and Minoda 1989). Our CO1 and nITS genes analysis of the Sakhalin population showed its maximum proximity to the *Eurytemora* population from Lake Ohnuma (Sukhikh et al. 2020).

What is more interesting is that all these Asian populations are closely related not to *E. affinis* species, but to the far situated Caspian Sea *Eurytemora caspica caspica* (Lee 2000; Makino 2018; Sukhikh et al. 2020). Altogether, these populations demonstrate Asian clade differed from all other studied populations of the *E. affinis* species complex.

Sewell (1956) was the first who analysed the distribution of various fresh- and brackish-water Copepoda in the light of concepts on the respective dispositions of the continents and the seas. The confirmation of Wegener's theory by later studies in plate tectonics led to a new approach to the distribution of planktonic marine Copepoda, taking into account the evolution of the continents. We also consider such an amazing similarity of very territorially separated populations in terms of the evolution of the Tethys Sea (Alekseev and Sukhikh 2020), which for a long time connected the modern regions of the Ponto–Caspian region and water bodies of the Far East (Scotese 2014a, b). A similar picture of the evolution of the copepod fauna associated with the Tethys Sea was traced in the papers by Carola et al. 1995 on Genus *Exumella* Fosshagen, 1970, Brodsky, 1972 for *Calanus sinicus* Brodsky, 1962; Vaupel Klein, 1984 for *Euchirella messinensis* (Claus, 1863) and Arcos F. and Fleminger 1986 for filter-feeding calanoid copepods, but concerned the distribution of exclusively marine species along the outer boundary of the Paratethys. Most of the papers on species indicative of continental drift and Tethys Sea evolution are dedicated to the

opening up of the Atlantic (Carola et al. 1995; Kolesnikova et al. 2017) and the closing of the Panama Isthmus (Sars 1924; Rose 1929; Stephan et al. 1990; Dercourt et al. 1993) or apply to the Pacific part (Wilson 1950; Grice and Hulsemann 1968; Markhaseva 1996). Regarding the continental fauna, several works on copepods have been published (Alekseev and Sukhikh 2020; Alekseev and Chaban 2021; Alekseev 2022). Genetic study of Far Eastern and Caspian *Eurytemora* is one more fact in supporting the theory about species-indicators showing continental drift.

Thereby this paper is dedicated to describing both Russian Far East and Japan populations as a new subspecies of *E. caspica* – *E. caspica thetysiana* subsp. nova. In addition, some aspects of the *Eurytemora* species evolution in the *affinis* group are discussed in connection with the Tethys Sea.

Material And Methods

Material And Methods Used For Morphological Analysis

The type material for this new species was selected from a sample collected on Lake Tunaicha, Sakhalin Isl., Russia (46°45'N 143°12'E), collected by D. Zavarzin, 01 July 2002 (Table 1, Fig. 1, point 9).

Table 1
Material, used in the subspecies *Eurytemora caspica thetysiana* subsp. nov. description

Species	Population	number F/ M	salinity at the sampling point, psu	Holotype/Paratype numbers	Date of collection	collector	coordinates
<i>E. caspica</i>	Lake Tunaicha	19/19	2.2–2.5	ZIN RN 55208- ZIN RN 55211	01 July 2002; summer of 2011	Dr D.S. Zavarzin;	46°45'N 143°12'E
	Lake Panitu	6/3	freshwater		16 August 1999	Dr D.S. Zavarzin	52°42'N 143°15'E
	Chaivo Bay	1/0	5–8		11 August 2004	Dr. D.S. Zavarzin	52°36'N 143°16'E
	Pil'tun Bay	1/1	5–8		17 September 2002	Dr D.S. Zavarzin	53°01'N 143°08'E
	Lake Medvezh'ye	2/2	freshwater		10 October 2021	Dr D.S. Zavarzin	53°54'N 142°91'E
	Lake Ohnuma	12/16	freshwater	ZIN RN55212- ZIN RN55213	October 2014	Dr S.Ban	41°59' N 140°40' E
	Caspian Sea	18/25		ZIN RN 55060- ZIN RN 55063	25 June 2011	FSUE "CaspNIRKh"	45°23'N 48°27'E
<i>E. affinis</i>	Elbe River Estuary	24/20	freshwater	ZIN RN 55080- ZIN RN 55082	2006 twice per season by	Dr G. Winkler	53°55'N 8°50'E
	Gironde Estuary	0/4	brackishwater	ZIN RN 55085- ZIN RN 55086	March 2005	Dr O. Glippa	45°31'N 0°57'W
	Gulf of Finland	4/3	1–3	ZIN RN 55083- ZIN RN 55084	15 July 2009	Dr N.M. Sukhikh	59°40'N 28°18'E
<i>E. carolleae</i>	Chesapeake Bay	25/25	marine	ZIN RN 55052- ZIN RN 55054	16 April 2008	Dr D. Kimmel	37°37'N 76°03'W
	Gulf of Finland	2/0	1.5–3	ZIN RN 55190	15 July 2009	Dr N.M. Sukhikh	59°40'N 28°18'E

For comparison, we used male and female specimens from other Sakhalin populations collected in Lakes Panitu and Medvezh'ye, Chaivo Bay and Pil'tun Bay, from Japanese Lake Ohnuma – 45 specimens altogether from Asia and from the Caspian Sea. Male and female specimens of the related species were also analysed: 44 specimens *terra typica* for *Eurytemora affinis* (Poppe, 1880) from the Elbe River, Germany; 50 specimens *terra typica* for *Eurytemora carolleae* Alekseev and Souissi 2011 from the Chesapeake Bay, Atlantic coastline of USA. The material from the Elbe River, from the Chesapeake Bay and from L. Ohnuma was fixed in 70% ethanol, from the Caspian Sea, Sakhalin waterbodies were fixed in a 4% formalin solution. Samples were collected with 100 or 230 µm mesh plankton nets by vertical tows from depth to surface in three replicates.

Species identification was made following the taxonomical keys presented in Alekseev and Souissi (2011), in Sukhikh and Alekseev (2013) and Kos (2016). The type material of *E. carolleae* and *E. caspica caspica* stored in the type collection of the Zoological Institute of the Russian Academy of Sciences under reference numbers 55052–55054 and 55060–55062 was used for this study.

The samples were sorted under stereomicroscopes (Olympus SZX2 and SZX10). Before dissection, copepod adults were measured with a camera and software packages Olympus CellSens Standard and LevenhukLite. The dissection was processed in glycerol. After dissection, the specimens were placed on slides in pure glycerol, covered with a cover slip and ringed with Canadian balsam. The slides were then examined at maximum resolution up to 1000 x (Plan

objective 100 x, oil immersion) under compound microscopes (Olympus BX51, Levenhuk D870T and Zeiss IMAGER equipped with the Nomarski system for differential interference contrast microscopy).

Photographs were performed using an Olympus BX51 microscope equipped with an Olympus DP25 camera and Olympus CellSens Standard 1.5 software. Final photos were made by combining 10–60 images at different focus depth using “CombineZP” software. Drawings were made from photographs and with a drawing tube.

To define the subspecies, both sexes were analysed. In total more than 20 different character (Table 2, Fig. 2) measurements were made, and in addition secondary sexual dimorphic characters typically used in copepod taxonomy, as well as mouth appendages, microcharacters of the fifth legs, seta structure and body shape were observed. After index calculations and their analysis, the most indicative indexes were chosen (Tables 3–6).

Table 2
Abbreviations of features used in the paper

Traits	Abbreviation
Caudal rami length	FrL
Caudal rami width	FrW
Caudal inner lateral seta length	Lseta
Distal exopod segment P1/P4 length	Lseg
The longest spine of P1/P4 length	LongSp
Spine 1 of P4 length	P4Sp1
Spine 2 of P4 length	P4Sp2
Distal segment P5 length	P5Lseg
Distal segment P5 width	P5Wseg
The longest spine of P5 length	P5LongSp
Small spine of P5 length	P5TSp
Spine 1 of P5 length	P5Sp1
Spine 2 of P5 length	P5Sp2
Spine 3 of P5 length	P5Sp3
Appendix 1 of P5 length	P5Ap1
Appendix 2 of P5 length	P5Ap2
P5 left basipodit length	P5L
P5 left basipodit width	P5W
P5 right basipodit width1	P5w1
P5 right basipodit width2	P5w2
Abdomen length without caudal rami and genital segment	Labd
A1 right spine of the 9-th segment	Sp9
A1 right spine of the 11-th segment	Sp11
A1 right spine of the 12-th segment	Sp12

Table 3

Morphometric indexes in males *Eurytemora caspica tethysiana* subsp. nov. *E. caspica caspica*, *Eurytemora carolleae* and *Eurytemora affinis* from the type localities. Mean \pm standard deviation (Min-Max)

Species/ Indexes	Caudal rami. L/W	P5 Basipodit left. L/W	P5 Basipodit right w1/w2	P4. LongSp/Lseg	P1. LongSp/Lseg	Labd/FrL	A1			Body size μm	
							Sp12/Sp9	Sp12/Sp11	Sp11/Sp9		
<i>E. affinis</i>	7.77 \pm 0.2	0.96 \pm 0.05	5.01 \pm 0.14	1.14 \pm 0.052	1.21 \pm 0.081	1.45 \pm 0.05	2.16 \pm 0.12	2.42 \pm 0.10	0.81 \pm 0.05	1905.19 + 14.59	
	(6.00- 10.45)	(0.91- 1.08)	(4.00- 6.3)	(1.03-1.25)	(1.06-1.38)	(1.02- 1.79)	(1.53- 2.73)	(1.60- 2.79)	(0.61- 1.11)	(1853.7- 1953.9)	
<i>E. carolleae</i>	9.76 \pm 0.2	1.43 \pm 0.03	4.23 \pm 0.23	1.06 \pm 0.060	1.1 \pm 0.117	1.08 \pm 0.04	2.77 \pm 0.18	3.09 \pm 0.20	0.89 \pm 0.07	1336.52 + 19.26	
	(8.06- 11.07)	(1.27- 1.6)	(2.78- 5.75)	(0.95-1.14)	(1-1.39)	(0.87- 1.36)	(1.67- 3.82)	(2.19- 4.08)	(0.46- 1.10)	(1285.9- 1386.1)	
<i>E. caspica caspica</i>	10.56 \pm 0.5	1.18 \pm 0.04	2.92 \pm 0.1	1.06 \pm 0.019	1.17 \pm 0.041	0.91 \pm 0.03	2.59 \pm 0.14	3.12 \pm 0.11	0.83 \pm 0.03	905.31 + 25.12	
	(8.58- 15.56)	(1.05- 1.4)	(2.14- 3.5)	(0.95-1.13)	(0.98-1.6)	(0.74- 1.00)	(2.00- 3.17)	(2.75- 3.71)	(0.67- 0.98)	(878.4-955.5)	
<i>E. c. tethysiana</i> subsp. nov.	8.32 \pm 0.30	1.54 \pm 0.05	4.04 \pm 0.83	1.13 \pm 0.03	1.09 \pm 0.04	1.13 \pm 0.02	3.27 \pm 0.11	3.02 \pm 0.09	1.09 \pm 0.05	1185.68 \pm 13.43	
	(6.83- 11.80)	(1.28- 1.88)	(2.57- 5.50)	(0.92-1.29)	(0.81-1.27)	(0.97- 1.31)	(2.69- 3.73)	(2.57- 3.41)	(0.85- 1.27)	(1080-1278)	

The following features were selected in both sexes: caudal rami length and width; the distal exopod segment; as well as distal spine lengths in the swimming legs 4.

For males only we measured the distal exopod segment as well as distal spine lengths in the swimming legs 1. For males, we additionally measured length and maximal width of the first segment of the right exopod of the leg 5, along with the maximal and minimal widths of the left exopod distal segment of the leg 5. We also measured the length of the first four urosome segments and the lengths of 9, 11, 12-th segment spines of right antennules (Tables 3-4, Fig. 2).

Table 4

Morphometric indexes in males *Eurytemora caspica tethysiana* subsp. nov. from terra typica. Mean \pm standard deviation (Min-Max)

Species/ Indexes	Caudal rami. L/W	P5 Basipodit left. L/W	P5 Basipodit right w1/w2	P4. LongSp/Lseg	P1. LongSp/Lseg	A1			Body size μm
						Sp12/Sp9	Sp12/Sp11	Sp11/Sp9	
L. Tunaicha	7.71 \pm 0.2	1.51 \pm 0.06	3.92 \pm 0.36	1.13 \pm 0.04	1.09 \pm 0.05	3.07 \pm 0.17	2.98 \pm 0.13	1.04 \pm 0.08	1167.92 + 11.72
	(7.0-8.5)	(1.28-1.65)	(2.57-5.0)	(0.92-1.29)	(0.81-1.27)	(2.69- 3.71)	(2.57-3.41)	(0.85- 1.26)	(1080.0- 1125.0)
L. Ohnuma	8.26 \pm 0.52	1.67 \pm 0.07	3.48 \pm 0.23	1.11 \pm 0.02	1.10 \pm 0.04	3.42 \pm 0.08	2.99 \pm 0.16	1.15 \pm 0.06	1110.89 + 17.31
	(6.8- 9.2)	(1.6-1.74)	(3.25-3.71)	(1.08-1.15)	(1.03-1.17)	(3.33- 3.67)	(2.63-3.33)	(1.00-1.27)	(1140.0- 1278.0)
L. Panitu	8.00 \pm 0.05	1.64 \pm 0.24	4.23 \pm 0.15			3.73	3.37	1.11	1256.71 + 0.71
	(7.85- 8.09)	(1.4-1.88)	(4.09-4.38)						(1256.0- 1258.13)
Piltun Bay		1.38	5.52						

In females, we measured additionally leg 5 exopod spine lengths, length and width of the distal segment and two lengths of appendices, as shown in the picture (Tables 5-6, Fig. 2). We also determined the length and width in the anterior (W1) and posterior (W2) sides of the somite for the genital double-somite (Fig. 2)

Table 5

Morphometric indexes in females *Eurytemora caspica tethysiana* subsp. nov. *E. caspica caspica*, *Eurytemora carolleeae* and *Eurytemora affinis* from the type

Species/ Indexes	Caudal rami. L/W	Genital somite. W1/W2	Leg 5								Leg 4
			sp2/sp3	LongSp/sp1	tiny spine/spine1	Ap1/Ap2	Ap1/Lseg	LongSp/Lseg	Sp1/Lseg	Lseg/Wseg	Long spine/ Segm
<i>E. affinis</i>	6.08 ± 0.638	1.24 ± 0.075	1 ± 0.02	2.63 ± 0.06	0.25 ± 0.053	0.71 ± 0.04	2.08 ± 0.13	2.44 ± 0.24	1.01 ± 0.04	1.51 ± 0.02	0.94 ±
	(5.2– 7.8)	(1– 1.39)	(0.75– 1.33)	(1.63–4.13)	(0.18–0.36)	(0.63– 0.76)	(1.86– 2.26)	(2.00–2.83)	(0.84– 1.28)	(1.30– 1.68)	(0.8–1
<i>E. carolleeae</i>	6.06 ± 0.531	1.54 ± 0.103	1.19 ± 0.02	2.51 ± 0.04	0.11 ± 0.029	0.79 ± 0.04	2.13 ± 0.13	3.07 ± 0.25	1.29 ± 0.17	1.37 ± 0.03	0.85 ±
	(5–7.4)	(1.35– 1.67)	(0.85– 1.54)	(1.42–3.21)	(0.06–0.20)	(0.86– 0.70)	(2.32– 1.9)	(2.57–3.39)	(1.02– 1.56)	(0.92– 1.56)	(0.70–
<i>E. caspica caspica</i>	7.65 ± 0.13	1.25 ± 0.01	1.03 ± 0.03	3.4 ± 0.10	0.09 ± 0.01	0.62 ± 0.05	1.97 ± 0.28	2.36 ± 0.19	0.68 ± 0.09	1.32 ± 0.04	0.92 ±
	(5.61– 9.45)	(1.15– 1.40)	0.46– 1.5	(2.59–4.91)	(0.06–0.17)	(0.53– 0.72)	(1.4– 2.47)	(2.06–2.83)	(0.5–0.9)	(1.08– 1.51)	(0.79–
<i>E. c. tethysiana</i> subsp. nov.	6.01 ± 0.13	1.51 ± 1.19	0.98 ± 0.10	3.15 ± 0.48	0.09 ± 0.04	0.75 ± 0.09	1.75 ± 0.14	2.53 ± 0.23	0.84 ± 0.1	1.64 ± 0.02	1.00 ±
	(4.82– 8.17)	(1.3– 2.04)	(0.77– 1.15)	(2.15–4.14)	(0.05–0.17)	(0.52– 0.94)	(1.55–2)	(2.13–2.91)	(0.65– 1.00)	(1.48– 1.88)	(0.91–

Table 6

Morphometric indexes in females *Eurytemora caspica tethysiana* subsp. nov. from terra typica. Mean ± standard deviation

Species/ Indexes	Caudal rami. L/W	Genital somite. W1/W2	Leg 5								Leg 4
			sp2/sp3	LongSp/sp1	tiny spine/spine1	Ap1/Ap2	Ap1/Lseg	LongSp/Lseg	Sp1/Lseg	Lseg/Wseg	Long spine/L Segme
L. Ohnuma	6.82 ± 0.23	1.35 ± 0.03	1.01 ± 0.05	3.43 ± 0.12	0.09 ± 0.01	0.68 ± 0.04	1.82 ± 0.08	2.39 ± 0.06	0.71 ± 0.02	1.59 ± 0.02	1.07 ± 0
	6.13– 8.2	1.32– 1.42	0.91– 1.13	2.82–3.78	0.06–0.17	0.52– 0.94	1.55– 2.39	2.13–2.64	0.56– 0.79	1.52–1.67	1–1.21
L. Tunaicha	6.30 ± 0.40	1.57 ± 0.07	0.93 ± 0.04	3.40 ± 0.13	0.11 ± 0.00	0.78 ± 0.02	1.75 ± 0.04	2.86 ± 0.11	0.84 ± 0.02	1.61 ± 0.02	1.05 ± 0
	4.83– 6.56	1.3– 2.04	0.77– 1.13	3.05–4.14	0.08–0.16	0.64– 0.86	1.62– 1.96	2.46–3.41	0.74– 0.92	1.47–1.81	0.91–1
L.Panitu	5.83 ± 0.12	1.51 ± 0.06	0.96 ± 0.03	2.77 ± 0.09	0.07 ± 0.01	1.09 ± 0.16	1.85 ± 0.16	2.54 ± 0.08	0.92 ± 0.04	1.74 ± 0.03	
	5.25– 6.37	1.35– 1.67	0.84– 1.13	2.49–3.16	0.06–0.09	0.77– 1.73	1.56– 2.79	2.32–2.77	0.74– 1.04	1.57–1.88	
Piltun Bay	5.36 ± 0.02	1.57	1.02 ± 0.14	2.55 ± 0.03	0.13 ± 0.03	0.81 ± 0.01	1.83 ± 0.00	2.53 ± 0.00	0.99 ± 0.01	1.57 ± 0.02	
	5.34– 5.38		0.88– 1.15	2.53–2.58	0.10–0.15	0.79– 0.82		2.52–2.53	0.98–1.00	1.56–1.59	
Chaivo Bay	5.17 ± 0.35	1.58									
	4.82– 5.51										

The type slides for *E. caspica tethysiana* subsp. nov. were placed in the type collection of the Zoological Institute of the Russian Academy of Sciences, St Petersburg, Russia (ZIN RAS) under reference numbers 55208–55213.

Material And Methods Used For Genetic Analysis

All sequences used have already been published so we retrieved them from the GenBank database (Table 7). Here we analyse all these sequences from a new point of view, with emphasis on newly described subspecies and their relationships with Caspian *E. caspica caspica*.

Table 7
Sequences with accession numbers used in the study.

Species name	CO1 Accession numbers	nITS Accession numbers	18SrRNA Accession numbers	No. of sequences CO1; nITS; 18SrRNA	Citation
<i>E. affinis</i>	KX400987- KX400993 KX401042-KX401328 HM473958–HM474028	KX401010- KX401023	KX400978- KX400986	347;14;9	Sukhikh et al. 2019 Sukhikh et al. 2016b Winkler et al. 2011
<i>E. affinis</i> from Northern Dvina River RF	MN256867 MT653564-MT653565	MT667429- MT667431		3; 3; 0	Sukhikh et al. 2020
<i>E.carolleeae</i>	KX400994-KX401004 HM474003. HM474012. HM474029. HM368364	KX401024- KX401041	KX400968- KX400977; MT676375- MT676380	15;18;16	Sukhikh et al. 2020 Sukhikh et al. 2019 Sukhikh et al. 2016b
<i>E.carolleeae</i> from St.Lawrence Estuary	MT653566-MT653568	MN541395- MN541397	MN567275 MT676373- MT676374	4;3;3	Sukhikh et al. 2020
<i>Eurytemora cf. affinis</i> from St.Lawrence Estuary	MT681192- MT681194	MN541398; KX401005	MT676370- MT676372	4;2;3	Sukhikh et al. 2020
<i>E. caspica thetysiana</i> subsp.nov. from Sakhalin (RF) and Japan	MN542734- MN542743 AB 699198- 699207	MN541399- MN541404	MN556098- MN556100	21;6;5	Sukhikh et al. 2020 Makino et al. 2018
<i>E.caspica caspica</i>	MN271657- MN271660; KC627339; KC627342	MT667435- MT667438	MT676381- MT676382	7;4;2	Sukhikh et al. 2019
<i>E. lacustris</i>	MH316160- MH316161 HM474030- HM474035 MT653592-MT653595		HM474030- HM474035 MN567281. MN567282	12;0;8	Ślugocki et al. 2019 Sukhikh et al. 2016
<i>E.gracilicauda</i>	MN256854 MT653569-MT653582	MN256854	MN270893 MT676386- MT676387	15;1;3	Sukhikh et al. 2016 Sukhikh et al. 2020
<i>E.arctica</i>	MT653583-MT653588	MT667432- MT667434	MT676383- MT676385	6;3;3	Sukhikh et al. 2020
<i>E.velox</i>	MT146445-MT146446 MZ400499–MZ400504	MZ373318– MZ373322	MZ373302	6;3;1	Sukhikh Lazareva 2022
<i>Centropages typicus</i>	GU132316	GU125729		1;1;0	Castellani et al. 2012
<i>Acanthocyclops vernalis</i>	MZ964940			1;0;0	GenBank. unpublished
<i>Mesocyclops edax</i>	MZ964924			1;0;0	GenBank. unpublished
<i>Cyclestheria hislopi</i>	JX999894 JX999912			1;0;0	GenBank. unpublished
<i>Chydorus pubescens</i>	KU315495			1;0;0	GenBank. unpublished
<i>Chydorus sp.</i>	KC617531			1;0;0	GenBank. unpublished

Genetic analysis was performed using the sequences of the mitochondrial cytochrome oxidase 1 (CO1) gene part and the nuclear nITS gene part published in different works, which had not previously been analysed together and which had not previously been used to estimate the time of species formation (Table 7). All DNA sequences were obtained by the authors, excluding material from Akan Lake AB 699198–699207, which was obtained in the work by Makino et al. (2018); no other sequences of the studied species were found in the database as of March 02, 2023. A total of 65 nucleotide sequences of the 545 bp CO1 gene fragment and 46 - ITS1 420 bp (for phylogenetic reconstruction) or nITS 824 bp long (for other analysis) were included in the analysis.

To study possible evolutionary paths, published models of the continental plates movement were used, which describe the changes in the Tethys Sea at the last stages of its existence and the closure of continental connections between faunal elements of western and eastern Eurasia (Scotese 2014 a,b).

Analysis Of Sequences And Phylogeny Reconstruction

Sequences were aligned using the algorithm CLUSTAL W (Thompson et al. 1994) implemented in BIOEDIT v.7.2 (Hall 1999) with manual editing of ambiguous sites. The number of polymorphic sites was estimated using DNASP v6 (Rozas et al. 2017). The level of nucleotide differences between the species was calculated using the Tamura–Nei model in MEGA 11 software package (Tamura et al. 2021). The best-fitting models of nucleotide substitution for both mitochondrial and nuclear data sets were selected in jModelTest v. 2.1.7, based on the likelihood scores for 88 different models and under the Akaike Information Criterion (AICc) (Posada 2008).

Phylogeny reconstruction was carried out as for each COI and ITS1 locus separately, and we reconstructed a joint consensus tree based on the combined date using the maximum likelihood (ML) or Bayesian (BI) methods. Because the main clades for CO1, ITS1 and consensus CO1-ITS1 were congruent, we presented only the last tree. For ML analysis we used the General Time Reversible model with gamma rate distribution (G) and without invariable sites (I) in MEGA 11 software package (Tamura et al. 2021). A Bayesian phylogeny was reconstructed in BEAST2 v.2.6 [Bouckaert et al. 2019], with all of the parameters of the substitution model determined using BEAUti [Drummond et al. 2012]. In each analysis, we conducted four independent runs of Markov chain Monte Carlo (MCMC, 50M generations, with selection of each 10 k generation), with effectiveness control in Tracer v.1.7 [Rambaut et al. 2018]. A consensus tree based on the maximum clade credibility (MCC) was obtained in TreeAnnotator (part of BEAST2) with a burn-in of 10%. Because the main clades for BI and ML were congruent, we presented the BI tree, with BI and ML branch support for key nodes.

Haplotype Diversity And Network

Genetic diversity was compared among populations using DNASP v6 (Rozas et al. 2017) including haplotype diversity (H_d , probability that two randomly chosen haplotypes are different in the sample) and nucleotide diversity (p , average number of nucleotide differences per location between two sequences). A maximum parsimony median-joining haplotype network was constructed with the Network 10.2 program (www.fluxus-engineering.com; Bandelt et al. 1999; Polzin and Daneschmand 2003).

Species Delimitation Methods

We applied three independent methods of species delimitation based on the combined CO1-ITS1 data sets : ASAP; mPTP; STACEY. The “assemble species by automatic partitioning (ASAP; Puillandre et al. 2012) method was implemented using the online ASAP server (<https://bioinfo.mnhn.fr/abi/public/asap>) with the following settings: fixed seed value=-1 and simple distance. The “multiple rate Poisson Tree Processes” (mPTP) model was run through the online mPTP server (<https://mptp.h-its.org/>). Analyses were performed on *Eurytemora spp.* sequences, with the exclusion of the outgroup.

As an alternative method, we used a Bayesian approach for the delimitation of multi-species coalescence model using molecular sequences from multiple loci in STACEY v.1.2.4 (Jones 2017) for BEAST2.

Estimation Of Possible Divergence Age

For estimation of possible divergence age of different clades, we used both paleontological information and points based on molecular phylogenetic data. To test the fit of our data to molecular clock models, we used a maximum likelihood test in MEGA 11 (Tamura et al. 2021; Takezaki et al. 1995). Nucleotide substitution parameters (using a maximum likelihood substitution model statistical method) were also made in MEGA 11, based on lowest BIC (Bayesian information criterion) scores. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level.

The divergence times of lineages were estimated using BEAST2 (ver. 2.6) (Bouckaert et al. 2014) with Bayesian inference, using the calibrated Yule model for the tree prior and the strict clock model. BEAST2 used a random tree with 6×10^6 generations and a sample frequency of 5×10^3 generations. We chose the rate of substitutions of 2.8% per Myr, as no properly calibrated rate estimates are available for copepods, in particular, and for other crustaceans estimates of COI rates in the range 1.4–4% divergence per Myr have been obtained (Knowlton and Weigt 1998; Zofkova and Timms 2009; Marino et al. 2011; Ketmaier et al. 2012; Sworobowicz et al. 2020). The chosen value gives the results which are in congruence with paleontological date.

As calibration points for the *E. affinis* group of the species and subspecies (with 15% standard deviations), fossil-based minimum age were applied for the split: Cyclestheria group 69.5 MYA; plus additional calibration points based on molecular phylogenetic data were used: within the outgroups: Chydorus group – 65.05 MYA, *Acanthocyclops-Mesocyclops* group – 75.24 MYA (Eyun 2017) and inner group *Eurytemora* aff. *affinis* – *Eurytemora carolleeae* – 11.5 MYA (Winkler et al. 2011). For evaluation of the genus *Eurytemora* and its species age calibration points based on fossil: Lepidurus/Triops 122 MYA (Kotov 2011; Schwentner 2013; Wolfe 2016) and on molecular phylogenetic data: *Acanthocyclops-Mesocyclops* group – 75.24 MYA (Eyun 2017).

Results

Analysis of the joined parts of genes CO1 and ITS1 showed expected close topology (Fig. 3) of the *E. affinis* group of species (*E. affinis*, *E. carolleae* and Caspian *E. caspica caspica* together with Far Eastern *Eurytemora*), gathered to common clade. Caspian *Eurytemora* joined with *E. caspica thetysiana* subsp. nov. to common subclade, which has high statistical supports.

Another clade formed by the species: European relict *E. lacustris* Poppe, 1887, brackish water *E. arctica* Wilson & Tash, 1966 and *E. gracilicauda* Akatova, 1949. *E. velox* Lilljeborg, 1853 stand apart from two these clades, but supports here and in some other nodes of the tree are rather low. Species delimitation analyses STACEY and ASAP performed for these joined parts of genes CO1 and ITS1 support all studied species and even the new described subspecies *E. caspica thetysiana* subsp. nov. The multirate Poisson Tree Processes (mPTP) algorithm combined all *E. affinis* species group as in a single species.

Median-joining network (Fig. 4) analysis revealed that Japanese *Eurytemora* consist of two lineages, one of which is closer to Caspian *Eurytemora*. At the same time, the Sakhalin population is close to another Japanese *Eurytemora* lineage. Both lineages formed a common clade, including Caspian *Eurytemora*. Nucleotide differences between Caspian (including 3 sequences from Akan Lake) and Far Eastern *Eurytemora* were 14.8% for CO1 and 1.1% for nITS genes.

The haplotype and nucleotide diversities were higher in *E. caspica thetysiana* subsp. nov. compared to *E. caspica caspica* (with the highest values in Akan Lake even excluding 3 sequences, which are close to caspian ones), values of Tajima's *D* and Fu's *F_s* indexes were calculated only for a few populations – they were insignificant, but mainly negative (Table 8).

Table 8
Sample size (*N*), nucleotide diversity ($\pi \pm SE$), haplotype diversity ($Hd \pm SE$), number of haplotypes (*h*), Tajima's *D* statistic and Fu's *F_s* statistic among all populations of the studied species based on CO1 gene sequences.

Species	Gene	Population	N of Sequences	π (%)	<i>Hd</i>	<i>H</i> Haplot number	Tajima's <i>D</i>	Fu's <i>F_s</i>
<i>E. caspica caspica</i>	CO1	Caspian Sea	1					
	CO1	Astrakhan	2					
	CO1	Saratov	1					
	CO1	Balakovo	1					
	CO1	Undory	1					
	CO1	Perm	1					
	CO1	all	7	0.002 ± 0.000	0.714 ± 0.127	3	0.206	-0.237
<i>E. caspica thetysiana</i> subsp. n.	ITS	all	4	0.002 ± 0.001	0.833 ± 0.222	3		
	CO1	Lake Akanko	8	0.007 ± 0.002	0.964 ± 0.008	7	-1.132	-2.529
	CO1	Lake Ohnuma	9	0.004 ± 0.002	0.583 ± 0.183	4	-1.823	0.391
	CO1	Lake Tunaicha	2					
	CO1	all	19	0.006 ± 0.001	0.784 ± 0.098	10	-1.780	-2.854
<i>E. caspica</i>	ITS	all	4	0.001 ± 0.000	0.667 ± 0.204	2		
	CO1	Lake Akanko	3	0.005 ± 0.002	1 ± 0.272	3		-0.341

Our molecular clocks suggested a Laurasian origin of *Eurytemora* genus (127–236 MYA), Mid Cretaceous differentiation of the ancient *Eurytemora* species (69.4–124.4 MYA) and Late Neogene–Paleogene differentiation *E. affinis* species complex. Speciation of *E. caspica* was possibly in Miocene about 11.9–24.1 MYA (Fig. 5). Two lineages of Japanese *E. caspica* were differentiated possibly in Early Miocene 7.5–17.3 MYA according to our molecular clock calibration (Fig. 5).

Systematics

Class Copepoda H. Milne Edwards, 1840

Order Calanoida Sars, 1903

Superfamily Diaptomoidea Baird, 1850

Family Temoridae Giesbrecht, 1893

Genus *Eurytemora* Giesbrecht, 1881

Eurytemora caspica tethysiana **subsp. nov.** (Tables 2–6, Figs. 6–13)

Etymology. The subspecies was named after the Tethys Sea, which probably was the initial area of *Eurytemora caspica* inhabiting.

Type material. Holotype, ZIN RN 55208, a female dissected on 1 slide; from the Lake Tunaicha, Sakhalin Isl., Russia (46°45' N 143°12' E), collected by D. Zavarzin, 01 July 2002.

Paratypes, ZIN RN 55209–55213 a male and females from a few lakes on the Sakhalin Isl., Russia and from the Ohnuma Lake, Japan given in the Table 6.

Description. Female (Fig. 6a). Body transparent, yellowish brown coloured. Full body length without caudal setae 1370 µm, with caudal setae 1573 µm.; cephalosome 207 µm and 4 free thoracic segments 1/2/3/4 = 120/118/109/103 µm. Urosome without caudal setae 530 µm, genital double-somite 165 µm, caudal rami 380 µm.

Cephalothorax as long as wide, with maximum width close to middle, frontal part of cephalothorax oval. Last thoracic somite with 1–2 small spines on wing-like outgrowths of lateral margin.

Genital double-somite (Fig. 2b, 6a, b) symmetrical with transverse constriction in the central part, 1.2 times as wide as long, due to wing-like outgrowths in anterior part of the somite, with two relatively long spines on both sides, with seminal receptacle.

Caudal rami (Fig. 2b, 6a, b) divergent, 5.4 times as long as wide, with long hair-setae on both sides, as well as on last abdominal somite. Caudal setae with segment like division. Length proportions of terminal setae, beginning from outermost caudal seta: 1/1.3/1.0/1.1. Length proportions of dorsal and lateral setae to outermost seta 0.3 and 0.8 times, respectively.

Antennules and antennae (Fig. 7a, b) look the same in all the representatives of this genus and, in particular, like in *E. caspica caspica* from the Caspian Sea.

Maxillula, maxilla, maxilliped, mandible (Fig. 7c, d, e, f), basically, like the same in other congeners of *Eurytemora* genus.

Swimming legs P1–4 (Figs. 8a, b, c, d.) consist of coxa and basis bearing 3-segmented exopod and 1 (P1) or 2-segmented endopod (P2–4). Swimming legs look the same in all the representatives of this genus. Length of P4 distal segment apical spine more than 1.2 times length of P4 distal segment.

Rudimentary P5 (Fig. 8e) uniramous and 4-segmented, narrow coxal plate bearing 1-segmented basis with big inner outgrowth and 2 spines. Big inner outgrowth has segment-like division from basis.

Distal segment with long apical seta and short lateral spine about 1/3 of apical seta. Tiny spine between spines on the distal segment about 10% of short spine length. Long apical spine has segment like division, which divided spine into 2 or 3 parts.

Eggs are packed in 1 sac.

Male (Fig. 9). Body length 1226 µm, with caudal setae 1493.

Cephalothorax 1.5 times as wide as long with maximum width close to caudal end, anterior part of cephalothorax round-shaped. Last thoracic somite without wings and spine on lateral margin. Abdomen 5-segmented.

Caudal rami without spines, with setae on inner sides. Length 8.5 times longer than width. Terminal setae ratio beginning from outermost caudal seta: 1/1.4/1.5/1.2.

Antennules, antenna, maxillula, maxilla, maxilliped, mandible (Fig. 10a, b, c, d, e, f) look the same in all the representatives of this genus and, in particular, in *E. caspica* from the Caspian Sea.

The structure of the swimming legs P1–4 (Figs. 11a, b, c, d) is typical for copepods and the same as in females. Formula for spine (Arabic) and seta (Roman) for distal exopod segments in P1–4 as follows: 3IV–3V–2V–3V. Some setae in swimming legs with not clear seen segment-like divisions.

Rudimentary legs P5 (Fig. 11e): right leg with basipodal segment cylindrical in shape and with small hill on inner side pointed with long spine, distal bent segment with 2 short spines in middle part. Shape of the bent segment closer to *E. carolleae*, than *E. caspica caspica*.

Left leg basipod like in *E. carolleae*, cylindrical, about 1.54 times as long as wide, next exopodal segment with two long spines in middle part, distal segment with strong long spine in middle, and hook in end similar in construction to *E. carolleae*.

Remarks. In our opinion, *Eurytemora caspica tethysiana* subsp. nov. like *Eurytemora caspica caspica* can be discriminated from two close-related species *E. affinis* and *E. carolleae* by a unique combination of characters that to some extent can be found in both other species but never coincide together in the same species. These features in both sexes include: in *E. caspica tethysiana* subsp. nov. as in *E. affinis* and *E. caspica caspica* mandible without a large outside orientated tooth and a big gap separating it from the other teeth as in *E. carolleae* (Figs. 7f, 10f). Also, *E. caspica caspica* adults, like *E. carolleae*, have segment-like division setae on swimming legs and on caudal rami, but the division is not so clear as in *E. carolleae* (Figs. 6b, 9).

At the same time, *E. caspica tethysiana* subsp. nov. females similarly to *E. carolleae* and *E. caspica caspica* equipped with outgrowths of the genital double-somite, but the outgrowths are more than in *E. caspica caspica* and have a wing-like shape; ratio W1/W2, as in *E. carolleae*, is about 1,5 (Fig. 2a, b).

Another difference of *E. caspica tethysiana* subsp. nov. from *E. caspica caspica* is the shape of the P5 distal segment, which is longer in *E. caspica tethysiana* subsp. nov. than in *E. caspica caspica*. P5 Lseg/Wseg is usually less than 1,5 in *E. caspica caspica* and more than 1,5 in *E. caspica tethysiana* subsp. nov.

In *E. caspica tethysiana* subsp. nov., females like *E. carolleae* and *E. caspica caspica* have P5 with a small, a tiny spine in the second exopodal segment placed between two distal spines (Alekseev and Souissi 2011). The length of this tiny spine is less than the width of the nearest spines, or about 10% of the short distal spine length, which definitely separates both species from *E. affinis* (Table 5).

In males of *E. caspica tethysiana* subsp. nov., the caudal rami are naked on both dorsal and ventral sides, as in *E. caspica caspica* and *E. carolleae*, in *E. affinis* males, caudal rami always have the sets of spines on the dorsal surface (sometimes only a few).

The left rudimentary P5 of *E. caspica tethysiana* subsp. nov., has an exopod of more or less cylindrical shape, like *E. carolleae* (Fig. 11e), whereas *E. caspica caspica* and *E. affinis* have exopod of more triangular shape with a length/width proportion of about 1 (Table 3).

The male *E. caspica tethysiana* subsp. nov., unlike the *E. caspica caspica* has a right rudimentary P5 with a special-shaped distal segment with W1/ W2 proportion of about 4, which is closer to both *E. affinis* and *E. carolleae* than to *E. caspica caspica* (Table 3). *E. caspica tethysiana* subsp. nov. is also different from *E. caspica caspica* in its L abdomen/ L caudal rami ratio, which is equal or more than 1 for *E. caspica tethysiana* subsp. nov. and equal or less than 1 for *E. caspica caspica*.

E. caspica tethysiana subsp. nov. differs from *E. affinis* by its strong and long spine 12 on A1, ratios 12spine/9spine are more than 2.7 and less than 2.7, correspondingly.

Distribution

According to our data and those of the literature, which we are able to check, *E. caspica tethysiana* subsp. nov. inhabits multiple waterbodies of Sakhalin Island: the lagoons of the north-east (Kafanov et al. 2003, Labay et al. 2016 (as *E. affinis*)); the freshwater Lake Sladkoe (Zavarzin and Safronov 2001, Zavarzin 2011 (as *E. affinis*)); the oligohaline lakes Ainskoe (Ueno 1935, 1935a, Borutzky and Bogoslovskii 1964), Tunaicha (Usova et al. 1980 (as *E. affinis*), Samatov et al. 2002, Zavarzin 2003, 2004, 2005 (as *Eurytemora* sp.), Labay et al. 2013, 2016 (as *E. affinis*)); the freshwater Lakes Panitu, Medvezh'ye and tundra lakes having a connection with the sea; the desalinated zones of the bays; and oxbows in the mouths of rivers from the north-western coast; and in the central part – in the oligohaline lakes Nevskoe and Protochnoe (Zavarzin 2021). The studied subspecies in Japan was observed by authors only from the Ohnuma Lakes. According to genetic studies by Makino et al. (2018) and Lee (2000), it also inhabits the freshwater Baratoko and Akan lakes and the Ishikari river, where a second Asian mitochondrial line were observed. These populations are not studied morphologically, but according to genetic data they belong to the *E. caspica* group anyway.

Variability among Far Eastern populations of *E. caspica*

In total there are no taxonomically significant differences among studied Asian populations of *E. caspica* maximal–minimal meanings of indices among studied populations overlap (Tables 4, 6). Mainly Japan population from the freshwater Lake Ohnuma and the Russian Sakhalin population from the brackishwater Lake Tunaicha were studied in the paper. Additionally, few exemplars of the Sakhalin populations from Lakes Panitu and Medvezh'ye (freshwater), and also from the Pil'tun and Chaivo brackish water bays were measured.

The female population from the freshwater Lakes Panitu and Medvezh'ye looks more different in accordance with all other studied Asian populations. In these lake *Eurytemoras* the long spine of P5 with strong notches (Fig. 12) is very different compared to other populations, where the long spine of P5 is rather smooth (Fig. 8e). The Panitu population, on average, has a longer distal segment of P5, and a shorter long spine of P5 distal segment (Table 6). Populations from Ohnuma and Tunaicha Lakes differed in P5 ratios: Tsp/Sp1, LongSp/Lseg and Sp1/Lseg (Ttest, $P \leq 0.05$) (Table 6).

The male Panitu population was not so well studied as females, and analysed features of caudal rami and P5 did not reveal differences from other populations. At the same time, populations of the Russian Tunaicha Lake and the Japanese Ohnuma Lake are most differed in the ratios 12vs9 of A1 and P5 L/W.

The Panitu and Medvezh'ye Lakes populations are not studied genetically, they possibly correspond to the second Asian line, which were observed in the freshwater Baratoko and Akan lakes of Japan with genetic analysis, but not studied morphologically.

Variability of newly studied morphological features for *E. affinis* group of species

The described below measurements were done specially to access the morphological variability among *E. caspica caspica* populations. The features previously have been not used in *E. affinis* group of the species before: length (Lseg) and width (Wseg) of P5 distal segment in females and for male's lengths of spines 9, 11, 12 of A1 as well as abdomen length. To use these new features for subspecies description we also measured them for different populations of related species *E. affinis* from the Elbe River Estuary, Gironde Estuary and the Gulf of Finland, previously supposed to be different morphological forms, and *E. carolleae* from the Chesapeake Bay and the Gulf of Finland (only females).

Female populations of all species and subspecies studied here differed statistically (T-test, $P \leq 0.05$) with each other with regard to the Lseg/Wseg ratio, excluding *E. caspica caspica* and *E. carolleae* (Table 5). Only the *E. affinis* population from the Gironde Estuary (1.41 ± 0.03 / min 1.30 – max 1.54) differed from both *E. affinis* from the Elbe and the Gulf of Finland, according to this index. *E. affinis* from the Elbe (1.53 ± 0.03 /1.33–1.68) and the Gulf of Finland (1.55 ± 0.04 /1.40–1.62) do not differ from each other using this parameter. *E. carolleae* from the Chesapeake Bay (1.31 ± 0.04 /0.92–1.44) and the Gulf of

Finland (place of its invasion) ($1.52 \pm 0.02/1.47-1.56$), which had not been compared morphologically previously, are different from each other in P5 Lseg/Wseg ratio.

Male populations of all species and subspecies studied here differed statistically (T-test, $P \leq 0.05$) with each other in relation to A1 Sp12/Sp9 ratio, excluding *E. caspica caspica* and *E. carolleae* (Table 3). *E. affinis* has no significant differences (T-test, $P \leq 0.05$) with any of the studied taxons in the A1 Sp12/Sp11 ratio, while all other taxons differ from each other in this ratio. *E. caspica tethysiana* subsp. nov. has significant differences (T-test, $P \leq 0.05$) with all studied taxons in the A1 Sp11/Sp9 ratio, while all other taxons showed no difference from each other in this ratio. Indices Labd/Lfurca (see Fig. 2 and Table 2) look useful for *affinis* group taxonomy as only *E. caspica tethysiana* subsp. nov. and *E. carolleae* had no significant differences according to this ratio (Table 2).

None of these indices were taxonomically significant in the interpopulation study within one species or subspecies, but could be used for the assessment of interpopulation variability.

Identification Key

Females

1. In leg 5, tiny spine about 10% or less of length of nearest lateral spine, shorter than width of nearest distal spine in insertion place 2
 - In leg 5, tiny spine about 15–30% of length of nearest lateral spine, equal to width of nearest distal spine in insertion place *E. affinis*
2. Mandible with more or less equal teeth; outside tooth not separated from neighbouring teeth by gap 3
 - Mandible with outside tooth clearly separated from neighbouring teeth by gap, caudal setae with clearly seen segment-like division, genital double-somite with strong wing-like outgrowths. *E. carolleae*
3. Distal leg 5 segment elongated with length/width ratio usually less than 1.5; genital double-somite with small outgrowths; caudal setae without clearly seen segment-like divisions *E. caspica caspica*
 - Distal leg 5 segment with length/width ratio usually more than 1.5; genital double-somite with strong wing-like outgrowths; caudal setae with clearly seen segment-like division. *Eurytemora caspica tethysiana* subsp. nov. (Fig. 13a)

Males

1. Caudal rami always naked on both dorsal and ventral sides. Left P5 basipodite with maximal width in the middle part, with length/width ratio usually more than 1,2 (Table 2, Fig. 11) 2
 - Caudal rami always with spines on dorsal surface (sometimes few). In left P5, basipodite with maximal width in anterior part of lateral outgrowth and with length/width ratio usually less than 1,1 *E. affinis*
2. Mandible with more or less equal teeth; outside tooth not separated from neighbouring teeth by gap 3
 - Mandible with outside tooth clearly separated from neighbouring teeth by gap, caudal setae with clearly visible segment-like division *E. carolleae*
3. Abdomen usually equal to or shorter than caudal branches; abdomen length/ caudal branches length ratio usually less than 1; caudal setae without clearly visible segment-like division. Ratio of caudal inner lateral seta length to caudal branches length less than 1,1 *E. caspica caspica*
 - Abdomen usually equal to or longer than caudal branches; abdomen length/ caudal branches length ratio usually less than 1; caudal setae with clearly visible segment-like division. Ratio of caudal inner lateral seta length to caudal branches length more than 2,8. *Eurytemora caspica tethysiana* subsp. nov. (Fig. 13b)

Discussion

A comprehensive study of the Caspian and Far Eastern *Eurytemora* populations showed that the Far Eastern population, both in terms of morphological features and the results of analysis of CO1 and ITS1 gene regions in this study, 18SrRNA according to Sukhikh et al. 2020, definitely refers to the species *Eurytemora caspica*. Perhaps this population even deserves a separate species status, given the significant genetic differences (14.8% for CO1 and 1.1% for nITS) and taking into account the results of the species delimitation algorithms STACEY and ASAP (Fig. 3). At the same time, the mPTP algorithm suggested all the species of *Eurytemora affinis* group are a single species, which is inconsistent with the results of our previous studies. It has been shown that *E. carolleae* and *E. affinis* live in sympatry for at least the last 18 years and have not mixed (Sukhikh et al. 2019), which indicates the validity of these species, at least.

As a result, having evaluated all the genetic analysis data, as well as based on the degree of morphological variability and our previous experience, we decided to assign the status of the Far Eastern populations, previously defined as *E. affinis*, as a subspecies. In light of the possible connection between the formation

of this taxon and the ancient Tethys Sea, the name *Eurytemora caspica tethysiana* subsp. nov. was chosen. It should be clarified that the status of populations (or at least parts of them) from the Ishikari river, Akanko and Baratoka lakes remains questionable due to the presence in these water bodies of another mitochondrial lineage closest to the Caspian population (Figs. 4, 5). The material from these waterbodies was not available to the authors for research.

Morphological comparison of the new subspecies *Eurytemora caspica tethysiana* subsp. nov. with the already described representatives of the *affinis* group show that, despite the obvious closeness of the new form to *E. caspica caspica*, a number of features also bring it closer to the American *E. carolleae* and the European *E. affinis*. The morphological identity of a new subspecies in this group of closely related species and subspecies is determined not by any one feature, but by a unique combination of relatively small differences. This approach is especially effective when separating morphologically close species (Alekseev and Souissi 2011; Sukhikh and Alekseev 2013).

Of undoubted interest is the very fact of finding two subspecies of *Eurytemora caspica* separated by a significant geographical barrier, which is the distance from the Far East to the Caspian Sea – about 6400 km by land. We consider such an amazing similarity of very territorially separated populations in terms of the evolution of the Tethys Sea (Alekseev and Sukhikh 2020), which for a long time connected the Ponto–Caspian region and water bodies of the Far East (Scotese, 2014a, b).

According to existing models, successive attachments of the African and Indian continental plates to the Eurasian continent led to the establishment of an internal intercontinental highly desalinated sea with several branches or straits connecting the Atlantic and Pacific coasts of Eurasia (Scotese 2014a, b). One such branch, which can be traced by modern finds of *Eurytemora caspica* populations in the Eurasian region, passes through the Caspian and Black seas and reservoirs of Kazakhstan (Fig. 1).

The further movement of the Indian subcontinent over the past 8–10 million years, according to geological data, has led to the successive closure of these straits and the isolation of populations separated by young mountain ranges of the Asian part of continent.

In all likelihood, the presence of three genetic lineages of the studied species in Japan and the Caspian Sea was the result of these geological events. Perhaps these lineages lived in different branches of the Tethys Sea. Molecular dating analysis showed similar results.

The existing models of the movement of tectonic plates at the last stage of the existence of the Tethys Sea allow us to correlate the proximity of the Far Eastern and Caspian populations with the possible connections of these regions, which finally ceased after the annexation of the Indian subcontinent. A similar pattern of isolated African and Ponto–Caspian species distribution in Eurasia has been observed for *Eucyclops* and *Mesocyclops* species (Mirabdullayev 1996; Karaytug 1999; Alekseev and Sanoamuang 2006; Alekseev and Chaban 2021). In our opinion, the distribution of the genus *Eurytemora* in the southern part of the area can act as a marker of the residual waterbodies of the Tethys Sea (Tethys sea belt, Alekseev Sukhikh 2020), which should be taken into account when conducting relevant studies of the relict fauna of this ancient water body. For instance, *E. composita* Keiser, 1929, can serve as an example of similar one species populations' distribution along the supposed axis of the continental straits closure. The species is described from the brackish lake Issyk-Kul in the mountains of Central Asia in Kirgizstan and subsequently found in in the Kuriles (Smirnov 1929), in South Korea, in North America in Alaska and St. Lawrence Island (Wilson 1953), and also noted on Sakhalin Isl. in the Lutoga river (Zavarzin 2021).

The populations of related *Epischura* and *Epischurella* genera species are distributed in similar ways (Bowman et al. 2019). *Epischurella* represented in the Asian Lake Baikal (Sars, G.O. 1900) and in two Far Eastern lakes Khanka (Rylov 1928) and Udyr (Borutzky 1947), in Kamchatka and *Epischura* widely represented in North America (Marsh 1933; Humes 1954).

It should be remembered that the relationship of the aquatic fauna of the Caspian Sea, Baikal, the Far East of Russia and North America was pointed out by experts in the field of zoogeography as early as the middle of the twentieth century (Vereshchagin 1940; Berg 1947). The reasons for this similarity were assumed to be the consequences of the Quaternary glaciations, since the theory of continental drift (Wegener 1912) at that time was not yet accepted by the scientific community.

The possibility of isolating the fauna of the Tethys Sea, along with the confirmation of the theory of continental drift by biological methods, also allows us to discuss the issue of much more conservative mechanisms of speciation and longer periods of existence of invertebrate species compared to vertebrates. Apparently, the age of at least some species of cosmopolitan Copepoda can be measured in tens and even hundreds of millions of years. At the molecular level, this assumption is seen in the different rates of evolution and, accordingly, the conservation of nuclear and mitochondrial DNA regions. In Copepoda invertebrates, the level of interspecies differences in mitochondrial DNA is high – on average 35% in the CO1 part of the gene. At the same time, nuclear DNA variability, for example, 18SrRNA, is practically absent in closely related Copepoda species (Hamrová et al. 2012; Vakati et al. 2019; Sukhikh et al. 2020; Kochanova et al. 2021). In closely related vertebrate species, the rate of evolution of which is much higher, and the age of the species is noticeably younger, the picture is very different, and the level of interspecific variability in mtDNA is much lower and usually does not exceed 2% (Pentinsaari et al. 2014; Huemer et al. 2018; Cox and Hebert 2001). In addition to clonal inheritance, short lifespan, fast generation time and peculiar life history of microcrustacean species (Rawson and Burton 2006; Kochanova et al. 2021), such differences in the levels of mitochondrial variability in vertebrates and lower crustaceans, in our opinion, reflect differences in the existence of species during the lifetime of which the invertebrate accumulates more changes in mitochondrial genes than in vertebrates. It is likely that invertebrates retain the ability to exchange nuclear DNA between isolated populations much longer. In addition, for aquatic organisms, especially those that live in marine waters or large long-lived lakes such as Baikal, living conditions in the aquatic environment are undoubtedly more stable than on land. This circumstance should slow down the variability and hence the rate of speciation in aquatic organisms and increase the lifetime of species.

Molecular dating showed that *E. affinis* species complex (*E. caspica* Sukhikh and Alekseev, 2013, *E. affinis* (Poppe, 1880), *E. carolleae* Alekseev & Souissi, 2011) would have divided from other *Eurytemora* species about 69.4–124.4 MYA. Speciation of *E. caspica* was possibly in Miocene (Fig. 5). Two lineages of Japanese *E. caspica* were differentiated possibly in Early Miocene according to our molecular clock calibration (Fig. 5). Thus, according to our estimates, the processes of speciation in the genus *Eurytemora*, began approximately in the Mid Cretaceous and ended in the Late Neogene-Paleogene, taking into account the data on the Miocene origin of the “young” species *E. carolleae* (Winkler et al. 2008). The processes of speciation during the Miocene period were also shown for several cryptic harpacticoid species and the *Eucyclops* species (Kochanova et al. 2021). Similar results have been obtained for some other Copepoda (Thum and Harrison 2009; Marrone et al. 2013; Previšić et al. 2016; Young et al. 2014).

On the whole, the genus *Eurytemora* has apparently Laurasian origin, given the results of our analysis of the time of its formation (127–236 MYA), its Holarctic distribution, and the absence of representatives of the genus on the continents that are descendants of the former Gondwana. For comparison, Eyun (2017) estimated the time of the genera *Eurytemora* and *Acartia* divergence at about 180 Ma (Jurassic).

Conclusion

1. Common origin, morphological and genetic affinity of the studied *affinis* group *Eurytemora* populations from Japan, Sakhalin Isl. and the Caspian Sea drainage basin (RF) enables us to identify all these populations as *E. caspica* Sukhikh and Alekseev, 2013, and describe Far Eastern *Eurytemora* as a new subspecies *Eurytemora caspica tethysiana* subsp. nov.
2. The morphological and genetic similarity of the two forms of *E. caspica*, molecular dating as well as the species distribution suggests that forms separation occurred in geologically recent times and is associated with the evolution of the Tethys Sea during the Miocene (about 8–20 MYA).
3. Our results indicate that the genus *Eurytemora* has apparently Laurasian origin and speciation in the genus *Eurytemora* had occurred during approximately the Mid Cretaceous and ended in the Late Neogene-Paleogene

Declarations

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Data availability. Accession numbers for sequences provided in Table 7, are uploaded to the GenBank (<https://www.ncbi.nlm.nih.gov>).

Conflicts of interest. The authors declare that they have no conflicts of interest.

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Figures



Figure 1

Distribution of *E. caspica* as a marker of the residual waterbodies of the Tethys Sea (Tethys sea belt Alekseev Sukhikh 2020). Literary date: Ban and Minoda 1989; Sukhikh and Alekseev 2013; Krupa et al. 2016; Krupa and Aubakirova, 2021; Zavarzin D.S. 2021.

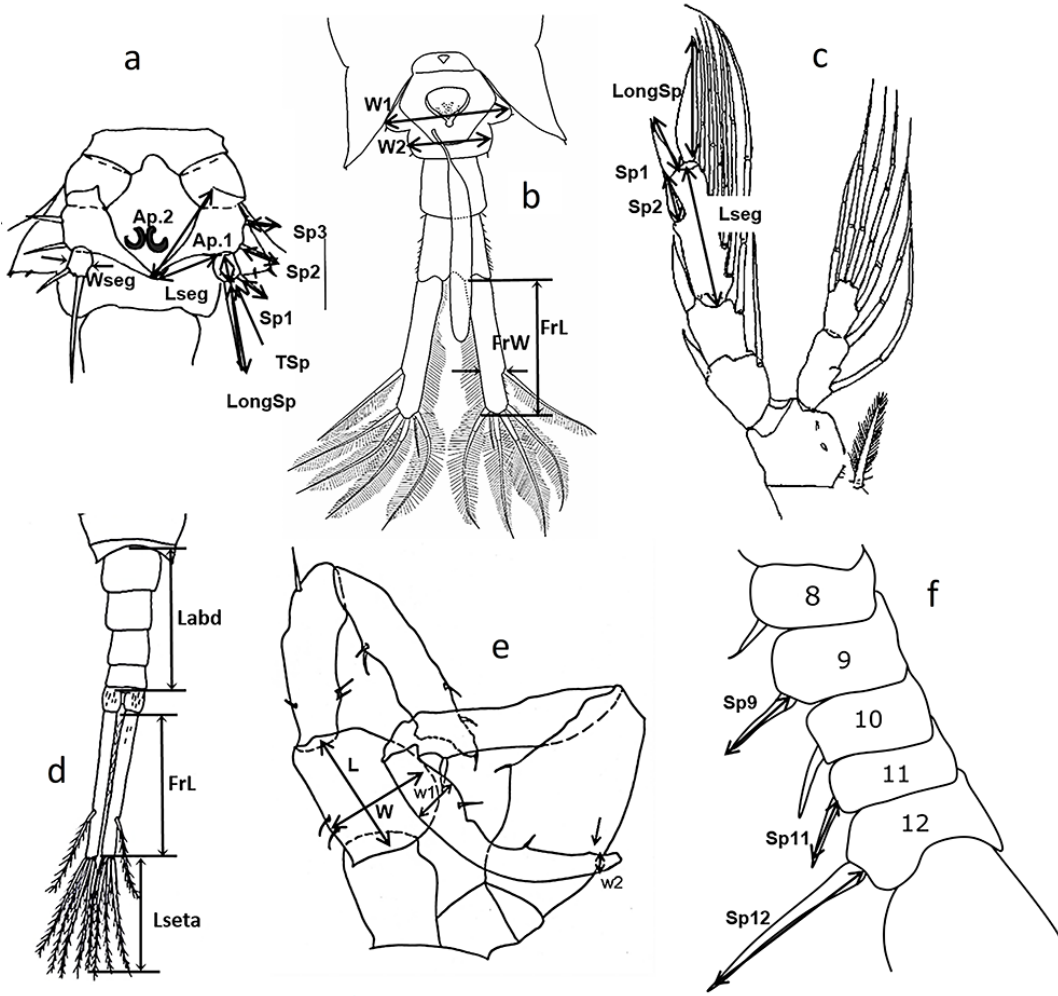


Figure 2

Morphological features used in present study for *Eurytemora* spp. Female measures a-c; male measures d-f.

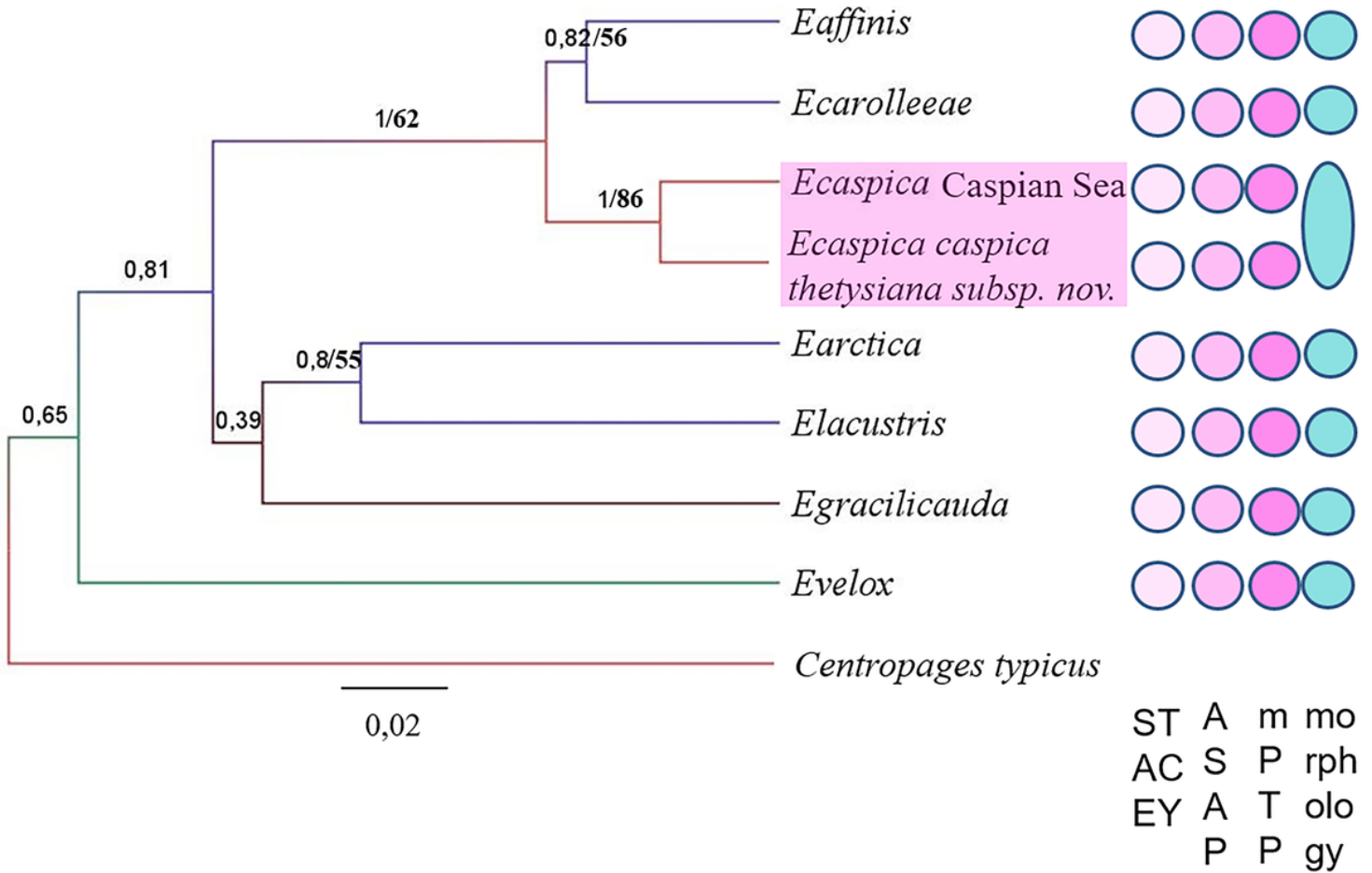


Figure 3

Bayesian phylogenetic tree of eight *Eurytemora* spp. on the concatenated CO1-ITS1 set based on 46 sequences constructed using the GTR+G model. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI)/bootstrap values (maximum likelihood, ML). Species separated by STACEY, ASAP and mPTP algorithms are marked by pink circles. Blue circles show results of morphological analysis.

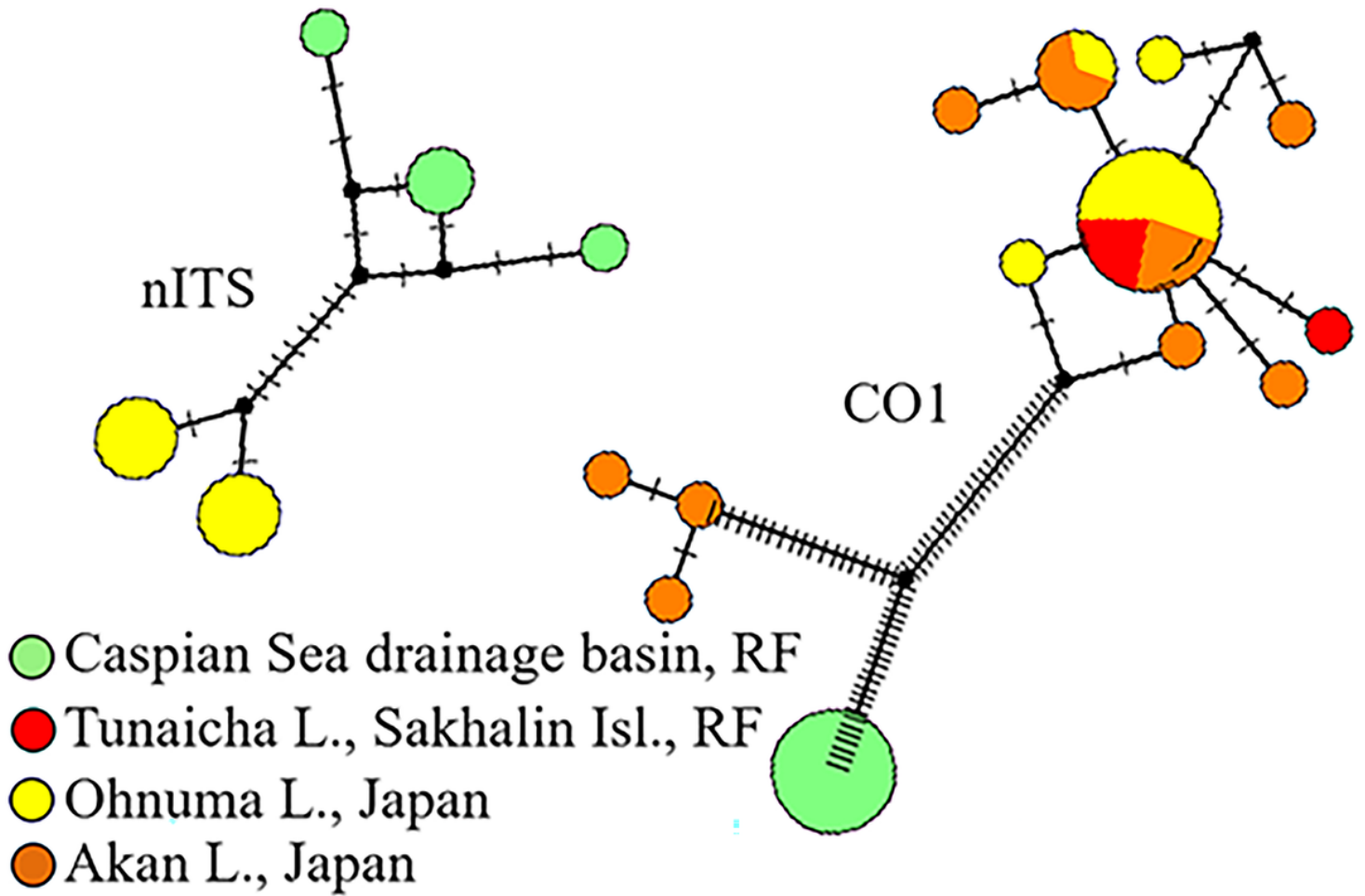


Figure 4

Median-joining network of haplotypes of *Eurytemora caspica thetysiana* subsp.nova., built on 10 and 29 nucleotide sequences of the nITS and CO1 part of genes. The lines indicate the number of mutations between haplotypes. In green-coloured haplotypes from the Caspian Sea (Russia) are given; in yellow – from Ohnuma Lake (Japan); in orange – from Akan Lake (Japan); and in red – from Lake Tunaicha (Sakhalin Isl. Russia).

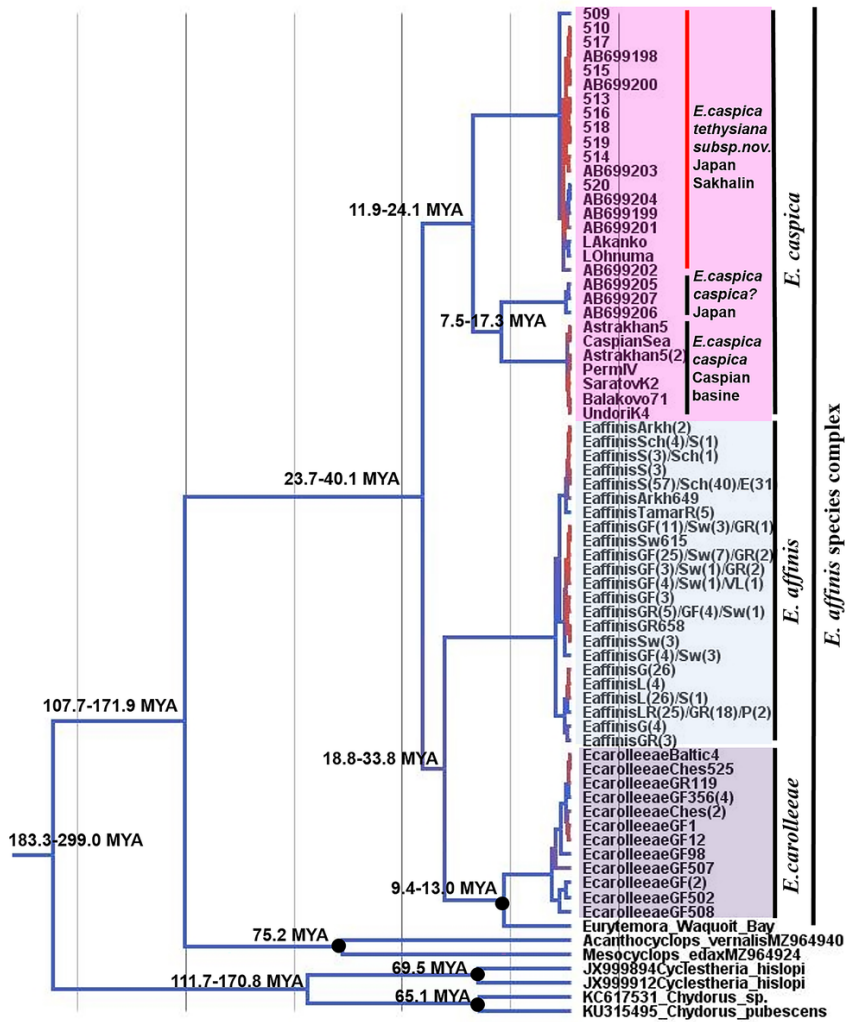


Figure 5

Phylogenetic ultrametric tree constructed in BEAST2 v.2.6 for the *Eurytemora affinis* group. Phylogenetic analysis of the mitochondrial data set (CO1) with strict clock estimates based on fossil calibration point (Cyclestheria group 69.5 MYA). Statistic support (posterior probabilities (BI)) of branches is coded by the colour gradient from violet (low) to blue (high). Probable time of divergence between clades (in MYA) based on the rate of substitutions of 2.8% per MYA given in nodes.

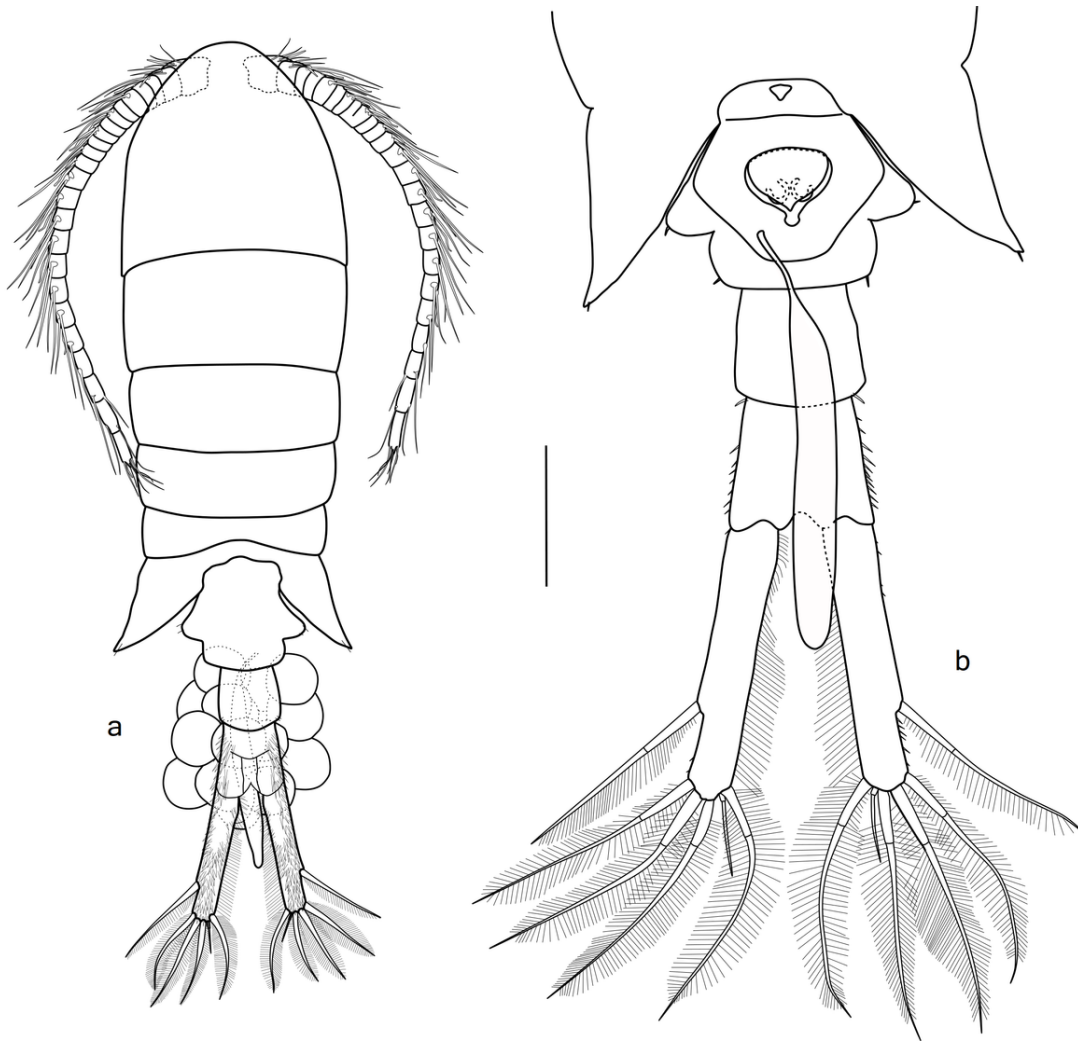


Figure 6

Eurytemora caspica tethysiana subsp. nov., female, holotype, ZIN RN 55208: a – habitus, dorsal view; b – urosome with genital double-somite, ventral view. Scale bar: a 200 μ m, b 100 μ m.

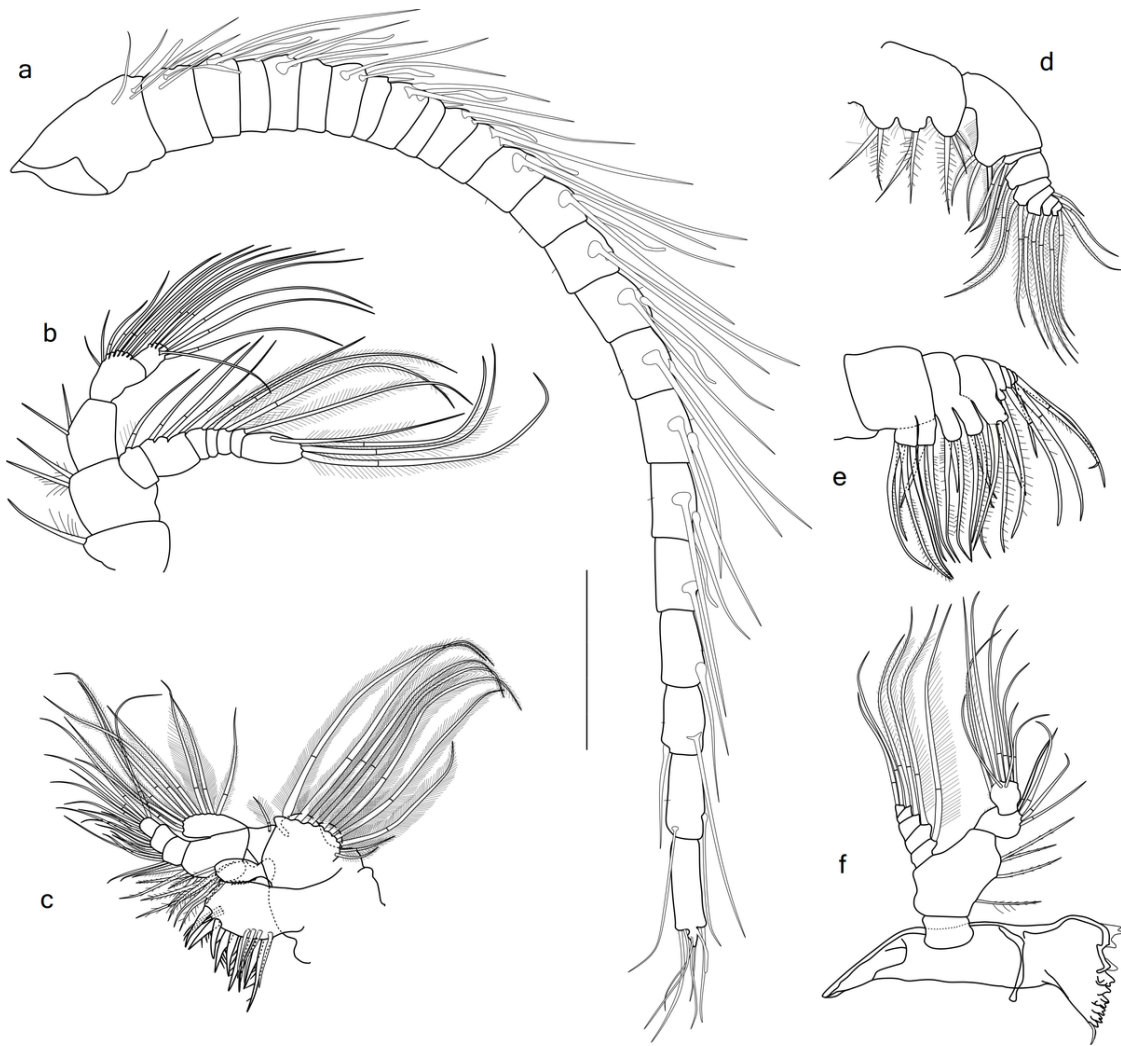


Figure 7

Eurytemora caspica tethysiana subsp. nov., female, holotype, ZIN RN 55208: a – right antennula; b – antenna; c – maxillula; d – maxilliped; e – maxilla; f – mandible. Scale bar: 100 μ m.

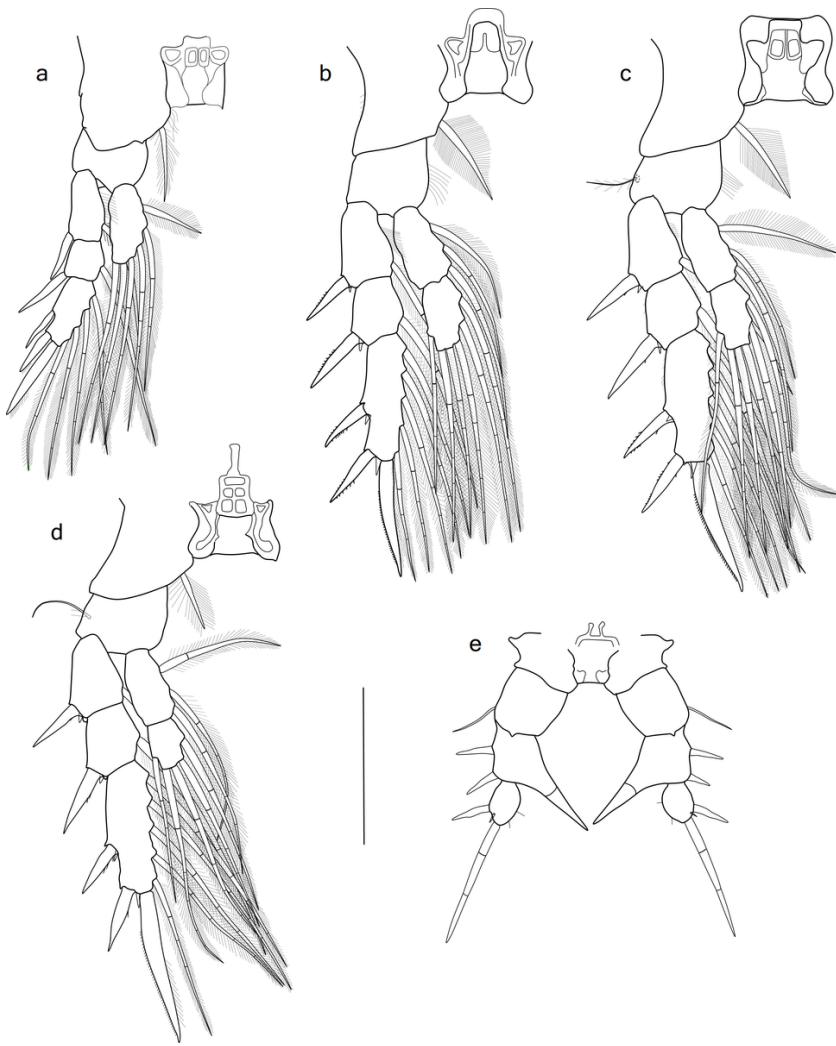


Figure 8
Eurytemora caspica tethysiana subsp. nov., female, holotype, ZIN RN 55208(a,c–e), ZIN RN 55209/2, paratype (b): a – right swimming leg 1, anterior view; b – right swimming leg 2, anterior view; c – right swimming leg 3, anterior view; d – right swimming leg 4, anterior view; e – rudimentary legs 5, anterior view. Scale bar: 100 μ m.

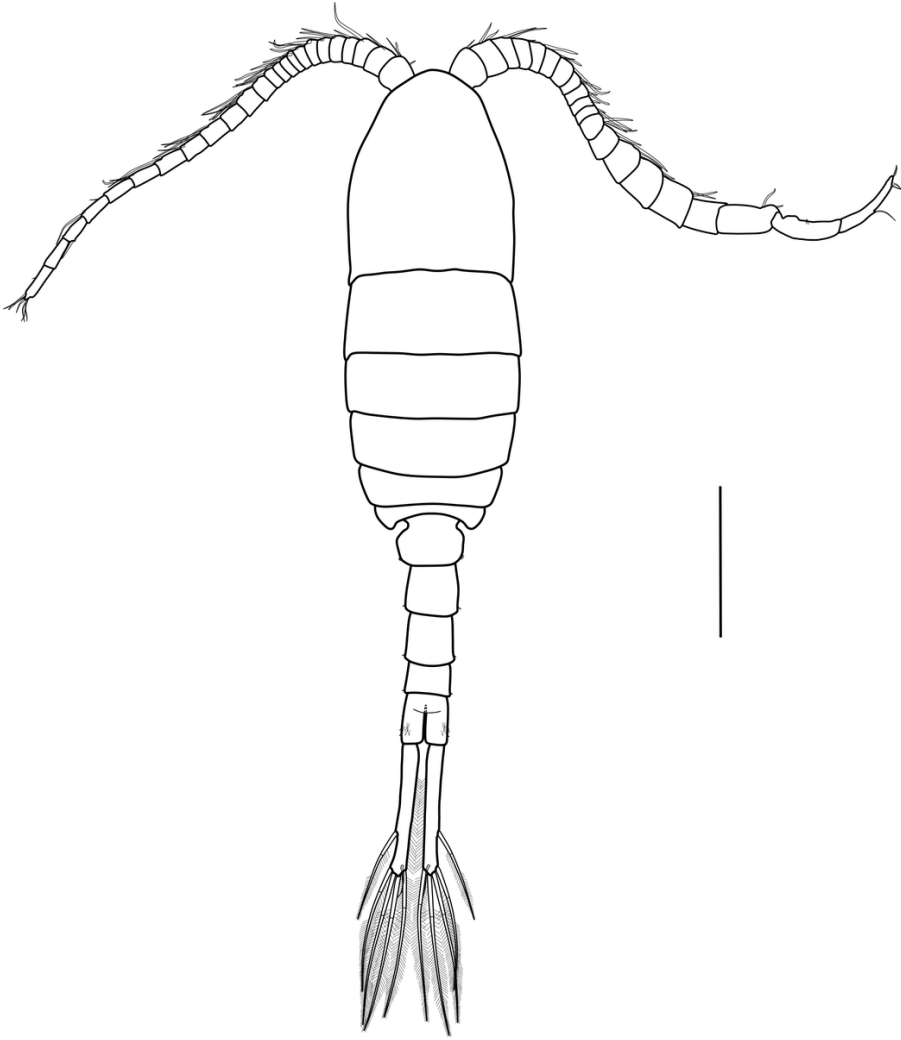


Figure 9

Eurytemora caspica tethysiana subsp. nov., male, paratype, ZIN RN 55210, habitus, dorsal view. Scale bar: 200 μ m.

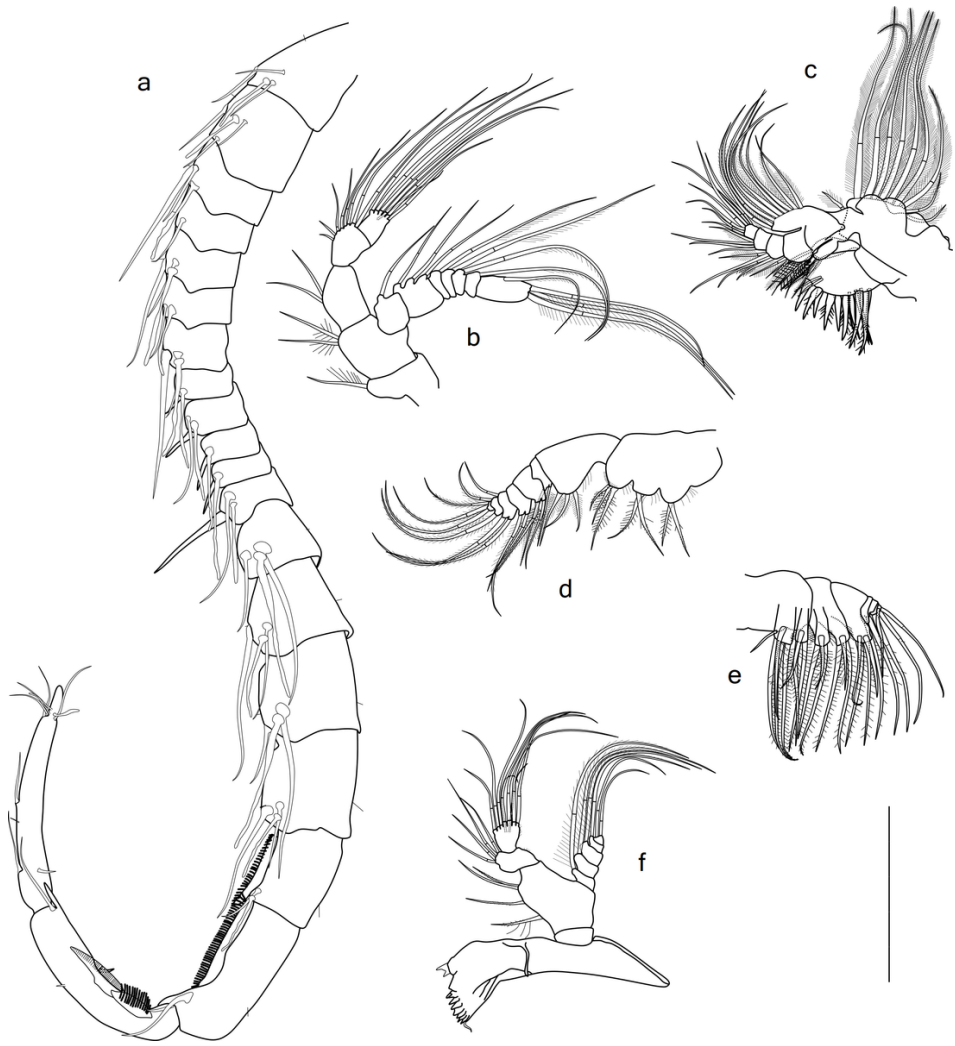


Figure 10

Eurytemora caspica tethysiana subsp. nov., male, paratype, ZIN RN 55210 (a), paratype, ZIN RN 55211/2 (b–f): a – right antennula; b – antenna; c – maxillula; d – maxilliped; e – maxilla; f – mandible. Scale bar: 100 μ m.

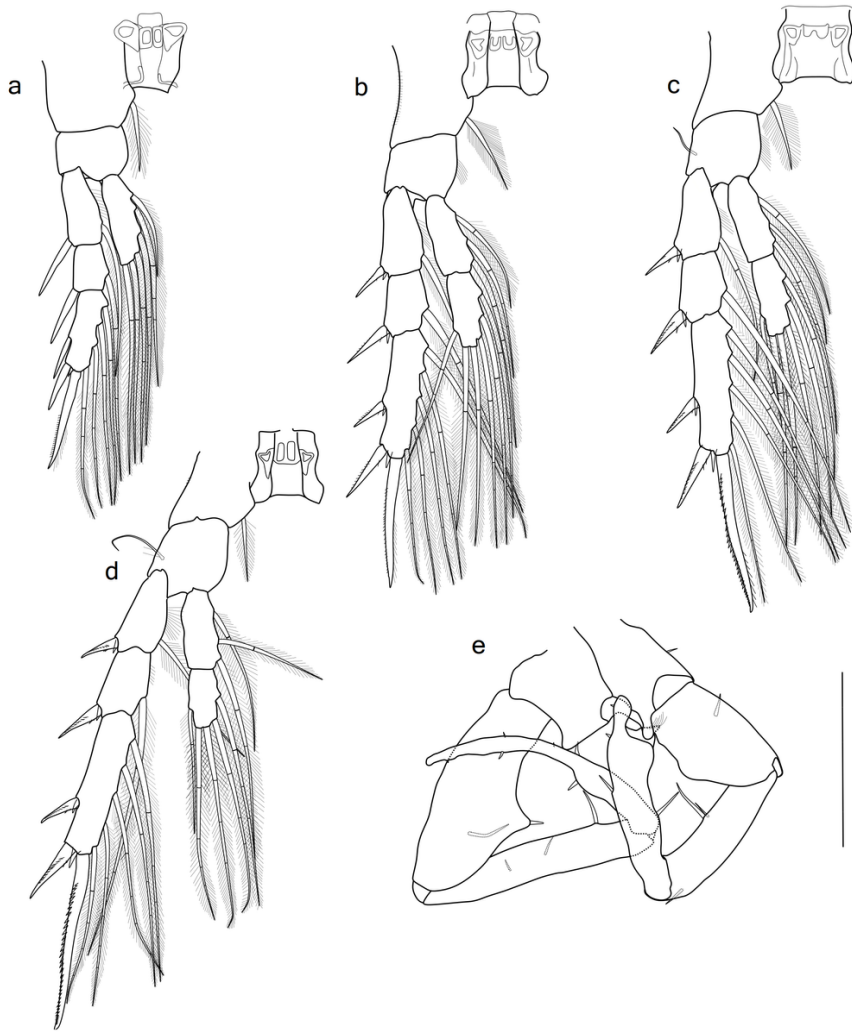


Figure 11

Eurytemora caspica tethysiana subsp. nov., male, paratype, ZIN RN 55211/3 (a–d), paratype, ZIN RN 55211/1 (e): a – right swimming leg 1, anterior view; b – right swimming leg 2, anterior view; c – right swimming leg 3, anterior view; d – rightswimming leg 4, anterior view; e – rudimentary legs 5, anterior view. Scale bar: 100 μ m.

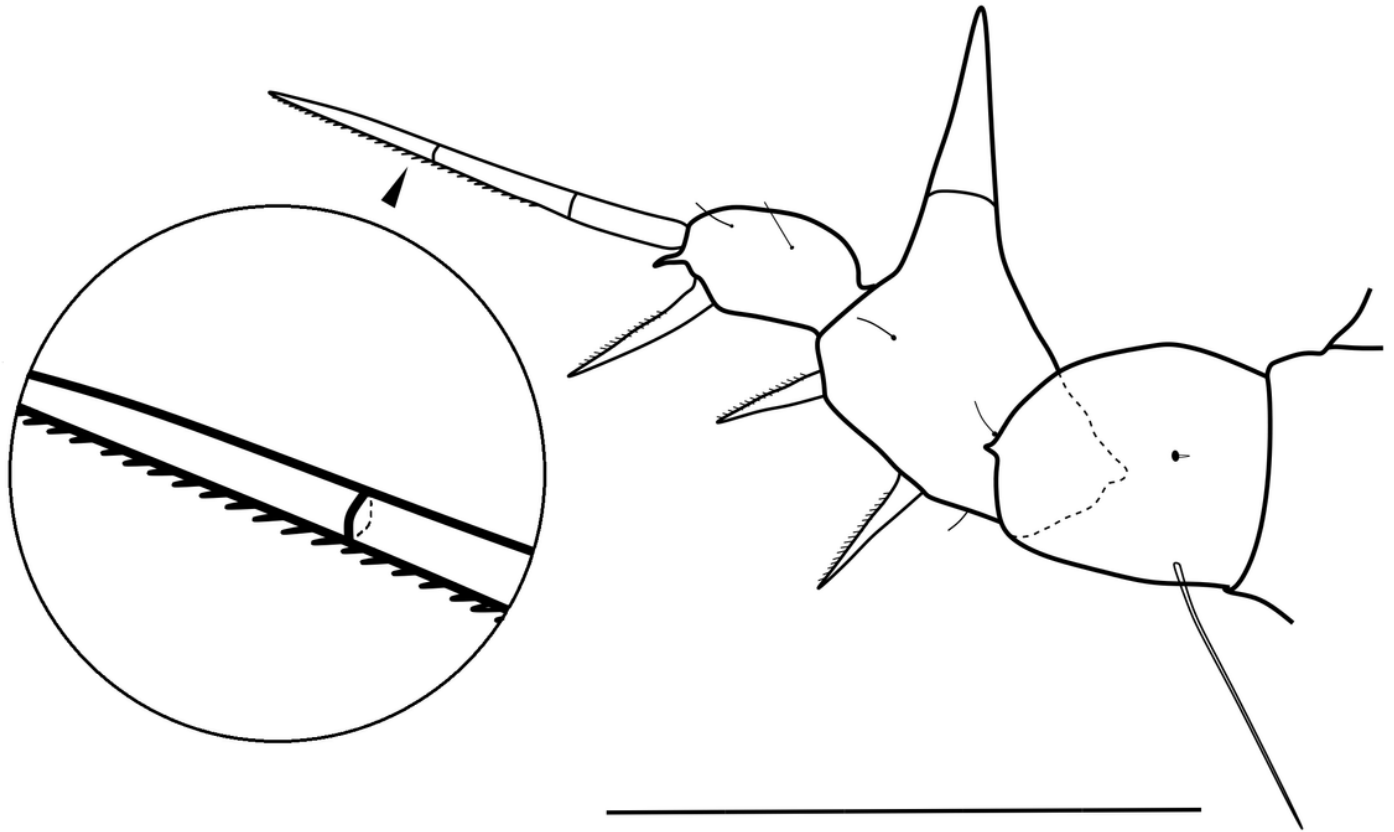


Figure 12

Eurytemora caspica tethysiana subsp. nov., female rudimentary left leg 5 from Lake Medvezh'ye, anterior view. Scale bar: 100 μ m.

Supplementary Files

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