Frontiers in Bioscience Scholar

Front. Biosci. (Schol Ed) **2023**; 15(4): 15
https://doi.org/10.31083/j.fbs1504015

Original Research

Characterization of the *Cambaroides wladiwostokiensis* Birstein & Vinogradov, 1934 (Decapoda: Astacidea) Mitochondrial Genome Using Genome Skimming and the Phylogenetic Implications within the Astacidea Infraorder

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Academic Editors: Khaleque N Khan and William Konigsberg
Submitted: 8 September 2023 Revised: 15 November 2023 Accepted: 24 November 2023 Published: 15 December 2023

Abstract

Background: The mitochondrial genome is a powerful tool for exploring and confirming species identity and understanding evolutionary trajectories. The genus *Cambaroides*, which consists of freshwater crayfish, is recognized for its evolutionary and morphological complexities. However, comprehensive genetic and mitogenomic data on species within this genus, such as *C. wladiwostokiensis*, remain scarce, thereby necessitating an in-depth mitogenomic exploration to decipher its evolutionary position and validate its species identity. **Methods**: The mitochondrial genome of *C. wladiwostokiensis* was obtained through shallow Illumina paired-end sequencing of total DNA, followed by hybrid assembly using both *de novo* and reference-based techniques. Comparative analysis was performed using available *Cambaroides* mitochondrial genomes obtained from National Center for Biotechnology Information (NCBI). Additionally, phylogenetic analyses of 23 representatives from three families within the Astacidea infraorder were employed using the PhyloSuite platform for sequence management and phylogenetic preparation, to elucidate phylogenetic relationships via Bayesian Inference (BI), based on concatenated mitochondrial fragments. **Results**: The resulting genome, which spans 16,391 base pairs was investigated, revealing 13 protein-coding genes, rRNAs (*12S* and *16S*), 19 tRNAs, and a putative control region. Comparative analysis together with five other *Cambaroides* mitogenomes retrieved from GenBank unveiled regions that remained unread due to challenges associated with the genome skimming technique. Protein-coding genes varied in size and typically exhibited common start (ATG) and stop (TAA) codons. However, exceptions were noted in ND5 (start codon: GTG) and ND1 (stop codon: TAG). Landscape analysis was used to explore sequence variation across the five available mitochondrial genomes of *Cambaroides*. **Conclusions**: Collectively, these findings reveal variable sites and contribute to a deeper understanding of the genetic diversity in this genus alongside the further development of species–specific primers for noninvasive monitoring techniques. The partitioned phylogenetic analysis of Astacidea revealed a paraphyletic origin of Asian cambarids, which confirms the data in recent studies based on both multilocus analyses and integrative approaches.

Keywords: mitochondrial genome; *Cambaroides wladiwostokiensis*; genome skimming; landscape variation; phylogenetics; partitioned analysis

1. Introduction

Molecular genetic techniques, such as polymerase chain reaction (PCR), DNA barcoding, and genomic sequencing, have significantly impacted the processes of species identification and classification. These techniques allow researchers to directly examine the genetic material of organisms, thereby providing a precise method to differentiate between closely related species, understand their evolutionary relationships, and perform accurate classifications [1–5]. Furthermore, they have transformed our understanding of mitochondrial genomes, allowing us to investigate their intricate details with unparalleled precision [6–10]. Within this context, the genus *Cambaroides* (Decapoda: Astacidea) has emerged as a focal point in research. Researchers are keen to use genetic markers to decipher the complex web of species identities and their interrelationships, thereby positioning this genus as a distinct avenue of study. Until now, only seven species have been described within this genus: the "Daurian crayfish" *C. dauricus* (Pallas, 1773), the "Schrenck's crayfish" *C. schrenckii* (Kessler, 1874), the "Korean crayfish" *C. similis* (Koelbel, 1892), the "Japanese crayfish" *C. japonicus* (De Haan, 1841), the "Sakhalin crayfish" *C. sachalinensis* (Birstein et Winogradow, 1934), the "Vladivostok crayfish" *C. wladiwostokiensis* (Birstein et Winogradow, 1934), and the "Kozhevnikov crayfish" *C. koshewnikowi* (Birstein

et Winogradow, 1934). However, their range is limited in the north by the Amur River basin, in the east by Sakhalin Island and the northern part of the Japanese islands, in the west by the lower Selenga River basin (Lake Baikal basin), and in the south by the southern part of the Korean Peninsula [11,12], in addition to personal observations. Notably, they also serve as intermediate hosts for the trematode Paragonimus westermani ichunensis, the causative agent of paragonimiasis—a severe parasitic disease [13].

These seven species of East Asian freshwater crayfish can be subdivided into three groups, each with unique ecological characteristics: The Daurian crayfish group (including Daurian, Japanese, Korean, and Vladivostok crayfish), which consists of stenobiotic rheophilic species that exclusively inhabit clean waters and can serve as indicators of a water body's purity; the Schrenk crayfish group (Schrenk's crayfish, Sakhalin crayfish), which are eurybiotic species, capable of inhabiting even polluted waters, small puddles, and swamps; Kozhevnikov's crayfish are ecologically distinct, are found only in the lower part of the Amur River—the estuarine zone—and are a stenobiotic species. Within the Daurian crayfish group, the Vladivostok crayfish (*Cambaroides wladiwostokiensis*) is a species with a narrow niche, meaning it requires special attention for its conservation within its range. This is particularly relevant considering its highly stressed state due to water body pollution, and its reduced ecological capacity for survival. Its range includes water bodies of the Sea of Japan basin from the northern part of the Korean Peninsula to the Black and Kievka rivers, situated north of Cape Povorotny. It is erroneously indicated to be present in the basin of the Mulinhè River in the territory of the People's Republic of China [11,14] through personal observations. Considering the fragmentation of the ranges of individual species, a revision of their taxonomic status is required. For this purpose, genetic analysis of various groupings is necessary as an auxiliary tool.

The use of genetic markers to delineate species boundaries within this genus holds promise for determining the taxonomic status of individual species. Moreover, it can reveal broader patterns of genetic variation and the evolutionary history among closely related taxa [11,15,16]. Such investigations underscore both the benefits and challenges of harnessing genetic data to distinguish species identities in a group marked by complex evolutionary trajectories and morphological similarities.

Freshwater crayfish have been proposed to form a monophyletic group closely related to clawed lobsters and are found on every continent except Antarctica [17]. From a taxonomic perspective, freshwater crayfish are divided into two monophyletic superfamilies: the northern hemisphere's Astacoidea and the southern hemisphere's Parastacoidea [17]. A comprehensive phylogenetic analysis, which encompassed representatives from 44 extinct and 27 extant crayfish families, including Polychelida, Achelata, Glypheidea, and Astacidea, culminated in the identification of a new superfamily—Glaessnericarioidea [11]. Additionally, three new families were recognized: Glaessnericaridae, Neoglypheidae, and Litogastroidae [11]. In another pivotal study, the debated relationships of major clades of reptant decapods were elucidated using a combined analysis of 16S, 18S, and 28S rRNA sequences, paired with morphological data [18]. The resulting optimal tree demonstrated that Glypheidea is the sister group to Astacidea. This relationship, in conjunction with the monophyletic Astacidea, which encompasses both freshwater crustaceans (Astacida) and marine clawed lobsters (Homarida), aligns with the findings of most previous studies.

Prior research into mitochondrial genomes has significantly contributed to our understanding of the evolutionary pathways of various species within the infraorder Astacidea [16–18]. Notably, these analyses have both reaffirmed the existence of conserved genetic elements and shone a light on structural variations, including gene rearrangements, thereby offering a deeper understanding of genome evolution processes [19].

Building on this foundation, the present study endeavors to validate the species identity of *C. wladiwostokiensis* through meticulous analysis of mitochondrial genetic markers. This involves elucidating the wider landscape of genetic diversity and evolutionary history within the genus and its sister lineages by leveraging mitochondrial genome sequences. The results of the genetic analysis will help to determine the place of *C. wladiwostokiensis* within the group of both the Daurian crayfish and East Asian River crayfish. Moreover, beyond the immediate taxonomic implications, our findings also have the potential to pave the way for more accurate species identification, which can improve the management of parasitic diseases linked with some members of this genus. Furthermore, by examining the mitochondrial genomes, we are laying a basis that can be instrumental for future conservation and management strategies, and for the broader understanding of evolutionary processes in freshwater crayfish.

2. Materials and Methods

2.1 Sample Collection, DNA Extraction, Sequencing, and Mitochondrial Genome Annotation

An individual *C. wladiwostokiensis* was captured in the area of Gerasimov Creek (Kievka River basin), Primorsky Krai, Russia, in May 2018. Species identification was conducted by leveraging descriptive data sourced from relevant literature [20–23]. After capture, the specimen was completely fixed in 95% ethanol. DNA extraction from the prefixed chela muscle tissue was performed using the "K-Sorb" kit (LLC "Sintol", Moscow, Russia). Total DNA sequencing was conducted on the Illumina NovaSeq 6000 platform (Novogen, Tianjin, China). Approximately 7.15 Gb of raw paired-end reads with a length of 150 bp were obtained. After using FastQC (version 0.12.0, https://

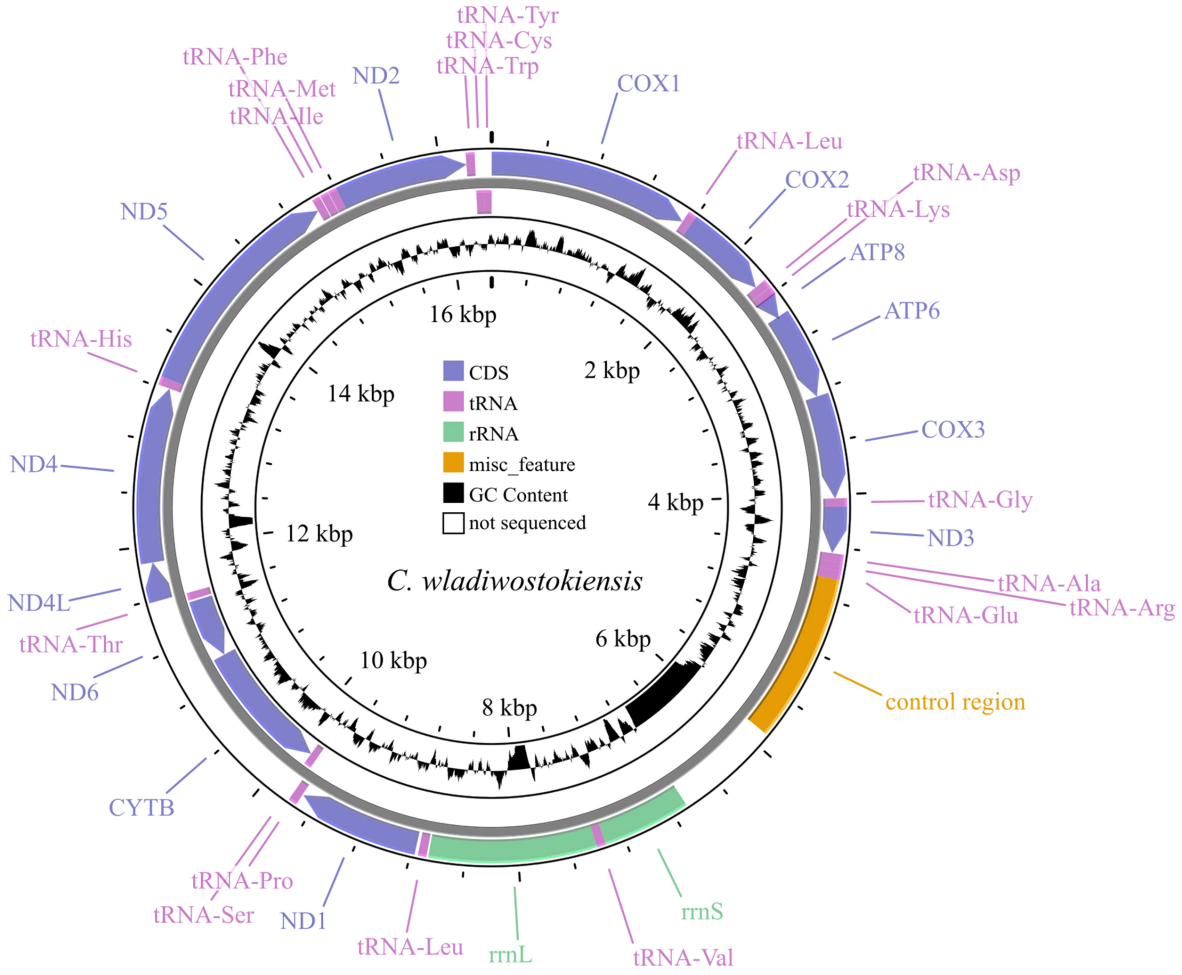

Fig. 1. Map of the *C. wladiwostokiensis* mitochondrial genome. Note that non-sequenced regions appear as "N" scaffolds, leading to GC view artifacts. Refer to Table 3 for details.

www.bioinformatics.babraham.ac.uk/projects/fastqc/) [24] to assess read qualities, AdapterRemoval (version 2.2.2, https://adapterremoval.readthedocs.io/en/stable/) [25] was employed to trim standard Illumina adapters. Mitochondrial genome assembly was performed using SPAdes (version 3.15.5, Saint Petersburg State University, Saint Petersburg, Russia) [26] and NOVOPlasty (version 4.3.3, https://github.com/ndierckx/NOVOPlasty) [27] in parallel since the preliminary runs of these assemblers did not yield the full expected genome length of the cyclic form. Initially, contigs were *de novo* assembled in SPAdes with default parameters and a kmer length of 21. A reference database was formed using the available complete mitochondrial genome sequences of *Cambaroides* representatives (Table 1, Ref. [16,19,28,29]) from GenBank (https://www.ncbi.nlm.nih.gov/). This reference was utilized to select the most homologous contigs from the SPAdes assembly to the target organism. Then, the selected contigs were used as seeds for the NOVOPlasty assembly, which can be considered reference-based. All contigs obtained in this manner, homologous to *Cambaroides* mitochondrial genomes, were aligned against *C. similis* and *C. dauricus* (considered to be the closest) using the MUSCLE algorithm [30] and implemented in MEGA (version 7, Mega Limited, Auckland, New Zealand) [31]. Manual curation was performed to form a consensus sequence.

Annotation of the obtained sequence was carried out using the MITOS Web Server [32] with reference sequences being manually cross-referenced. The annotated sequence was deposited in GenBank under accession number: OR353741. To estimate assembly parameters and exclude possible artifacts, we mapped reads onto both the newly assembled genome and the C. dauricus (OL542521) genome using Bowtie 2 (version 2.3.4.1, https://bowtie-bio.sourceforge.net/bowtie2/manual.shtml) [33], and then sorted and indented the reads in SAMtools (version 1.7, https://github.com/samtools/samtools) [34]. Coverage and assembly quality were also assessed using SAMtools (depth and flagstat functions). Visualization of reads per reference was performed in the Tablet alignment viewer version 1.21.02.08 [35]. Genome map visualization (Fig. 1) was conducted using the web-implemented CGView program [36], with a sliding window width of 50 bases.

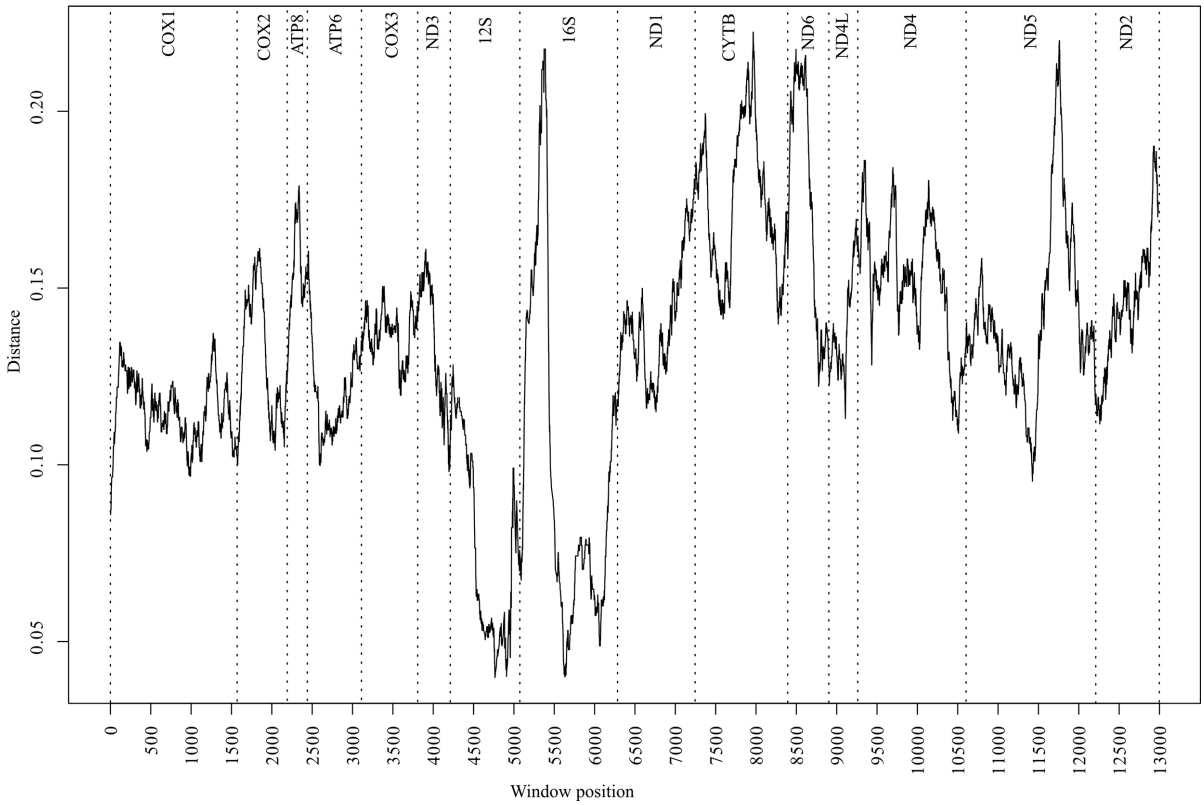

Fig. 2. Distribution of divergence values (*p*-distance) along the matrix of 15 mitochondrial genome fragments from five representatives in the genus *Cambaroides*. The analysis was conducted using the sliding window algorithm. Vertical dashed lines indicate fragment boundaries. Fragment names are provided at the top.

2.2 Confirmation of Species Identity for C. wladiwostokiensis

To confirm the species identity for the genetic material obtained from the specimen, an additional analysis was conducted on a 502 bp fragment of *COX1* and a 524 bp fragment of *16S* [12,19] from *Cambaroides* representatives; *A. astacus* was used as an outgroup. We selected these fragments because they are the only ones that constitute a reliable reference, which follows the results of studies that used an integrative approach in the systematics of this genus. Sequences for comparison were downloaded from GenBank. Alignment was performed by MUSCLE [30], and genetic *p*-distance calculations (Table 2) were conducted for species groups using MEGA [31]. In addition, we performed distance-based NJ-phylogenetic sequence analyses using MEGA.

2.3 Mitochondrial Genome Variation Landscape in Cambaroides

To identify variation landscapes of mitochondrial genome sequences of five *Cambaroides* representatives, a sliding window analysis was implemented in the Spider package (version 1.4-2, https://rdrr.io/rforge/spider/) [37]. The sequence matrix was concatenated from 15 fragments, totaling 13,288 bases. The control region was not included in the matrix due to its absence in *C. similis*. Unread regions in the sequenced fragments of *C. wladiwostokiensis* were substituted with "-". The window width was set to 500 bases with a 1-base interval. For each window, average genetic *p*-distances were calculated using the *dist.dna* function in the APE package version 5.5 [38]. Then, the distances were visualized (Fig. 2) using base R tools version 4.1.0 (https://cran.r-project.org/bin/windows/base/old/4.1.0/).

2.4 Phylogenetic Relationships of Cambaroides and the Position of the Genus in the Infraorder Astacidea

To define the position of *C. wladiwostokiensis* within the genus and of *Cambaroides* in the Astacidea infraorder, we conducted a phylogenetic analysis of 23 representatives spanning three families in this infraorder. Complete mitochondrial genome sequences for these representatives were sourced from GenBank (as detailed in Table 1). Parsing sequences, calculating basic statistics, aligning fragments, concatenating them into a supermatrix, and preparing them for phylogenetic analysis were carried out using the PhyloSuite platform version 1.1.14 (https://github.com/dongzhang0725/PhyloSuite/releases) [39,40]. Alignment of all fragments was performed using MAFFT version 7.505 [41] with default parameters. The concatenated matrix consisted of 15 fragments and encompassed 13,612 bases. Simultaneous determination of partitions and the selection of op-

Table 1. Species names, accession numbers, capture locations, and sources of comparative material used in this study.

Family	Species	NCBI accession numbers	Capture localities	Source
Astacidae	*Astacus astacus*	MT862440	-	GenBank
	Austropotamobius pallipes	KP205430	France: Lucelle, Alsace region	Grandjean *et al*., 2017 [16]
	A. torrentium	KX268734	Germany: Kammel, Bavaria	Grandjean *et al*., 2017 [16]
	Pacifastacus leniusculus	KX268740	United Kingdom: Greenwich Ecology Park, South London	Grandjean *et al*., 2017 [16]
Cambaroididae	*Cambaroides dauricus*	OL542521	Qingdingzi forest farm (Huinan County, Tonghua City, China)	Luo *et al*., 2023 [28]
	C. japonicus	KX268736	Japan: Bibai, Hokkaido	Grandjean *et al*., 2017 [16]
	C. schrenckii	KX268737	Russia: Southeast Russia	Grandjean *et al*., 2017 [16]
	C. similis	JN991196	ravine in the Gwanak Mountain in South Korea	Kim *et al*., 2012 [19]
	C. wladiwostokiensis	OR353741	Russia: Primorsky Krai: Kievka River basin: Gerasimov Creek	Original data (this study)
Cambaridae	*Cambarus robustus*	KX268738	USA: Oberlin, Ohio	Grandjean *et al*., 2017 [16]
	Orconectes rusticus	KU239994	USA	GenBank
	O. limosus	KP205431	France: Vonne, Poitou-Charentes region	Grandjean *et al*., 2017 [16]
	O. luteus	KX268739	USA: Fouche Renault, Missouri	Grandjean *et al*., 2017 [16]
	O. punctimanus	KX119150	Oriskany, Virginia, USA	GenBank
	O. sanbornii	KU239995	USA	GenBank
	Procambarus acutus	KX268741	USA: Prairie Fork Pond, Missouri	Grandjean *et al*., 2017 [16]
	P. alleni	KT074363	See ref	Vogt *et al*., 2015 [29]
	P. clarkii	OL542520	Yangtze River Fisheries Research Institute of Chinese Academy of Fishery Sciences	Luo *et al*., 2023 [28]
	P. clarkii	JN991197	pet market in Incheon, South Korea	Kim *et al*., 2012 [19]
	P. fallax	KT074364	See ref	Vogt *et al*., 2015 [29]
Parastacidae	*Cherax quinquecarinatus*	HG799091	Australia: Dunsborough, southwest Western Australia	GenBank
	Engaeus cunicularius	HG942173	Australia: Robbins Ck, South of Naracoopa, King Island, Tasmania	GenBank
	Geocharax gracilis	HG942174	Australia: Yaloak Ck, East of Panmure, Victoria	Grandjean *et al*., 2017 [16]

timal substitution models for them were performed using PartitionFinder version 2 [42], based on the Bayesian information criterion. Bayesian analysis (tree inference) using the previously determined scheme by PartitionFinder was conducted in MrBayes version 3.2.7 [43]. Tree topology searching and marginal posterior probability values were generated by two parallel runs of four Markov chains for 2,000,000 generations. The sampling frequency of topologies and parameters by the Metropolis-coupled algorithm was 1 per 1000 generations. The first 25% of trees corresponding to the burn-in step were discarded as non-optimal. A consensus tree was generated based on the remaining 3002 trees. Convergence indices (ESS, PSRF) indicated sufficient sampling across all parameters. The average standard deviation of split frequencies approached 0.000047 at the end of the run. Maximum likelihood analysis was performed in IQ-TREE [44] with simultaneous model selection for designated partitions and bootstrap support assessment using 50,000 ultrafast bootstrap replicates [45]. The Bayesian phylogeny was chosen as the basis for presenting the results of the phylogenetic analysis (Fig. 3).

3. Results

3.1 Mitochondrial Genome Assembly and Annotation

The assembled mitochondrial genome of *C. wladiwostokiensis* comprises 16,391 base pairs. We identified a total of 13 protein-coding genes, *12S* (rrnS) and *16S* (rrnL) rRNAs, 19 tRNAs, and a putative control region (Tables 3,4 and Fig. 1). Through alignment with other available *Camboroides* mitogenomes, we discovered regions that were not successfully sequenced and remain unretrievable from the raw reads obtained. These regions include a 784 bp fragment between the control region and rrnS, where tRNAs *Gln*, *Ser*, and *Asn* might be located (see Table 3 and [28]), 18 bases between tRNA-*Val* and rrnL, 154 bases between rrnL and tRNA-*Leu*, and 107 bases within the *ND4* gene. As a result, 1063 bases, or 6.5% of the genome remained unread. We did not detect any rearrangements in the genome. The non-sequenced regions did not have any reads to be covered with, and are not artifacts of assembly, as was proved by mapping the reads onto the genomes of *C. wladiwostokiensis* and *C. dauricus* (see **Supplementary Figs. 1,2**).

Table 2. Matrix of mean genetic *p*-distances calculated between *COX1* (bottom left) and *16S* (top right) sequence fragments from *Cambaroides* representatives.

Species names and GenBank accession numbers	*C. wladiwostokiensis*	*C. dauricus* OL542521, DQ666837	*C. similis* JN991196, DQ666841	*C. schrenckii* KX268737, DQ666835	*C. japonicus* KX268736, DQ666839	*A. astacus* KX268736
C. wladiwostokiensis	-	0.02	0.06	0.03	0.06	0.13
C. dauricus AY820883, OL542521	0.08	0.01/0.02	0.07	0.04	0.06	0.13
C. similis AY820880, JN991196	0.09	0.09	0/0.06	0.07	0.06	0.15
C. schrenckii AY820882, KX268737	0.11	0.1	0.11	0.01/0	0.06	0.14
C. japonicus AY820881, KX268736	0.11	0.11	0.11	0.11	0.03/0	0.14
A. astacus MT862440	0.14	0.17	0.18	0.17	0.15	-

Along the diagonal, average intraspecific distances are presented for *COX1*/*16S* fragments. Lack of intraspecific sampling is indicated as "-".

The mean coverage was 26.9 reads per position. From the total 20,861,158 reads that were obtained from the sequencing run, 3152 reads (0.02%) were successfully mapped to the reference genome. Among the mapped reads, 2994 (0.01%) were properly paired, indicating the correct alignment of read pairs. Additionally, 114 reads were identified as singletons, where the mate did not map to the reference genome.

Protein-coding genes vary in size from 159 (*ATP8*) to 1731 (*ND5*) base pairs. ATG is the most common start codon, and TAA is the most common stop codon. Notably, the *ND5* fragment contains a unique start codon (GTG), and *ND1* contains a unique stop codon (TAG). A putative transcription exception was observed, where the TAA stop codon might be completed by the addition of 3' A residues to the mRNA. This feature was detected in the *COX2* and *CYTB* genes. Most protein-coding genes are located on the "+" strand, except for *ND6* and *CYTB*. The large rRNA spans 1255 base pairs, while the small rRNA covers 661 base pairs, both on the "+" strand, although the small rRNA appears unfinished (Table 3). The tRNA fragment lengths vary from 61 (tRNA-*Ala*, tRNA-*Arg*, and tRNA-*Phe*) to 68 (tRNA-*Glu* and tRNA-*Val*) base pairs, with a common length of 64 bases. Only four tRNAs are located on the "-" strand: tRNA-*Ser*, tRNA-*Thr*, tRNA-*Cys*, and tRNA-*Tyr*. The control region, situated between tRNA-Glu and rrnS, spans 1262 base pairs. Features that are common to nucleotide content in the obtained genome are presented in Table 4.

3.2 Confirmation of Species Identity for C. wladiwostokiensis

Following alignment, the *COX1* fragment matrix consisted of 502 bases. Among them, 132 sites were variable, including 90 parsimony informative sites and 42 singletons. The *16S* matrix, aligned to 524 bases, had 105 variable sites, including 48 parsimony informative sites and 57 singleton sites. Intraspecific variation for the *COX1* marker in *Cambaroides* representatives ranged from 0 (*C. similis*) to 0.03 (*C. japonicus*). Interspecific divergence varied from 0.08 (between *C. wladiwostokiensis* and *C. dauricus*) to 0.11 (between *C. japonicus* and all other representatives of the genus) (Table 2). The outgroup exhibited the highest differentiation between all species. The variability in the *16S* marker showed greater heterogeneity. Intraspecific variability ranged from 0 (*C. japonicus* and *C. schrenckii*) to 0.06 (*C. similis*). No clear interspecific threshold was identified based on this marker. Divergence ranged from 0.02 (between *C. wladiwostokiensis* and *C. dauricus*) to 0.07 (*C. similis*–*C. dauricus* and *C. similis*–*C. schrenckii*). The outgroup was distant from all species, with the highest divergence values. The phylogenetic NJ-trees (see **Supplementary Figs. 3,4**) showed separate clusters for each species on the *16S* tree, except for *C. similis*, and an individual branch for *C. wladiwostokiensis*, yet express low bootstrap support on interspecific nodes. The outgroup naturally forms a basal position on both trees.

3.3 P-distance Variability Landscape along Cambaroides Mitochondrial Genome Sequences

A comprehensive analysis of the *p*-distance landscapes was conducted across the aligned mitochondrial genomes of five *Cambaroides* representatives (Fig. 2). The resulting profiles depicted local sequence variations between all genomes, with the most conserved regions identified within the *12S* and *16S* fragments. Conversely, the *16S* fragment displayed the most uneven variability, with a peak in the first half. Among the protein-coding fragments, *CYTB*, *ND6*, and *ND5* exhibited the highest variability, whereas the *COX1* fragment did not show high variability, thereby limiting the divergence profile to 0.09–0.13.

3.4 Phylogenetic Relationships of Cambaroides and its Position in Astacidea

The phylogenetic tree (Fig. 3) shows a strongly supported clade comprising representatives of Astacidae and Cambaridae. The chosen external group, Parastacidae, occupied a naturally separated position and was supported in both algorithms. Within this designated macroclade, representatives of the genus *Cambaroides* from the family Cam-

Table 3. Mitochondrial genome structure of *C. wladiwostokiensis*.

Region	Strand	Position (bp)	Size (bp)	Start/Stop codons
COX1	+	1–1536	1536	ACG/TAA
TRNA-*Leu*	+	1538–1602	65	-
COX2	+	1603–2290	688	ATG/TAA*
TRNA-*Lys*	+	2288–2351	64	-
TRNA-*Asp*	+	2353–2416	64	-
ATP8	+	2417–2575	159	ATG/TAA
ATP6	+	2569–3243	675	ATG/TAA
COX3	+	3243–4031	789	ATG/TAA
TRNA-*Gly*	+	4030–4091	62	-
ND3	+	4092–4445	354	ATT/TAA
TRNA-*Ala*	+	4447–4507	61	-
TRNA-*Arg*	+	4508–4568	61	-
TRNA-*Glu*	+	4569–4636	68	-
Control region	+	4637–5898	1262	-
Non-sequenced region	n/a	5899–6682	784	-
rrnS	+	6683–7343	661	-
Non-sequenced region	n/a	6893–6961	69	-
TRNA-*Val*	+	7344–7411	68	-
Non-sequenced region	n/a	7358–7375	18	-
rrnL	+	7412–8666	1255	-
Non-sequenced region	n/a	7812–7965	154	-
TRNA-*Leu*	+	8680–8744	65	-
ND1	+	8769–9710	942	ATA/TAG
TRNA-*Pro*	+	9718–9782	65	-
TRNA-*Ser*	-	9786–9848	63	-
CYTB	-	9849–10983	1135	ATG/TAA*
ND6	-	10983–11501	519	ATT/TAA
TRNA-*Thr*	-	11518–11580	63	-
ND4L	+	11583–11876	294	ATG/TAA
ND4	+	11876–13216	1341	ATG/TAA
Non-sequenced region	n/a	12093–12199	107	-
TRNA-*His*	+	13216–13279	64	-
ND5	+	13280–15010	1731	GTG/TAA
TRNA-*Phe*	+	15010–15070	61	-
TRNA-*Ile*	+	15077–15140	64	-
TRNA-*Met*	+	15144–15207	64	-
ND2	+	15208–16200	993	ATG/TAA
TRNA-*Trp*	+	16200–16265	66	-
TRNA-*Cys*	-	16265–16328	64	-
TRNA-*Tyr*	-	16329–16391	63	-

The asterisk (*) indicates the exception where the TAA stop codon is completed by the addition of 3' A residues to the mRNA. Non-sequenced regions are inferred based on alignment with the closest taxa. n/a, not applicable.

baridae occupied an external position, with slightly less support following in the ML topology, a division into two clades based on belonging to the Astacidae and Cambaridae subfamily Cambarinae. The latter subfamily includes two additional clades, one containing the genus *Procambarus* and the other containing *Orconectes* with *Cambarus robustus*. Thus, representatives of the family Cambaridae exhibited a paraphyletic position in this topology. Within the genus *Cambaroides*, *C. japonicus* held an external position, followed by a sequential branching of *C. similis*, *C. schrenckii*, and the closest grouping of *C. dauricus* with *C. wladiwostokiensis*. The support for this topology was absolute in both algorithms. Representatives of the genus *Procambarus* displayed an additional bifurcation into *P. alleni* + *P. fallax* and *P. clarkii* + *P. acutus*. Notably, independently sequenced *P. clarkii* sequences clustered together.

Table 4. Nucleotide composition of the obtained genome.

Regions	Strand	Size (bp)	GC (%)	AT skewness	GC skewness
Full genome	n/a	16,391	26.0	–0.079	0.200
PCGs	all	11,154	29.0	–0.191	0.116
PCGs	+	9501	28.8	–0.184	0.181
PCGs	-	1653	30.4	–0.234	–0.239
tRNAs	all	1215	25.1	0.026	0.204
tRNAs	+	962	25.5	0.007	0.208
tRNAs	-	253	23.3	0.093	0.186
rRNAs	all	1916	22.9	0.015	0.338
rRNAs	+	1916	22.9	0.015	0.338

n/a, not applicable; PCGs, protein coding genes.

In the adjacent clade, *C. robustus* held an external position, followed by sequential bifurcations within *F. punctimanus* > *O. luteus* > *O. rusticus* > *O. sanbornii* + *O. limosus*. In the external group (Parastacidae family), *C. quinquecarinatus* occupied the basal position.

4. Discussion

This study presents, for the first time, data on the mitochondrial genome sequence of *C. wladiwostokiensis* crayfish and compares it with other available *Cambaroides* genomes. Various methods have been previously employed to obtain mitochondrial genomes in this genus, such as assembly from multiple fragments after Sanger sequencing, including the primer walking technique [19], and shallow sequencing of total DNA, known as genome skimming [16]. Although we employed a relatively shallow sequencing depth in our research, it was still nine times more comprehensive than the data garnered in a comparable study [10]. However, in our study, this approach did not allow for the complete reading of certain genome regions (in total 1063 bases) (Table 3). Genome skimming has also proven to be effective for obtaining complete mitochondrial genomes of fishes [46]; nevertheless, the development of other methods to reduce the costs of obtaining mitochondrial genomes continues [47].

A plausible explanation for the existence of unread regions (specifically, the missed tRNAs) in the crayfish genome in this study might stem from the contamination of the total DNA from the target organism with genetic material sourced from the microbiome. Such contamination is not uncommon and has been previously documented in both genomic (including metagenomics) and transcriptomic sequencing endeavors [48,49]. However, higher sequencing depth usually mitigates these issues. In theory, labor-intensive approaches based on pulling the complete genome sequence by pieces via PCR should eliminate this disadvantage. Additionally, it is important to note that the copy number of mitochondrial DNA varies significantly across different tissues [50,51], which might potentially substitute for enrichment procedures. Furthermore, this circumstance implies that when comparing genome skimming results, it is necessary to specify tissue types and, ideally, the number of mitochondrial DNA copies in the study methodology.

The genome structure (Fig. 1, Table 3) and nucleotide composition (Table 4) of *C. wladiwostokiensis* are quite similar to those of other *Cambaroides* representatives (see **Supplementary Table 1**). The main differences relate to nucleotides. Furthermore, single-nucleotide indels are present within the regions of transfer RNA compared to the nearest species, *C. dauricus*. According to the comparison data with reference sequences of *COX1* and *16S* [12,19], the identity of *C. wladiwostokiensis* can be confirmed, albeit relatively. Genetic distances indicate that the specimen from which the genome was obtained does not correspond to any of the available reference species—*C. dauricus*, *C. japonicus*, *C. schrenckii*, or *C. similis*—differing from them by 0.08, which is comparable to interspecies differences within this genus. Another species in this genus, *C. koshewnikowi* Birstein and Vinogradov, 1934, is missing from the analysis. However, the assignment of our specimen to this species is doubtful due to the more northern range of *C. koshewnikowi*, as well as the fact that the species is extremely rare and only known from a few records in the lower part (estuarine zone) of the Amur River [11,52,53].

We examined landscape variation data to identify genome regions that could be used to develop future species-specific primers and probes. Typically, for the development of species-specific assays for crayfish, researchers use mitochondrial DNA [54–56] or a combination of nuclear and mitochondrial fragments [57,58]. This is driven by the fact that mitochondrial DNA is present in significantly higher copy numbers in cells than nuclear DNA. Moreover, the ecological characteristics of mitochondrial DNA suggest that it is less prone to degradation [59]. In this case, we are limited by the available resources of the mitochondrial genome. Additionally, we need to exclude the control region (CR) from consideration since it is also unread in the species *C. similis*. Based on the landscape data (Fig. 2), the *COX1* fragment is the most conserved among the mitochondrial protein-coding regions of *Cabraroides*, while also having the most uniform distribution in variability. The *COX1* encodes one of the essential components in

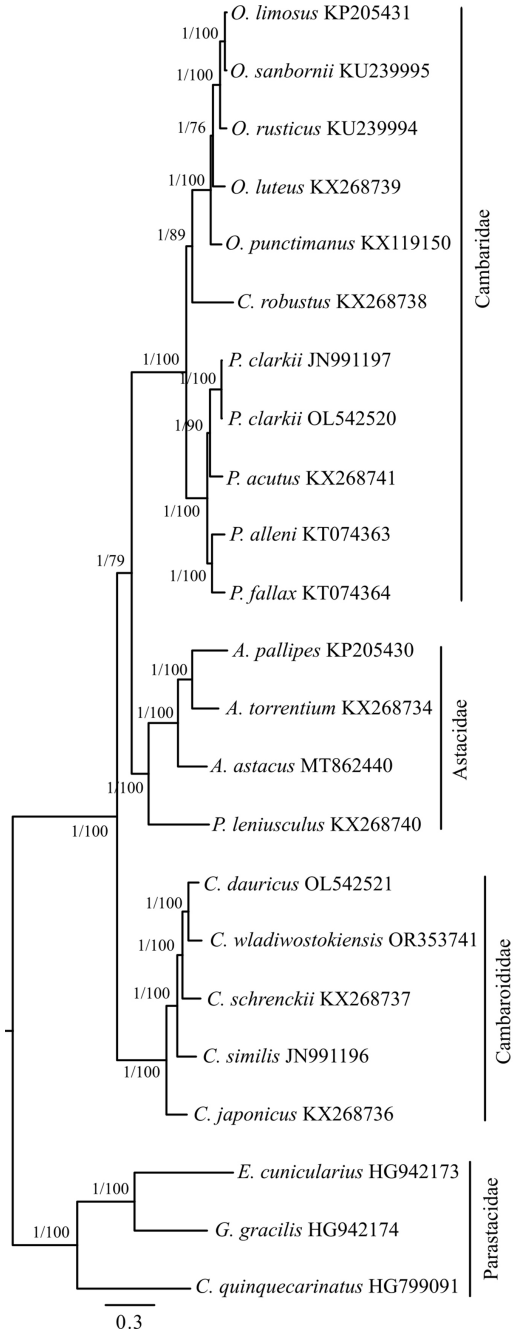

Fig. 3. Bayesian inference (BI) phylogenetic tree illustrating the relationships among representatives of the genus *Cambaroides* and the position of the genus among other groups within the Astacidea infraorder. Construction based on the concatenated matrix of 15 mitochondrial genome fragments (detailed in the Materials and Methods). The tree is rooted at the midpoint. Node support values are provided as Bayesian posterior probabilities (BI) and as percentages of 50,000 replicates in the ultrafast bootstrap test (ML). They are denoted as BI/ML.

the complexes of the electron transport chain [60,61]. Thus, strong purifying selection may define its conservative nature [62], forming an expected threshold for distinguishing between intraspecific and interspecific variability, thereby making this marker suitable for species delimitation of most multicellular organisms [2], including many known crustaceans [63]. The observed pattern in our case is likely to be a candidate for probe design; however, it may have limitations when searching for primers for haplotype-specific PCR. The *16S*, *CYTB*, *ND6*, and *ND5* fragments are more likely to be suitable for this purpose since they showed the highest peaks in variability.

Based on an independent partitioned phylogenetic analysis of 15 mitochondrial fragments in the Astacidea infraorder, we have shown that the family Cambaridae is not monophyletic, yet more precisely, it exhibits properties of paraphyly (Fig. 3) when considered alongside representatives in Astacidae. Accordingly, our results support the view that East Asian cambarids (Cambaroididae) occupy a basal position relative to Astacidae and North American Cambaridae [16], thus, representing a naturally monophyletic group. This view is inconsistent with data based on an integrative approach [64], although it had a limited sample from the group under discussion. Topologically, similar results were obtained when analyzing new mitochondrial genomes of *P. clarkii* and *C. dauricus* from China [28], where East Asian cambarids also form a group external to the others. There is another view according to which "the genus *Cambaroides* continues to fall outside the Cambaridae, but clusters with different taxa depending on the data set used" [14]. Our results also indicate that *C. wladiwostokiensis* is the closest to *C. dauricus*, and together they form the cluster most recently diverged from all other *Cambaroides*.

5. Conclusions

This study introduces the mitochondrial genome of *C. wladiwostokiensis* crayfish and compares it with other *Cambaroides* genomes. Shallow sequencing revealed certain unread regions, possibly due to contamination or tissue-specific DNA copy variations, coupled with insufficient sequencing depth. Despite these gaps, we show that the structure and composition of the genome resemble other *Cambaroides*. While the genetic analysis affirmed the identity of *C. wladiwostokiensis*, it also underscored its distinctiveness from previously known species. Landscape variation data identified potential regions for species-specific primer development, excluding the unread control region. Partitioned phylogenetic analysis showed the paraphyletic origin of the Cambaridae family. The basal position of East Asian cambarids (Cambaroididae) supports its monophyletic status and is consistent with previous studies. This contributes to discussions about *Cambaroides* taxonomic placement and enriches insights into crayfish evolutionary relationships. Overall, this research sheds light on the genetic characteristics of *C. wladiwostokiensis* and its *Cambaroides* relatives, providing a foundation for future studies in crayfish genomics and contributing to a broader understanding of the evolutionary history of this diverse group.

Further exploration and integration of additional data could refine the conclusions drawn from this study.

Availability of Data and Materials

Raw data that support the findings of this study have been deposited in NCBI SRA archive with the identifier SRR26399148.

Author Contributions

EB performed the collection, identification and conceptualization of the taxonomic position and ecology of *C. wladiwostokiensis*. ST designed the research, performed genetic analysis and analyzed the data. ST wrote the manuscript. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The experiments on crayfish in this study are consistent with the current Russian and international standards of law and regulations for research involving animals and were approved by the Commission of biomedical ethics of A.V. Zhirmunsky National Scientific Center of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences (the record # 1-080923 from the Meeting # 2, September 6, 2023).

Acknowledgment

The authors are grateful to Dr Alexey Boyko for the help in working on the "Umnik" computing cluster at the NSCMB FEB RAS.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbs1504015.

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