

# Analysis of Partial Mitochondrial COI and 16S rRNA Gene Sequences Variation of *Pagurus brachiomastus* and *Pagurus proximus* (Decapoda: Paguridae) Populations from the South of Russian Far East

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## Abstract

Mitochondrial COI and 16S rRNA gene partial sequences were generated for *Pagurus brachiomastus* from two locations and *P. proximus* from single location of the south of Russian Far East. Genetic variation and differentiation of *P. brachiomastus* Russian and South Korean (GenBank data) populations were estimated and compared on the basis of sequence data on two mitochondrial genes separately. Additionally, *P. simulans* from South Korea was analyzed. Results show *P. brachiomastus* from two locations of the south of Russian Far East and South Korea and Korean *P. simulans* differs at intraspecific level based on molecular markers. Median-Joining analysis indicate presence of two main groups of haplotypes, representing *P. proximus* and *P. brachiomastus* + *P. simulans*, respectively. These data support that *P. brachiomastus* and *P. simulans* are conspecific. Mismatch distribution analysis showed population growth for *P. brachiomastus* at the south of Russian Far East. Sequence pairwise variations of both molecular markers appear differently in two far eastern samples of *P. brachiomastus*, that can be partially explained by active gene flow through hermit crab migrations between two localities after their isolation.

## Introduction

*Pagurus brachiomastus* (Thallwitz 1891) is the coastal sublittoral hermit crab species of the genus *Pagurus* Fabricius, 1775, common for shallow waters of Japan, Korea and Russian Far East. This hermit crab represents a significant element of marine shallow water biotopes, which is interact closely with other marine benthos, forming symbiotic relationships or feed links. Moreover, this is a mass species – a feature that allow to use it for studies of microevolutionary processes of decapods. *Pagurus brachiomastus* was described from a single specimen from Japan; however,

the holotype was lost so far and the neotype was designated from material from Pacific coast of Hokkaido (Komai 2000). Two additional morphologically distinct species, *P. proximus* Komai, 2000 and *P. simulans* Komai, 2000, were distinguished from *P. brachiomastus* in Japan Sea with detailed morphological descriptions (Komai 2000). Recent studies on DNA barcoding of *Pagurus* species in South Korea showed that *P. brachiomastus* and *P. simulans* differs from each other at intraspecific level (Jung and Kim 2020) Authors of this study proposed *P. simulans* as subspecies of *P. brachiomastus*. and showed high intraspecific molecular diversity for *P. brachiomastus* in the South Korea. At the

present study we provide mitochondrial 16S rRNA and COI gene partial sequences of *P. brachiomastus* and *P. proximus* from shallow waters of south of Russian Far East and compared our data with Korean material on *P. simulans* from GenBank to estimate intraspecific variation of *P. brachiomastus* on larger territory and interspecific molecular diversity of three above-mentioned species with taxonomical interpretation.

## Material and Methods

### Samples Collection

Two species of the genus *Pagurus*: *P. brachiomastus* (33 specimens), *P. proximus* (7 specimens) were collected from Kievka bay and Vostok Bay in the south of Russian Far East (Table 1). First morphological identification of the species was carried out according to the description given by Komai (2000). Tissue samples were fixed in 96% ethanol by 24 hour with following ethanol refresh.

### DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from muscle tissue of claws and walking legs using phenol-chloroform extraction technique (Sambrook et al. 1989). Amplification of COI gene fragment was performed using polymerase chain reaction with primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TT 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AA 3'), as described earlier (Folmer et al., 1994). Amplification of the 16S gene fragment was performed using the polymerase chain reaction with primers 16S ar (5' CGCCTGTTTATCAAAAACAT 3') and 16S br (5'CCGGTCTGAFCTCAGATCACGT 3'), as described previously (Schubart et al. 2000). PCR contamination control was performed by including negative controls alongside positive controls, using both primers. PCR products were directly sequenced using the ABI Big Dye Terminator v.3.1 Cycle Sequencing kit (as instructed by manufacturer) using primers 16S ar and 16S br. Reading of the sequence products was performed with an ABI

3130xl genetic analyzer at the Department of Cell Biology and Genetics, FEFU. The resulting sequences were submitted to the GenBank under the accession numbers presented in Table 1.

### DNA Assembling, Alignment and Analysis

The sequences were initially assembled with SeqScape v.2.6 software and aligned using MEGA 7.1 alignment explorer with default options (Kumar et al. 2016). Genetic divergence was estimated by calculating the genetic *p*-distances. Genetic variation and differentiation parameters and mismatch distribution analysis were performed with DnaSP v. 6 software (Rozas et al. 2017). Median-Joining reconstructions were generated with Network 10.2.0.0 software (www.flexus-engineering.com) using our data and the nucleotide sequences of mitochondrial COI and 16S rRNA genes from different species of the genus *P. brachiomastus*, *P. proximus* and *P. simulans* from the NCBI GenBank database (Table 1).

## Results

Mitochondrial COI gene fragment 522 bp in length and 16S rRNA gene fragment 406 bp in length were generated for *P. brachiomastus* and *P. proximus* from Kievka Bay and Vostok Bay, Primorsky Region, south of Russian Far East (Table 1). Additional data on these species and *P. simulans* from Korea and Japan were used from Gene Bank database (Jung and Kim 2020).

### Mitochondrial COI Gene-based Analysis

Genetic *p*-distance value between *P. brachiomastus* and *P. simulans* by COI gene sequence data was  $1.39\pm 0.25\%$ , whereas between these two species and *P. proximus* *p*-distance values were considerably higher, representing  $9.014\pm 1.2\%$  and  $8.86\pm 1.2\%$ , respectively. Intraspecific *p*-distance values for *P. brachiomastus*, *P. simulans* and *P. proximus* were  $1.55\pm 0.25\%$ ,  $1.18\pm 0.29\%$  and  $0.44\pm 0.17\%$ , respectively.

**Table 1.** List of taxa incorporated into analysis

Species	N	Location	Author	GenBank Accession number	
				COI gene	16S rRNA gene
<i>Pagurus brachiomastus</i>	19/8	Kievka Bay, Primirsky Region, Russia	Original data	JN590063 - JN590080	KX037064- KX037070, 83
<i>P. brachiomastus</i>	14/12	Vostok Bay, Primirsky Region, Russia	Original data	KC347543 - KC347556	KX037071 - KX037082
<i>P. brachiomastus</i>	7/7	South Korea	Jung & Kim, 2020	MN549986- MN549992	MN545761- MN545767
<i>P. brachiomastus</i>	1/-	Japan: Hokkaido, Akkeshi	Sultana et al., 2017 (unpublished)	LC222530	
<i>Pagurus proximus</i>	2/7	Vostok Bay, Primirsky Region, Russia	Original data	KC347562 - KC347563	KX037057 - KX037063
<i>P. proximus</i>	8/8	South Korea	Original data	MN549993- MN550000	MN545768- MN545775
<i>Pagurus simulans</i>	8/8	South Korea	Jung & Kim, 2020	MN550001- MN550008	MN545776- MN545783

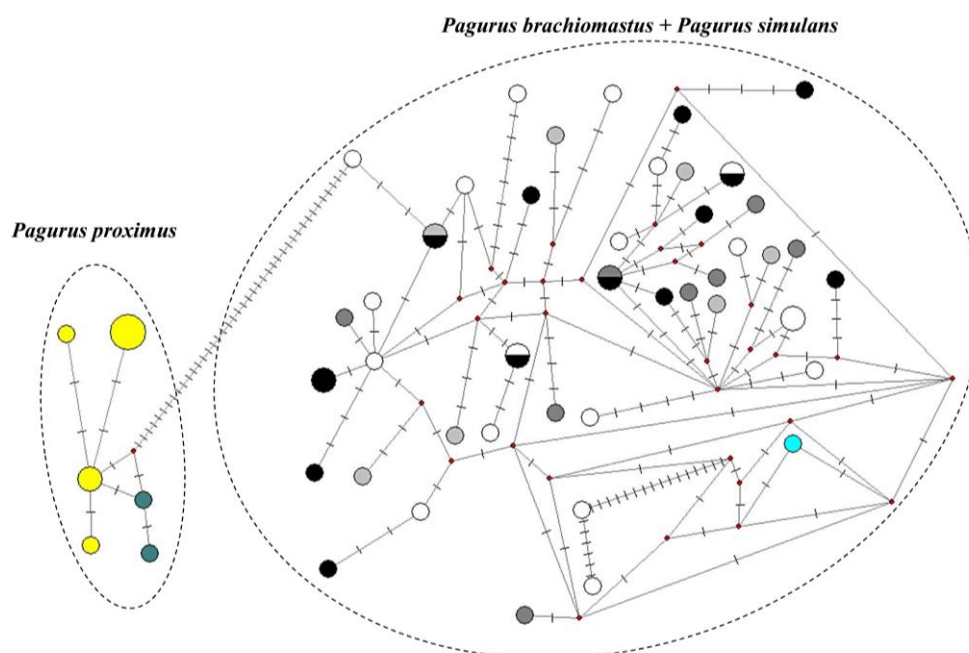
Genetic variation and differentiation parameters were calculated for sample of *P. brachiomastus* from two locations of south of Russian Far East and one from South Korea (Table 2). Samples from Kievka Bay and Vostok Bay possess similar haplotype diversity, whereas *P. brachiomastus* from Kievka Bay characterized by higher nucleotide diversity in comparison to specimens from Vostok Bay. This result supported by analysis of variable nucleotide positions along sequence (Figure 1). Sample from South Korea has similar values of Hd and Pi with sample from Vostok Bay. Genetic differentiation between two samples of *P. brachiomastus* from Russian Far East, revealed through p-distance calculation, was  $1.59 \pm 0.27\%$ , and Slatkin's differentiation index Fst was extremely low (0.00115, see Table 2). Parameter Nm was 216.47, indicating high level of gene flow between two studied communities of *P. brachiomastus*. Genetic p-distances between specimens from Korea and Vostok Bay were lower than between Korean sample and specimens from Kievka Bay. However, differentiation index Fst and gene flow parameter Nm between Korean specimens and two samples from south of Russian Far East were negative. Hudson et al. (1992) provide an

explanation for such result, stating that "the estimator is undefined for that sample". Constructively, negative Nm occurs because of estimate of average divergence time of genes sampled between subpopulations lesser than estimate of average divergence time of genes sampled within subpopulations. Another words, genetic divergence between Korean and Russian samples of *P. brachiomastus* lesser than within each one. On the other hand, divergence between two populations from south of Russian Far East higher than within each of these samples. Thus, it cannot be excluded that situation with Korean sample is a result of molecular convergence of mitochondrial COI gene.

Median-Joining analysis indicate presence of two main groups of haplotypes (Figure 1). The first group includes specimens of *P. proximus* from the Russian Vostok Bay and from South Korea; this group was star-like and contains six haplotypes with ancestral from Korea. Each of two specimens of *P. proximus* from Vostok Bay possess unique closely related haplotype. Second group contained all other analyzed hermit crabs, including *P. brachiomastus* from different localities of southern Russian Far East, South Korea and Japan and

**Table 2.** DNA polymorphism parameters and differentiation indexes of *Pagurus brachiomastus* local population based on partial nucleotide sequences of mitochondrial COI gene. N – number of specimens.

DNA polymorphism	N	h	Hd	Pi	K	d, %
Vostok	14	13	0.989±0.031	0.0154±0.00135	8.022	1.29±0.29
Kievka	19	19	1.0±0.017	0.0216±0.00375	11.287	1.87±0.3
South Korea	7	7	1.0±0.017	0.0141±0.00199	7.238	1.41±0.3
Molecular differentiation	N	d, %	Fst	Nm		
Vostok/Kievka	33	1.59±0.27	0.00115	216.47		
Vostok/S.Korea	21	1.28±0.24	-0.132	-38.37		
Kievka/S.Korea	26	1.51±0.25	-0.0073	-68.9		



**Figure 1.** Median-Joining network generated on the basis of mitochondrial COI gene partial sequences of three *Pagurus* species from Russian Far East, South Korea and Japan.

Korean *P. simulans*. This group characterized by high haplotype diversity and complex network structure without obvious ancestral haplotype being identified. *Pagurus brachiomastus* and *P. simulans* were difficult to be delimited on the COI gene-based MJ network topology. Single Japanese specimen of *P. brachiomastus* was relatively different from other individuals, possessing unique haplotype.

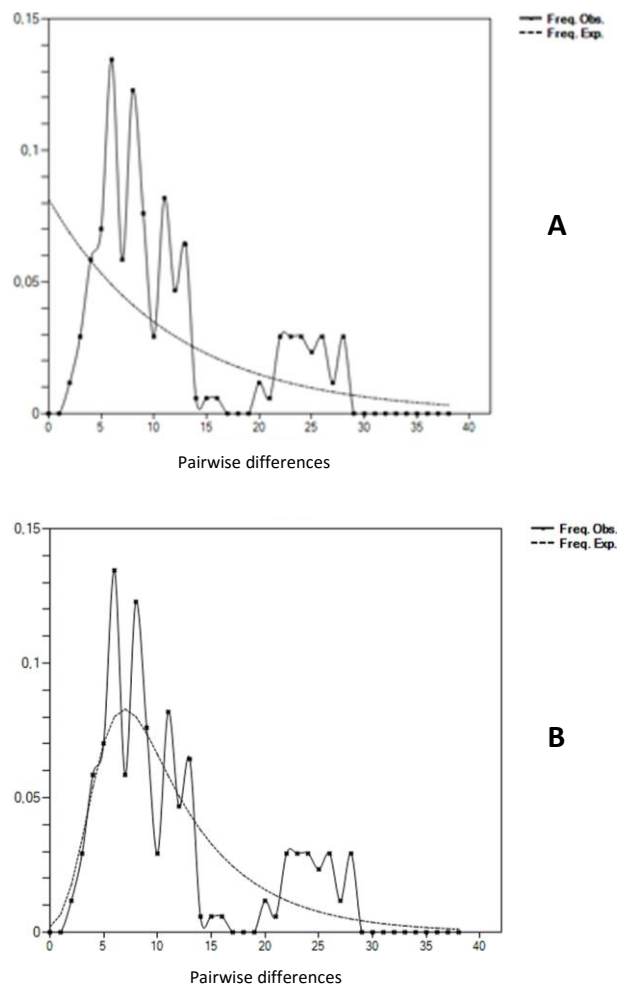
Mismatch distribution analysis for all local samples of *P. brachiomastus* showed multimodal distribution with trend towards population growth model (Figures 2-5). Mismatch distribution of the sample of *P. brachiomastus* from Kievka possesses a hiatus within range from 15 to 20 differences (Figure 2). Analysis of pairwise differences within the group "*P. brachiomastus* + *P. simulans*", revealed in the MJ network topology, showed distribution close to unimodal, corresponding to population growth model (Figure 5).

### Mitochondrial 16S Gene-based Analysis

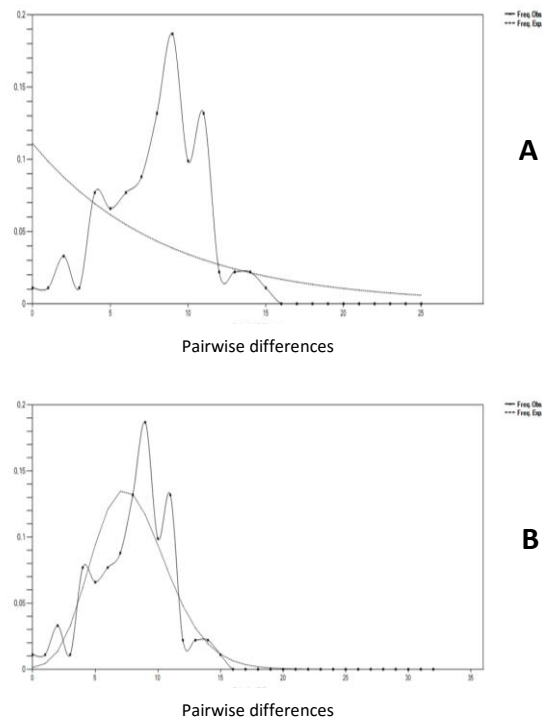
Genetic p-distance value between *P. brachiomastus* and *P. simulans*, calculated on the basis

of 16S rDNA sequences, was  $0.327 \pm 0.14\%$ , whereas between these two species and *P. proximus* p-distance values were considerably higher, representing  $3.67 \pm 0.85\%$  and  $3.62 \pm 0.86\%$ , respectively. Intraspecific p-distance values for *P. brachiomastus*, *P. simulans* and *P. proximus* were  $0.345 \pm 0.14\%$ ,  $0.31 \pm 0.17\%$  and  $1.1 \pm 0.25\%$ , respectively.

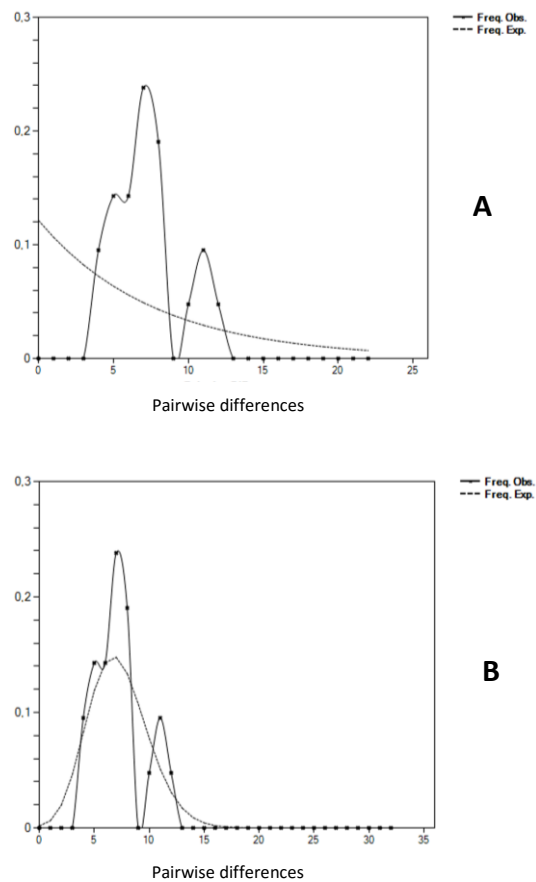
Samples of *P. brachiomastus* from Vostok Bay and Kievka Bay possess similar values of haplotype diversity (Hd) index, whereas Korean sample characterizes by lower value of this parameter (Table 3). Alongside this, nucleotide diversity of *P. brachiomastus* from Vostok Bay was in two times higher than that for *P. brachiomastus* from Kievka Bay and in almost five times higher relative to hermit crabs from Korea. Same rates of average number of nucleotide differences and p-distance values observed for these three samples (Table 3). Differentiation index and gene flow parameter for all three samples of *P. brachiomastus* were negative, being difficult to be interpreted well. Genetic p-distance values between samples from Vostok Bay and Kievka Bay as well as between Vostok Bay and South Korea were in two times higher relative to p-distance value



**Figure 2.** Results of mismatch distribution analysis based on mitochondrial COI gene sequence data for *P. brachiomastus* population from Kievka Bay, Russian Far East, for constant-size (A) and growth/decline (B) population models.



**Figure 3.** Results of mismatch distribution analysis based on mitochondrial COI gene sequence data for *P. brachiomastus* population from Vostok Bay, Russian Far East, for constant-size (A) and growth/decline (B) population models



**Figure 4.** Results of mismatch distribution analysis based on mitochondrial COI gene sequence data for *P. brachiomastus* population from South Korea for constant-size (A) and growth/decline (B) population models

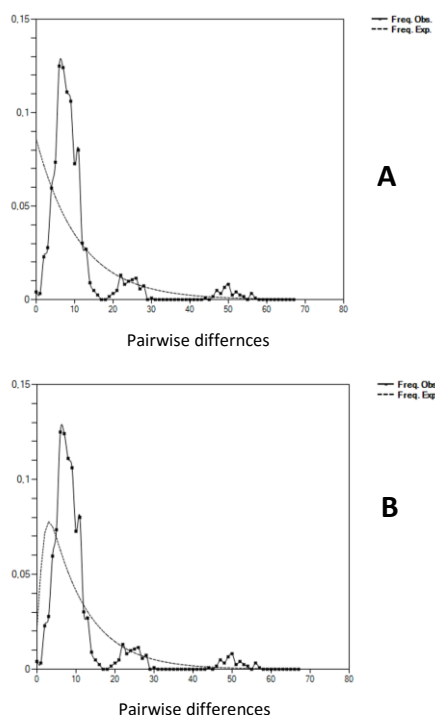
between samples from Kievka Bay and South Korea (Table 3).

Results of Median-Joining network analysis indicate obvious differentiation of *P. proximus* from other two *Pagurus* species. Specimens of *P. proximus* from Vostok Bay and South Korea formed star-like group of haplotypes, contained four haplotypes with one ancestral observed for five Korean and one Russian individuals. Hermit crabs of *P. brachiomastus* and *P. simulans* formed another group of haplotypes, containing two main haplotypes that differ from each other with one mutational step (Figure 6). Both haplotypes were observed for specimens of *P. brachiomastus* from Russian Vostok Bay, Kievka Bay, for specimens from South Korea and for Korean *P. simulans*. Several specimens of *P. brachiomastus* from Russian Vostok Bay, Kievka Bay and South Korea possess unique haplotypes. Single specimen, firstly identified as *P. proximus* from South Korea was close to one main haplotype of *P. brachiomastus* + *P. simulans*. For this reason, taxonomical status of this specimen is questionable.

Mismatch distribution of *P. brachiomastus* local samples was different, indicating unimodal distribution for Korean sample, slightly multimodal for sample from Vostok Bay and unimodal with a cavity at low-value area that is characteristic of bottleneck event (Figures 7 – 10). Despite these differences, all distributions most likely correspond to growth / decline population dynamics model. Group of species *P. brachiomastus* and *P. simulans*, revealed with MJ analysis, possesses a unimodal distribution of pairwise differences, which corresponds to the growth-decline population dynamics model (Figure 10).

## Discussion

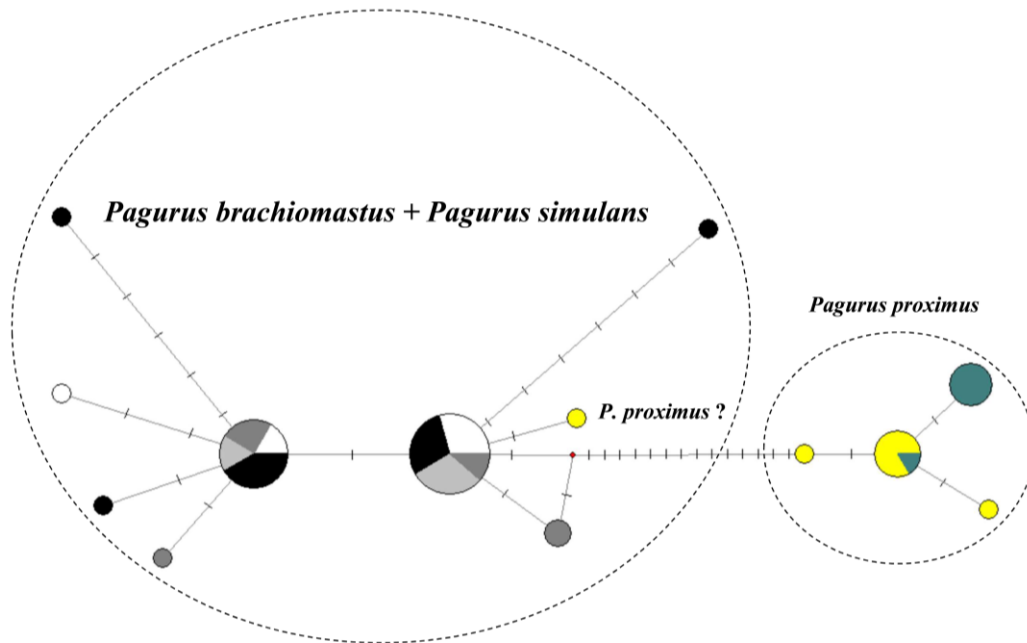
*Pagurus brachiomastus* was thought to be valid species at least at the coastal waters of Japan Sea, until Komai (2000) has separated two additional new species from this taxon, *P. proximus* and *P. simulans*, in the Russian Far East and Japan on the basis of results of detailed morphological analysis (Komai, 2000). Recent



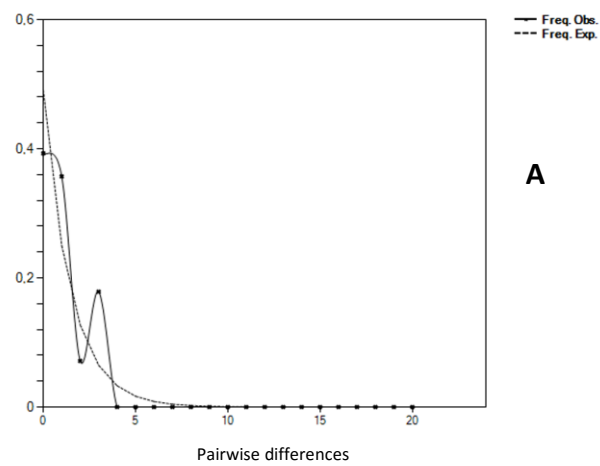
**Figure 5.** Results of mismatch distribution analysis based on mitochondrial COI gene sequence data for the group “*P. brachiomastus* + *P. simulans*”, revealed in the MJ network topology for constant-size (A) and growth/decline (B) population models

**Table 3.** DNA polymorphism parameters and differentiation indexes of *Pagurus brachiomastus* local population based on partial nucleotide sequences of mitochondrial 16S rRNA gene. N – number of specimens.

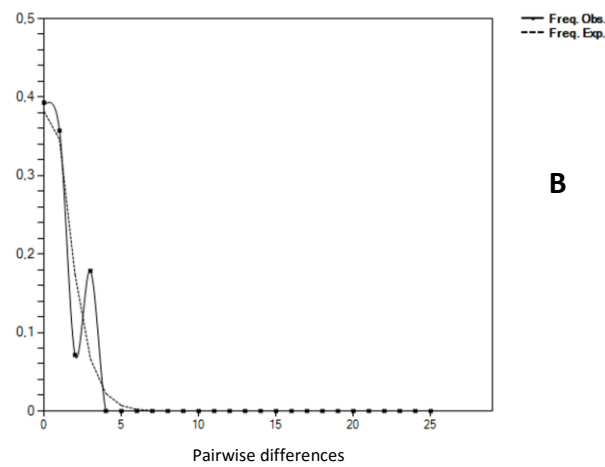
DNA polymorphism	N	h	Hd	Pi	K	d, %
Vostok	12	5	0.758±0.093	0.00548±0.002	2.212	0.548±0.176
Kievka	8	3	0.607±0.164	0.00256±0.001	1.036	0.256±0.159
South Korea	7	2	0.476±0.171	0.00118±0.0004	0.476	0.118±0.118
Molecular differentiation	N	d, %	Fst	Nm		
Vostok/Kievka	20	0.392±0.142	-0.02563	-10.00		
Vostok/S.Korea	19	0.33±0.136	-0.00812	-31.05		
Kievka/S.Korea	15	0.172±0.116	-0.8547	-3.18		



**Figure 6.** Median-Joining network generated on the basis of mitochondrial 16S gene partial sequences of three *Pagurus* species from Russian Far East, South Korea and Japan

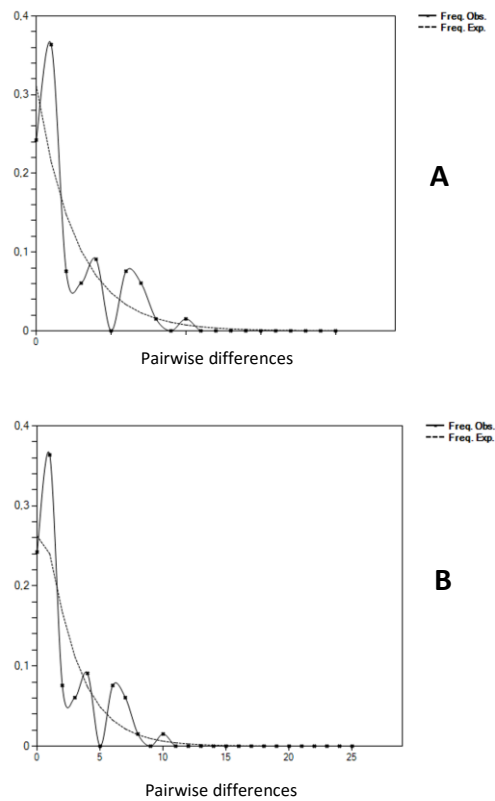


**A**

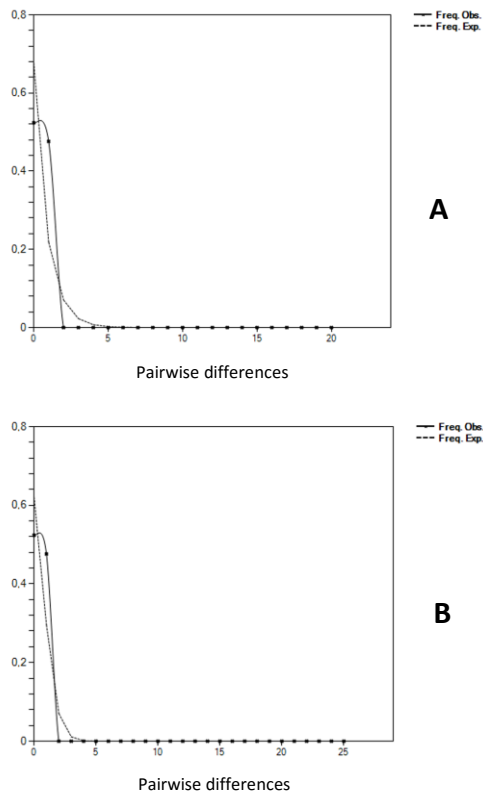


**B**

**Figure 7.** Results of mismatch distribution analysis based on mitochondrial 16S gene sequence data for *P. brachiomastus* population from Kievka Bay, Russian Far East, for constant-size (A) and growth/decline (B) population models



**Figure 8.** Results of mismatch distribution analysis based on mitochondrial 16S gene sequence data for *P. brachiomastus* population from Vostok Bay, Russian Far East, for constant-size (A) and growth/decline (B) population models



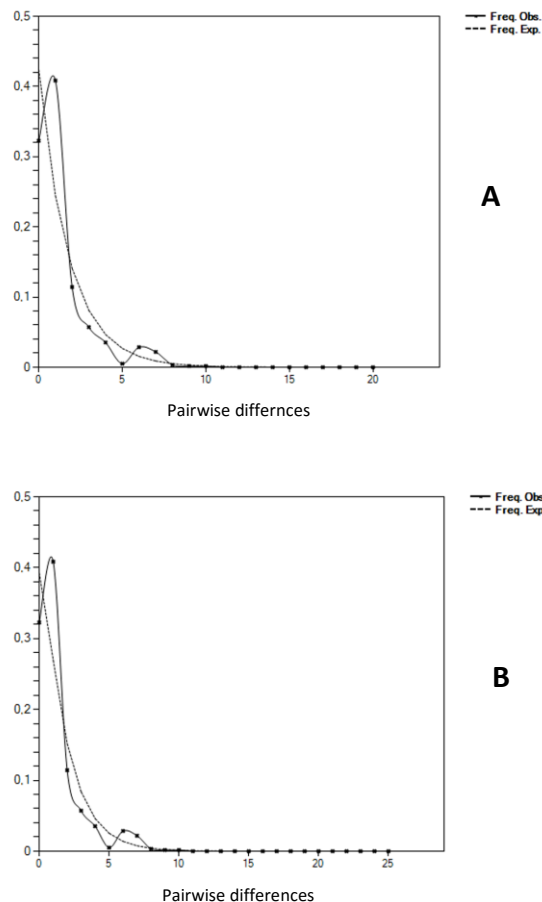
**Figure 9.** Results of mismatch distribution analysis based on mitochondrial COI gene sequence data for *P. brachiomastus* population from South Korea for constant-size (A) and growth/decline (B) population models



molecular studies indicate that *P. brachiomastus* and *P. simulans* from coastal waters of South Korea have insignificant differences from each other by COI and 16S mitochondrial DNA sequence data (Jung and Kim 2020). The last year study on diversity and molecular phylogeny of Pagurus species also showed an absence of clear differentiation of *P. brachiomastus* and *P. simulans* relative to other species of this genus (Sultana et al., 2022). Our results indicate, that *P. brachiomastus* from two locations of the south of Russian Far East and South Korea and Korean *P. simulans* differs at intraspecific level with both COI gene and 16S rDNA sequence data: genetic p-distances between samples of *P. brachiomastus* are compatible with that between *P. brachiomastus* and *P. simulans*. Additionally, results of median-joining analysis based on both COI gene and 16S rDNA sequences showed that specimens of both species form single homogenous group of haplotypes, whereas *P. proximus* formed separate haplotype group. Mismatch distribution analysis indicate unimodal distribution of pairwise differences for group, including *P. brachiomastus* and *P. simulans* that confirm absence of clear interspecific molecular differentiation between these hermit crabs as well. Moreover, Fst index between each pair of samples is close to zero, indicating absence

of significant differentiation between samples. Thus, our data confirm conspecificity of Far Eastern *P. brachiomastus* and *P. simulans*, revealed earlier (Jung and Kim 2020). Also, our data confirm validity of *P. proximus* from the south of Russian Far East and South Korea by p-distance estimation and results of MJ analysis on the basis of both COI and 16S rDNA mitochondrial gene sequence data.

Intraspecific genetic diversity of *P. brachiomastus* from two localities of the Russian Far East and from South Korea, revealed through estimation of COI and 16S rDNA mitochondrial gene sequences variation, characterized by relative complexity. Haplotype diversity of each sample of *P. brachiomastus* from COI gene sequences data is quite high and nucleotide diversity for sample from the Kievka Bay at two times higher in comparison with samples from Vostok Bay and South Korea (Table 2). On the other hand, haplotype diversity estimated on the basis of 16S rDNA gene sequence data, markedly lesser than that estimated with COI gene sequences data, highest Hd value revealed for the sample from Vostok Bay. Nucleotide diversity index has highest value in Vostok Bay sample as well. Same tendency for average pairwise differences (k) and internal p-distance (d) values observed for COI



**Figure 10.** Results of mismatch distribution analysis based on mitochondrial 16S gene sequence data for the group “*P. brachiomastus* + *P. simulans*”, revealed in the MJ network topology for constant-size (A) and growth/decline (B) population models

and 16S rRNA sequence dataset: highest values of these two parameters revealed for sample from Kievka Bay with COI gene sequence data and from Vostok Bay by 16S rRNA gene sequence data. These results can be explained by different variability of COI and 16S gene sequences, which was demonstrated in other population genetic studies of decapods (Marra et al. 2015; Jung and Kin 2020; Shahdadi et al. 2022). On the other view, sequence pairwise variations of these two molecular markers appear differently in two far eastern samples of *P. brachiomastus*, reflecting in highest values of nucleotide diversity and average pairwise differences indexes and p-distances for Kievka Bay sample revealed with COI gene sequence data and for Vostok Bay sample revealed with 16S rRNA gene sequence data. This feature demonstrated at the first time for decapods in the present study and it can be partially explained by active gene flow through hermit crab migrations between two localities after their isolation; high value of the gene flow index, calculated on the basis of COI gene sequence differentiation confirm this suggestion. Isolation is proposed by hypothetically different mutational rate for COI and 16S rRNA genes within hermit crab communities in isolated territories. However, this assumption has no strong basis to be confirmed unambiguously and should be clarified with additional studies on mitochondrial genes variation of hermit crab species from these regions.

### Ethical Statement

Not applicable.

### Funding Information

Not available

### Author Contribution

The author of this study performed all experimental works, data analyses and writing the MS.

### Conflict of Interest

The authors declare no conflict of interest and compliance with all relevant ethical standards. All

original molecular data are verified and can be approved with protocols and raw data.

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