



Phylogenetic relationships and historical population reconstruction of Asian members of the genus *Epischura* (Copepoda, Calanoida)

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ABSTRACT

We have reconstructed major demographic and phylogenetic events for two closely related copepod species: *Epischura baikalensis* Sars, 1900 (Copepoda, Calanoida), a key endemic species of the plankton community from Lake Baikal, and *E. chankensis* Rylov, 1928, a key zooplankton species from Lake Khanka. The reconstructions were based on the nucleotide sequences of mitochondrial DNA (mtDNA) cytochrome oxidase subunit 1 (CO1) using the BEAST software package and recently identified calibrations for the Baikal amphipods *Gmelinoides fasciatus*. The time span to a common ancestor for *E. baikalensis* and *E. chankensis* is 13.7 million years (95% confidence interval is 8.3–21.4 million years). Both species studied have experienced abrupt changes in the effective population size in the past. These changes are likely associated with climate and geological shifts in their habitat areas. For *E. baikalensis*, these changes can be associated with alternating glacier and interglacial periods as well as an increase in depth of Lake Baikal; for *E. chankensis* with formation and increase in the area of Lake Khanka, which was also due to climate and geological shifts in the Khanka basin.

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Introduction

Geological and climatic changes directly influence the dispersal and survival of species. The structure of the nucleotide sequences in the genome can therefore indicate past events, such as immigration of species to new areas, the passage through a bottleneck, the occurrence and disappearance of the geographical isolation, etc.

The genus *Epischura*, described in 1882, includes nine species, six of which are found in North America and three in Asia. The current natural habitats of members of the genus *Epischura* indicate its wider former distribution in the fresh waters of the Northern Hemisphere. According to Borutskii (1947), these are remnants of a group that was previously spread across North America and East Asia (East Siberia and the Far East), from which separate relict colonies survived and evolved *E. baikalensis*, *E. chankensis* and *E. smirnovi* Borutsky, 1961 are the Asian species. The latter species (*E. smirnovi*) was recorded in Lake Chlya and the lower Amur River near the Tyr settlement. This species is mainly known from the study in which it was described (Borutskii, 1961).

Epischura baikalensis inhabits the ancient Lake Baikal. This lake is located (Fig. 1) in the south of East Siberia and is the largest and

deepest freshwater lake in the world, containing approximately 20% of the world's surface fresh waters. The surface area of the lake is 31,722 km², and its maximum depth is 1642 m. *E. baikalensis* is an endemic species that occupies the entire water column of the pelagic zone throughout the year (Afanasyeva, 1998; Kozhov, 1963). It is one of the main components in the diet of pelagic fish and invertebrates and an important link in the biotic cycling of matter and energy in the lake.

Epischura chankensis is widespread in the basin of the Amur River and is known from Lake Khanka and the Amur floodplain lakes; it was also recorded in the Amur itself. This species is found in the southern part of the Sea of Okhotsk, the brackish water of the Amur estuary as well as in freshwater bodies of China (Borutskii et al., 1991). This study analyzed the population from the Far Eastern Lake Khanka (Fig. 1), where it is one of the key species in the zooplankton community (Barabanschikov, 2004). Khanka is the largest lake inhabited by *E. chankensis*. Lake Khanka is also the largest in Primorsky Territory and located in the center of the Khanka Lowland. Its area is approximately 4000 km², and the maximum depth is approximately 6 m (Korotky, 1989). In summer, *Epischura chankensis* is one of the predominant zooplankton species; in winter, it is the main zooplankton species in the lake.

The analysis of the intraspecific genomic structure of both species without divergence dates was carried out previously: *E. baikalensis* (Zaidikov et al., 2015) and *E. chankensis* (Zaidykov

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Fig. 1. Map of the general location of Lake Baikal and Lake Khanka.

et al., 2018). The mentioned papers describe the features of the DNA extraction, PCR amplification and purification of the amplification product, as well as the structure of the selected specific primers. It was shown that *E. baikalensis* from Lake Baikal is represented by a single panmictic population with an apparently large effective population size whereas *E. chankensis* from Lake Khanka identified traces of geographical subdivision in the past in the current single population. The sequence data were placed in GenBank.

Materials and methods

The present study has determined the approximate dates of phylogenetic and demographic events for *E. baikalensis* from Lake Baikal and *E. chankensis* from Lake Khanka (Fig. 1), as well as determining the history of changes in the effective population size. The analysis used the COI sequences from *E. baikalensis* (KC683830–KC683889) and *E. chankensis* (KR704356–KR704407) that we previously placed in the GenBank. For the third positions of the codon in COI, we did a marker test on substitutions saturation (Xia et al., 2003) using Dambe v.6.4. software (Xia and Lemey, 2009).

The DNA evolution model was selected using the Bayesian information criterion (BIC) in the JModelTest-2.1.7 software program (Darriba et al., 2012). The phylogenetic tree was reconstructed based on unique haplotypes of the COI gene using the Bayesian analysis with the molecular clock in the BEAST v. 1.8.4 software package (Drummond and Rambaut, 2007). The following speciation models were used for the analysis: yule process (Gernhard, 2008) with strict clock, yule process with uncorrelated lognormal relaxed clock (Drummond et al., 2006), birth-death process (Gernhard, 2008) with strict clock, birth-death process with uncorrelated lognormal relaxed clock. The best evolutionary models were chosen by comparison of likelihood indicators and AICm information criterion values in the Tracer v1.6 program.

For dating the phylogenetic tree, substitutions rate of 1.7% per million years was used, which was calculated for the endemic Baikal amphipod *Gmelinoides fasciatus* (Bukin et al., 2018) and is close to the values obtained for snapping shrimp of the genus *Alpheus* (Knowlton and Weight, 1998) and Jamaican crabs of the family Grapsidae (Schubart et al., 1998). In our case, the rate calculated for the Baikal amphipods is more relevant due to the higher similarity in living conditions of the latter to the studied organisms.

For reconstructing the history of changes in the effective population size Bayesian skyline plot was used (Drummond et al., 2005) in BEAST v1.8.4 software package. Bayesian skyline plot gives the dependence of the population parameter θ on time. Since for mitochondrial markers $\theta = 2Ne\lambda$, where Ne is the effective number of females, and λ is the probability of a mutation per genetic marker for generations, changes in θ will be determined by changing the

effective population size. For analysis, we used the strict molecular clock and the same model of DNA evolution as in the phylogenetic analysis. The most recent common ancestor (TMRCA) for populations in Bayesian skyline plot was taken from the phylogenetic analysis.

For each isolated population, the genetic diversity parameters θ_π and θ_s (Tajima, 1996) were calculated. The parameter θ_π is the average number of mismatched nucleotides between the sequences of organisms in a population, the parameter θ_s is calculated based on the number of polymorphic sites in the alignment. A large difference between the values of θ_π and θ_s indicates a non-neutral character of the evolutionary process in a population (abrupt changes in population size or the effect of natural selection). To determine the evolutionary mechanisms leading to the formation of the observed genetic diversity in populations, we used Tajima's D (Tajima, 1989) to test for the neutral mechanism of the substitutions accumulation in the population and R2 (Ramos-Onsins and Rozas, 2002) to test for an expansion or fast increase in population size. Calculations of the θ parameters and population-statistical criteria (Tajima's D test and R2 test) were performed using the «pegas» package (Paradis, 2010) for the programming language R.

Results and discussion

Statistical testing has shown that third codon positions in the studied marker are not oversaturated with substitutions (Table 1). All observed “Iss” is significantly lower than “Iss.c” which indicates little saturation and suitability for phylogenetic analysis. Therefore, for further analysis, we used a full data array with all three codon positions.

We have determined that the nucleotide substitution model HKY + I + G yielded the lowest BIC indicating it was the the best model of DNA evolution for the data set. We used this model in all subsequent calculations.

The phylogenetic reconstructions have indicated speciation birth-death process with strict molecular clock (the highest likelihood value and minimum value of the AICm information criterion) as the most optimal model (see Table 2). We used the phylogenetic tree constructed by this model for further analysis and conclusions.

In the phylogenetic tree (Fig. 2), the time span to the common ancestor for *E. baikalensis* and *E. chankensis* is 13.7 million years (95% confidence interval is 8.3–21.4 million years), which indicates the existence of the members of the genus *Epischura* on the Asian territory for more than 8 million years. It is most likely that animals migrated between the continents through Beringia, which connected Eurasia and North America into a joint supercontinent (Gladenkov and Gladenkov, 2004). This connection between continents lasted for more than 100 million years, from the Early Cretaceous to the Late Miocene (approximately 5 million years ago), when the Bering Strait began to form (Marincovich et al., 1990).

In the phylogenetic tree, unique *E. baikalensis* haplotypes form a single cluster without an internal subdivision having a divergence

Table 1

Results of Test on substitution saturation (Xia et al., 2003; Xia and Lemey, 2009) in Dambe v.6.4. software. Testing whether the observed “Iss” is significantly lower than “Iss.c”. “Iss” is index of substitution saturation; “Iss.c” is critical Iss value at which the sequences will begin to fail to recover the true tree; “Iss.cSym” is “Iss.c” assuming a symmetrical topology; “Iss.cAsym” is “Iss.c” assuming an asymmetrical topology.

NumOTU	Iss	Iss.c Sym	Iss.c Asym
4	0.100	0.799	0.766
8	0.094	0.756	0.645
16	0.092	0.729	0.519
32	0.090	0.707	0.379

Table 2
Comparison of evolutionary models.

Evolutionary model	Natural logarithm of Likelihood value	AICm value	Tree root age in million years (with 95% confidence interval)
Speciation yule process + Strict clock	−2335.32	5003.64	6.04 (4.34–7.94)
Speciation yule process + Uncorrelated relaxed clock	−2335.64	4984.68	6.01 (4.27–7.96)
Speciation birth–death process + Strict clock	−2321.13	4936.81	13.71 (8.34–21.43)
Speciation birth–death process + Uncorrelated relaxed clock	−2321.57	4942.11	15.19 (8.31–23.25)

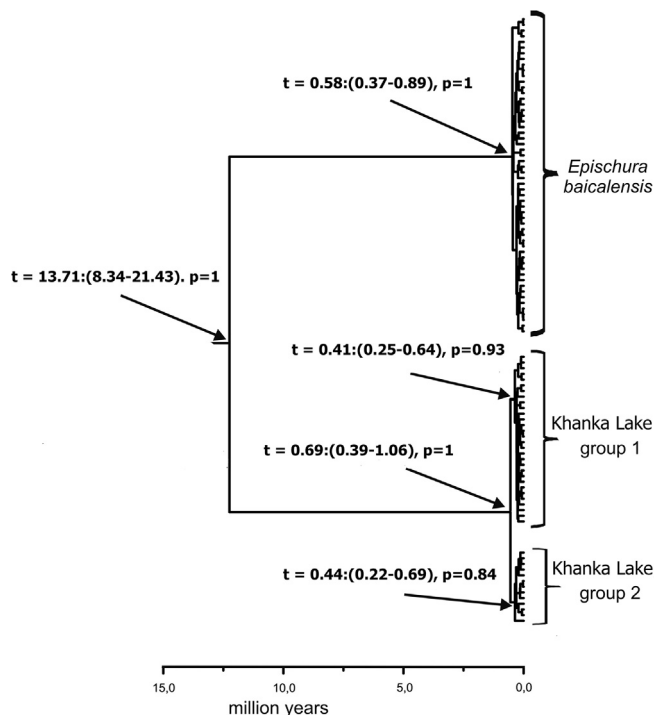


Fig. 2. A tree with a time scale constructed from the nucleotide sequences of mtDNA COI gene in the BEAST program. There are dates of taxa divergence in the main sites; 95% confidence intervals for dating are shown in parentheses. The same sites are provided with statistical support of P value, a posteriori probability.

time of 0.58 million years and a 95% confidence interval of 0.37–0.89 million years. This confirms the conclusions of the previous work (Zaidykov et al., 2015) that this species consists of a single panmictic population in Lake Baikal. The unique *E. chankensis* haplotypes have divided into two clusters (Khanka Lake group 1 and Khanka Lake group 2), which confirms the conclusions in (Zaidykov et al., 2018) about the existence of reproductive, most probably geographic isolation somewhere in the past. The divergence of the groups took place 0.69 million years ago with a 95% confidence interval of 0.39–1.06 million years. The divergence of haplotypes within the cluster of Khanka Lake group 1 occurred 0.41 million years ago with a 95% confidence interval of 0.25–0.64 million years. The divergence of haplotypes within the cluster of Khanka Lake group 2 occurred 0.44 million years ago with a 95% confidence interval of 0.22–0.69 million years. Populations of both species under study here have similar dates of the onset of their existence, which may indicate the common processes that influenced their dispersal or survival, as well as the formation of genetic population structure. Global climatic and geological changes, causing changes in the living conditions of this species, undoubtedly played an important role.

Currently, Khanka Lake group 1 and Khanka Lake group 2 of the *E. chankensis* are not geographically separated from each other. It is likely that the genetic differentiation is due to geographic isolation in the past. Significant changes in the structure of lake and river system in the Khanka basin and lake area during the alternating glacier and interglacial periods of the Pleistocene, as well as other geological rearrangements, can explain such subdivision (Korotky, 1989; Korotkii et al., 2007, 1980; Mikishin et al., 2007). The available reconstructions of the regional history based on the geological data (Korotky, 1989; Korotkii et al., 2007, 1980; Mikishin et al., 2007) suggest that a single water body appeared in the Khanka basin during the Middle Pleistocene, namely, the cold Shmakov period (Korotky et al., 2007) of 0.26–0.29 million years. Prior to that time, this territory was a network of river valleys with small lakes, which were gradually filled with water and expanded. In these previously isolated water bodies, highly differentiated genetic lineages of *E. chankensis* were likely formed. Later, simultaneous expansion of the lakes in the Khanka basin during the Middle Pleistocene (0.26–0.29 million years) formed a single water body with the modern appearance of the lake. Our dating shows (Fig. 2) that the separation of the *E. chankensis* groups occurred in the period before the formation of the present single water body. After the formation of the single, large lake and until now, *E. chankensis* has retained the imprint of the past geographical subdivision.

The historical reconstruction of change in the effective population size (history of change θ parameter) (Fig. 3) has shown that during the entire period of existence of *E. baikalensis* and the *E. chankensis* Khanka Lake group 1 showed the highest growth. The population of the *E. chankensis* Khanka Lake group 2 also showed an increase in the effective size, but on a much smaller scale (considering the logarithmic scale in the graphs). Unlike the *E. baikalensis*, in which the maximum growth rate of the effective size was in the period from 0.18 to 0.1 million years ago, both *E. chankensis* populations increased in size with the maximum rate in the period from 0.35 to 0.25 million years ago. By the present time, the effective sizes have stabilized.

The results of the population genetic calculations have shown (Table 3) that *E. baikalensis* and *E. chankensis* group 2 have the highest values of the θ_π parameter; the *E. chankensis* group 1 has a lower value of this parameter. The populations of *E. baikalensis* and *E. chankensis* group 1 have the maximum θ_s value. In all populations, the θ_π value is lower than the θ_s value, which indicates a violation in the character of neutral molecular evolution. For mitochondrial genomes, the parameter is $\theta = 2Ne\lambda$ (Ne is the number of females involved in breeding, and λ is the mutation probability). Because λ was a constant in the study (phylogeny supported the strict molecular clock hypothesis), θ changes and an increase in neutrality at the population level could be only due to abrupt changes in Ne . The Ne parameter is proportional to the effective population size; therefore, the shifts in θ_π and θ_s values are due to the change in the effective population size. Population tests

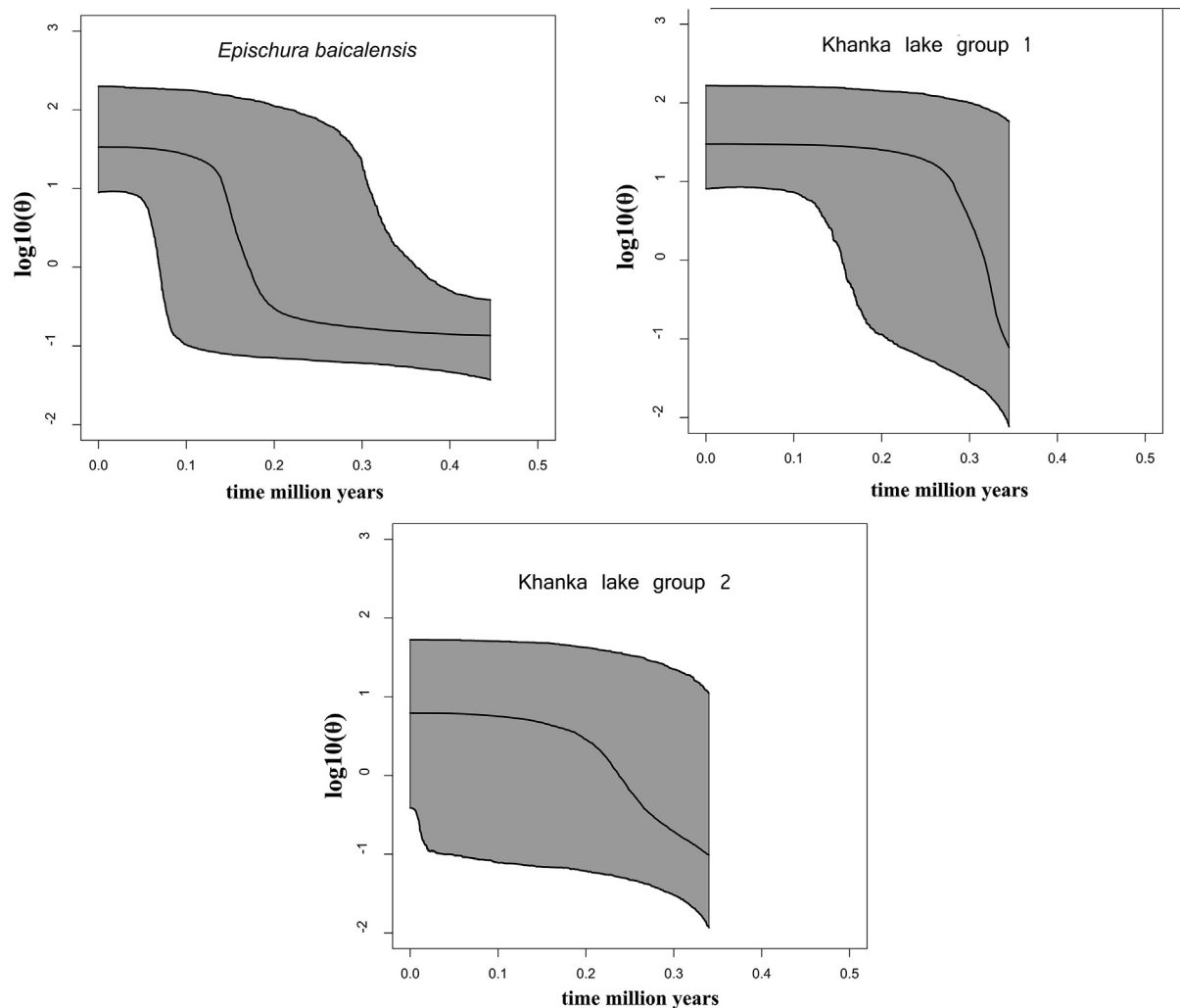


Fig. 3. Results of historical reconstruction of change in the population parameter θ (effective population size change) of *E. baicalensis* and *E. chankensis* (Khanka Lake group 1 and Khanka Lake group 2 populations) using Bayesian Skyline Plot. The grey area in the graphs shows 95% confidence interval for the estimated effective population size. The intermediate curve is the median value for θ . The parameter $\theta = 2Ne\lambda$, where Ne is the effective number of females, and λ is the probability of a mutation to a genetic marker per generations. Changes in θ are associated with changes in Ne .

Table 3
Results of the population genetic analysis.

Population name	Number of sequences	Number of haplotypes	TMRCAs strict clock	θ_π	θ_s	Tajima's D test	R2 test
Baikal	60	56	0.36 (0.19–0.95)	5.21	13.29	D = –2.07 P_value = 0.016	R = 0.034 P_value = 0.00
Khanka group 1	38	30	0.63 (0.40–1.00)	2.92	8.56	D = –2.31 P_value = 0.00	R = 0.028 P_value = 0.00
Khanka group 2	13	13	0.42 (0.24–0.80)	4.29	6.44	D = –1.42 P_value = 0.15	–

for *E. baicalensis* and *E. chankensis* group 1 confirm this fact. The statistic D values for them are significantly negative ($P < 0.05$) (Table 3), which indicates the expansion (abrupt increase in the number) or the action of directional selection. For the same populations (Table 3) the R2 test yielded a significant deviation from neutrality ($P < 0.05$) in favor of the population growth hypothesis. For *E. chankensis* group 2, population test did not deviate from the neutrality hypothesis during the formation of the population diversity. This may be due to the fact that the numbers of this population might have increased in the past but insignificantly. The *E. chankensis* group 1 has shown the highest relative differences between θ_π and θ_s , which probably indicate this group increased

the most in the past. The results of the population tests coincide with those obtained by the Bayesian Skyline Plot analysis. For the *E. chankensis* the past increase in the numbers is associated with the formation of the lake and a corresponding expansion of the ecological niche for this species.

An abrupt increase in the number of *E. baicalensis* is ambiguous because the modern shape of Lake Baikal has existed for more than 8 million years. Phylogenetic analysis has shown that *E. baicalensis* (or its ancestor) inhabited the lake throughout this time. At the same time, the population expansion of *E. baicalensis* occurred 0.18 to 0.1 million years ago. The number could also increase, if the population first declined due to the deterioration of the envi-

ronmental conditions (bottleneck) and, then, became normal, again expanding the ecological niche. Periodic climate cooling (glacial periods) could cause the abrupt decrease in the number of *E. baikalensis*. During the glacial periods, the inflow of nutrients to Lake Baikal was significantly reduced due to a decrease in river flow; consequently, the primary production in the pelagic zone of the lake was reduced. Unlike the other Baikal copepods, *E. baikalensis* was able to survive under these conditions, maintaining a minimum population size due to the ability to feed on various phytoplankton species as well as bacterioplankton, filtering large water volumes for food (Afanasyeva, 1998). *E. baikalensis* has a lipid storage mechanism to reduce dependence on the current nutritional conditions. Possibly, it can also maintain reproduction during the periods with low primary production (Bowman et al., 2017). Such biological features of *E. baikalensis* could play a significant role in the survival during global climate cooling. During warming and restoration of productivity in the pelagic zone of Lake Baikal, the number of *E. baikalensis* increased according to the population expansion scenario.

There could be also another mechanism, which caused the population expansion of *E. baikalensis*. Over the past 150 thousand years, the maximum depth of Lake Baikal have increased by more than 600 m due to raising the sides of the depression while lowering the bottom (Mats et al., 2001). At the same time, the ratio of the surface area of the lake to its water volume has reduced. This has decreased the water heating during summer as well as its average annual temperature. Under such conditions, *E. baikalensis*, as a psychrophilic species, may displace more thermophilic zooplankton species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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