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VARIABILITY OF NUCLEOTIDE SEQUENCES IN TWO GENE MARKERS (*COI* AND *H3*) OF *LITOROGAMMARUS KARADAGIENSIS* (GRINTSOV, 2009) (AMPHIPODA, GAMMARIDAE) AND THE SYSTEMATICS OF THE GENUS *LITOROGAMMARUS*

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A. Zolotova[1], V. Grintsov[2], V. Ryabushko[2], and Yu. Kartavtsev[1]

[1]A. V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS, Vladivostok, Russian Federation
[2]A. O. Kovalevsky Institute of Biology of the Southern Seas of RAS, Sevastopol, Russian Federation
E-mail: *anna.o.zolotova@gmail.com*

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We investigated intra- and interspecific variability of mitochondrial DNA cytochrome *c* oxidase subunit I gene (*COI*) and nuclear DNA histone *H3* gene in *Litorogammarus* representatives. Our results indicate that histone *H3* molecular marker is not suitable for species identification in this genus due to its insufficient sequence variability. The taxonomy of the studied species was refined based on the analysis of *COI* gene marker. Comprehensive analysis of genetic distances (*p*-distances) performed involving ABGD and ASAP algorithms suggested that *COI* gene fragment is a diagnostic marker for species identification in *Litorogammarus*. Phylogenetic reconstruction based on four methods and analysis of the resulting gene trees for *COI* revealed close genetic relationship between *Litorogammarus karadagiensis* and *L. glareophilus*. For these two species, we found several common morphological features: the presence of plumose setae on the mandibles, first maxillae, maxillipeds, and on the articles of the first and second gnathopods. The combined use of molecular and morphological data proved to be highly valuable for species identification and for resolving taxonomic issues within this group of amphipods.

Keywords: mtDNA, nDNA, *COI*, histone *H3*, *p*-distances, *Litorogammarus karadagiensis*, *Litorogammarus*, Gammaridae, Amphipoda

Litorogammarus karadagiensis (Grintsov, 2009) inhabits the splash zone of pebble-sand beaches. It was first described from the beaches off the Karadag (the Eastern Crimea), Batiliman, and the Laspi Bay (southern coast of the Crimea, the Black Sea) [Grintsov, 2009]. *L. karadagiensis* is the gammarid life form and the kryvophile ecological form (from Greek words 'kryvo' meaning 'to hide' and 'phileo' meaning 'to love') [Grintsov, 2023]. These animals form dense aggregations, up to 1,000 ind. *per* 1 m^2 in beach habitats with a substrate particle size of at least 5–6 mm. This species occurs year-round and is frequently found along with representatives of the genera *Chaetogammarus* Martynov, 1924 and *Pectenogammarus* Reid, 1940. In the paper [Grintsov, 2009], the members of these genera were mentioned as *Echinogammarus ischnus behningi* (Stebbing, 1899), *Echinogammarus foxi* (Schellenberg, 1928), and *Echinogammarus olivii* (H. Milne Edwards, 1830). To date, *E. ischnus behningi* is classified under the genus *Chaetogammarus*, while *E. foxi* and *E. olivii* are attributed to *Pectenogammarus* (https://www.marinespecies.org/). Molecular phylogenetic analyses [Copilaș-Ciocianu et al., 2025; Marin & Palatov, 2021; Marin et al., 2023] indicate that *Litorogammarus karadagiensis* is a close relative to several recently described species: *Litorogammarus mazestiensis* (Marin & Palatov, 2021), *Litorogammarus dursi* Marin, Palatov & Copilaș-Ciocianu, 2023, *Litorogammarus samuricus* Palatov, Copilaș-Ciocianu & Marin, 2025, and *Litorogammarus glareophilus* Marin, Palatov & Copilaș-Ciocianu, 2025. Its range covers the entire Ponto-Caspian region where adaptive radiation of gammaroids has occurred [Grintsov, 2023]. This evolutionary radiation exhibits remarkable morphological and ecological diversity, comparable to unique amphipod radiations reported for the Lake Baikal [Copilaș-Ciocianu, Sidorov, 2022].

L. karadagiensis (the species originally described as *Echinogammarus karadagiensis* [Grintsov, 2009]), is now classified within the genus *Litorogammarus* [Marin et al., 2023], the family Gammaridae Latreille, 1802, and the superfamily Gammaroidea Latreille, 1802. Molecular markers have prompted a reassessment of the systematic position of the family Gammaridae [Copilaș-Ciocianu, Sidorov, 2022]. The phylogenetic analysis of four molecular markers (*COI*, *28S* rRNA, *18S* rRNA, and histone *H3*) revealed a new position for the monophyletic superfamily Gammaroidea *Echinogammarus* s. l. among the Ponto-Caspian gammarids [Copilaș-Ciocianu et al., 2020]. This arrangement contrasted with the results obtained in previous morphology-based phylogenetic analysis [Lowry, Myers, 2013]. The conducted molecular genetic analysis provided strong evidence for establishing the genus *Litorogammarus* [Marin et al., 2023].

The aim of this study was to test the hypothesis about the species affiliation of a gammarid sample to a previously described *Litorogammarus karadagiensis* based on analysis of nucleotide sequence variation in the mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (*COI*) genetic marker and the nuclear DNA (nDNA) histone *H3* genetic marker. Histone *H3* marker, which was informative for distinguishing *L. mazestiensis* and *L. karadagiensis* [Copilaș-Ciocianu et al., 2023], and the *COI* marker, known for its diagnostic significance in amphipod identification [Copilaș-Ciocianu et al., 2022], were selected to achieve the aim of this work.

MATERIAL AND METHODS

We studied six *L. karadagiensis* specimens [Grintsov, 2009] sampled in Batiliman (southern coast of the Crimea, N44°25′12″, E33°41′48″) on 05.06.2022. The material was sampled in the splash zone of a pebble-sand beach. Sequences from the genetic database GenBank NCBI (https://www.ncbi.nlm.nih.gov/) were additionally used for the analysis.

DNA was extracted from all the samples using the DNA-Extran kit (DNA-Extran-2, Syntol, Moscow, Russia) according to the manufacturer's protocol and stored at −20 °C. Amplification for the mtDNA *COI* marker and the nDNA histone *H3* marker was performed using universal primers (Table 1) according to the protocol as follows: the initial denaturation at +94 °C (5 min); 35 cycles including denaturation at +94 °C (30 s), primer annealing at +45 °C (30 s), and elongation at +72 °C (60 s); and a final cycle at +72 °C (10 min). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, the USA) in accordance with the manufacturer's protocol. Nucleotide sequencing was carried out on an ABI 3500 Genetic Analyzer (Applied Biosystems).

The obtained sequences were deposited in BOLD Systems (https://boldsystems.org/) and then in GenBank NCBI (Table 2). All available sequences of *Litorogammarus* representatives [Copilaș-Ciocianu et al., 2025] from GenBank NCBI were included in the analysis, with *Chaetogammarus ischnus* [Marin et al., 2023] selected as the outgroup. Following alignment, the sequence lengths were 638 base pairs (bp) for *COI* and 354 bp for histone *H3*.

Table 1. Primers used for amplification and sequencing of the genetic markers studied

Primer	DNA fragment	Sequence	Primer authors
LCO1490-JJ	*COI*	CHACWAAYCATAAAGATATYGG	Astrin, Stüben, 2008
HCO2198-JJ	*COI*	AWACTTCVGGRTGVCCAAARAATCA	Astrin, Stüben, 2008
H3F	Histone *H3*	ATGGCTCGTACCAAGCAGACVGC	Colgan et al., 2000
H3R	Histone *H3*	ATATCCTTRGGCATRATRGTGAC	Colgan et al., 2000

Table 2. List of *Litorogammarus karadagiensis* samples and accession numbers of DNA sequences deposited in BOLD Systems and GenBank NCBI databases

Litorogammarus karadagiensis	BIN number in BOLD Systems	Sequence for *COI* gene in GenBank NCBI	Sequence for histone *H3* gene in GenBank NCBI
01	BOLD:AGE7470	PV053540	PV053546
02	BOLD:AGE7470	PV053541	PV053547
03	BOLD:AGE7470	PV053542	PV053548
04	BOLD:AGE7470	PV053543	PV053549
05	BOLD:AGE7470	PV053544	PV053550
06	BOLD:AGE7470	PV053545	PV053551

Note: BIN stands for Barcoding Index Number, a unique identifier of a DNA barcode in BOLD Systems.

Sequences were analyzed for each genetic marker separately. Phylogenetic (gene) trees were reconstructed by the following methods: neighbor joining (NJ), maximum likelihood (ML), maximum parsimony (MP), and Bayesian analysis (BA). MEGA11 [Tamura et al., 2021] and MrBayes 3.2 [Ronquist, Huelsenbeck, 2003] software was used. Phylogenetic trees were constructed for each gene separately. For tree reconstruction, the most appropriate evolutionary models were calculated (jModelTest) [Posada, 2008]. Based on the Akaike information criterion, the optimal evolutionary models were the Tamura–Nei ones [Tamura, Nei, 1993]: TrN + G for *COI* and TrN for histone *H3*. For BA reconstruction, runs were considered to have converged when the standard deviation of split frequencies was below 0.01 [Ronquist, Huelsenbeck, 2003]. The number of generations was set to 2,000,000, with the first 25% discarded as "burn in." Samples were taken for every 100th generation.

For species identification based on molecular sequences, the automatic barcode gap discovery procedure was applied in software packages ABGD (Automatic Barcode Gap Discovery, https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) [Puillandre et al., 2012] and ASAP (Assemble Species by Automatic Partitioning, https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) [Puillandre et al., 2021].

RESULTS

Analysis of genetic distances (*p*-distances) ([Nei, Kumar, 2000], p. 33) confirmed the presence of specimens representing five species within the studied sample: *L. dursi*, *L. glareophilus*, *L. mazestiensis*, *L. samuricus*, and *L. karadagiensis*. Interspecific *p*-distances ranged within 13–19.5% (*COI*) (with the exception for *L. mazestiensis* and *L. samuricus*, $p = 4.3\%$). Between our specimens and *L. karadagiensis* from GenBank NCBI, *p*-distance was 1.5% (*COI*) (Table 3), which falls within a range of intraspecific *p*-distances. No differences were detected for histone *H3* gene.

Based on *COI* sequences, both software packages, ABGD and ASAP, allowed revealing four species-level clusters for all the analysis settings: 1) *L. dursi*; 2) *L. glareophilus*; 3) *L. karadagiensis*; 4) *L. mazestiensis* + *L. samuricus*. Histone *H3* gene sequences available for analysis, those for *L. karadagiensis* and *L. mazestiensis*, were grouped into a cluster.

Table 3. Inter- and intraspecies *p*-distances for *COI* genetic markers studied

	LD	*LG*	*LK*	*LK* (our specimens)	*LM*	*LS*	*CI*
LD	**0.0063**	0.0153	0.0153	0.0153	0.0148	0.0147	0.0160
LG	0.1951	**0.0026**	0.0135	0.0134	0.0147	0.0146	0.0165
LK	0.1803	0.1348	**0.0000**	0.0046	0.0143	0.0144	0.0159
LK (our specimens)	0.1787	0.1301	0.0141	**0.0000**	0.0144	0.0144	0.0159
LM	0.1839	0.1708	0.1765	0.1718	**0.0147**	0.0070	0.0158
LS	0.1740	0.1661	0.1689	0.1665	0.0428	**0.0104**	0.0162
CI	0.2241	0.2124	0.2038	0.2053	0.2044	0.2038	**n/c**

Note: below the bold-highlighted diagonal, there are the values of interspecific *p*-distances for *COI*; along the diagonal, there are the values of intraspecific *p*-distances; above the diagonal, there are the values of standard errors for *COI*. *LD*, *Litorogammarus dursi*; *LG*, *Litorogammarus glareophilus*; *LK*, *Litorogammarus karadagiensis*; *LM*, *Litorogammarus mazestiensis*; *LS*, *Litorogammarus samuricus*; *CI*, *Chaetogammarus ischnus*. N/c, not computable.

Phylogenetic reconstructions (BA, ML, NJ, and MP) showed identical topologies for *COI* gene. In the resulting dendrograms, the studied specimens formed five clades with high node support values. These values were 100% for four species. The exception was the *L. samuricus* clade (BA = 63%; ML = 66%; NJ = 97%; and MP = 70%). The clades *L. mazestiensis* + *L. samuricus* and *L. glareophilus* + *L. karadagiensis* formed sister groups, with *L. dursi* as an external clade (Fig. 1).

None of the phylogenetic methods allowed distinguishing the species *L. karadagiensis* and *L. mazestiensis* using histone *H3* gene sequences. The dendrograms showed a random distribution of the studied specimens, with no discernible clade formation.

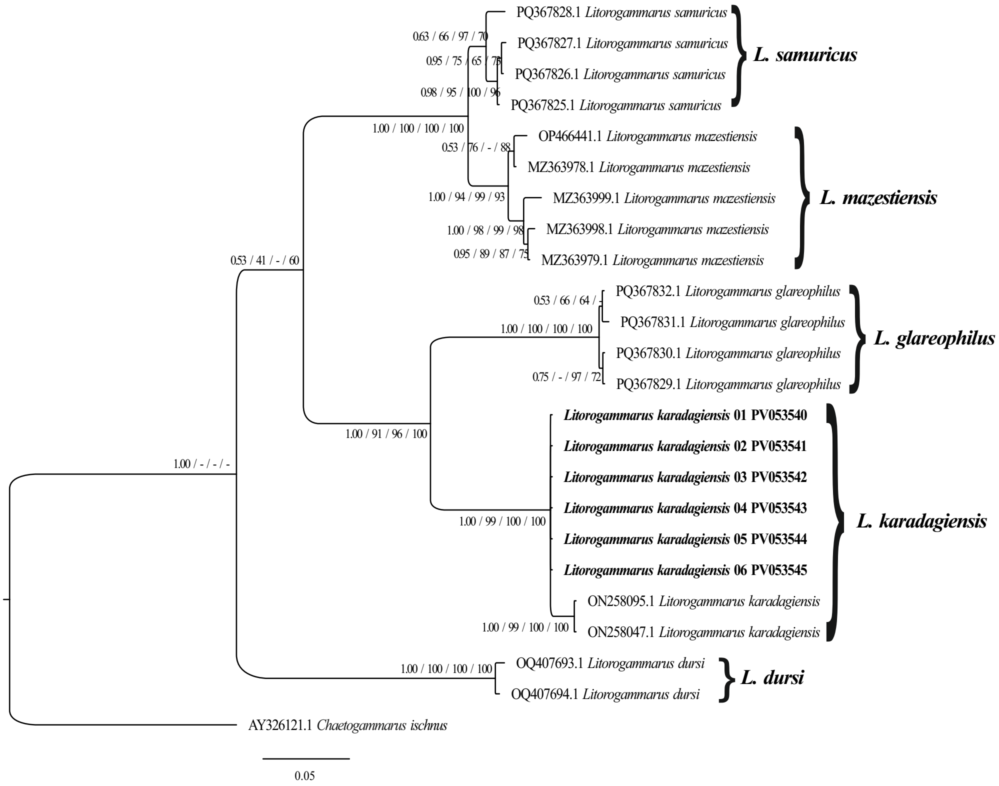

Fig. 1. Phylogenetic relationships among representatives of the genus *Litorogammarus* reconstructed based on *COI* gene sequences. Nodal support values (BA, Bayesian posterior probabilities; ML, maximum likelihood bootstrap; NJ, neighbor-joining bootstrap; MP, maximum parsimony bootstrap) are indicated in the following order: BA / ML / NJ / MP. Our six sequences are highlighted in bold

DISCUSSION

Based on the results of phylogram analysis, calculation of *p*-distances, and analysis involving ABGD and ASAP software packages, no differences were revealed between the histone *H3* gene sequences of the studied samples. Consequently, this nDNA region lacks sufficient resolution for species identification within the genus *Litorogammarus*.

The results of phylogenetic analysis based on the mtDNA *COI* gene nucleotide sequences confirmed the differentiation of *Litorogammarus* species into several clades identified earlier [Copilaș-Ciocianu et al., 2025]. Furthermore, the gene trees show distinct clusters for *L. mazestiensis* and *L. samuricus* representatives supported by topological differentiation. The tree topologies suggest that these species are evolutionarily young, as their clades diverged more recently than clades of *L. glareophilus* and *L. karadagiensis*. *L. mazestiensis* and *L. samuricus* may be young species. However, despite the high support values for tree topologies in both this study (see Fig. 1) and the paper [Copilaș-Ciocianu et al., 2025], the data on *p*-distance analysis and ABGD and ASAP analyses do not support *L. samuricus* identification as a separate species. This conclusion follows from the low interspecific variability between *L. mazestiensis* and *L. samuricus* (4.3%). Interspecific values for other *Litorogammarus* representatives are 10% higher. It may be more appropriate to consider *L. samuricus* as a subspecies of *L. mazestiensis*. Such a conclusion would require a thorough morphological analysis of a sufficiently large sample (meeting statistical requirements), focusing on trait variability to clarify taxonomic status. Currently, the suggested diagnosis [Copilaș-Ciocianu et al., 2025] is the only one distinguishing *L. samuricus* and *L. mazestiensis*. All the differences concern the armament of appendages or body parts and certain proportions. Research on the closely related genus *Pectenogammarus* Reid, 1940 [Grintsov, 2009] has shown that the armament of appendages and body parts (spines, setae, and denticles) are subject to individual variability. This may also apply to body proportions that are known to be variable [Guryanova, 1951].

L. karadagiensis specimens sampled from the species type locality form the closest clades on the phylograms (Fig. 1). Those have minimal *p*-distances (Table 3), and are assigned to a single cluster by both ABGD and ASAP analyses. The sequences of these specimens are close to *COI* sequences of *L. karadagiensis* in GenBank NCBI: ON258095 and ON258047. All these evidences confirm their assignment to the same species-level taxon, which is supported by comparative data for the hierarchy of various taxa (see the concluding paragraph below). Our results reinforce that *L. karadagiensis* is a sister species to *L. glareophilus*.

The morphology of the two *Litorogammarus* species, *L. karadagiensis* and *L. glareophilus*, is pretty similar. Comprehensive morphological descriptions of *L. karadagiensis* are provided in [Grintsov, 2009, 2022], and of *L. glareophilus*, in [Copilaș-Ciocianu et al., 2025]. Unlike other representatives of the genus (*L. dursi*, *L. mazestiensis*, and *L. samuricus*), both *L. karadagiensis* and *L. glareophilus* possess long, plumose setae on the following mouthparts and pereopods: mandibles, first maxillae, maxillipeds, and the articles of the first and second gnathopods. The molecular genetic analysis corroborates the morphological similarity of *L. karadagiensis* and *L. glareophilus* thus suggesting these are sister species (Fig. 1).

According to [Copilaș-Ciocianu et al., 2025], there are few distinguishing morphological traits between these species. *L. karadagiensis* is described as having a small sub-marginal spine on the first epimeral plate, whereas *L. glareophilus* lacks this spine. On the second epimeral plate, *L. karadagiensis* is reported to possess one sub-marginal spine, while *L. glareophilus* possesses two. However, the study [Grintsov, 2024] emphasizes that the armament of epimeral plates with spines and setae exhibits significant individual variability, rendering these traits unreliable for distinguishing the two species. As noted in [Copilaş-Ciocianu et al., 2025], *L. karadagiensis* has a nearly rounded basipodite on the fifth pereopod, whereas in *L. glareophilus*, the basipodite is narrower and more elongated. This distinction is corroborated by *L. karadagiensis* analysis. Differences in the armament of urosomite 2 are also mentioned [Copilaş-Ciocianu et al., 2025]. Thus, *L. karadagiensis* is reported to have two marginal spines and one lateral spine, while *L. glareophilus* has two marginal spines and two lateral spines. However, research on the armament of two *Pectenogammarus* representatives [Grintsov, 2024] showed that the armament of all urosomal segments is also subject to individual variation and cannot serve as a reliable species marker. Consequently, only one trait remains consistently diagnostic: the shape of the basipodite of the fifth pereopod.

In a summary of genetic distances for more than 20,000 species of vertebrates and invertebrates, *p*-distances for the mtDNA *COI* and *Cyt-b* genes were determined for five hierarchical groups: populations within a species (1); taxa of various ranks, including subspecies and semispecies (2); congeneric species (3); confamilial species (4); and species from different families within the same order (5). The mean unweighted *p*-distances for these five groups are:

- *Cyt-b* for (1), 1.46 ± 0.34; for (2), 5.35 ± 0.95; for (3), 10.46 ± 0.96; for (4), 17.99 ± 1.33; for (5), 26.36 ± 3.88;
- *COI* for (1), 0.72 ± 0.16; for (2), 3.78 ± 1.18; for (3), 10.87 ± 0.66; for (4), 15.00 ± 0.90; for (5), 19.97 ± 0.80.

Notably, the values for subspecies and semispecies are 5.35 ± 0.95 (*Cyt-b*) and 3.78 ± 1.18 (*COI*), while the values for species are 10.46 ± 0.96 (*Cyt-b*) and 10.87 ± 0.66 (*COI*) [Kartavtsev, 2009a]. This is consistent with our findings regarding the taxonomic status of *L. samuricus*. The results of our nucleotide divergence analysis are in line with other data on genetic divergence across taxonomic hierarchies, including non-protein coding genetic markers and complete mitochondrial genomes [Kartavtsev, 2021; Kartavtsev, Masalkova, 2024; Redin, Kartavtsev, 2022], thereby supporting the taxonomic inferences presented in this article. Moreover, the data leads to the general conclusion that at the molecular level, phyletic evolution predominates in the animal world, where speciation primarily follows geographic speciation model [Kartavtsev, 2009a, b, 2011a, b, 2013, 2021].

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ИЗМЕНЧИВОСТЬ НУКЛЕОТИДНЫХ ПОСЛЕДОВАТЕЛЬНОСТЕЙ МАРКЕРОВ ДВУХ ГЕНОВ (*CO-1* И *H3*) *LITOROGAMMARUS KARADAGIENSIS* (GRINTSOV, 2009) (AMPHIPODA, GAMMARIDAE) И СИСТЕМАТИКА РОДА *LITOROGAMMARUS*

А. О. Золотова[1], В. А. Гринцов[2], В. И. Рябушко[2], Ю. Ф. Картавцев[1]

[1]Национальный научный центр морской биологии имени А. В. Жирмунского ДВО РАН,
Владивосток, Российская Федерация
[2]ФГБУН ФИЦ «Институт биологии южных морей имени А. О. Ковалевского РАН»,
Севастополь, Российская Федерация
E-mail: *anna.o.zolotova@gmail.com*

Исследована внутри- и межвидовая изменчивость гена субъединицы I цитохромоксидазы *c* митохондриальной ДНК (*Co-1*) и гена гистона *H3* ядерной ДНК представителей рода *Litorogammarus*. Показано, что молекулярный маркер гена гистона *H3* не подходит для видовой идентификации *Litorogammarus* ввиду своей недостаточной изменчивости. Таксономия изученных видов рода уточнена на основании анализа молекулярного маркера гена *Co-1*. Результаты комплексного анализа генетических расстояний (*p*-расстояний), выполненного с помощью ABGD и ASAP, подтверждают значимость участка гена *Co-1* как маркера для идентификации видов рода *Litorogammarus*. На основе информации для четырёх филогенетических реконструкций и анализа топологии соответствующих генных деревьев для *Co-1* выяснена генетическая близость видов *L. karadagiensis* и *L. glareophilus*. Для этих двух видов выявлены общие морфологические признаки — перистые щетинки на мандибулах, максиллах первой пары, максиллипедах и члениках гнатопод первой и второй пар. Использование молекулярных и морфологических данных в интегративном подходе представляется перспективным для идентификации видов и для решения таксономических вопросов в пределах анализируемой группы амфипод.

Ключевые слова: мтДНК, яДНК, *Co-1*, гистон *H3*, *p*-расстояния, *Litorogammarus karadagiensis*, *Litorogammarus*, Gammaridae, Amphipoda