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Genome characterization and comparative analysis among three swimming crab species

Ming Liu[1]*, Shanshan Ge[2]*, Shivish Bhandari[1], Chunlei Fan[1], Yu Jiao[3], Chunlei Gai[2], Youhong Wang[2] and Hongjun Liu[2]*

[1]Patuxent Environmental and Aquatic Research Laboratory, Morgan State University, St. Leonard, MD, United States, [2]Key Laboratory of Disease Control in Mariculture, Marine Science Research Institute of Shandong Province, Qingdao, China, [3]Fishery College, Guangdong Ocean University, Zhanjiang, China

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EDITED BY
Libin Zhang,
Institute of Oceanology (CAS), China

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Zhejiang Wanli University, China
Louis V. Plough,
University of Maryland, United States

*CORRESPONDENCE
Ming Liu
ming.liu@morgan.edu
Shanshan Ge
ge_gss@163.com
Hongjun Liu
hongjunl@126.com

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In this study we sequenced the genomes of three economically important swimming crabs *Portunus trituberculatus, Charybdis japonica*, and *Callinectes sapidus* using the next-generation sequencing approach and made a basic assembly. The genomes of the three species are characterized with high heterozygosity (>1.2%) and high repeat content (>50%). Genome comparative analysis revealed 40 long conserved fragments (>5,000 bp) among the three species, most of them are involved in cardiac-related biological process. Relative higher genome similarity was found between *P. trituberculatus* and *C. japonica* that are belong to different subfamilies, compared to that between *P. trituberculatus* and *C. sapidus* which are from the same subfamily. It is inconsistent with their phylogenetic evolutionary trees inferred from previous mitochondrial DNA coding fragments and a conserved ANK2 protein fragment from this study. We speculated that the high genome similarity between *P. trituberculatus* and *C. japonica* might be attributed to their same inhabit range in which the genome is subject to the same environment selection, and the inconsistence between genome similarity and phylogenetic relationship is caused by the different evolutionary rates of coding DNA and non-coding DNA under environment selection.

KEYWORDS
***Portunus trituberculatus*, *Charybdis japonica*, *Callinectes sapidus*, genome heterozygosity, genome repeat sequences, genome similarity, environment selection, ankyrin-2**

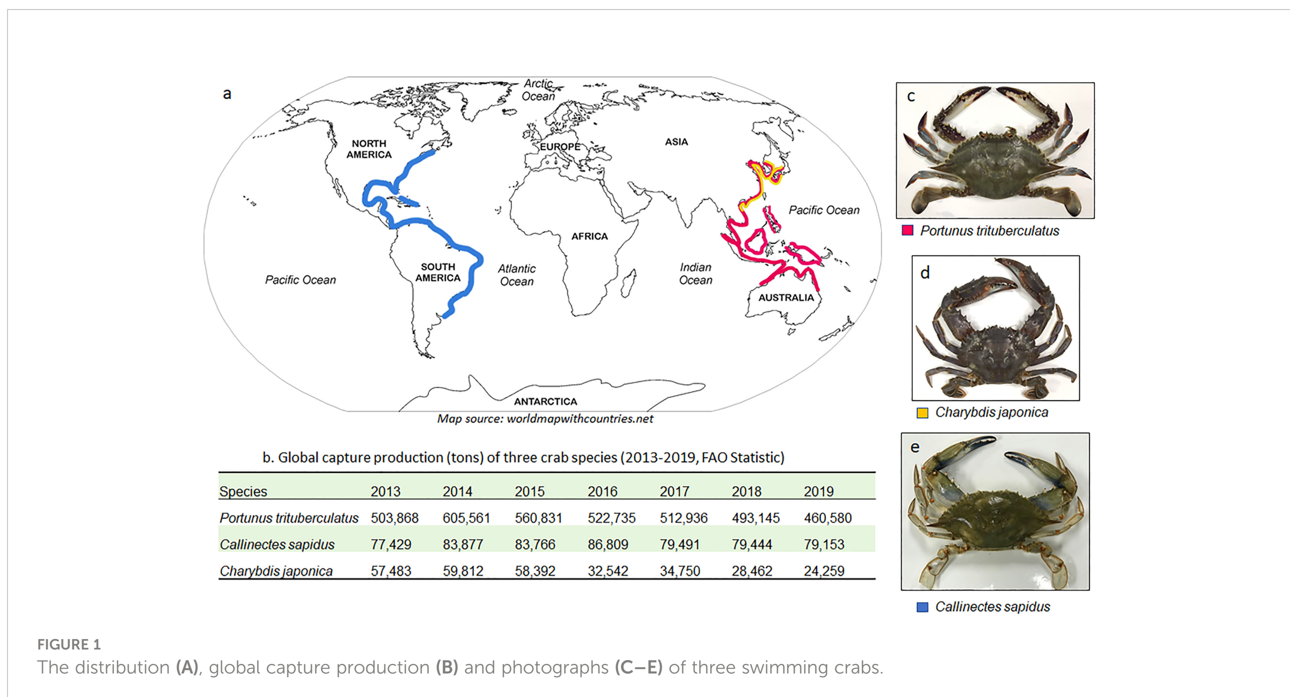
Introduction

Crabs are decapod crustaceans of the infraorder Brachyura, typically with a very short tail hidden entirely under the thorax, a thick shell and a single pair of pincers. They are comprised of 98 families with over 7,000 described species that inhabit in oceans, freshwaters, as well as land (Ng et al., 2008; de Grave et al., 2009; Tsang et al., 2014).

Crabs are a significant part of the fishery abundance of many countries and are a vital protein source (Lohani, 2011; Anderson and Alford, 2014; Krisfalusi-Gannon et al., 2018). Commercial crabs make up 26% of all crustaceans caught and farmed worldwide, with about 1.5 million tons being consumed annually (Food and Agriculture Organization (FAO), 2021). In most estuaries, the crab plays a key ecological role as a keystone predator on numerous species of smaller invertebrates and serves as prey for sea birds, turtles, and large fish species (Hines, 2007; Lipcius et al., 2007; Long et al., 2011).

The Gazami crab (*Portunus trituberculatus*), Asian paddle crab (*Charybdis japonica*) and blue crab (*Callinectes sapidus*) are three swimming crabs (belong to Portunidae family) that are characterized by the flattening of the fifth pair of legs into broad paddles which are used for swimming. All the three crabs are commercially important species. *P. trituberculatus* are widely distributed in the eastern coasts of Asia and North Australia (Figure 1A). It is the world's most heavily fished crab species, with over 450,000 tons being caught annually (Figure 1B). *C. japonica* has a similar distribution with *P. trituberculatus* but mostly inhabits in the coasts of China, Japan and Korea, its capture production is about one tenth of *P. trituberculatus*. Both resources of *P. trituberculatus* and *C. japonica* declined largely recent years due to overfishing, farming practice of these two species have started in China to reduce the fishery pressure (Liu and Dai, 2000; Gu and Qiao, 2012). *C. sapidus* is native to the eastern coasts of America from Argentina to Cape Cod in MA, US (Williams, 1974). It is the dominant crab fishery in the US with the landing s of 71,400 tons in 2019, which is valued at $199.3 million (National Oceanic and Atmospheric Administration (NOAA) Fisheries, 2022). Both *P. trituberculatus* and *C. sapidus* belonged to Portuninae subfamily. They have similar morphological appearance. *C. japonica* is from a different subfamily, Thalamitinae, and its shell is rounder and thicker, and the sawtooth on the edge of the shell is larger compared to the other two Portuninae crabs (Figures 1C–E).

The genome is all the nucleotide sequences of DNA (or RNA in viruses) in organisms (Roth, 2019), which is highly associated with biological processes and cell function and contains the information of evolution as well (Gao et al., 2018). With the rapid development of molecular biotechnologies, more and more organisms' genomes are revealed, and they are playing important roles in modern medicine and agriculture such as disease diagnosis (Manolio, 2013; Yu et al., 2020) and genomic selection (Varshney et al., 2014; Meuwissen et al., 2016). The high-resolution intra-or-inter specific genomic data also brings new perspectives in species conservation and evolution (Primmer, 2009; Benestan et al., 2016). In the present study, we sequenced the genomes of the three swimming crab species and performed a comparative analysis. These genome sequences, despite a basic assembly, are useful for resource conservation and genetic breeding of crabs. Through the genome comparative analysis, it will help further understand the phylogenetic evolution and taxonomy relationship of the three species, and the conserved regions that may be part of the "homologous genes" can be identified. The homologous genes are important in shaping the characterized phenotypes of related species and adapting to climate and environment changes (Yuan et al., 2021). Our results may bring new insights in understanding the scenarios of speciation and adaptive evolution of genome in Decapoda species.



b. Global capture production (tons) of three crab species (2013-2019, FAO Statistic)

Species	2013	2014	2015	2016	2017	2018	2019
Portunus trituberculatus	503,868	605,561	560,831	522,735	512,936	493,145	460,580
Callinectes sapidus	77,429	83,877	83,766	86,809	79,491	79,444	79,153
Charybdis japonica	57,483	59,812	58,392	32,542	34,750	28,462	24,259

FIGURE 1
The distribution **(A)**, global capture production **(B)** and photographs **(C–E)** of three swimming crabs.

Materials and methods

Sampling and sequencing

C. japonica and *P. trituberculatus* were collected from a marine benthic survey at the coast of Yantai City (Yellow Sea, China) in June 2018. C. *sapidus* was purchased from local seafood market of St. Leonard, Maryland, US. The muscles of crab pincers were sampled and preserved in 95% ethanol for DNA extraction. The genomic DNA were extracted using traditional phenol-chloroform method. The DNA was broken into fragments of 350 bp using an ultrasonicator (Covaris Inc. Woburn, US), then the library was constructed through the steps of end repair, addition of A at the 3' end, addition of connector, target fragment recovers using electrophoresis, PCR amplification of the target fragments. The libraries were sequenced on the Illumina HiSeq X platform at Gene Denovo Co. (Guangzhou, China). The raw data were generated after base calling from the sequencing with each base a quality score. The ratios of bases with quality score above 20 and 30 called Q20 and Q30 respectively were calculated. The raw data also include the adapter bases and unknown bases (N). The clean reads were obtained by removing those reads containing adaptor, the reads of containing >10% N, and low-quality reads (containing >40% base with Q <= 10).

Estimate and assembly of the genomes

The K-mer analysis was performed to predict the genome size, heterozygosity and repeat sequences. K-mers are substrings of length *k* contained within a biological sequence in bioinformatics. In sequence assembling, the term K-mer refers to all of a sequence's subsequences of length *k*. The genome size can be estimated using the equation below:

$$\text{Genome size} = \frac{\text{K} - \text{mer number}}{\text{K} - \text{mer depth}}$$

Where the K-mer number is the total number of K-mer in the read set, the K-mer depth is the expected value. The K-mer depth frequency distribution is assumed to follow a Poisson distribution, therefore the expectation of K-mer depth can be estimated from the depth frequency distribution curve. The heterozygosity ratio and repeat sequences were estimated based on the methods described by Liu et al. (2013). All the K-mer analysis were performed using Jellyfish (Marçais and Kingsford, 2011) and GenomeScope (Vurture et al., 2017; http://qb.cshl.edu/genomescope/). In this study, we used $k = 21$ for K-mer analysis which is most frequently used. The clean reads were assembled into contigs in software SOAPdenovo2 by applying the de Bruijn graph structure (Luo et al., 2012), and then the contigs were spliced into scaffolds. Genome-wide Microsatellite Analyzing Tool Package (GMATA, Wang and Wang, 2016) was used to identify all the microsatellite loci, statistics the location, length, repetitions of the repeat motif, and design the primers for ideal microsatellite makers. The parameters were set as below: min-length of motif is 2, the max length is 6, and the min-repeat times is 5. The maker design parameters are min amplicon size = 120 bp, max amplicon size = 400 bp, optimal annealing temperature = 60°C.

Genome comparative analysis

We employed two approaches two measure the genome similarity among the three crab species, 1) the Average Nucleotide Identity (ANI, Ciufo et al., 2018; Jain et al., 2018) and 2) the number and cumulative length of conserved regions. The algorithm of ANI is described by the formula:

$$\text{ANI} = \frac{\sum(\text{ID}\,\% \times \text{Length of Alignment})}{\sum \text{Length of the shorter fragemet}}$$

The ID%, Length of the Alignment, and Length of the shorter fragment terms refer to the individual blast hits from genome–genome comparisons (Gosselin et al., 2022). The ANI was calculated using FastANI v1.33 (Jain et al., 2018) with the parameter set-up of K-mer size = 16 and Fragment Length = 3,000. The genome conserved regions were identified using MUMmer 4.0 (Delcher et al., 2003; Marçais et al., 2018). The minimum length of a cluster of matches is 65, the minimum length of a single exact match is 20, and the maximum gap between two adjacent matches is 90. The alignment results were further filtered using identify > 85% and minimum aligned length > 1,000 bp and >5,000 bp respectively.

The nucleotide sequence of the identified conserved regions (>5,000 bp) that appear in all the three species were extracted using BEDTools (Quinlan and Hall, 2010). Because a chromosome-level assembled genome of *P. trituberculatus* has been available (Tang et al., 2020; NCBI Reference No. ASM1759143v1), the gene names of those conserved regions can be acquired through BLAST (Altschul et al., 1997), and then the gene ontology (GO) terms were obtained using gene name from NCBI database (https://www.ncbi.nlm.nih.gov). The GO enrichment analysis was performed using the OmicShare tools, a free online platform for data analysis (https://www.omicshare.com/tools).

The gene that has the longest conserved region in all three crab species was selected for further analysis. The gene characteristics and the location of the conserved region on the gene were identified by BLAST with *P. trituberculatus* genome (Tang et al., 2020) shown in NCBI Sequence Viewer (Rangwala et al., 2021). The protein domains were predicted using SMART (a Simple Modular Architecture Research Tool; Letunic et al., 2021; http://smart.embl-heidelberg.de). The homeotic gene fragments from other known-genome Decapoda species were obtained by BLAST the coding sequence of the similar region. A phylogenetic

tree was constructed using MEGA 11 (Tamura et al., 2021) based on Neighbor-joining method (Saitou and Nei, 1987).

Results

Genome characterization of the three crab species

The summary for the sequencing data is shown in Table 1. The minimum Q20 and Q30 are 95.09% and 89.02% which meet the sequencing accuracy requirement of illumine platform that the Q20 and Q30 should be at least 90% and 85%. The estimated genome sizes based on the 21-mer analysis (Figure S1, Table 2) are 737.6 Mb, 994.4 Mb and 993.4 Mb for *P. trituberculatus*, *C. japonica*, and *C. sapidus* respectively. The observed heterozygous rate is larger than 1% for all three species. The repeat sequence content ranges from 58.4% to 62.6% (Table 2). The preliminary genome assembly results are shown in Table 3. Total of 485,821 scaffolds with the total length of 762 Mb were obtained for *P. trituberculatus*, 655,217 scaffolds with the total length of 1Gb were obtained for *C. japonica*, and 706,990 scaffolds with the total length of 858 Mb were obtained for *C. sapidus*. The N50 length is short for all three species, ranging from 1,397 (*C. sapidus*) to 2,131 (*P. trituberculatus*). The GC content for the assembled genome sequences ranges from 39% - 42% for the three species, which fall into the GC preference range of Illumina sequencing (25% - 65%). Total of 1.3 million, 1.0 million and 1.7 million microsatellite loci were identified from the assembled genome sequences of *P. trituberculatus*, *C. japonica* and *C. sapidus* (Table S1). Of the five motif repeat types, the dinucleotide repeat occurs most frequently in all the three species. The top grouped dinucleotide motifs are GT/AC in both *P. trituberculatus* and *C. japonica*, while it is different in *C. sapidus* (TC/GA, Figure S2). The microsatellite markers and their primers developed from these repeat motifs are listed in Table S2.

Genome similarity and conserved fragments among three crab species

The ANI between *P. trituberculatus* and *C. japonica* (82.3%) is close with that between *P. trituberculatus* and *C. sapidus* (82.6%), while the ANI between *C. japonica* and *C. sapidus* is relative smaller (80.3%, Table 4). The MUMmer results revealed 16,414 conserved fragments (alignment length > 1,000 bp, identity > 85%) with total length of 26.5 Mb between *P. trituberculatus* and *C. japonica*, 12,745 conserved fragments with total length of 20 Mb between *P. trituberculatus* and *C. sapidus*, and 9,366 conserved fragments with total length of 14.7 Mb between *C. japonica* and *C. sapidus* (Table S3). The results were further filtered using alignment length > 5,000 bp, the numbers of conserved fragments are 126, 67, and 58 respectively (Table 4). Among these similar fragments, total of 40 were found in all three crab species. There are 34 fragments from 28 genes that have been annotated. The gene names are shown in Table 5. These genes are mostly involved in 22 biological processes, 6 molecular functions and 13 cellular components (Figure 2A). The top 20 significantly enriched ontologies are shown in Figure 2B, including regulation of cardiac conduction, circulatory system process, heart contraction, blood circulation, muscle structure development, synaptic membrane, etc.

The gene of ankyrin-2

The longest conserved region among the three species was found in the gene of ankyrin-2 (ANK2, Table 5). The whole length of *P. trituberculatus* ANK2 (*PtANK2*) is 391,211 bp, which has 34 isoforms with total of 25 - 43 exons (Tang et al., 2020). The identified conserved region in ANK2 consists of two separate fragments. The longer fragment (CF1) locates from 29,981,076 to 29,994,164 on Chromosome 46, which mostly locates in the intron area and contain 1 – 2 short exons. The shorter fragment (CF2) is a sequence of 10,602 bp that contains the longest exon of *PtANK2*. That exon appears in all isoforms (Figure 3). The PtANK2 protein contains a string of ankyrin repeats, a ZU5 domain and a DEATH domain in the first 1/3 region, the rest region is characterized by low complexity proteins where both CF1 and CF2 locate (Figure 3).

The protein sequences translated from the long exon of CR2 were found highly conserved in other known-genome Decapoda species. Protein sequences from other 9 species including the Chinese mitten crab *Eriocheir sinensis* (Accession No.: PRJNA636904), the snow crab *Chionoecetes opilio* (PRJNA602365), the blue king crab *Paralithodes platypus* (PRJNA555178), the coconut crab *Birgus latro* (PRJNA704570), the American lobster *Homarus americanus* (PRJNA655509), the red swamp crayfish *Procambarus clarkii* (PRJNA727411), the Kuruma shrimp *Penaeus japonicus* (PRJDB11151), the whiteleg

TABLE 1 Sequencing data statistics for three crab species.

Species	Raw base (bp)	HQ Clean Base (bp)	Q20 (%)	Q30 (%)	GC (%)	HQ Clean Reads Num
P. trituberculatus	131,227,130,100	128,638,819,259	95.09	89.02	41.10	862,246,354
C. japonica	144,113,920,500	140,998,228,122	95.38	89.34	42.94	946,429,526
C. sapidus	127,800,956,400	125,451,487,843	96.06	90.77	40.61	838,881,390

TABLE 2 K-mer statistics and genome estimates of three crab species.

Species	Kmer			Genome Size (Mb)	Heterozygous Ratio (%)	Repeat (%)
	Length	Number	Depth			
P. trituberculatus	21	80,904,234,922	99.86	737.6	1.2592	58.4
C. japonica	21	91,399,966,726	81.82	994.4	1.9725	62.6
C. sapidus	21	78,116,783,104	72.42	993.4	1.7039	62.4

shrimp *Penaeus vannamei* (PRJNA438564), and Chinese white shrimp *Penaeus chinensis* (PRJNA691453) were extracted from their genome database in NCBI. The phylogenetic tree of the 12 species based on neighbor-joining method coincides with the current taxonomy and evolutionary relationship of Decapods (Figure 4). The *P. trituberculatus* and *C. sapidus* clustered first then connected to *C. japonica*. The three swimming crabs then grouped with the other two Brachyura crabs *C. opilio* and *E. sinensis* successively. *B.latro* and *P. platypus* forms a clade that belongs to Anomura. *H. americanus* and *P. clarkia* make up a cluster of Astacidea that consists of lobsters and crayfish. All Brachyura, Astacidea are infraorders of Pleocyemata suborder. The three prawn species belong to the other suborder Dendrobranchiata of Decapoda. The genetic distance reflected by tree branch length between Astacidea and prawns is smaller than that between either crab infraorder and prawns, indicating the lobsters and crayfish first diverged from prawn, this is consistent with "Carcinization" process (McLaughlin and Lemaitre, 1997).

Discussion

High genome heterozygosity and repeat sequence content

The heterozygosity is one of important characteristics of diploid or polyploid organism's genome, which is caused by the genetic variations between different chromosomes. High heterozygosity has been a challenge for the estimation of genome size and genome assembly (Kajitani et al., 2014; Gao et al., 2018). For diploid or polyploid organisms, only the sequences from one set of chromosomes should be counted and assembled. However, the sequences from high heterozygous regions will be also included, therefore may double the region. On the K-mer depth frequency distribution curve, it often manifests as multiple peaks or tailing, which will cause deviation when estimating the expectation of the K-mer depth. The heterozygosity is usually used to divide genomes into low heterozygosity (0.5% ≤ heterozygous ratio <0.8%) and high heterozygosity (heterozygous ratio ≥0.8%) (Li et al., 2019). In this study, high heterozygosity was detected in all three crab species (>1.2%). The genome heterozygosity is generally high (ranging from 1% to 4%) for most crabs and bivalves according to the existing records (Cai et al., 2019; Bachvaroff et al., 2021; Penaloza et al., 2021; Tang et al., 2021), compared to fish genome with usually lower than 0.5% (Chen et al., 2020; Xu et al., 2020; Choi et al., 2021).

Organisms' genome is also characterized of a certain fraction of repetitive sequences that may originated from DNA duplication and recombination during the evolution. The content of the repeat sequences increases from virus (<1%) to maize (77%; Gao et al., 2018). The human genome of about 66%–69% is repetitive (de Koning et al., 2011). When the repeat content is larger than 50%, it is recognized as high repetitive genome (Tang et al., 2015). In this study, the repeat sequences of all the three crab species are above 50%, the *C. japonica* and *C. sapidus* are larger than 60%. The assembly becomes very difficult

TABLE 3 Statistics of the assembled genome sequences for the three crab species.

Species	Contigs					Scaffolds					GCcontent
	Total number	Total length (bp)	Maximum length (bp)	N50 (bp)	N90 (bp)	Total number	Total length (bp)	Maximum length (bp)	N50 (bp)	N90 (bp)	
P. trituberculatus	1,055,667	721,142,604	26,745	919	318	485,821	761,962,573	68,884	2,131	681	41.14%
C. japonica	1,320,719	962,126,970	26,326	999	340	655,217	1,009,259,456	45,888	2,002	694	41.68%
C. sapidus	1,460,505	785,420,294	25,472	716	256	706,990	857,633,810	52,502	1,397	624	39.74%

TABLE 4 Pairwise results of Mummer (lower diagonal) and Average Nucleotide Identity (upper diagonal) among three crab species.

	P. trituberculatus	*C. japonica*	*C. sapidus*
P. trituberculatus	–	82.3404	82.6388
C. japonica	805,904 (126)	–	80.2685
C. sapidus	432,779 (67)	366,058 (58)	–

Mummer filtration criteria are identify > 85% and alignment length > 5,000 bp. The values in brackets are total number of similar regions, the values out brackets are the total length of similar regions in the species of first row.

for high repetitive genome when only using the next-generation short-read sequencing. The high heterozygosity and high repeat content of all the three crab species resulted in that the assembled scaffolds are very short. For high-quality assembly of a high heterozygosity and high repeat content genome, the combination method of second-generation short-read sequencing (Illumina) and third-generation long-read sequencing (PacBio) supplemented with Hi-C technology are usually used (Li et al., 2019; Tang et al., 2020; Bachvaroff et al., 2021).

Microsatellites is one kind of tandem repeat sequences typically with motif of 1–6 nucleotides (Richard et al., 2008). It is widely distributed on the whole genome, characterized by high polymorphism, co-dominant inheritance and easy to capture (Bhattarai et al., 2021). The microsatellites are very dense in the genomes of all the three species (~1,320/Mb). The microsatellite loci developed from this study provide useful tools for population genetics and selective breeding, such as fingerprinting, linkage mapping, parentage identification, or marker assisted selection etc.

Inconsistency between genome similarities and their taxonomy relationship

In taxonomy, both *P. trituberculatus* and *C. sapidus* are from Portuninae subfamily and they have more similar morphological appearance, compared to *C. japonica* that is from a different subfamily - Thalamitinae. The previous phylogenetic evolutionary trees constructed using mitochondrial DNA coding sequences also showed that *P. trituberculatus* and *C. sapidus* clustered together with small genetic distance and were in the Portuninae branch, which had an explicit separation with the Thalamitinae species that *C. japonica* is included (Mantelatto et al., 2018; Evans, 2018). Moreover, our phylogenetic tree constructed using a highly conserved ANK2 protein fragment is consistent with the phylogenetic and taxonomy relationship in which *P. trituberculatus* is closer with *C. sapidus* rather than *C. japonica*. However, for the comparison of genome similarity among three crab species based on the genome draft of this study, a high ANI was observed between *P. trituberculatus* and *C. japonica* that are from different subfamilies. It is very close to the ANI between *P. trituberculatus* and *C. sapidus*, the species within subfamily. In contrast, the *C. sapidus* genome is less similar with *C. japonica* that may reflect a normal subfamily-level differentiation. In addition, both the number and the cumulative length of the identified conserved regions (>1,000 bp and >5,000 bp) between *P. trituberculatus* and *C. japonica* are larger than those between *P. trituberculatus* and *C. sapidus*, which may indicate the genome of *P. trituberculatus* is more similar with *C. japonica* compared to *C. sapidus*.

We speculate that the high genome similarity between *P. trituberculatus* and *C. japonica* might be attributed to their similar living environments. Both *P. trituberculatus* and *C. japonica* occupy the same areas across the western coast of Pacific, while *C. sapidus* lives in the west coasts of Atlantic which is totally separate in geography from *P. trituberculatus'* inhabit range, where the hydrological conditions, climate and biological communities are different. The environment cannot change the genome directly but may play as a selection pressure to impact the genome evolution. Based on the theories of natural selection (Darwin, 1859) and modern synthesis (Fisher, 1958; Wright, 1932; Dobzhansky, 1937), mutations randomly occurred in the genome of organisms, the genome evolved by retaining the mutations that adapt to selection pressures and wiping out the ones that do not adapt.

However, the inconsistency between entire genome similarities and phylogenetic evolutionary relationship inferred from previous studies might be caused by the difference of rate of evolution between coding DNA (cDNA) and non-coding DNA (ncDNA). The organism's genome consists of coding cDNA and ncDNA sequences. The cDNA encodes proteins that are the basic component for the cell structure, function, and regulation of the body's tissues and organs, but only comprises 1% of the entire genome (Rands et al., 2014), whereas the other 99% does not encode any proteins which had been ever thought as useless "junk" (Ohno, 1972). The cDNA has much slower mutation rate than ncDNA (Kimura, 1983) because they are so related to the life activities of the organisms and therefore the mutations are hardly retained. Compared to cDNA, the super high occupation ratio of ncDNA on the genome means much higher mutation occurring frequency and more mutations. Because they do not encode protein, their mutations would have higher possibility to be retained. Despite the mutations are considered occurring neutrally based on the neutral theory (Kimura, 1983), they are subject to selection as long as the genome region where they are

TABLE 5 Top 40 similar regions among the three crab species and their gene prediction .

P. trituberculatus			C. japonica			C. sapidus			Gene prediction
ID	Start	End	ID	Start	End	ID	Start	End	
scaffold343209	528	13618	scaffold455259	5412	18509	C26831641	13264	164	ankyrin-2
scaffold365022	86	5127	scaffold372161	16766	11724	C26829463	263	5304	ankyrin-2
scaffold365022	5184	10688	scaffold372161	11671	6166	C26829801	5508	8	ankyrin-2
scaffold12757	666	6566	scaffold90461	843	6775	C26830719	6488	591	mucin-17
scaffold338169	5	7330	scaffold341668	10722	3358	C26831539	10652	3323	mucin-17
scaffold343046	2654	7771	scaffold494143	2808	7969	C26830341	5068	1	mucin-17
scaffold358440	3064	8292	scaffold116476	5237	3	C26831653	14125	8948	nesprin-1
scaffold358440	9884	17218	C19960886	7398	33	C26831653	7575	292	nesprin-1
scaffold286885	9795	14882	scaffold273050	5655	568	C26829621	304	5391	titin
scaffold10409	8299	15223	scaffold11177	3859	10862	C26830939	6894	1	titin
scaffold157321	5279	14174	scaffold507714	10751	1859	C26831647	10116	1214	protein split ends
scaffold51958	9678	17913	scaffold27248	13711	5494	C26831339	1	8251	protein tramtrack, beta isoform-like
scaffold363752	1731	9637	scaffold195673	7946	27	C26831279	7972	14	histone-lysine n-methyltransferase 2c
scaffold62273	2502	9981	scaffold94622	7668	139	C26831523	2134	9602	MAM and LDL-receptor class A domain-containing protein 2
scaffold162095	1992	9254	scaffold410282	7664	373	C26831603	11287	4023	epidermal growth factor receptor substrate 15
scaffold122804	981	8214	scaffold499967	3525	10759	C26831083	20	7241	serine/threonine-protein kinase smg1
C13917025	4742	11309	scaffold28529	9788	3179	C26831481	2843	9441	rna polymerase ii elongation factor ell
scaffold365021	6	6389	scaffold257705	869	7237	C26830653	10	6392	protein unc-13 homolog a
scaffold361444	818	7175	scaffold223093	10223	3934	C26831295	1709	8072	zinc finger homeobox protein 3
scaffold61223	2602	8556	scaffold113949	8308	2206	C26830229	2	5934	armadillo repeat protein
scaffold266012	4166	10100	scaffold414482	12029	6085	C26830865	6008	37	golgin subfamily a member 4
scaffold69305	1406	7278	scaffold264200	434	6305	C26830255	108	5955	cadherin-related tumor suppressor
scaffold271828	4686	10496	scaffold114821	3108	8914	C26830141	30	5833	pre-mrna-processing-splicing factor 8
scaffold81629	9180	14966	scaffold515163	9418	3553	C26830979	6650	862	sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1
scaffold101886	3467	9074	scaffold482293	9963	4365	C26829945	113	5624	xyloside xylosyltransferase 1
scaffold346721	5567	11113	scaffold401141	548	6108	C26830551	6300	715	serine-rich adhesin for platelets
scaffold358632	1537	6995	scaffold516611	1027	6450	C26830467	234	5696	polypyrimidine tract-binding protein 1
scaffold252510	2070	7452	scaffold481478	5377	2	C26829923	5475	117	rho gtpase-activating protein 100f
C13914143	10	5324	scaffold515683	6507	1154	C26829645	5396	42	potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 3
scaffold342451	1321	6561	scaffold415814	1383	6503	scaffold24598	7078	1871	band 7 protein agap004871
scaffold81327	9768	14852	scaffold9884	14528	9487	C26830665	249	5296	zinc finger swim domain-containing protein 4
scaffold329301	4009	8752	scaffold26313	15872	20571	C26830747	5478	731	maternal effect protein staufen
scaffold75986	3014	7193	C19960242	4130	1	C26829979	1	4183	small subunit processome component 20 homolog
scaffold364198	4476	7596	scaffold4554	8671	5615	C26831415	8681	5586	plasma membrane calcium-transporting ATPase 2
scaffold15423	48	6259	scaffold263292	735	6949	C26831639	321	6539	uncharacterized protein
scaffold38621	4960	10258	scaffold57094	8799	3589	C26829291	5193	1	uncharacterized protein
scaffold38753	4979	11426	scaffold181360	8171	14631	C26830771	1	6442	uncharacterized protein
scaffold60656	3276	11020	scaffold283774	5016	12767	C26831365	7	7765	uncharacterized protein
scaffold32874	8679	13838	scaffold384374	8330	13462	C26831423	5794	697	uncharacterized protein
scaffold48410	2778	8200	scaffold217569	3426	8860	C26831009	7065	1636	uncharacterized protein

located are functional. It is becoming clear that the ncDNA plays essential roles in the regulation of gene expression (Ludwig, 2016). A comparative genome study between *D. melanogaster* and its related sibling species *D. simulans* demonstrated that a large fraction of ncDNA regions is functionally important and subject to both purifying selection and adaptive evolution. Adaptive changes to ncDNA might have been considerably more common in the evolution of *D. melanogaster* (Andolfatto, 2005). High mutation occurrence and subjecting to selection for ncDNA make it possible that the similarity in the

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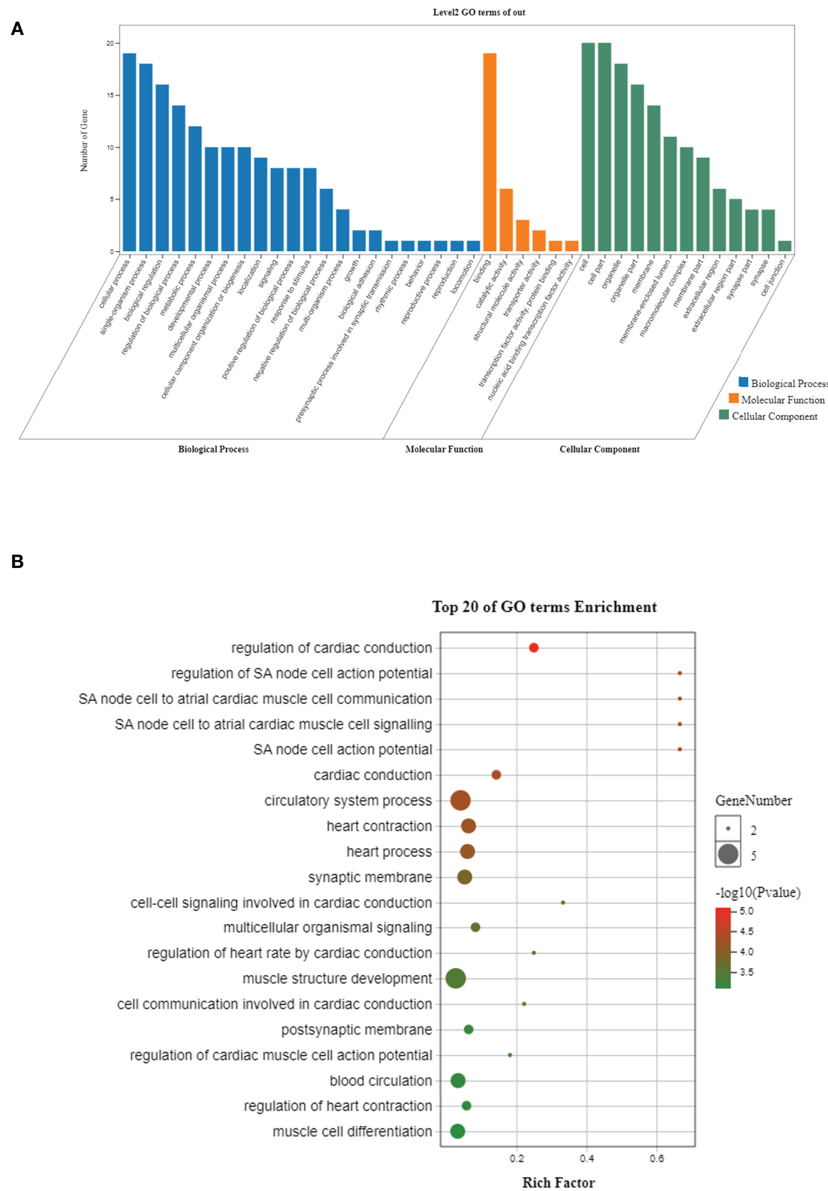

FIGURE 2
Histogram **(A)** and bubble plot **(B)** of Gene Ontology enrichment for the annotated similar regions.

entire-genome is higher than some conserved cDNA sequences between related species that live in the same environment.

Arkyrin-2 gene and its conservative protein fragment in Decapoda

The identified longest conserved region in all three crab species is contained in the arkyrin-2 gene (*ANK2*). The *ANK2* encodes a member of the ankyrin protein family that are adapters facilitating the local organization of integral membrane proteins with cytoskeletal elements (Cunha and Mohler, 2008; National Center for Biotechnology Information (NCBI), 2022). The ankyrin protein also play important roles in activities such as cell motility, activation, proliferation, contact and the maintenance of specialized membrane domains (National Center for Biotechnology Information (NCBI), 2022). This protein is active in many cell types, particularly in the brain and in cardiac muscle. It interacts with ion channels and ion transporters that generates the electrical signals controlling the heartbeat and maintain a normal heart rhythm (Mohler and Bennett, 2005; Cunha and Mohler, 2008). Such function of ANK2 coincides with the GO enrichment result those the most significant gene ontologies are cardiac-related.

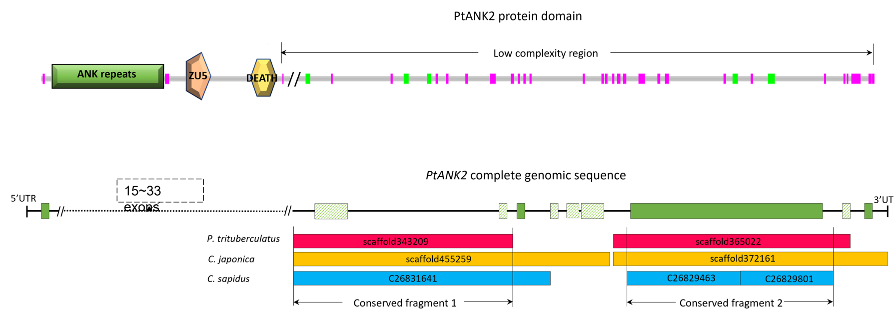

FIGURE 3
The illustration of the PtANK2 protein domain structure and the location of the two longest conserved region on PtANK2 gene.

Most ankyrins are typically composed of three structural domains: a consecutive ANK repeats (EMBL-EBI ID: IPR002110) that associate with ion channels, transporters, and cell adhesion molecules, such as the Na+/Ca2+ exchanger and Na/K-ATPase; a central region with a highly conserved spectrin binding domain (ZU5, EMBL-EBI ID: IPR000906) that participates in induction of apoptosis and binding of melanoma-associated antigen; and an Alpha-helical domain (Death, EMBL-EBI ID: IPR000488) that is related in sequence and structure to the death effector domain and the caspase recruitment domain. None of the similar regions that we identified spans the domain area, this is because the proteins of this area are encoded by very short exons, the genetic variations in introns are more than those in exons, the longer exons mainly distribute in the second half area, thus the similar regions (>1,000 bp) are mostly detected in this area. The mRNA

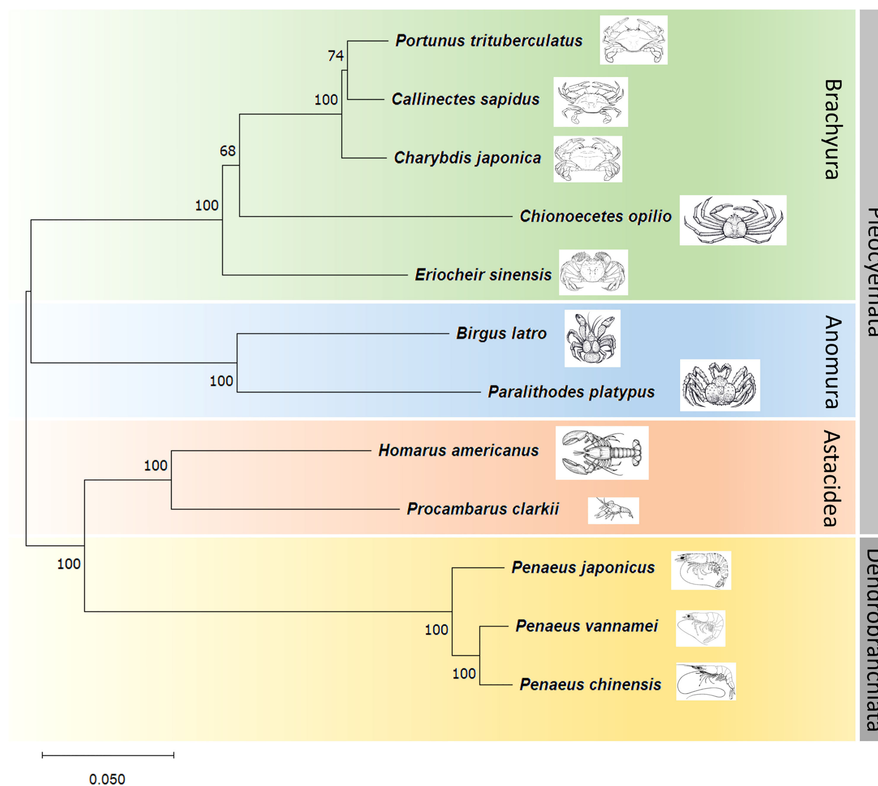

FIGURE 4
Neighbor-Joining tree based on the conserved ANK2 protein fragments of 12 Decapoda species.

of *ANK2* contain multiple isoforms. The number of isoforms is very high in some known Decapoda genomes (*P. trituberculatus*: 38; *H. americanus*:32; *P. japonicus*: 34). These isoforms are generated by alternative splicing. Alternative splicing is the primary mechanism through which the genome generates mRNA and protein diversity from a given coding repertoire. Alternative splicing plays important roles in regulating development, physiology and homeostatis, providing an alternative program in response to environmental stress (Mastrangelo et al., 2012; Kelemen et al., 2013; Liu and Guo, 2017). The *P. trituberculatus ANK2* contains 25 ~ 43 exons, almost all of which are missing in some isoforms except the longest exon close to the 3' end. That exon is included in one of the two longest conserved regions (CR2, Figure 3).

The protein sequence translated by this exon is found very conserved in other Decapoda species. The phylogenetic analysis using this protein fragment from 12 Decapoda species revealed a clear taxonomy and evolution relationship. The specification of crabs referring to a "Carcinization" or "Brachyurization" (McLaughlin and Lemaitre, 1997). Under that hypothesized scenario, the prawns (Dendrobranchiata) first diverged from Decapod ancestors, then a group evolved from swimming to crawling to walking (Reptantia, the ancestors of lobster/crayfish and crabs), during this process, their abdomen and tail gradually become short and flat, and start folding toward below the carapace, finally a clade of this group formed the nowadays' crab shape. In the phylogenetic tree, the genetic relationship between lobster/crayfish and crabs is closer than that between lobster/crayfish and prawns or between crabs and prawns, indicating later divergence of these two clades, however, the genetic distance between lobster/crayfish and prawns is smaller than that between crabs and prawns indicating the lobster/crayfish first diverged from prawns and their appearance is also more like the spawns than crabs. The evolution of this *ANK2* gene may occur accompanying the evolution process of the species.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/, accession numbers are SAMN26565628, SAMN26565629 and SAMN26565630.

Author contributions

Conceptualization: ML and SG; methodology: ML, SG, and SB; software: ML; formal analysis: ML, SG, and SB; data interpretation: ML and YJ; writing original draft preparation: ML and SB; writing review and editing: all authors; visualization: CG and YW; supervision: CF and HL; funding acquisition: HL and CF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.895119/full#supplementary-material

SUPPLEMENTARY TABLE 1
Statistics of microsatellite loci for the three crab species

SUPPLEMENTARY TABLE 2
The primer information of identified unique microsatellite makers.

SUPPLEMENTARY TABLE 3
The identified similar regions of alignment length of >1,000 bp among three crab species.

SUPPLEMENTARY FIGURE 1
K-mer depth distribution curves of three crab species that are generated by GenomeScope. The X-axis is the coverage (depth) of K-mer and the Y-axis is the frequency of the K-mer at that depth.

SUPPLEMENTARY FIGURE 2
The top 20 microsatellite motifs with highest occurrence frequencies **(A)**, and the top 9 scaffolds with most microsatellite distribution **(B)** in the three crab species.

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