The Crown Pearl: a draft genome assembly of the European freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758)

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**Abstract**

Since historical times, the inherent human fascination with pearls turned the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) into a highly valuable cultural and economic resource. Although pearl harvesting in *M. margaritifera* is nowadays residual, other human threats have aggravated the species conservation status, especially in Europe. This mussel presents a myriad of rare biological features, e.g. high longevity coupled with low senescence and Doubly Uniparental Inheritance of mitochondrial DNA, for which the underlying molecular mechanisms are poorly known. Here, the first draft genome assembly of *M. margaritifera* was completed and is presented herein.
produced using a combination of Illumina Paired-end and Mate-pair approaches. The genome assembly was 2.4 Gb long, possessing 105,185 scaffolds and a scaffold N50 length of 288,726 bp. The \textit{ab initio} gene prediction allowed the identification of 35,119 protein-coding genes. This genome represents an essential resource for studying this species’ unique biological and evolutionary features and ultimately will help to develop new tools to promote its conservation. 

\textbf{Key words:} Margaritifera margaritifera, freshwater mussel, pearls, unionida genome, whole genome

1. Introduction

Pearls are fascinating organic gemstones that have populated the human beauty imaginary for millennia. Legend says that Cleopatra, to display her wealth to her lover Marc Antony, dissolved a pearl in a glass of vinegar and drank it. The human use of pearls or their shell precursor material, nacre, is ancient. The earliest known use of decorative nacre dates to 4200 BC in Egypt, with pearls themselves only becoming popular around 600 BC. Before the arrival of marine pearls to Europe, most were harvested from a common and widespread freshwater bivalve, the freshwater pearl mussel \textit{Margaritifera margaritifera} L. 1758 (Fig. 1), where generally one pearl is found per 3,000 mussels leading to massive mortality.\footnote{During the Roman Empire period, pearls were a desirable luxury, so that it is believed that one of the reasons that persuaded Julius Caesar to invade Britain was to access its vast freshwater pearl resources.} \textit{M. margaritifera} freshwater pearls were extremely valuable being included in many royal family jewels, such as the British, Scottish, Swedish, Austrian, and German crown jewels and even in the Russian city’s coat of arms.\footnote{Although over-harvesting represented a serious threat to the species for centuries, there has been a decrease in interest and demand for freshwater pearls in the 20th century. However, the global industrialization process introduced stronger threats to the survival of the species. In fact, \textit{M. margaritifera} belongs to one of the most threatened taxonomic groups on earth, the Margaritiferidae. The species was once abundant in cool oligotrophic waters throughout most of northwest Europe and northeast North America. However, habitat degradation, fragmentation, and pollution have resulted in massive population declines. Consequently, the Red List of Threatened Species from the International Union for Conservation of Nature has classified \textit{M. margaritifera} as Endangered globally and Critically Endangered in Europe. Besides being able to produce pearls, \textit{M. margaritifera} presents many other remarkable biological characteristics, \textit{e.g.} is among the most longest-living invertebrates, reaching up to 280 years\footnote{\textit{M. margaritifera} freshwater pearls were extremely valuable being included in many royal family jewels, such as the British, Scottish, Swedish, Austrian, and German crown jewels and even in the Russian city’s coat of arms. Although over-harvesting represented a serious threat to the species for centuries, there has been a decrease in interest and demand for freshwater pearls in the 20th century. However, the global industrialization process introduced stronger threats to the survival of the species. In fact, \textit{M. margaritifera} belongs to one of the most threatened taxonomic groups on earth, the Margaritiferidae. The species was once abundant in cool oligotrophic waters throughout most of northwest Europe and northeast North America. However, habitat degradation, fragmentation, and pollution have resulted in massive population declines. Consequently, the Red List of Threatened Species from the International Union for Conservation of Nature has classified \textit{M. margaritifera} as Endangered globally and Critically Endangered in Europe.}^ {10}.\textit{M. margaritifera} displays very weak signs of senescence, referred as the concept of ‘negligible senescence’;\footnote{has an obligatory parasitic larval stage on salmonid fishes used for nurturing and dispersion; and, like many other bivalves (see Gusman et al.\footnote{Although these biological features are well described, the molecular mechanisms underlying their regulation and functioning are poorly studied and practically unknown. Thus, a complete genome assembly for \textit{M. margaritifera} is critical for developing the molecular resources required to improve our knowledge of such mechanisms.} for a recent enumeration), shows an unusual mitochondrial DNA inheritance system, called Doubly Uniparental Inheritance or DUI.\footnote{To date, several Mollusca genomes are currently available and new assemblies are released every year at an increasing trend (reviewed in Ref.\cite{16–18})}^ {11,12} although these biological features are well described, the molecular mechanisms underlying their regulation and functioning are poorly studied and practically unknown. Thus, a complete genome assembly for \textit{M. margaritifera} is critical for developing the molecular resources required to improve our knowledge of such mechanisms.}

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Jellyfish v.2.2.10 and GenomeScope223,24 using Mollusca genomes (e.g. Ref.16) they should be identified and masked. The assembly was produced by running Meraculous v.2.2.6 and both PE and MP cleaned reads were used for whole-genome assembly. Long range Illumina MP quality processing was as described above.

2.3. Genome assembly and quality assessment
Cutadapt.22 Clean reads were used for genome size estimation with adapter sequences and removal of low-quality reads using formatics.babraham.ac.uk/projects/fastqc/) and raw reads were quality evaluated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The overall characteristics of the genome were accessed using PE reads. Reads quality was evaluated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and raw reads were quality trimmed with Trim Galore v.0.4.0 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), allowing the trimming of adapter sequences and removal of low-quality reads using Cutadapt.22 Clean reads were used for genome size estimation with Jellyfish v.2.2.10 and GenomeScope23,24 using k-mers lengths of 25 and 31.

2.3. Genome assembly and quality assessment
Long range Illumina MP quality processing was as described above and both PE and MP cleaned reads were used for whole-genome assembly. The assembly was produced by running Meraculous v.2.2.6 with several distinct k-mer sizes (meraculousising).21 This allowed determining the optimal k-mer size of 101. Genome assembly metrics were estimated using QUAST v5.0.2.26 Assembly completeness, heterozygosity, and collapsing of repetitive regions were evaluated through analysis of k-mer distribution using PE reads, with K-mer Analysis Toolkit.27 Furthermore, PE reads were aligned to the genome assembly using BBMap.28 BUSCO v. 3.0.229 was used to provide a quantitative measure of the assembly completeness, with a curated list of eukaryotic (n = 303) and metazoan (n = 978) near-universal single-copy orthogonal. Finally, in order to inspect the genome for possible contamination, we used BlobTools30 (Additional File 1).

The whole mitochondrial genome was assembled using the PE reads with MitoBim v.1.9.0171 and its annotation performed using MITOS232 web server and manually validated against other Margaritiferidae mitogenomes.

2.4. Repeat sequences, gene models predictions, and transcriptome alignment
Given the generally high composition of repetitive elements in Mollusca genomes (e.g. Ref.14) they should be identified and masked before proceeding to genome annotation. A de novo library of repetitive elements was created for M. margaritifera genome assembly, using RepeatModeler v.2.0.131 (excluding sequences <2.5 kb). Soft masking of the genome was performed with RepeatMasker v.4.0.734 combining the de novo library with the ‘Bivalvia’ libraries from Dfam_consensus-20170127 and RepBase-20181026.

BRAKER2 pipeline v.2.1.535,36 was used for gene prediction in the genome. First, all RNA-seq data of M. margaritifera73,38 available on GenBank were downloaded, assessed with FastQC v.0.11.8, quality trimmed with Trimmomatic v.0.3839 (Parameters, LEADING: 5 TRAILING: 5 SLIDINGWINDOW: 4:20 MINLEN: 36) and error corrected with Rockcorrector v.1.0.3.40 Afterwards, the RNA-seq data were aligned to the masked genome assembly, using Hisat2 v.2.2.0 with the default settings.21 The complete proteomes of 13 mollusc species, one Chordata (Ciona intestinalis) and one Echinodermata (Stronglylocentrotus purpuratus) were downloaded from distinct public databases (Supplementary Table S1) and used as additional evidence for gene prediction. The BRAKER2 pipeline was applied with the parameters (–etpmode; –softmasking; –UTR-off; –crf; –cores =30) and following the authors’ instructions.35,36 The resulting gene predictions (i.e. gff3 file) were renamed, cleaned, and filtered using AGAT v.0.4.0,35 correcting coordinates of overlapping gene prediction, removing predicted coding sequence regions (CDS) with <100 amino acid (in order to avoid a high rate of false-positive predictions) and removing incomplete gene predictions (i.e. without start and/or stop codons). Functional annotation was conducted by searching for protein domain information using InterProScan v.5.44.8033 and protein blast search using DIAMOND v. 0.9.324 against SwissProt (Download at 2/07/2020), TREMBL (Download at 2/07/2020), and RefSeq-NCBI (Download at 3/07/2020).45,46 BUSCO v. 3.0.229 scores for the predicted proteins were accessed using the eukaryotic (n = 255) and metazoan (n = 954) curated lists of near-universal single-copy orthogonal.

Finally, the M. margaritifera transcriptome assembly from Bertucci et al.37 downloaded from NCBI (BioProject: PRJNA369722) was aligned to the masked genome with blastl v.2.5,47 specifying the option ‘-fine -q=rna’ while maintaining the remaining parameters as default. Alignment stats were calculated with isoblat_v0.3148 using default parameters.

2.5. Phylogenetic analyses
For the phylogenetic assessment, the proteomes of 12 mollusc species were downloaded from distinct public databases (Supplementary Table S2), which included 11 Autobranchia bivalves and 2 outgroup species, i.e. the Cephalopoda Octopus bimaculoides and Gastropoda Biomphalaria glabrata (Fig. 3). Single-copy orthologous between these 12 species and M. margaritifera were retrieved using OrthoFinder v2.4.049 specifying multiple sequence alignment as the method of gene tree inference (-M). The resulting 118 single-copy orthologous sequences were individually aligned using MUSCLE v3.8.3150 with default parameters and subsequently trimmed with TrimAl v.1.251 specifying a gap threshold of 0.5 (-gt). Trimmed sequences were then concatenated using FASconCAT-G (https://github.com/PatrickKueck/FASconCAT-G). The best molecular evolutionary model was estimated using ProTest v.3.4.1.52 Phylogenetic inferences were conducted in IQ-Tree v.1.6.1253 for Maximum-Likelihood analyses (with initial tree searches followed by 10 independent runs and 10,000 ultra-bootstrap replicates) and MrBayes v.3.2.6614 for Bayesian Inference (2 independent runs, 1,000,000 generations, sampling frequency of 1 tree per 1,000 generations). All phylogenetic analyses were applied using the substitution model LG+I+G.
2.6. Hox and ParaHox gene identification and phylogeny

To identify the repertoire Hox and ParaHox genes in *M. margaritifera*, a similarity search by BLASTn of the CDS of *M. margaritifera* genome, was conducted using the annotated homeobox gene set of *Crassostrea gigas*. Candidate CDSs were further validated for the presence of the homeodomain by CD-Search. Finally, each putative CDS identity was verified by BLASTx and BLASTp searches in Nr-NCBI nr database and phylogenetic analyses. Since the search was conducted in the annotated genome (i.e. scaffolds over 2.5 kb), when genes were not found, a new search was conducted in the remaining scaffolds. At the end, any genes still undetected were search in the Transcriptome assembly of the species (Bioproject: PRJNA369722). Due to the phylogenetic proximity and for comparative purposes, Hox and ParaHox genes were also searched in the genome assembly of *M. nervosa*.

For phylogenetic assessment of Hox and Parahox genes, amino acid sequences of homeodomain of the genes from *M. nervosa* and *M. margaritifera* were aligned with other Mollusca orthologous. Molecular evolutionary models and Maximum-Likelihood phylogenetic analyses were obtained using IQ-TREE v.1.6.12.

3. Results and discussion

3.1. Sequencing results

A total of 494 Gb (~209×) of raw PE and 76 Gb (~32×) of raw MP data were generated, which after trimming and quality filtering were reduced by 0.3% and 10%, respectively (Supplementary Table S3). GenomeScope2 model fitting of the k-mer distribution analysis estimated a genome size between 2.31–2.36 Gb and very low heterozygosity between 0.127–0.105% (Fig. 2A). Although larger than the genome of *V. ellipsiformis* (i.e. 1.80 Gb), the size estimation of the *M. margaritifera* genome is in line with the recently assembled Unionida mussel *M. margaritifera* (i.e. 2.38 Gb). The estimated heterozygosity is the lowest observed within Unionida genomes and one of the lowest in Mollusca, which is remarkable considering it refers to a wild individual. This low value is likely a consequence of population bottlenecks during glacialization events, which have been shown to shape the evolutionary history of many freshwater mussels (e.g. 19,62,63) and may also be enhanced by recent human-mediated threats.

3.2. *Margaritifera margaritifera* de novo genome assembly

The Meraculous assembly and scaffolding yield a final genome size of 2.47 Gb with a contig N50 of 16,899 bp and a scaffold N50 of 288,726 bp (Table 1). Both N50 values are significantly higher than *V. ellipsiformis* genome assembly, i.e. 3,117 and 6,523 bp, respectively. Presently, this *M. margaritifera* genome assembly reveals one of the highest scaffold N50 of the Unionida genomes currently available. On the other hand, *M. nervosa* genome assembly contig N50, i.e. 51,552 bp, is higher than *M. margaritifera*, which is expected given the use of Oxford Nanopore ultra-long reads libraries in the assembly produced by Rogers et al. BUSCOs scores of the final assembly indicate a fairly complete genome assembly (Table 1) and although the contiguity is lower when compared with other recent Bivalve genome assemblies, the low percentage of fragmented genes (i.e. 5.9% for Eukaryota and 4.9% for Metazoa) gives further support to the quality of the genome assembly. Similarly, the slight difference observed between the genome size and the initial size estimation is unlikely to be a consequence of erroneous assembly duplication, as duplicated BUSCOs scores are also low (i.e. 1% for Eukaryota and 1.1% for Metazoa). The quality of the genome assembly is further supported by the high percentages of PE reads mapping back to the genome (i.e. 97.75%, Table 1), as well as the KAT k-mer distribution spectrum (Fig. 2B), which demonstrates that almost no read information was excluded from the final assembly. Additionally, around 99% of the transcripts of the *M. margaritifera* transcriptome assembly aligned to the genome assembly (Supplementary Table S4). Overall, these statistics indicate that the *M. margaritifera* draft genome assembly here presented is fairly complete, non-redundant, and useful resource for various applications.

The whole mitochondrial genome obtained with MitoBim is 16,124bp long and its gene content is the expected for Margaritiferidae female type mitogenomes with 13 protein-coding genes, 22 transfer RNA, and 2 ribosomal RNA.

3.3. Repeat identification and masking and gene models prediction

The use of the custom repetitive library combined with the RepBase ‘Bivalvia’ library, resulted in masking repetitive elements in more than half of the genome assembly, i.e. 59.07% (Table 2).

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**Figure 2.** (A) GenomeScope2 k-mer (25 and 31) distribution displaying estimation of genome size (len), homozygosity (aa), heterozygosity (ab), mean k-mer coverage for heterozygous bases (kcov), read error rate (err), the average rate of read duplications (dup), k-mer size used on the run (k), and ploidy (p). (B) Margaritifera margaritifera genome assembly assessment using KAT comp tool to compare the Illumina PE k-mer content within the genome assembly. Different colours represent the read k-mer frequency in the assembly.
Most of the annotated repetitive elements were unclassified (31.86%), followed by DNA elements (16.00%), long interspersed nuclear elements (6.13%), long terminal repeats (3.72%), and short interspersed nuclear elements (0.79%). After masking, gene prediction resulted in the identification of 35,119 protein-coding genes, with an average gene length of 25,712 bp and average CDS length of 1,287 bp (Supplementary Table S5). Furthermore, 26,836 genes were functionally annotated by similarity to at least one of the three databases used in the annotation (Table 1). The number of predicted genes is in accordance to those observed in other bivalves (and Mollusca) genome assemblies, which although highly variable, in average have around 34,949 predicted genes (calculated from Table 2 of Gomes-dos-Santos et al.16) Although the number of genes predicted within the three Unionida genomes is highly variable, i.e. 123,457 in V. ellipsiformis, 49,149 in M. nervosa, and 35,119 in M. margaritifera, a direct comparison should be taken with caution, given the considerable differences in genome qualities and the different gene predictions strategies applied in the three assemblies.

### 3.4. Single copy orthologous phylogeny

Both Maximum-Likelihood and Bayesian Inference phylogenetic trees revealed the same topology with high support for all nodes (Fig. 3). The phylogeny recovered the reciprocal monophyletic groups Pteriomorphia (represented by Orders Ostreida, Mytilida, Pectinida, and Arcida) and Heteroconchia (represented by Orders Unionida and Venerida). These results are in accordance with recent comprehensive bivalve phylogenetic studies.38,66–68 The only difference is observed within Pteriomorphia, where two sister clades are present, one composed by Arcida and Pectinida and the other by Mytilida and Ostreida (Fig. 3), while accordingly to the most recent phylogenomic studies, Arcida appears basal to all other Pteriomorphia.38,67,68 It is noteworthy that Arcida and Pectinida clade is the less supported in the phylogeny, which together with the fact that many Pteriomorphia clades are missing in this study, should explain these discrepant results. Heteroconchia is divided into monophyletic Palaeoheterodonta and Heterodonta (here only represented by two Euheterodonta bivalves). As expected, the two Unionida

### Table 1. Margaritifera margaritifera genome assembly, read alignment, gene prediction, and annotation general statistics

| Total number of sequences (≥1,000 bp) | 265,718 | 105,185 |
| Total number of sequences (≥10,000 bp) | 66,019 | 15,384 |
| Total number of sequences (≥25,000 bp) | 18,725 | 11,583 |
| Total number of sequences (≥50,000 bp) | 4,284 | 9,265 |
| Total length (≥1,000 bp) | 2,230,011,992 | 2,472,078,101 |
| Total length (≥10,000 bp) | 1,523,143,239 | 2,293,496,118 |
| Total length (≥25,000 bp) | 789,559,702 | 2,236,013,546 |
| Total length (≥50,000 bp) | 299,796,296 | 2,152,307,394 |
| N50 length (bp) | 16,899 | 288,726 |
| GC content, % | 35.42 | 35.42 |

**Clean paired-end (PE) reads alignment stats**

| Percentage of mapped PE (%) | — | 97.754 |
| Percentage of proper pairs PE (%) | — | 90.653 |
| Average PE sequence coverage | — | 181.968 |
| Percentage of scaffolds with any coverage (%) | — | 100.00 |

**Total BUSCOS for the genome assembly (%)**

| #Euk database | — | C: 86.8% (S: 85.8%, D: 1.0%), F: 5.9% |
| #Met database | — | C: 84.9% (S: 83.8%, D: 1.1%), F: 4.9% |

**Gene prediction and annotation stats**

| Protein-coding genes (CDS) | — | 35,119 |
| Transcripts (mRNA) | — | 40,544 |
| Protein-coding genes functional annotated | — | 26,836 |
| Transcripts functional annotated | — | 31,584 |
| Total gene length (bp) | — | 902,994,752 |
| Total mRNA length (bp) | — | 1,101,526,909 |
| Total CDS length (bp) | — | 52,211,391 |
| Total exon length (bp) | — | 52,211,391 |
| Total intron length (bp) | — | 1,024,450,311 |

**Total BUSCOS for the predicted proteins (%)**

| +Euk database | — | C: 90.6% (S: 81.2%, D: 9.4%), F: 3.9% |
| +Met database | — | C: 92.6% (S: 82.3%, D: 10.3%), F: 3.2% |

C: complete; S: single; D: duplicated; F: fragmented.

#Euk: from a total of 303 genes of Eukaryota library profile.

#Met: from a total of 978 genes of Metazoa library profile.

**a**

Euk: from a total of 255 genes of Eukaryota library profile.

**b**

Met: from a total of 954 genes of Metazoa library profile.

**All statistics are based on contigs/scaffolds of size ≥1,000 bp.**

**All statistics are based on contigs/scaffolds of size ≥2,500 bp.**
Table 2. Statistics of the content of repetitive elements in the *M. margaritifera* genome assembly

|                | Number of elements | Length occupied (bp) | Percentage of sequence (%) |
|----------------|--------------------|----------------------|-----------------------------|
|                | Marmar + Bivalvia  | Marmar + Bivalvia    | Marmar + Bivalvia           |
| SINEs:         |                    |                      |                             |
| ALUs           | 108,986            | 17,810,092           | 0.79                        |
| MIRs           | 51,807             | 7,321,859            | 0.33                        |
| LINEs:         |                    |                      |                             |
| LINE1          | 395,376            | 137,422,770          | 6.13                        |
| LINE2          | 108,179            | 29,801,298           | 1.33                        |
| L3/CR1         | 13,806             | 3,697,570            | 0.17                        |
| LTR elements:  |                    |                      |                             |
| ERVL           |                    |                      |                             |
| ERVL-MaLRs     |                    |                      |                             |
| ERV_classI     | 2,849              | 481,472              | 0.02                        |
| ERV_classII    | 1,072              | 286,047              | 0.01                        |
| DNA elements:  |                    |                      |                             |
| hAT-Charlie    | 1,208,077          | 358,545,022          | 16.00                       |
| TcMar-Tigger   | 22,178             | 3,778,430            | 0.17                        |
| Unclassified:  | 54,446             | 15,068,283           | 0.67                        |
| Total interspersed repeats: | 3,057,728          | 713,890,849       | 31.86                        |
| Small RNA:     | 51,767             | 1,311,085,924        | 58.51                        |
| Satellites:    | 24,005             | 4,250,110            | 0.34                        |
| Simple repeats:| 64,021             | 8,534,185            | 0.38                        |
| Low complexity:| 970                | 115,583              | 0.01                        |
| Total masked   |                    | 1,323,560,844        | 59.07                       |

Values were produced by RepeatMasker using a RepeatModeler’s custom build *M. margaritifera* repeat library (abbreviated with ‘Marmar’) combined with the RepBase Bivalve repeat library (RepeatMasker option -lib).

Figure 3. Maximum likelihood phylogenetic tree based on concatenated alignments of 118 single-copy orthologous amino acid sequences retrieved by OrthoFinder. *Above the nodes refer to bootstrap and posterior probabilities support values above 99%.
species, i.e. *M. nervosa* and the newly obtained *M. margaritifera*, are placed within Palaeoheterodonta.

### 3.5. Hox and ParaHox gene repertoire and phylogeny

Homeobox genes refer to a family of homeodomain-containing transcription factors with important roles in Metazoan development by specifying anterior–posterior axis and segment identity (e.g. Refs 69,70). Many of these genes are generally found in tight evolutionary conserved physical clusters (e.g. Refs 71,72). Hox genes are typically arranged into tight physical clusters, showing temporal and spatial collinearity.73 Consequently, Hox genes provide useful information for understanding the emergence of morphological novelties, understanding the historical evolution of the species, infer ancestral genomic states of genes/clusters, and even study genome rearrangements, such as whole-genome duplications (e.g. Refs 69,70,74). Given the disparate body plans in molluscan classes, the study of Hox cluster composition, organization and gene expression has practically become a standard in Mollusca genome assembly studies.60,75–88

Homeobox genes are divided into four classes, of which the Antennapedia (ANTP)-class (Hox, ParaHox, NK, Mega-homeobox, SuperHox) is the best studied, particularly the Hox and ParaHox clusters.60,74,84 The number of genes from these two clusters is relatively well conserved across Lophotrochozoa, with Hox cluster being composed of 11 genes (3 anterior, 6 central, and 2 posterior) and ParaHox cluster composed of 3 genes. Although several structural and compositional differences have been observed within Mollusca ANTP-class (e.g. Bivalvia,80 Cephalopoda,81 Gastropoda,82 and Polychaeta),77 most Bivalvia seem to retain the gene composition expected for lophotrochozoans: Hox1, Hox2, Hox3, Hox5, Lox, Antp, Lox4, Lox2, Post2, and Post1 for the Hox cluster and Gsx, Xlox, and Cdx for the ParaHox cluster.78 Consequently, the identification of these genes on a bivalve genome assembly represent further validation of the genome completeness and overall correctness. Furthermore, to the best of our knowledge, this study reports for the first time the Hox and ParaHox genes were identified Unionida. A single copy of the 3 ParaHox and 10 Hox genes were found in the *M. margaritifera* genome assembly (Supplementary Table S6). Despite an intensive search, no evidence of the presence of Hox4 was detected. However, the gene was identified in the *M. margaritifera* transcriptome, thus confirming its presence in the species. All genes, apart from *Antp* and *Lox5*, were scattered in different scaffolds, with Hox5, Post1, and Gsx being present in scaffolds smaller than 2.5 kb (Supplementary Table S6). Both the small proximity between *Antp* and *Lox5* and the fact that both

*Figure 4. Hox and ParaHox maximum likelihood gene tree constructed using Mollusca homeodomain amino acid sequences. Bootstrap values are presented above the nodes. Red squares highlight the position of *M. margaritifera* Hox and ParaHox.*
genes are expressed in the same direction are in accordance with the results observed in other bivalves, including in the phylogenetically closest species (from which Hox cluster has been characterized), i.e. the Venerida clam Cyclina sinensis (Gmelin, 1791).60 The fact that the remaining genes were scattered in the different scaffolds is likely a consequence of the low contiguity of the genome assembly since the distances between Bivalvia Hox genes within a cluster can be as high as 9.9 Mb.60 Conversely, three Hox and one ParaHox genes were found in the *M. margaritifera* transcriptome assembly and nine Hox and one ParaHox gene were found in *M. nervosa* genome assembly (Supplementary Table S6). Finally, to further validate the identity of the identified Hox and ParaHox genes, a phylogenetic analysis using the homeodomains (encoded 60–63 amino acid domain) of several Mollusca species was conducted (Fig. 4). All Hox and ParaHox genes of *M. margaritifera* (as well as *M. nervosa*) were well positioned within their respective orthologous genes from other Mollusca species (Fig. 3), thus confirming their identity.

### 3.6. Conclusion and future perspectives

Unionida freshwater mussels are a worldwide distributed and diverse group of organisms with 6 recognized families and around 800 described species.59,60 These organisms play fundamental roles in ecosystems, such as water filtration, nutrient cycling, and sediment bioturbation and oxygenation,61,62 allowing to maintain and support freshwater communities.12 However, as a consequence of several anthropogenic threats, freshwater mussels are experiencing a global-scale decline.12,13 *M. margaritifera* belongs to the most threatened of the 6 Unionida families, i.e. Margaritiferidae. Despite all this, our understanding of the genetics of this species is still to date restricted to a few mtDNA markers phylogenetic and restricted phylogeographical studies63–96 as well as neutral genetic markers,93,97,98 making the availability of the present genome a timely resource with application in multiple fields. The characterization of genetic features and identification of genomic novelties (such as single genes or gene families, genomic pathways, single-nucleotide polymorphism, among others) may provide guidance understanding molecular and cellular mechanisms of biomineralization in freshwater mussel shells that may facilitate the use of shell material as environmental and metabolic archives99 and even help clarify the formation of new mineralized tissue following extracorporeal shock wave therapy in humans.100 Being the first representative genome of the family Margaritiferidae, it will help launch both basic and applied genomic-level research on the unique biological and evolutionary features characteristic of this emblematic group.

### Accession numbers

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### Supplementary data

Supplementary data are available at NARES online.

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### Data availability

All the raw sequencing data are available from GenBank via the accession numbers SRR13091478, SRR13091479, and SRR13091477. The assembled genomes are available in the accession number JADWMO0000000, under the BioSample PRJNA678877 and BioSample SAMN16815977 (Supplementary Table S7). The whole mitogenome is available in GenBank under the accession number MW556443. Fasta alignment of homeodomain amino acid sequences from Hox and ParaHox genes used in gene tree construction is available in Additional File 2. The scaffolds in which homeodomains were detected (as described in Supplementary Table S6) are available as Additional File 3. The repeat masked genome assembly, BRAKER2 prediction statistic and prediction gff files, as well as all predicted genes, transcripts and amino acid sequence files are available at Figshare: 10.6084/m9.figshare.1333841.

### Conflict of interest

None declared.

### Additional File 1. BlobBTools contamination screening methods’ description and results.

### Additional File 2. Fasta alignment of homeodomain amino acid sequences from Hox and ParaHox genes used in gene tree construction. Sequences used include the Hox and ParaHox homeodomain sequences retrieved from Refs.59,60 Additional File 3. Scaffolds fasta sequences in which homeodomains were detected (as described in Supplementary Table S6).

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