High-quality reannotation of the king scallop genome reveals no ‘gene-rich’ feature and evolution of toxin resistance

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The king scallop, Pecten maximus is a well-known, commercially important scallop species and is featured with remarkable tolerance to potent phytotoxins such as domoic acid. A high-quality genome can shed light on its biology and innovative evolution of toxin resistance. A reference genome has recently been published for P. maximus, however, it is suspicious that over 67,700 genes are annotated in this genome, which is unexpectedly larger than its close relatives of pectinids. Herein, we provide an improved high-quality chromosome-level reference genome assembly and annotation for the king scallop P. maximus. A final set of 26,995 genes is annotated after carefully checking and curation of the predicted gene models, which significantly improves the accuracy of gene structure information. The large number of gene duplicates in the previous genome is mainly distorted by the fragmented annotation. Through integrated genomic, evolutionary and transcriptomic analyses, we reveal that the Phi subfamily of ionotropic glutamate receptors (iGluRs) are well preserved in molluscs, and P. maximus experienced the rapid expansion of the Phi class of iGluR (GluF) gene family. The GluF genes exhibit ubiquitously high expression and altered sequence characteristics for ligand selectivity, which may contribute to the remarkable tolerance to neurotoxins in P. maximus. Taken together, our study disapproves the previous claim of the ‘gene-rich’ genome of this species and provides a high-quality genome assembly for further understanding of its biology and evolution of toxin resistance.

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1. Introduction

The king scallop Pecten maximus is widely distributed along the Northeast Atlantic coast and is one of the major commercial fisheries in Europe [1]. In recent years, the king scallop has been ranked consistently in the top five fishery species in UK, with over 48,800 tonnes landing in 2018 [2]. Besides its important commercial value, the king scallop fishery also serves as an excellent model for the study of neurotoxin resistance. When king scallops are exposed to harmful algal blooms (HABs) in their habitat, they can accumulate and retain a fairly high level of domoic acid (DA) for a long period [3], which makes them distinguished from other bivalves (e.g., mussels) that usually possess a toxin ‘saturation’ point [4,5]. It is suggested that genomic features may be present and contribute to its unparalleled toxin resilience [6].

The rapid developments of high-throughput sequencing technologies facilitate the accumulation of vast amount of genomic data in molluscs [7]. Nevertheless, gene information encoded by the genomic assembly needs to be accurately retrieved to help us understand the organism biology and the evolution in a broad picture. Despite that numerous genomic annotation methods have been developed to integrate evidences from multi-omic datasets, accurate genome annotation remains a major challenge for large and complex genomes [8,9]. Recently, a reference genome sequence of P. maximus has been assembled and annotated with 67,741 genes, which is about 3-fold more than its close relatives of the Pectinidae family and was interpreted as unique ‘gene-rich’ genomic feature [6]. Considering that the genome size and

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gene cassette is generally conservative across the Pectinidae species [7], the suspicious over-inflated number of genes could be potentially resulted from the artifacts or errors in genome annotation.

As the accuracy of reference genome sequences is of vital importance for genetic and genomic analysis, we generated an de novo genome assembly of P. maximus and annotated a set of 26,995 genes after manual curation of automated gene structural prediction. The high-quality reference genome sequences and transcripts enable a comprehensive analysis of the ionic glutamate receptors (iGluRs) in the king scallop, and lay the foundation for deep understanding of the innovative evolution of molluscan toxicant resistance.

2. Results and discussion

2.1. Genome sequencing and assembly

A total of 139.16 Gb bases were used for the assembly of the king scallop genome, including 46.62 Gb Illumina reads and 92.54 Gb Pacbio reads, representing ~ 128 × genome coverage (Supplementary Table S1). The genome of king scallop was estimated with a heterozygous ratio of 1.24% and repeat rate of 56.48%. We assembled a genome sequence with a total size of 1,059.85 Mb and a contig N50 of 50.6 Mb, which is consistent with the previous karyotype analysis of this scallop species [10]. The chromosomes of P. maximus exhibited a 1:1 perfect correspondence to that of Muzihopexten yessoensis [11], despite that P. maximus has a closer phylogenetic relationship with the Argopexen genus where 16 haploid chromosomes were observed (Fig. 1B; Supplementary Fig. S2).

2.2. Evaluation of genome annotation

In Kenny et al. (2020), a BUSCO evaluation was performed in the genome mode. Despite that 95.5% of the BUSCO Metazoa dataset could be predicted in the genome assembly, whether or not they have been successfully annotated is unknown [6]. Therefore, we conducted the analysis in the protein mode with default parameters, and identified only 601 (61.4%) complete BUSCOs in the previous one and is more in line with the k-mer analysis (1.083.96 Mb) and previous assessments (1.025–1.150 Mb) [6] (Supplementary Fig. S1). The improved completeness was also revealed by the alignment rate of whole genomic sequencing reads. The mapping rate of Illumina and Pacbio reads reached 98.6% and 97.28% for the new assembly, which is higher than the previous one (~94%) [6]. We only detected 9,291 (0.0009%) conflicting sites in the genome assembly (Fig. 1A), indicating a high degree of consistency with the Illumina reads. The contigs were anchored into 19 chromosomes with a scaffold N50 of 50.6 Mb, which is consistent with the previous karyotype analysis of this scallop species [10]. The chromosomes of P. maximus exhibited a 1:1 perfect correspondence to that of Muzihopexten yessoensis [11], despite that P. maximus has a closer phylogenetic relationship with the Argopexen genus where 16 haploid chromosomes were observed (Fig. 1B; Supplementary Fig. S2).

2.3. Evolution of iGluR gene family

We checked the distribution of exon numbers per gene across seven molluscan species and noticed that the gene models of the former P. maximus genome assembly exhibited a distinguished pattern compared with the remaining sets (Fig. 1D). The number of genes with eight or more exons were obviously reduced in the previous annotation of P. maximus genomic assembly [6], while genes with four or fewer exons were sharply expanded, suggesting that a considerable number of large genes were mis-annotated as fragmented models. We further checked the protein length of 7,544 single copy orthologues in four scallops. It clearly shows that the previous version of gene models was incomplete (Fig. 1E). Even several well-conserved genes across metazoans, including Hox1, Lox5 and Post2, have been identified as two adjacent separate genes and mistaken as tandem duplication (Fig. 1F; Supplementary Fig. S4). We further checked the alignment of RNA-seq data from different tissues and developmental stages. The new version of reference genome sequences and annotation improved about 8% and 5% of the unique and overall mapping rate on average, respectively (Table S8).

Identified the full sets of iGluR family genes from the genomes of seven species covering major molluscan lineages, and performed a phylogenetic study with those identified from 28 additional representative species across the metazoans. The number of iGluR genes ranged from 19 (Hapalochlaena maculosa) to 36 (P. maximus) within the molluscs (Supplementary Table S9). Ramos-Vicente et al. (2018) revealed that the iGluR genes could be clustered into four major subfamilies that diverged prior to the split of metazoan lineages, including Lambda, N-methyl-D-aspartate (NMDA), Epsilon, and AKDF [15]. Our phylogenetic analysis recovered the monophyly of the groups defined by the four subfamilies and placed Lambda at the deepest lineage (Fig. 2A).

Almost all the molluscan iGluR genes were grouped into the clade of NMDA and AKDF, suggesting the loss of Lambda and Epsilon in molluscs. Intriguingly, despite that the Phi class of iGluRs genes (GluF) of the AKDF subfamily were previously considered to be restricted in Echinodermata, Hemichordate, and non-vertebrate Chordata, we identified the Phi class in all the seven molluscs. A novel class Zeta was identified exclusively in molluscs as a sister group of Phi, suggesting a molluscan specific expansion in the AKDF subfamily. Phylogenetics of GluF genes from 21 molluscs revealed four clades (subclass I to IV). Here we show that the subclass I and IV is present in most of the molluscs, whereas the subclass II and III is phylogenetically restricted in gastropods and bivalves, respectively (Fig. 2B). Notably, the king scallop possesses the largest number of GluF genes among the 21 analyzed molluscs. The flanking genes of two GluFIV genes are highly conservative, suggesting that the rapid expansion is driven by tandem duplication (Supplementary Fig. S5).

Gene expression profiling facilitates the understanding of the function and evolution of iGluR families. Beside the king scallop, we collected another eight molluscs with the best availability of comprehensive transcriptome datasets to analyze the expression
profiles in various adult tissues/organs (Fig. 3A). The iGluR genes generally exhibited diverse tissues/organs-preferential expression patterns across the nine molluscs. Interestingly, compared with the cephalopod and gastropods, the GluF genes in *P. maximus* and the other two scallops were ubiquitously expressed with the most prominent expression levels among all the iGluRs (Fig. 3A). It is suggested that GluF genes may be important receptors in response to neurotransmitters and control the signal transmission in the scallops.

Proteins of the GluF genes in molluscs present well-conserved transmembrane domains and residues for tetramerization (Supplementary Fig. S6). Three-dimensional models of the GluFIII of *P. maximus* indicate that its general fold is well preserved (Fig. 3B). Previous studies have revealed that the most important residues for fixing the amino acid backbone are Arg485 and an acidic residue at position 705 [16,17,18]. These two positions are well conserved in nine of the ten proteins of GluF genes in scallops, suggesting that their intrinsic ligand is an amino acid. Ligand selectivity of iGluRs is mainly determined by residues at 653 and 655 [19]. For typical glutamate-binding receptors, these two sites are occupied by glycine and threonine, whereas, for glycine-binding iGluRs, they are serine and a non-polar residue, respectively. In ctenophore, the residue 653 could be substitute to serine or threonine for glutamate-binding iGluRs, and to arginine for glycine-binding subunits [19]. Notably, despite that GluFi, GluFIV3, and GluFIV4 possess residues of typical glutamate-binding iGluRs, high variability was observed in residues 653 and 655 of the remaining GluF genes in *P. maximus*. The king scallop GluFII, GluFIV1, and GluFIV2 genes, which possess a glycine or valine at residue 653, and a non-polar residue at residue 655, are thus candidates for glycine-binding receptors (Fig. 3C). As the span of domain opening is much smaller in the ligand-binding core of glycine-binding iGluRs, they usually are not affected by glutamate agonists, such as DA and kainic acid (KA) [20]. The presence of these glycine-binding iGluRs may explain scallop’s amazing ability to tolerate neurotoxin DA. Our bioinformatic analyses of GluF genes would provide important protein structural insights and guidance for further experiment-oriented investigations and deepen our understanding of their functional roles in the evolution of toxin resistant.

3. Conclusion

Our study provides a high-quality chromosome-level reference genome sequence for the king scallop *P. maximus*. A final set of 26,995 genes were annotated on the genome after carefully check and curation of the predicted gene models, which significantly improved the accuracy of gene structural information. We proved that the large numbers of gene duplicates in *P. maximus* were distorted by the fragmented annotation in the previous genomic study. The number of genes in *P. maximus* is actually comparable to those of other Pectinidae species with high-quality genomic assembly. Through integrated genomic, evolutionary and transcriptomic analyses, we revealed for the first time that the Phi subfamily of iGluRs were well preserved in molluscs, and the *P. maximus* experienced a rapid expansion in the GluF gene family. The GluF genes exhibited a ubiquitously high expression and altered sequence characteristics for ligand selectivity, which may contribute to the remarkable tolerance to neurotoxins in *P. maximus*. Taken together, our study disapproves the previous claim of the ‘gene-rich’ genome of this species (mostly due to inaccurate gene annotation) and provides a high-quality genome assembly for further understanding of its biology and evolution in toxin-resistance.
4. Materials and methods

4.1. Samples preparation and sequencing

The king scallops, *P. maximus* were purchased commercially from Seashell AS (Norddyrøy, Norway). The shell and DNA samples were deposited at the Key Laboratory of Marine Genetics and Breeding (Ministry of Education), Ocean University of China (Specimen code: OUC-MGB-2019-PMA-03). Soft tissues were dissected and frozen in liquid nitrogen immediately after sampling. Genomic DNA was extracted from the adductor muscle using phenol/chloroform alcohol method [21]. A paired-end Illumina library with an insert size of 350 bp was prepared and sequenced on an Illumina HiSeq X-Ten system. Genomic DNA from the same sample was also used to construct a library for PacBio sequencing on a PacBio Sequel Single-molecule Real-time (SMRT) platform. Mantle and kidney of three adult individuals were collected for transcriptome sequencing. Tissues were stored at -80°C after being flash-frozen in liquid nitrogen. Total mRNA was extracted with phenol/chloroform alcohol and all the libraries were constructed by VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme Biotech Co., Ltd) and sequenced on an Illumina HiSeq system. All of the sequencing data have been deposited at NCBI under the accession of PRJNA719586.

4.2. Genome size estimation and genome assembly

The Illumina reads were trimmed to remove adaptors and reads with >10% ambiguous first using Trimmomatic [22]. The paired reads were filtered when the number of low quality bases (Q < 5) in a single-ended sequencing read exceeds 20%. Then Jellyfish (version 2.2.5) [23] was used to count the k-mer frequency with a k-mer size of 17. Genome size was estimated according to the formula: genome size = k-mer number/k-mer depth [24]. Self-correction was first performed on the Pacbio data which were then assembled with Overlap-Layout-Consensus algorithm. Pacbio long reads were mapped to the contigs with Minimap2 (version 2.12) [25]. The assembly was then polished by Racon (version 1.4.13) [26] with default parameters. Illumina short reads were mapped to the polished assembly by Burrows-Wheeler Alignment tool (BWA, version 0.7.17) [27] and the results were used to conduct another round of polish by Pilon (version 1.23) [28]. Redundant haplotigs were purged with purge_Dups (v1.2.5) [29] using default parameters. To ensure high accuracy and integrity of the genome assembly, Illumina paired-end clean reads were aligned to it using BWA (version 0.7.17) [27]. The Core Eukaryotic Genes Mapping Approach (CEGMA) [30] was applied to evaluate the completeness of the gene set in the draft genome. The BUSCO (version 3) [31] was also used to perform the parameters of “-i $inputfile -o $outputfile -l $metazoa_odb9 -m proteins -c 8". Hi-C reads from the previous genome sequencing project of *P. maximus* were used for scaffolding with Juicer [6,32]. Mis-joins, order and orient were corrected by 3D-DNA [33], and contigs were anchored into pseudo-chromosomes. Finally, the candidate assembly was manually corrected in Juicebox Assembly Tools [34,35].

4.3. Genome annotation

Repetitive sequences in the genome assembly were identified through de novo identification and homologous alignment of
sequences. De novo repetitive sequence database was built by LTR_FINDER [36], RepeatScout (version 1.0.5) and RepeatModeler (version 1.0.11). Repbase database [37] was used together for homology-based searches by Repeatmasker (version 4.0.9). The gene structure was predicted by de novo prediction, homology-based searches combined with transcriptome data alignment. Firstly, Augustus (version 3.3.2) [38], Glimmer HMM [39], Semi-HMM-based Nucleic Acid Parser (SNAP, v2013.11.29) [40], Geneid (version 1.4) [41] and Genescan (version 1.0) [42] were used to generate a de novo gene library based on the frequencies of codon usage and distribution of exons. Then protein coding sequences of *M. yessoensis*, *Crassostrea gigas*, *Homo sapiens*, *Drosophila melanogaster*, *Octopus bimaculoides* and *Lottia gigantea*, were aligned to the genome assembly using TBLATN and GeneWise [43]. The Illumina RNA-seq reads were also aligned to the assembly and all gene model evidences were integrated with EVidenceModeler [44]. The Program to Assemble Spliced Alignments (PASA) pipeline [45] was used to correct the integrated gene set. Finally, the predicted gene models were visualized via IGV and manually checked with TBtools [46]. The predicted proteins were aligned to public databases, including Swissprot, NCBI-NR and KEGG to perform gene functional annotation. InterProScan (version 4.8) [47] was used to search for domains or motifs in InterPro, Pfam and Gene Ontology (GO) database. The noncoding RNA genes, including rRNAs, tRNAs, snRNAs and miRNAs were annotated in the *P. maximus* genome. The miRNAs and snRNAs were screened using INFERNAL 1.1.2 against the Rfam database (version 14.1) [48] with default parameters. Transfer RNA were predicted by tRNAscan-SE (version 1.4) [49] with parameters for eukaryotes. The gene models of annotation used in this study and by Kenny et al. (2020) were compared with the other four scallops (*M. yessoensis*, *Azumapecten farreri*, *A. irradians*, and *A. purpuratus*) for quality evaluation. Briefly, orthologues from the six genome assemblies were identified with Orthofinder. All the single copy orthologues in *M. yessoensis*, *A. farreri*, *A. irradians*, and *A. purpuratus* were retrieved. Their corresponding orthologues were extracted from the two versions of *P. maximus* genome annotation, and the Z-scores were calculated for the protein lengths of each orthologue.

### 4.4. Gene family identification and phylogenetic analysis

The gene sets from 16 eumetazoan species were used to analyze the gene clustering, including *Nematostella vectensis*, *Branchiostoma floridae*, *H. sapiens*, *O. bimaculoides*, *L. gigantea*, *Aplysia californica*, *Biomphalaria glabrata*, *C. gigas*, *Saccostrea glomerata*, *Scapharca broughtonii*, *M. yessoensis*, *A. farreri*, *A. irradians*, *A. purpuratus* and *P. maximus*. OrthoFinder (version 2.3.3) [50] was used to assign the gene family clusters with default parameters. The longest protein sequence was selected as representative when a gene possesses multiple splicing isoforms. Phylogeny of *P. maximus* was inferred by one-to-one orthologous gene families detected from the result of Orthofinder. Multiple alignments of the genes were performed using Mafft (version 7.221) [51] and the conserved blocks of alignments were selected by Gblocks [52] to concatenate a supergene for tree construction. Single-copy orthologues were used for maximum-likelihood (ML)
4.5. Phylogenetic analysis and classification of molluscan iGlurS

The iGlur genes were identified in the *P. maximus* genomes by HHSearch with an E-value threshold of 1e-5 against the iGlur hmm files (PF10613 and PF00060) from pfam database and were further confirmed by comparing to the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd) and SMART (https://smart.embl-heidelberg.de/). In addition, the iGlur genes were screened through BLAST searches and manual literature inspections. The identified iGlurS were classified based on molecular phylogeny and manual inspection of conserved residues. The same approach was used to identify iGlurS genes in other six molluscan species, including *Simonovacula constricta*, *O. mimaculoides*, *B. glabrata*, *H. maculosa*, *P. canaliculata*, *M. yessoensis*. Phylogenetic relationships of iGlur amino acid sequences were estimated using maximum-likelihood (ML) analyses with IQ-TREE (v1.6.12). Branch supports were evaluated with 10,000 pseudo-replicates of the ultrafast bootstrap procedure [57]. ModelFinder from IQ-TREE was used to select the best-fitting model (LG + R9). Plant iGlurS from *Arabidopsis thaliana* were used as an outgroup. The sequence alignment file for phylogenetic analysis is stored on FigShare [58]. GluF of 21 mollusc species (*A. california*, *Tegillarca granosa*, *Cyclina sinensis*, *Eliesia chlorotica*, *Pinctada fucata* martensis, *S. brounii*, *S. constricta*, *B. bathymodiolus* platifrons, *C. gigas*, *L. gigantea*, *Modiolus philippinarum*, *O. mimaculoides*, *Octopus minor*, *B. glabrata*, *A. farreri*, *Chrysomallon squamiferum*, *Gigantopelta aegis*, *H. maculosa*, *P. canaliculata*, *M. yessoensis*) were identified and then used to build phylogenetic tree with the same method mentioned above. The protein structures of iGlurS in the *P. maximus* were predicted by PHYRE2 [59]. Alignment of the amino acid sequences and protein structures were performed using Jalview (version 2.11.1.4) [60] and TM-align [61], respectively.

4.6. Transcriptome analysis

Transcriptome data from *T. granosa*, *S. constricta*, *C. gigas*, *B. glabrata*, *A. farreri*, *C. squamiferum*, *G. aegis*, *H. maculosa*, *P. canaliculata* and *M. yessoensis* were retrieved from the MolluscDB. Raw reads were first trimmed to remove those containing undetermined bases (“N”) and excessive numbers of low-quality positions (>10 positions with quality scores < 10). Hisat2 was used for the alignment of RNA-seq data [62]. The index for reference genomes were built with the annotation file using the default parameters. The high-quality reads were mapped to the reference index with the parameters of “Hisat2 -p 24 -x Sindex_filename -1 Sforward_fq -2 Sreverse_fq -U Ssingle_fq --summary-file $summary_filename -S output_filename”. The counts of aligned read for each gene model were calculated with FeatureCounts [63], and were normalized by calculating the transcripts per kilobase of exon model per million mapped reads (TPM).

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