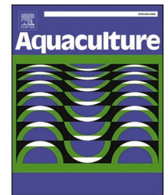
Aquaculture 598 (2025) 742044

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Development of a sex-specific molecular marker reveals the ZW/ZZ sex-determination system in *Babylonia areolata* (link 1807)

Yu Zou [a,d,1], Yuan Liang [a,d,1], Xuan Luo [a,d], Weiwei You [a,d], Minghui Shen [c], Jingqiang Fu [a,b,d,*], Caihuan Ke [a,d,*]

[a] State Key Laboratory of Mariculture Breeding, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, PR China
[b] School of Marine Biology, Xiamen Ocean Vocational College, Xiamen 361100, PR China
[c] Hainan Academy of Ocean and Fisheries Sciences, Hainan 570216, PR China
[d] Fujian Key Laboratory of Genetics and Breeding of Marine Organisms, Xiamen, 361102, PR China

ARTICLE INFO

Keywords:
Babylonia areolata
Sex-specific marker
Re-sequencing
Sex determination

ABSTRACT

The ivory shell (*Babylonia areolata*) is an economically significant marine gastropod that is widely distributed in southeast Asian countries. Although *B. areolata* is a dioecious species, there is a substantial deficiency in fundamental biological research on its sex. Furthermore, the increasing prevalence of marine pollution has led to severe imposex in some gastropod species, including the ivory shell. This shows that the female individual develops clear penile tissue before sexual maturity, which has affected researchers' ability to accurately determine the sex of ivory shells and hampered the development of crossbreeding and family breeding of ivory shells. Based on re-sequencing data from 42 ivory shell individuals, 4574 sex-linked SNPs/InDels loci were identified. Data analysis showed that the sex-determination type in the ivory shell was of the ZW/ZZ system, characterized by females being heterozygous and males being homozygous, with chromosome 3 as the sex chromosome. Based on a putative W chromosome-specific deletion fragment, a pair of sex-specific molecular markers was designed and validated by PCR. The primer was applied to four cultured populations of *B. areolata*, including Fujian, Hainan, and Thailand populations, as well as a hybrid population. Validation in individuals with known phenotypic sexes revealed an accuracy of 100 %. These results will be useful in future research on the molecular mechanism of sex determination and also provide a necessary tool to accurately identify the sexes in the breeding of ivory shells.

1. Introduction

Sex determination is an important and fundamental biological issue in life sciences, known as the "queen of evolutionary biology", which is related to the occurrence, survival, adaptation, and evolution of species (Bell, 1982). Therefore, the mechanism of sex determination has always been a hot topic in life science research (Mei and Gui, 2015).

There are many kinds of shellfish, and the number of species is second only to that of the phylum Arthropods. Owing to their special evolutionary status, lifestyle, and extremely diverse habitats, shellfish exhibit a variety of sex types, including hermaphroditic, simultaneous, and sequential hermaphroditism (Tworzydlo and Bilinski, 2019), which is a good material for sex-determination research. However, the study of the sex-determination mechanism of shellfish is limited to a few species, meaning that, compared with other animal phyla, research is significantly lagging behind. In the early stages, the main research methods were sex and karyotype analysis (Thiriot-Quiévreux, 2003). At present, the understanding of the mechanism of sex determination in shellfish remains relatively limited.

In aquatic species, the mechanism of sex determination of fish and molluscs can be divided into three types encompassing genetic sex determination, environment sex determination, and a combination of both, and many of them with no distinct alienation of sex chromosomes, which increase the difficulty to identify sex and study the mechanisms of sex identification (Kitano et al., 2024; Martínez and Viñas, 2014; Tworzydlo and Bilinski, 2019). In recent years, with the rapid development of sequencing and bioinformatics, even homomorphic sex chromosomes with very low differentiation levels can be analyzed and identified (Wang et al., 2022a, 2022b; Weng et al., 2022). This has improved the ability of researchers to identify sex chromosomes and

* Corresponding authors at: State Key Laboratory of Mariculture Breeding, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, PR China.
E-mail addresses: jingqiangfu@xmu.edu.cn (J. Fu), chke@xmu.edu.cn (C. Ke).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquaculture.2024.742044
Received 16 April 2024; Received in revised form 20 November 2024; Accepted 14 December 2024
Available online 16 December 2024
0044-8486/© 2024 Elsevier B.V. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

promoted the determination of sex-determination types and the study of sex-determination mechanisms (Palmer et al., 2019). For example, sex locus mapping has been performed in *Crassostrea gigas*, *Chlamys farreri*, and *Haliotis discus hannai* (Han et al., 2021; Jiao et al., 2014; Luo et al., 2021), and some genes associated with sex determination have been found in oyster, scallop, pearl oyster, freshwater pearl mussel, and abalone by transcriptome sequencing or gene cloning analysis. (Klinbunga et al., 2009; Li et al., 2016; Wang et al., 2021; Yu et al., 2011; Yue et al., 2018). In addition, molecular markers have been developed in several types of molluscs, namely *Busycon carica*, *H. discus hannai*, *H. diversicolor*, and *Mizuhopecten yessoensis* (Avise et al., 2004; Luo et al., 2021; Nagasawa et al., 2019; Weng et al., 2022). And the application of sex-special marker has facilitated the progress of some breeding programs and aquaculture management, especially for aquaculture species with long sexual maturation period and non-sexual dimorphism (Martínez and Viñas, 2014).

The ivory shell belongs to the phylum Mollusca, Gastropoda, Neogastropod, Babyloniidae, and *Babylonia*, which are distributed from the coast of the Indian ocean to the coastal areas of southeast Asia (Fu et al., 2024a; Petsantad et al., 2020). China is the main country for breeding ivory shell, where it is cultivated in the coastal areas of southern China, and its main species is *B. areolata* (Fu et al., 2024b). The ivory shell is a dioecious species, in that the male and female have a vagina and a penis, respectively, and internal fertilization is performed for heterotopic mating. The male penis is located behind the right antenna and is clearly visible as it crawls. The female's vagina is on the inner edge of the right mantle and is covered with a shell that cannot be observed during crawling. In crossbreeding and family breeding of ivory shells, it is necessary to accurately identify the sex of the ivory shells and isolate them before they reach sexual maturity and mating. However, imposex is a common phenomenon in neogastropods because of environmental pollutants such as organotin, which shows that female individuals develop abnormal male characteristics, such as the formation of a penis and vas deferens (Sternberg et al., 2009). Cases of severe imposex lead to the loss of a female's reproductive ability, resulting in population decline and even local extinction (Horiguchi et al., 2006). As early as 20 years ago, Liu and Suen investigated the imposex of five species of gastropods on the west coast of Taiwan and found that the imposex rates of wild *B. areolata* in Kaohsiung were close to 100 %, while those of two wild ivory shell species in Pingtung County were 7 % and 12 %, respectively (Liu and Suen, 1996). In addition, the ivory shell aquaculture industry has observed a serious imposex phenomenon, on which we have conducted a field investigation over the past 2 years. Two cultured populations of *B. areolata* were randomly selected, and we found that the imposex rate of sexually mature females reached 100 % (unpublished data), suggesting that this phenomenon is very serious. Because of the imposex, it is difficult to distinguish the male and female ivory shells from external morphology, which seriously affects the implementation of relevant breeding program of ivory shells. Therefore, an effective method for identifying the sex of ivory shells is urgently required.

At present, there is a lack of understanding and research on the mechanism of sex determination of ivory shells. In this study, we identified sex-specific markers by genome sequencing and then employed them to perform the identify of molecular sexing in *B. areolata*. We developed a reliable and rapid approach for sex identification using polymerase chain reaction (PCR) amplification and agarose gel electrophoresis. Our results provide new evidence for elucidating the sex-determination system and improving the breeding efficiency of *B. areolata*.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 42 samples, including 21 females and 21 males (Supplementary Table 1), were collected from an ivory shell breeding farm (24.21 N, 118.02 E, Zhangzhou, China), which were originally from a wild population in Zhangzhou, China (23.54 N, 117.44E) in 2021, then they were transferred to the above farm for breeding. Foot muscles were collected from each individual, frozen immediately with liquid nitrogen, and stored in a − 80 °C refrigerator. Under the protection of dry ice, the frozen muscle tissue was then sent to Novogene (Beijing, China) for DNA extraction and sequencing in the Illumina sequencing platform, which was performed according to the manufacturer's instructions. The concentration of extracted DNA was tested using a Qubit Fluorometer, and agarose gel electrophoresis was used to detect the integrity and purity of the DNA. DNA samples that passed the test were randomly broken into 300–350 bp fragments using an ultrasonic processor, and the paired-end library was constructed through the following steps: end repair, poly(A) addition, barcode addition, purification, and PCR amplification. The Illumina Hiseq X platform was used to sequence the constructed library in the 150 PE mode. The raw reads were filtered using the software fastp (version 0.20.0) (Chen, 2023) and used for downstream analysis.

2.2. Variation detection and sex-linked position identification

The filtered clean data were aligned to the chromosome-level genome of *B. areolata* (Size: ~1.6 Gb, NCBI GenBank: GCA_041734735.1) (Zou et al., 2024) using BWA-MEM2 (version 2.2.1) (Vasimuddin et al., 2019) with default parameters. Then, SAMtools (version 1.10) (Danecek et al., 2021) was used for filtering and sorting bam files, and Picard toolkit 2.9.0 (https://broadinstitute.github.io/picard/) was used for duplicate marking and filtering.

Variant calling was conducted using the Genome Analysis Toolkit (GATK, version 4.2.6.1) (McKenna et al., 2010). Variants were further filtered using VCFtools (version 0.1.17) (Danecek et al., 2011) with the criteria "–min-alleles 2 –maf 0.05 –min-meanDP 3 –max-missing 0.75" to ensure a minimum allele count of 2, a minor allele frequency of at least 5 %, a minimum mean depth of 3, and a maximum missing rate of 25 %. In addition, specific filters with GATK were applied to SNPs and InDels using the following parameters: for SNPs, "QD < 2.0 || FS > 60.0 || MQ < 40.0 || SOR > 3.0 || ReadPosRankSum < –8.0 || MQRankSum < –12.5"; and for InDels, "QD < 2.0 || FS > 200.0 || SOR > 10.0 || ReadPosRankSum < –20.0 || MQRankSum < –12.5." These filters were applied to remove variants with low quality, high strand bias, low mapping quality, and abnormal read position or mapping quality rank sum scores.

2.3. Identification of the sex-determination system and sex-linked region

Sex-linked regions were identified by analyzing sexually dimorphic SNPs and InDels using a custom script. The primary criterion for selection was the presence of SNPs/InDels with identical genotypes across all female and male individuals (Han et al., 2022). This approach allowed us to pinpoint loci exhibiting consistent sex-specific patterns with significantly fewer false positives than Genome-wide association studies (GWAS) methods that excel in quantitative trait analysis. Subsequently, the distribution of these sex-linked sites across all chromosomes and chromosome 3 was quantified and visualized using the ggplot2 package (Wickham, 2011) in R language (Ihaka and Gentleman, 1996). The Hi–C interaction matrix from the genome data of *B. areolata* in chromosome 3 was generated using 3D-DNA (version 180,419) (Dudchenko et al., 2017). A custom Hi–C plot was created using the Matplotlib library (Hunter, 2007) in Python.

Sequencing depth analysis in the re-sequenced samples was obtained using the SAMtools depth command. This involved filtering for genomic loci where the sequencing depth was zero in all male individuals and non-zero in females and vice versa. Drawing on methods used to assess genome characteristics during the genome survey step. In results that conform to a Poisson distribution (Sims et al., 2014), loci near the primary peak of the haplotype, specifically those from the lowest depth before the haplotype peak to 1.8 times the depth of the haplotype peak,

are identified as male or female-specific loci. This method effectively reduces the likelihood that the sex-specific loci we identify are affected by individual differences, sequencing errors, or interference from repetitive sequences. The distribution of sites meeting these criteria across the genome was statistically analyzed, and all graphs were plotted using the ggplot2 package.

2.4. Development and detection of sex-specific molecular makers

Sexually dimorphic InDels over 20 bp located in sex-linked regions were screened as target regions for sex-specific molecular markers, and then the reads alignment of male and female individuals was manually checked on Integrative Genomics Viewer (IGV, https://www.broadinstitute.org/software/igv/) (Thorvaldsdóttir et al., 2013). PCR primers based on genome sequence were designed using Primer Premier 6.0 (https://premierbiosoft.com/primerdesign/index.html) and synthesized by BGI Genomics (Shenzhen, China). PCR detected sex-specific molecular markers with 228 adult ivory shells, including the Fujian population (FJ: 30 individuals) that was originally from a wild population in Zhangzhou, China (23.54 N, 117.44E), Hainan population (HN: 27 individuals) that was originally from a wild population in Sanya, China (18.40 N, 109.75E), Thailand population (TL: 36 individuals) that was originally from a wild population in Rayong, Thailand (12.67 N, 101.27E), and a hybrid population (Hainan *B. areolata* ♀ × *Thailand B. areolata* ♂, HT: 21 individuals) that was the offspring of Hainan population and Thailand population by crossbreeding. All populations were cultured in a same ivory shell farm (24.21 N, 118.02 E, Zhangzhou, China). After collecting foot muscles and extracting DNA with a Tissue Kits (Qiagen, Shanghai, China), the PCR reaction was performed according to the following conditions: initial denaturation at 94 °C for 4 min, 35 cycles for 30 s at 94 °C for denaturation, 30 s for annealing at the Tm, and 30 s at 72 °C for extension, followed by a final extension at 72 °C for 4 min. The amplified products were separated using 1 % agarose gel electrophoresis.

3. Results

3.1. Re-sequencing and variant detection

As a result of whole-genome re-sequencing on the Illumina Hiseq X platform, 490.82 Gb of raw data and 464.77 Gb of clean data were retained with an average depth of 5.33 to the genome after quality filtering (Supplementary Table 1). Finally, through SNP calling and quality control from the re-sequencing data of 42 individuals (21 females and 21 males), 14,067,338 high-quality SNPs and 3,743,308 high-quality InDels were obtained for downstream analysis.

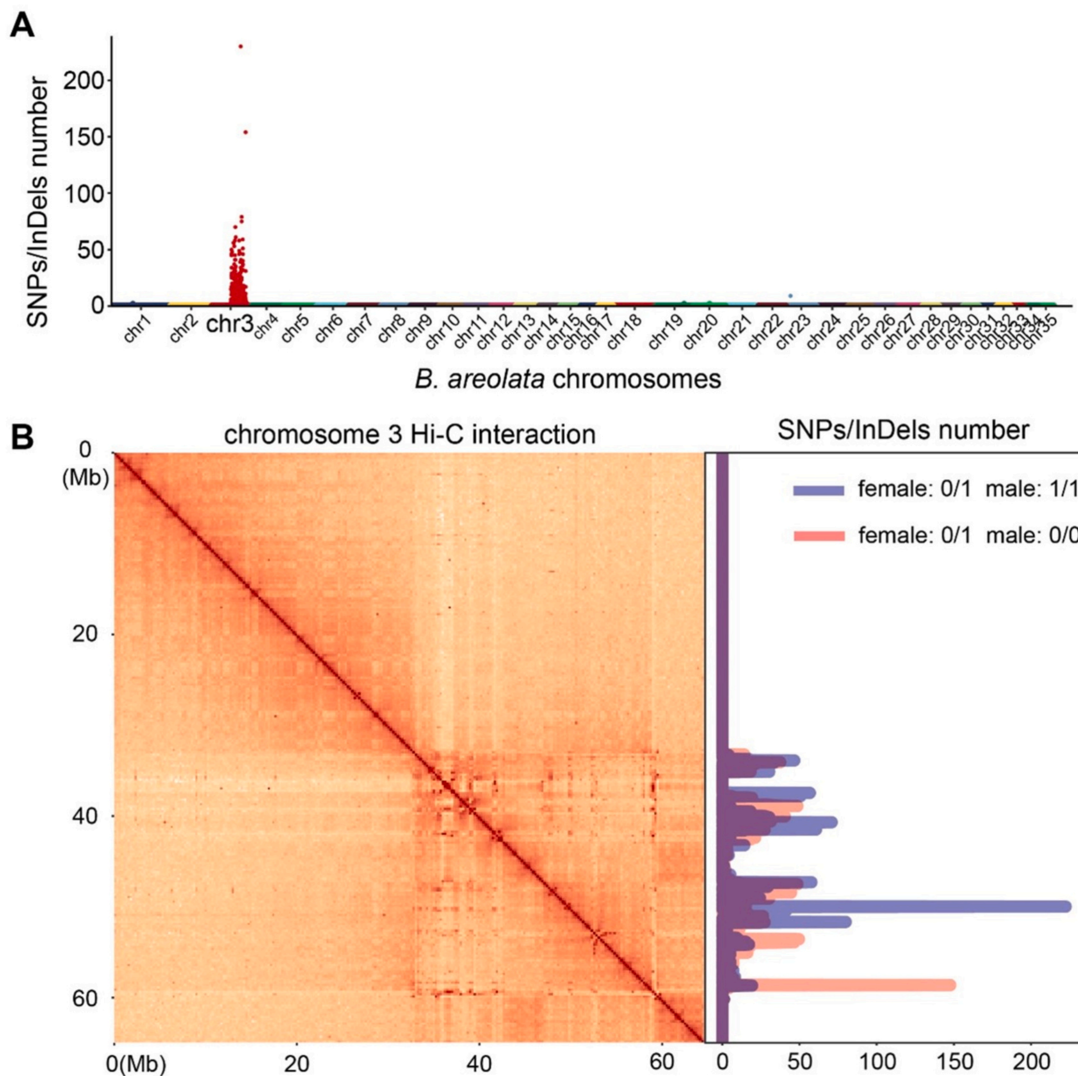


Fig. 1. Distribution of sex-linked dimorphic loci on chromosomes of *Babylonia areolata* (resolution: 50 kb). A. Distribution of dimorphic loci on chromosomes. B. Distribution of dimorphic sites on chromosome 3 and Hi–C interaction at corresponding positions with 250 kb resolution.

3.2. Variant distribution and sex-linked regions

Through statistics on the combination and number of all nine possible sexual dimorphism types in the entire genome, 4119 sexually dimorphic SNPs and 455 sexually dimorphic InDels were screened out (Supplementary Table 2). Except for heterozygous loci (0/1) for both sexes, all sexually dimorphic loci were heterozygous (0/1) for females and homozygous (0/0 or 1/1) for males (Supplementary Table 3). More importantly, 99.74 % of the sexually dimorphic loci were clustered in the 27.28 Mb region from 33.25 to 60.53 Mb on chromosome 3 (Fig. 1A, Table 1, Supplementary Table 4), indicating a close relationship with sex phenotype. Therefore, we considered this sequence segment to be the sex-linked region.

We examined the distribution of sexually dimorphic loci with the Hi-C interaction on chromosome 3 (Fig. 1B). In this region, the clustering of sexually dimorphic loci aligns with areas exhibiting complex Hi–C interactions, further supporting the significance of this region in sex determination. Furthermore, by analyzing the relationship between the distribution of sex-specific sites and sequencing depth, we found that sex-specific sites approximate a Poisson distribution only in females, and 98.40 % of these sites with depths of 39–173 were situated within the region spanning 32.98–59.97 Mb on chromosome 3 (Supplementary Fig. 1, Supplementary Tables 5–7). This observation aligns with the sex-determining region delineated through the analysis of sex-dimorphic sites, thereby corroborating the hypothesis of a female heterogametic chromosome system and underscoring the pivotal role of this region in sex determination.

3.3. Development and detection of sex-specific molecular makers

Eight sexually dimorphic InDels over 20 bp were screened out earlier (Supplementary Table 8), all of which were in the sex-linked region. After checking the read comparison of male and female individuals, the longest InDel fragment chr3_58920957 (81 bp) was selected to develop PCR-based sex-specific molecular markers, and one pair of primers (Bv3s1F: 5'-CCGCCACCACCACAACAAAGAG-3′ and Bv3s1R: 5'-TCA-CAATGAGAAACCTGCTGTGACAAA-3′) was designed according to the genome sequence containing the putative female-specific deletions (Fig. 2). The PCR detection of sex-specific molecular markers based on 228 individuals from four *B. areolata* populations was consistent with the expected results. All female samples were heterozygous with two electrophoretic bands, and all male samples had only one band, indicating homozygosity (Fig. 3, Table 2).

4. Discussion

In the past, research on the localization of sex-linked regions was typically based on two commonly used methods: quantitative trait locus (QTL) analysis and GWAS. Sex-specific markers are necessary to discover the sex-determining region (Lv et al., 2018; Wang et al., 2022a, 2022b). Traditional techniques such as restriction fragment length polymorphism, amplified fragment length polymorphism, simple sequence repeats, and random amplified polymorphic DNA have been successfully used to develop sex-specific markers in many aquaculture species, but these methods are inefficient and inaccurate (Gong et al., 2022; Wang et al., 2022a, 2022b). Compared with traditional techniques, the whole genome re-sequencing technology is a more efficient genotyping strategy (Jiao et al., 2014; Luo et al., 2021). Based on the distribution of sex-dimorphic sex-linked loci, this approach offers several advantages, including lower sample size, increased cost-effectiveness, higher precision, and faster analysis, and can accurately determine the sex-determination mechanism for most species that do not have heteromorphic sex chromosomes (Chen et al., 2022; Gong et al., 2023; Han et al., 2022; Lin et al., 2017; Luo et al., 2021). In recent years, thanks to the advances in genome sequencing technologies, the genomes of many aquatic animals have been assembled (Huang et al., 2024; Yang et al., 2020). Based on genomic information and resequencing data, researchers have developed sex-specific molecular markers in many aquatic species (Wu et al., 2021; Lin et al., 2017; Xue et al., 2020; Luo et al., 2021), which provide an accurate and rapid identification method for sex identification. In the present study, for the first time, we used whole genome re-sequencing and sexually dimorphic SNPs and indels analysis to successfully screen out a sex-specific marker in *B. areolata*. The coincidence of diagnosis was 100 % in all four tested populations, which strongly suggests that the sex marker can be used to accurately and efficiently identify the sex of young offspring. This provided a powerful molecular tool for relevant breeders and researchers to more easily and accurately identify the sex of *B. areolata*.

As low invertebrates, marine molluscs have a variety of reproductive types, which are easily affected by environmental factors. Hermaphroditism is relatively common, and some species also demonstrate dynamic sex changes (Collin, 2013; Nagasawa et al., 2019). Complex types and the influence of multiple factors make it difficult to analyze the sex-determination mechanism, which is the main reason for the slow development of research on the genetic mechanism of sex determination in shellfish (Weng et al., 2022). *B. areolata* is the main maricultured ivory shell species with important economic value on the southeast coast of China. The ivory shell is a gonochoristic species but exhibits a severe imposex phenomenon (Horiguchi et al., 2006; Muenpo et al., 2010), and ovarian spermatogenesis and suppressed ovarian maturation were observed in the females that exhibited imposex (Horiguchi et al., 2006). Several investigations provide converging evidence that tributyltin (and derivatives) pollution results in the induction of imposex in marine gastropods species by altering the retinoid X receptor (RXR) signaling pathway (Giulianelli et al., 2020), which could also lead to shell abnormalities, mortality, and population decline (Lesoway and Henry, 2021). Therefore, the degree of imposex can provide a reference for the monitoring and evaluation of seawater organotin pollution. An investigation of imposex in ivory shell should be completed by using sex-specific marker before selecting the site of farm and hatchery, and the areas with high levels of organotin pollution should be avoided.

Sex-associated markers are very useful for precocious sex identification, especially in those species lacking morphological sexual dimorphism. This can aid to identify the sex of potential broods in genetic breeding programs and to avoid sex bias in the selected population (Martínez et al., 2014). Under the breeding conditions, ivory shell reaches sexual maturity in one year, and imposex can be found 3–4 months after larvae metamorphosis. With the extension of farming period, the ratio of imposex will become higher and higher, and even reach 100 % at maturity. It is necessary to identify and isolate the male and female parents before ivory shell maturation in their hybridization and family breeding programs. The availability of a sex-special marker has enabled assessment of the genotypic sex of ivory shell for precocious sex identification just after about three months when some haemocytes can be obtained without affecting survival (Di et al., 2013), thus ensuring the reliability of the breeding program. Moreover, the females with severe imposex that may suffer from reproductive difficulties should be screened out to maintain appropriate sex ratios in the breeding

Table 1
Quantity and distribution of sexually dimorphic SNPs and InDels in the *Babylonia areolata* genome.

Type	Chromosome	No. of sexually dimorphic loci	Position	
			Start (bp)	End (bp)
SNPs	1	1 (0.02 %)	–	–
	3	4108 (89.81 %)	33,257,255	60,484,491
	19	1 (0.02 %)	–	–
	20	1 (0.02 %)	–	–
	23	8 (0.17 %)	7,960,920	7,961,304
InDels	3	454 (9.93 %)	33,460,331	58,995,800
	23	1 (0.02)	–	–

Fig. 2. *Re*-sequence alignment between females and males across female-specific deletion. A red box represented the putative female-specific 81 bp deletions on the *Babylonia areolata* genome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

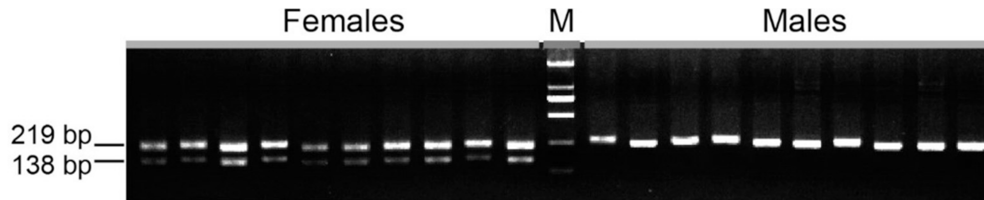


Fig. 3. Representative electrophoresis results with female and male individuals of *Babylonia areolata* using the primer pairs. M indicates the DNA marker.

Table 2
Coincidence rate in sex diagnosis of *Babylonia areolata* by the sex-specific molecular marker.

Population	Female	Male
FJ	30/30 (100 %)	30/30 (100 %)
HN	27/27 (100 %)	27/27 (100 %)
TL	36/36 (100 %)	36/36 (100 %)
HT	21/21 (100 %)	21/21 (100 %)

Note: FJ: Fujian *Babylonia areolata*, HN: Hainan *B. areolata*, TL: Thailand *B. areolata*, HT: Hainan *B. areolata* ♀ × Thailand *B. areolata* ♂.

programs of ivory shell.

In addition, the genomic location of sex-specific markers also provides important clues for sex chromosome identification, especially in species lacking heteromorphic sex chromosomes (Wen et al., 2024). Although most fish and invertebrates do not have significantly different sex chromosomes, some species have developed sex-determining regions (Luo et al., 2021; Wen et al., 2022). In some species, these genetic differences were large, such as ~12 Mb in *Takifugu bimaculatus* (Wang et al., 2022), ~17.9 Mb in *Sarotherodon melanotheron* (Gammerdinger et al., 2016), and ~ 17.4 Mb in channel catfish (Bao et al., 2019). By contrast, there are also many species whose sex loci are restricted to a small region (< 1 Mb) (Feron et al., 2020; Qu et al., 2021; Sun et al., 2023). However, distinguishing morphological differences between sex chromosomes or sex-determining regions in these species through cytological analysis is often challenging. In recent years, genetic differences have been easily identified through comparative genomic analysis between males and females (Palmer et al., 2019). Among them, detection and location of sexually dimorphic SNPs was the most sensitive method, which allowed the identification of sex chromosomes with differentiation for only several SNPs (Shi et al., 2018). In the present study, we identified a large sex-determination region on chromosome 3 (~27.28 Mb), in which 99.74 % of the sexually dimorphic loci were clustered, indicating that Chr. 3 is the sex-related chromosome of *B. areolata*. Moreover, the development and segregation pattern of female-specific markers in *B. areolata* suggested that its sex determination is female heterogamety (ZZ/ZW system). For example, there was only one 219-bp band in ZZ males, whereas there were two bands (219 and 138 bp) in ZW females. These results will be helpful to further explore the sex determination mechanism of *B. areolata*.

5. Conclusion

Our results confirmed the heterogametic ZZ/ZW sex-determination system in *B. areolata* by identifying chromosome 3 as the sex

chromosome and pinpointing the sex-determining region. Moreover, we successfully developed a female-specific marker based on PCR and agarose gel electrophoresis for rapid, accurate, and cost-efficient genetic sex identification in these species. These findings provide crucial fundamental data for the study of sex-determination mechanisms and represent a necessary tool for elevating the efficiency of breeding practices in ivory shell farming industries.

CRediT authorship contribution statement

Yu Zou: Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Yuan Liang:** Validation, Methodology, Investigation, Writing – review & editing. **Xuan Luo:** Validation, Supervision, Investigation. **Weiwei You:** Validation, Supervision, Investigation. **Minghui Shen:** Methodology, Investigation. **Jingqiang Fu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Caihuan Ke:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32202900), the Key Research and Development Program of Hainan Province (ZDYF2022XDNY234), the Outstanding Postdoctoral Scholarship, State Key Laboratory of Marine Environmental Science at Xiamen University, and the Project of Hainan Provincial Key Laboratory of Tropical Maricultural Technologies (HNTMTOF202302).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.742044.

Data availability

Data will be made available on request.

References

Avise, J.C., Power, A.J., Walker, D., 2004. Genetic sex determination, gender identification and pseudohermaphroditism in the knobbed whelk, *Busycon carica* (Mollusca: Melongenidae). Proc. Biol. Sci. 271 (1539), 641–646. https://doi.org/10.1098/rspb.2003.2533.

Bao, L., Tian, C., Liu, S., Zhang, Y., Elaswad, A., Yuan, Z., Khalil, K., Sun, F., Yang, Y., Zhou, T., Li, N., Tan, S., Zeng, Q., Liu, Y., Li, Y., Li, Y., Gao, D., Dunham, R., Davis, K., Waldbieser, G., Liu, Z., 2019. The Y chromosome sequence of the channel catfish suggests novel sex determination mechanisms in teleost fish. BMC Biol. 17 (1). https://doi.org/10.1186/s12915-019-0627-7.

Bell, G., 1982. The masterpiece of nature: the evolution and genetics of sexuality. The University of California Press, Berkeley. https://doi.org/10.4324/9780429322884.

Chen, S., 2023. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. iMeta e107. https://doi.org/10.1002/imt2.107.

Chen, J., Shao, X., Ye, S., Xiao, J., Zou, Y., Ye, K., Zhang, L., Wang, Z., Xiao, S., Cai, M., 2022. Development of sex-specific markers in Spinyhead croaker. *Collichthys lucidus*. Aquaculture 547. https://doi.org/10.1016/j.aquaculture.2021.737424.

Collin, R., 2013. Phylogenetic patterns and phenotypic plasticity of molluscan sexual systems. Integr. Comp. Biol. 53 (4), 723–735. https://doi.org/10.1093/icb/ict076.

Danecek, P., Auton, A., Abecasis, G., Albers, C.A., Banks, E., DePristo, M.A., Handsaker, R.E., Lunter, G., Marth, G.T., Sherry, S.T., 2011. The variant call format and VCFtools. Bioinformatics 27 (15), 2156–2158. https://doi.org/10.1093/bioinformatics/btr330.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and BCFtools. GigaScience 10 (2). https://doi.org/10.1093/gigascience/giab008.

Di, G., Zhang, Z., Ke, C., 2013. Phagocytosis and respiratory burst activity of haemocytes from the ivory snail, *Babylonia areolata*. Fish Shellfish Immunol 35 (2), 366–374. https://doi.org/10.1016/j.fsi.2013.04.032.

Dudchenko, O., Batra, S.S., Omer, A.D., Nyquist, S.K., Hoeger, M., Durand, N.C., Shamim, M.S., Machol, I., Lander, E.S., Aiden, A.P., 2017. De novo assembly of the *Aedes aegypti* genome using hi–C yields chromosome-length scaffolds. Science 356 (6333), 92–95. https://doi.org/10.1126/science.aal3327.

Feron, R., Zahm, M., Cabau, C., Klopp, C., Roques, C., Bouchez, O., Eché, C., Valière, S., Donnadieu, C., Haffray, P., Bestin, A., Morvezen, R., Acloque, H., Euclide, P.T., Wen, M., Jouano, E., Schartl, M., Postlethwait, J.H., Schraidt, C., Christie, M.R., Larson, W.A., Herpin, A., Guiguen, Y., 2020. Characterization of a Y-specific duplication/insertion of the anti-Mullerian hormone type II receptor gene based on a chromosome-scale genome assembly of yellow perch. *Perca flavescens*. Molecular Ecology Resources 20 (2), 531–543. https://doi.org/10.1111/1755-0998.13133.

Fu, J., Liang, Y., Shen, M., Lü, W., Luo, X., You, W., Ke, C., 2024a. Survival and immune responses of two populations of *Babylonia areolata* and their hybrids under pathogenic *Vibrio* challenge. Aquaculture 584. https://doi.org/10.1016/j.aquaculture.2024.740646.

Fu, J., Nie, L., Xia, G., Lü, W., Liu, H., Liang, Y., You, W., Ke, C., Luo, X., Shen, M., 2024b. Changes in low salinity and hypoxia tolerance in F1 hybrids of the ivory shell. *Babylonia areolata*. Aquaculture Reports 36. https://doi.org/10.1016/j.aqrep.2024.102131.

Gammerdinger, W.J., Conte, M.A., Baroiller, J.-F., D'Cotta, H., Kocher, T.D., 2016. Comparative analysis of a sex chromosome from the blackchin tilapia, *Sarotherodon melanotheron*. BMC Genomics 17 (1). https://doi.org/10.1186/s12864-016-3163-7.

Giulianelli, S., Primost, M.A., Lanari, C., Bigatti, G., 2020. RXR expression in marine gastropods with different sensitivity to imposex development. Sci. Rep. 10 (1). https://doi.org/10.1038/s41598-020-66402-1.

Gong, J., Li, B., Zhao, J., Zhou, Z., Ke, Q., Zhu, Q., Xu, D., Zhou, T., Xu, P., 2022. Sex-specific genomic region identification and molecular sex marker development of rock bream (*Oplegnathus fasciatus*). Marine Biotechnol. 24 (1), 163–173. https://doi.org/10.1007/s10126-022-10095-2.

Gong, G., Xiong, Y., Xiao, S., Li, X.-Y., Huang, P., Liao, Q., Han, Q., Lin, Q., Dan, C., Zhou, L., Ren, F., Zhou, Q., Gui, J.-F., Mei, J., 2023. Origin and chromatin remodeling of young X/Y sex chromosomes in catfish with sexual plasticity. Natl. Sci. Rev. 10 (2). https://doi.org/10.1093/nsr/nwac239.

Han, Z., Li, Q., Xu, C., Liu, S., Yu, H., Kong, L., 2021. QTL mapping for orange shell color and sex in the Pacific oyster (*Crassostrea gigas*). Aquaculture 530. https://doi.org/10.1016/j.aquaculture.2020.735781.

Han, W., Liu, L., Wang, J., Wei, H., Li, Y., Zhang, L., Guo, Z., Li, Y., Liu, T., Zeng, Q., Xing, Q., Shu, Y., Wang, T., Yang, Y., Zhang, M., Li, R., Yu, J., Pu, Z., Lv, J., Lian, S., Hu, J., Hu, X., Bao, Z., Bao, L., Zhang, L., Wang, S., 2022. Ancient homomorphy of molluscan sex chromosomes sustained by reversible sex-biased genes and sex determiner translocation. Nature Ecology & Evolution 6 (12), 1891–1906. https://doi.org/10.1038/s41559-022-01898-6.

Horiguchi, T., Kojima, M., Hamada, F., Kajikawa, A., Shiraishi, H., Morita, M., Shimizu, M., 2006. Impact of tributyltin and triphenyltin on ivory shell (*Babylonia japonica*) populations. Environ. Health Perspect. 114 (Suppl. 1), 13–19. https://doi.org/10.1289/ehp.8047.

Huang, Y., Li, Z., Li, M., Zhang, X., Shi, Q., Xu, Z., 2024. Fish genomics and its application in disease-resistance breeding. Rev. Aquac. https://doi.org/10.1111/raq.12973.

Hunter, J.D., 2007. Matplotlib: a 2D graphics environment. Computing in science & engineering 9 (03), 90–95. https://ieeexplore.ieee.org/document/4160265.

Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. J. Comput. Graph. Stat. 5 (3), 299–314. https://doi.org/10.1080/10618600.1996.10474713.

Jiao, W., Fu, X., Dou, J., Li, H., Su, H., Mao, J., Yu, Q., Zhang, L., Hu, X., Huang, X., Wang, Y., Wang, S., Bao, Z., 2014. High-resolution linkage and quantitative trait locus mapping aided by genome survey sequencing: building up an integrative genomic framework for a bivalve mollusc. DNA Res. 21 (1), 85–101. https://doi.org/10.1093/dnares/dst043.

Kitano, J., Ansai, S., Takehana, Y., Yamamoto, Y., 2024. Diversity and convergence of sex-determination mechanisms in teleost fish. Annual Review of Animal Biosciences 12 (1), 233–259. https://doi.org/10.1146/annurev-animal-021122-113935.

Klinbunga, S., Amparyup, P., Khamnamtong, B., Hirono, I., Aoki, T., Jarayabhand, P., 2009. Isolation and characterization of testis-specific DMRT1 in the tropical abalone (*Haliotis asinina*). Biochem. Genet. 47 (1–2), 66–79. https://doi.org/10.1007/s10528-008-9207-1.

Lesoway, M.P., Henry, J.Q., 2021. Retinoids promote penis development in sequentially hermaphroditic snails. Dev. Biol. 478, 122–132. https://doi.org/10.1016/j.ydbio.2021.06.013.

Li, Y., Zhang, L., Sun, Y., Ma, X., Wang, J., Li, R., Zhang, M., Wang, S., Hu, X., Bao, Z., 2016. Transcriptome sequencing and comparative analysis of ovary and testis identifies potential key sex-related genes and pathways in scallop *Patinopecten yessoensis*. Mar. Biotechnol. (N.Y.) 18 (4), 453–465. https://doi.org/10.1007/s10126-016-9706-8.

Lin, A., Xiao, S., Xu, S., Ye, K., Lin, X., Sun, S., Wang, Z., 2017. Identification of a male-specific DNA marker in the large yellow croaker (*Larimichthys crocea*). Aquaculture 480, 116–122. https://doi.org/10.1016/j.aquaculture.2017.08.009.

Liu, L.L., Suen, I.J., 1996. Prosobranch gastropod imposex in the west coast of Taiwan. Venus (Japanese Journal of Malacology) 55 (3), 207–214.

Luo, H., Xiao, J., Jiang, Y., Ke, Y., Ke, C., Cai, M., 2021. Mapping and marker identification for sex-determining in the Pacific abalone. *Haliotis discus hannai* Ino. Aquaculture 530. https://doi.org/10.1016/j.aquaculture.2020.735810.

Lv, J., Sun, D., Huan, P., Song, L., Liu, P., Li, J., 2018. QTL mapping and marker identification for sex-determining: indicating XY sex determination system in the

swimming crab (*Portunus trituberculatus*). Front. Genet. 9. https://doi.org/10.3389/fgene.2018.00337.

Martínez, P., Viñas, A.M., Sánchez, L., Díaz, N., Ribas, L., Piferrer, F., 2014. Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture. Front. Genet. 5. https://doi.org/10.3389/fgene.2014.00340.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., 2010. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20 (9), 1297–1303. https://genome.cshlp.org/content/20/9/1297.long.

Mei, J., Gui, J.F., 2015. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. Sci. China Life Sci. 58 (2), 124–136. https://doi.org/10.1007/s11427-014-4797-9.

Muenpo, C., Suwanjarat, J., Klepal, W., 2010. Ultrastructure of oogenesis in imposex females of *Babylonia areolata* (Caenogastropoda: Buccinidae). Helgol. Mar. Res. 65 (3), 335–345. https://doi.org/10.1007/s10152-010-0227-y.

Nagasawa, K., Thitiphuree, T., Osada, M., 2019. Phenotypic stability of sex and expression of sex identification markers in the adult yesso scallop *Mizuhopecten yessoensis* throughout the reproductive cycle. Animals (Basel) 9 (5). https://doi.org/10.3390/ani9050277.

Palmer, D.H., Rogers, T.F., Dean, R., Wright, A.E., 2019. How to identify sex chromosomes and their turnover. Mol. Ecol. 28 (21), 4709–4724. https://doi.org/10.1111/mec.15245.

Petsantad, P., Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., Chaitanawisuti, N., Karnchanatat, A., 2020. The antioxidant potential of peptides obtained from the spotted babylon snail (*Babylonia areolata*) in treating human colon adenocarcinoma (Caco-2) cells. RSC Adv. 10 (43), 25746–25757. https://doi.org/10.1039/d0ra03261a.

Qu, M., Liu, Y., Zhang, Y., Wan, S., Ravi, V., Qin, G., Jiang, H., Wang, X., Zhang, H., Zhang, B., Gao, Z., Huysseune, A., Zhang, Z., Zhang, H., Chen, Z., Yu, H., Wu, Y., Tang, L., Li, C., Zhong, J., Ma, L., Wang, F., Zheng, H., Yin, J., Witten, P.E., Meyer, A., Venkatesh, B., Lin, Q., 2021. Seadragon genome analysis provides insights into its phenotype and sex determination locus. Sci. Adv. 7 (34), 5196. https://doi.org/10.1126/sciadv.abg5196.

Shi, X., Waiho, K., Li, X., Ikhwanuddin, M., Miao, G., Lin, F., Zhang, Y., Li, S., Zheng, H., Liu, W., Aweya, J.J., Azmie, G., Baylon, J.C., Quinitio, E.T., Ma, H., 2018. Female-specific SNP markers provide insights into a WZ/ZZ sex determination system for mud crabs *Scylla paramamosain*, *S. Tranquebarica* and *S. Serrata* with a rapid method for genetic sex identification. BMC Genomics 19 (1). https://doi.org/10.1186/s12864-018-5380-8.

Sims, D., Sudbery, I., Ilott, N.E., Heger, A., Ponting, C.P., 2014. Sequencing depth and coverage: key considerations in genomic analyses. Nat. Rev. Genet. 15 (2), 121–132. https://www.nature.com/articles/nrg3642.

Sternberg, R.M., Gooding, M.P., Hotchkiss, A.K., LeBlanc, G.A., 2009. Environmental-endocrine control of reproductive maturation in gastropods: implications for the mechanism of tributyltin-induced imposex in prosobranchs. Ecotoxicology 19 (1), 4–23. https://doi.org/10.1007/s10646-009-0397-z.

Sun, D., Wen, H., Qi, X., Li, C., Sun, C., Wang, L., Zhu, M., Jiang, T., Zhang, X., Li, Y., 2023. Comparative study of candidate sex determination regions in snakeheads (*Channa argus* and *C. Maculata*) and development of novel sex markers. Aquaculture 575. https://doi.org/10.1016/j.aquaculture.2023.739771.

Thiriot-Quiévreux, C., 2003. Advances in chromosomal studies of gastropod molluscs. J. Moll. Stud. 69 (3), 187–202. https://doi.org/10.1093/mollus/69.3.187.

Thorvaldsdóttir, H., Robinson, J.T., Mesirov, J.P., 2013. Integrative genomics viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14 (2), 178–192. https://doi.org/10.1093/bib/bbs017.

Tworzydlo, W., Bilinski, S.M., 2019. Evo-Devo: non-model species in cell and developmental biology. Springer. https://doi.org/10.1007/978-3-030-23459-1.

Vasimuddin, M., Misra, S., Li, H., Aluru, S., 2019. Efficient architecture-aware acceleration of BWA-MEM for multicore systems, 2019 IEEE international parallel and distributed processing symposium (IPDPS). IEEE 314–324. https://ieeexplore.ieee.org/document/8820962/authors#authors.

Wang, Y., Wang, X., Ge, J., Wang, G., Li, J., 2021. Identification and functional analysis of the sex-determiner transformer-2 homologue in the freshwater pearl mussel. *Hyriopsis cumingii*. Front Physiol 12, 704548. https://doi.org/10.3389/fphys.2021.704548.

Wang, M., Li, L., Lin, H., Zhou, Z., Liu, B., Zhong, J., Pu, F., Shi, Y., Zhou, T., Xu, P., 2022a. Genome-wide association study identifies genomic loci of sex determination, gonadal weight and gonadosomatic index traits in *Takifugu bimaculatus*. Aquaculture 546. https://doi.org/10.1016/j.aquaculture.2021.737389.

Wang, Y., Yang, Y., Li, Y., Chen, M., 2022b. Identification of sex determination locus in sea cucumber *Apostichopus japonicus* using genome-wide association study. BMC Genomics 23 (1). https://doi.org/10.1186/s12864-022-08632-3.

Wen, M., Zhang, Y., Wang, S., Hu, F., Tang, C., Li, Q., Qin, Q., Tao, M., Zhang, C., Zhao, R., Wang, S., Liu, Q., Tang, C., Liu, S., 2022. Sex locus and sex markers identification using whole genome pool-sequencing approach in the largemouth bass (*Micropterus Salmoides* L.). Aquaculture 559. https://doi.org/10.1016/j.aquaculture.2022.738375.

Wen, M., Wang, S., Zhu, C., Zhang, Y., Liu, Z., Wu, C., Wang, S., Wang, Y., Ren, L., Tao, M., Qin, Q., Zhang, C., Hu, F., Liu, Q., Tang, C., Liu, S., 2024. Identification of sex locus and a male-specific marker in blunt-snout bream (*Megalobrama amblycephala*) using a whole genome resequencing method. Aquaculture 582. https://doi.org/10.1016/j.aquaculture.2024.740559.

Weng, X., Xu, Y., Dong, X., Luo, X., You, W., Ke, C., Cai, M., 2022. Sex-specific markers developed by next-generation sequencing confirmed a male heterogametic sex determination in small abalone. *Haliotis diversicolor*. Aquaculture 555. https://doi.org/10.1016/j.aquaculture.2022.738256.

Wickham, H., 2011. ggplot2. Wiley interdisciplinary reviews: computational statistics 3 (2), 180–185. https://www.deepdyve.com/lp/wiley/ggplot2-obx16U9wio.

Wu, X., Zhao, L., Fan, Z., Lu, B., Chen, J., Tan, D., Jiang, D., Tao, W., Wang, D., 2021. Screening and characterization of sex-linked DNA markers and marker-assisted selection in blue tilapia (*Oreochromis aureus*). Aquaculture 530. https://doi.org/10.1016/j.aquaculture.2020.735934.

Yang, Z., Zhang, L., Hu, J., Wang, J., Bao, Z., Wang, S., 2020. The evo-devo of molluscs: insights from a genomic perspective. Evol. Dev. 22 (6), 409–424. https://doi.org/10.1111/ede.12336.

Yu, F.F., Wang, M.F., Zhou, L., Gui, J.F., Yu, X.Y., 2011. Molecular cloning and expression characterization of Dmrt2in akoya pearl oysters, *Pinctada martensii*. J. Shellfish. Res. 30 (2), 247–254. https://doi.org/10.2983/035.030.0208.

Yue, C., Li, Q., Yu, H., 2018. Gonad transcriptome analysis of the pacific oyster *Crassostrea gigas* identifies potential genes regulating the sex determination and differentiation process. Mar. Biotechnol. (N.Y.) 20 (2), 206–219. https://doi.org/10.1007/s10126-018-9798-4.

Zou, Y., Fu, J., Liang, Y., Luo, X., Shen, M., Huang, M., Chen, Y., You, W., Ke, C., 2024. Chromosome-level genome assembly of the ivory shell *Babylonia areolata*. Scientific Data 11 (1). https://doi.org/10.1038/s41597-024-04001-9.