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Molecular evidence for the adaptive evolution in euryhaline bivalves

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ABSTRACT

Marine bivalves inhabiting intertidal and estuarine areas are frequently exposed to salinity stress due to persistent rainfall and drought. Through prolonged adaptive evolution, numerous bivalves have developed eurysalinity, which are capable of tolerating a wide range of salinity fluctuations through the sophisticated regulation of physiological metabolism. Current research has predominantly focused on investigating the physiological responses of bivalves to salinity stress, leaving a significant gap in our understanding of the adaptive evolutionary characteristics in euryhaline bivalves. Here, comparative genomics analyses were performed in two groups of bivalve species, including 7 euryhaline species and 5 stenohaline species. We identified 24 significantly expanded gene families and 659 positively selected genes in euryhaline bivalves. A significant co-expansion of solute carrier family 23 (*SLC23*) facilitates the transmembrane transport of ascorbic acids in euryhaline bivalves. Positive selection of antioxidant genes, such as *GST* and *TXNRD*, augments the capacity of active oxygen species (ROS) scavenging under salinity stress. Additionally, we found that the positively selected genes were significantly enriched in KEGG pathways associated with carbohydrates, lipids and amino acids metabolism (*ALDH*, *ADH*, and *GLS*), as well as GO terms related to transmembrane transport and inorganic anion transport (*SLC22*, *CLCND*, and *VDCC*). Positive selection of *MCT* might contribute to prevent excessive accumulation of intracellular lactic acids during anaerobic metabolism. Positive selection of *PLA2* potentially promote the removal of damaged membranes lipids under salinity stress. Our findings suggest that adaptive evolution has occurred in osmoregulation, ROS scavenging, energy metabolism, and membrane lipids adjustments in euryhaline bivalves. This study enhances our understanding of the molecular mechanisms underlying the remarkable salinity adaption of euryhaline bivalves.

1. Introduction

Salinity is a crucial environmental factor that has significant influence on the distribution, survival, development, and physiology metabolism of aquatic animals (Navarro and Gonzalez, 1998). Marine bivalves are osmoconformers, exhibiting variations in body fluid osmolality corresponding to changes in environmental salinity (Larsen et al., 2014; Sokolov and Sokolova, 2019). Consequently, bivalves lack the ability to regulate osmotic osmolarity and are thus susceptible to osmotic stress in response to changes in environmental salinity (Berger and Kharazova, 1997; Lin et al., 2016). Osmoregulation in bivalves primarily relies on the rapid transmembrane transport of inorganic ions and organic osmolytes, which plays a pivotal role in avoiding excessive cellular swelling and shrinkage under salinity stress (Berger and Kharazova, 1997; Kube et al., 2006). During exposure to hypersaline environments, a significant elevation in Na^+ concentration was observed in the gills of *Venerupis philippinarum* (Carregosa et al., 2014). Free amino acids (FAAs) serve as the predominant constituents of organic osmolytes

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(Pourmozaffar et al., 2020). During salinity stress, regulation of intracellular FAAs has been documented in various bivalve species (Sokolowski et al., 2003; Hosoi et al., 2007; Huo et al., 2014).

Natural selection refers to the process by which certain genotypes are preserved or eliminated as a result of survival competition. Marine bivalves inhabiting intertidal and estuarine areas frequently experience salinity stress of varying degrees due to fluctuating patterns of rainfall and drought (Gagnaire et al., 2006; Bussell et al., 2008). Through a prolonged period of natural selection, numerous intertidal bivalves have developed euryhalinity, which enables them to endure a broad spectrum of salinity changes through the sophisticated regulation of physiological metabolism (Zhang et al., 2012). Adequate energy supply is indispensable for supporting cellular stress responses and maintaining homeostasis (Meng et al., 2018). Due to the additional energy expenditure associated with the transport of inorganic ions and FAAs, a reallocation of energy towards osmoregulation was observed in bivalves under salinity stress (Berger and Kharazova, 1997). For instance, a substantial variation in the levels of numerous metabolites associated with energy metabolism, including lactate, succinate, and acylcarnitines, was observed in *Mercenaria* under acute hyposalinity stress (Zhou et al., 2022). Additionally, to mitigate the oxidative damage induced by salinity stress, bivalves are capable of initiating a cascade of antioxidant responses, encompassing the up-regulation of antioxidant enzymes activities and the metabolites with properties of active oxygen species (ROS) scavenging (Freitas et al., 2017; Zhou et al., 2022). Moreover, euryhaline bivalves have been reported to modulate their heart rate (Pourmozaffar et al., 2020), oxygen consumption and ammonia excretion (Nie et al., 2018), endocrine response (Lacoste et al., 2001), and programmed cell death (Song et al., 2021), in response to changes in environmental salinity.

Adaptive evolution typically refers to the species' ability to modify its own traits in response to alterations in external environmental conditions over the course of its extensive evolutionary history (Ackerly, 2003). Mutations, which are categorized as synonymous mutations and non-synonymous mutations, serving as the molecular basis of adaptive evolution. Non-synonymous mutations possess the potential to fundamentally modify the amino acids composition, thereby significantly impacting the structure and function of proteins. Natural selection plays a pivotal role in preserving these non-synonymous mutations that augment a species' adaptability to environmental factors. Current studies commonly investigated the adaptive evolution of gene sequences by assessing the ratio (ω) between non-synonymous mutations (dN) and synonymous mutations (dS). The value of ω exceeding 1 suggests that the gene has experienced positive selection and exhibited adaptive evolutionary characteristics, whereas a value of ω below 1 indicates that the gene was under purifying selection (Yang, 2007; Gerrish et al., 2013; Gu et al., 2014). Consequently, the analysis of the sites difference in orthologous sequences within a specific group of species is crucial for comprehending the adaptive evolutionary characteristics of target species. Most current studies have predominantly focused to investigate the physiological responses of bivalves to short-term salinity stress, leaving a significant gap in our understanding of the adaptive evolutionary characteristics in the genomes of euryhaline bivalves. In recent years, the increasing assembly and publication of the reference genomes of bivalves have provided valuable resources for adaptive evolution analysis.

In the present study, we conducted comparative genomics analyses in two groups of bivalve species, including 7 euryhaline species and 5 stenohaline species. We identified the significantly expanded gene families and positively selected genes in euryhaline bivalves. The gene functional enrichment analysis was performed to elucidate the pivotal biological processes or pathways in which the positively selected genes are involved. Given the dedicated focus of our research team on unraveling the molecular mechanisms underlying salinity adaptation in hard clams (*M. mercenaria*), we selected this euryhaline species to investigate the physicochemical properties, structural characteristics, and tissue expression patterns of the significantly expanded gene family members. Additionally, we explored the expression variation of eight critical positively selected gene in hard clams under acute and chronic salinity stress using qRT-PCR analysis. Results of this study enhance our understanding of the adaptive evolutionary characteristics in euryhaline bivalves and provide novel insights into the molecular mechanisms underlying their remarkable salinity adaptation.

2. Materials and methods

2.1. Genome data collection

Seven euryhaline bivalves (*Argopecten irradians*, *Crassostrea gigas*, *Crassostrea hongkongensis*, *Crassostrea virginica*, *Cyclina sinensis*, *Sinonovacula constricta* and *M. mercenaria*) and five stenohaline bivalves (*Dreissena polymorpha*, *Mizuhopecten yessoensis*, *Modiolus philippinarum*, *Pecten maximus* and *Pinctada fucata*) were selected for comparative genomics analyses (Chauvaud et al., 2005; Meng et al., 2013; Eierman and Hare, 2014; Liu and Wang, 2016; Le et al., 2021; Li et al., 2021; Song et al., 2021; Silina, 2023). The reference genome sequence and annotation files (in GFF format) of theses species were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/), MolluscDB (http://mgbase.qnlm.ac/home), Figshare (https://figshare.com/), and Dryad (https://datadryad.org/stash). The download link of these files are presented in Table S1. The coding sequence (CDS) and protein sequence files of each species were obtained using TBtools software (v1.1043) (Chen et al., 2020).

2.2. Phylogenetic tree construction and orthologues identification

The protein sequence of the 12 bivalve species were utilized as input files for OrthoFinder software (v2.5.5) to identify the orthologues with default parameters. The single-copy sequences were identified and utilized for the construction of a phylogenetic tree. Firstly, the single-copy sequences from 12 bivalves were aligned using MUSCLE software (v5.1). Subsequently, the conserved region of these single-copy sequences was extracted using Gblocks software. Finally, multiple conserved sequences belonging to the same species were merged into a single sequence and employed as input files for IQ-Tree software (v2.1.4). The phylogenetic tree was constructed using the maximum-likelihood (ML) algorithm, and 1000 bootstraps were performed to obtain branch support values.

According to the study conducted by Yuan et al. (2020), we further identified additional orthologous genes from the 12 bivalves due to the limited number of single-copy genes available for adaptive evolution analysis. Firstly, gene families containing at least one gene per species were filtered from the results of gene family clustering. In each multi-copy gene family, the longest protein sequence of *M. mercenaria* was selected as the query sequence. Subsequently, a BLASTP search with an e-value threshold of 1e-20 was performed to identify the best match in other 11 species. Orthologue groups (OGs) defined as those gene families that retained only one representative gene per species were obtained and utilized for subsequent analyses.

2.3. Gene family expansion and contraction analysis

Identification of expanded and contracted gene families in 7 euryhaline bivalves was accomplished by comparing them with 5 stenohaline bivalves, using a Student's t-test to compare the gene numbers between the two groups of species (Yuan et al., 2020). The gene families exhibiting *P* values below 0.05 are deemed to have undergone significant expansion or contraction. Additionally, we selected the co-expanded gene families (i.e., those exhibiting dramatically higher gene numbers in multiple euryhaline species) for subsequent analyses. The ML phylogenetic trees of the significantly expanded gene families in euryhaline bivalves were constructed using FastTree software with default parameters (v2.1.11). According to the genome annotation

information, TBtools software was employed for visualizing the chromosomal localization of the significantly expanded gene family members. The ExPASy-ProtParam tool (https://web.expasy.org/protparam/) was utilized for predicting the molecular weights (MWs), isoelectric points (pI), and grand averages of hydropathicity (GRAVY) of each gene family member. Additionally, WoLF PSORT online software (https://wolfpsort.hgc.jp/) was employed to predict the subcellular localization of these genes. The conserved motif analyses were performed to gain a better understanding of the structural diversity of these genes using MEME online tool (v5.5.0) (https://meme-suite.org/meme/tools/meme).

2.4. Tissue expression profiles of the significantly expanded gene family members in M. mercenaria

The Illumina paired-end cDNA libraries of various tissues, including gills, mantles, feet, adductor muscles, hemolymph, liver, stomach, intestines, and ovaries were constructed in our previous study (Song et al., 2021). Raw data were obtained from the SRA database in NCBI (accession number: PRJNA596049). The adapters, poly-N, and low-quality reads were removed using Cutadapt software(v3.3). Data was checked using FastQC software(v0.11.8), and subsequently mapped to the reference genome of *M. mercenaria* using TopHat software (v2.0.12). The HTSeq software (v0.6.1) was utilized for quantifying the read counts mapped to each gene, and subsequently, the fragments per kilobase of exonmodel per million mapped fragments (FPKM) value was calculated for each gene. We utilized the heatmap tool available in OmicShare (https://www.omicshare.com/tools/) to visualize the expression levels of the significantly expanded gene family members in different tissues of hard clams.

2.5. Positive selection analysis

The nonsynonymous/synonymous substitution ratio ($\omega = dN/dS$) is commonly employed to assess whether a gene has undergone adaptive evolution, with $\omega = 1$, $\omega < 1$, and $\omega > 1$ denoting neutral evolution, purifying selection, and positive selection, respectively. In each OG, the sequences of 12 bivalves were aligned using MUSCLE software, followed by the construction of a phylogenetic tree using FastTree software. We further extracted the CDS sequence and eliminated the stop codons and gaps. Codon alignments were performed using Translatorx software. The fasta format alignment files were converted to phylip format using a perl script (FASTAtoPHYL.pl) available on GitHub (https://github.com/). In the tree file of each OG, we removed the genetic distance and appended '' #1'' to the sequences of 7 euryhaline species as a distinctive label for foreground branches. The PAML software (v4.6) was utilized to predict the positively selected orthologues and positively selected amino acid sites in euryhaline species using the branch-site model (Yang, 2007; Zhang et al., 2005; Yuan et al., 2020). In the null hypothesis model, a constant ω value of 1 was assumed, while the alternative hypothesis model allowed for variation of ω across different branches. The parameters of the null hypothesis are set as follows: seqtype = 1, ndata = 1, clock = 0, model = 2, Nssites = 2, fix_kappa = 0, kappa = 2, fix_omega = 1, omega = 1. The parameters of the alternative hypothesis are set as follows: seqtype = 1, ndata = 1, clock = 0, model = 2, Nssites = 2, fix_kappa = 0, kappa = 2, fix_omega = 0, omega = 2. We obtained the lnL values from both null and alternative hypotheses, and conducted a likelihood ratio test (LRT) to evaluate the difference between the two models. To determine significance, we derived *P* value for each LRT using the Chi-square test provided by PAML software. OGs with *P* values below 0.05 were considered as exhibiting positive selection. Furthermore, we employed the Bayes empirical Bayes approach (BEB) to identify the positively selected amino acid sites. Sites with probability values exceeding 95% were deemed as positively selected and subsequently validated using the false discovery rate method.

2.6. Gene annotation and function enrichment analysis

The orthologues were annotated using the OmicShare gene annotation tool based on the BLASTP results against Swiss-prot and NR databases. Additionally, gene function enrichment analyses of the positively selected orthologues were performed using the KEGG and GO enrichment analyses tool in OmicShare. KEGG pathways and GO terms exhibiting a *P* value less than 0.05 were deemed significantly enriched by the positively selected orthologues.

2.7. Salinity stress experiments

The hard clams (shell length 4–5 cm) were collected from a pond in Tianjin, China. Clams were acclimated in 30 practical salinity units (psu) seawater in our laboratory for two weeks before salinity stress experiments. In the control group, 50 clams were placed into a tank filled with 30-psμ seawater (normal seawater, NS). During short-term acute salinity stress experiments, a total of 50 clams were promptly placed into two separate tanks filled with 10-psμ (acute hyposalinity, AL) and 45-psμ seawater (acute hypersalinity, AH) for 5 days, respectively. During chronic salinity stress experiments, 50 clams were subjected to gradual decrease and increase in salinity (at a rate of 1 psμ per day), ultimately maintaining at 10 psμ (chronic hyposalinity group, CL) and 45 psμ (chronic hypersalinity group, CH) for a duration of 50 days. Changes of salinity levels were achieved through the addition of distilled water and artificial seawater salt. During acclimation and salinity stress experiments, *Spirulina* powder was utilized as the dietary source for clams, and seawater with specific salinity levels was exchanged on a daily basis. Gills are the primary tissues involved in osmoregulation in bivalves, which directly interacted with surrounding environments (Hosoi et al., 2007). The gills of 18 clams in the NS, AL, AH, CL, and CH groups were dissected and washed with phosphate-buffered saline, respectively. Six clams' gills were pooled into one sample to minimize the interference of individual differences on experiments. A total of 15 gills samples (5 groups × 3 biological replicates) were stored at −80 °C and utilized for RNA extraction.

2.8. RNA extraction and qRT-PCR analysis

TRIzol kit (Invitrogen, USA) was used to extract the RNA from each sample following the manufacturer's instructions. RNA degradation was monitored using 1% agarose gels electrophoresis (buffer: 1 × TAE, voltage: 300 V, time: 15 min). RNA purity and concentrations were detected using Nanodrop (Thermo Scientific, USA). TaKaRa Prime Script™RT reagent Kit (TaKaRa Bio, Japan) was used to obtain cDNA. Eight positively selected genes were selected to investigate the vatiation in gene expression levels under salinity stress using qRT-PCR analysis. Primers of these genes were designed using a online tool provided by Sangon Biotech (https://www.sangon.com/primerDesign), and validated using 1.5 % agarose gels electrophoresis (buffer: 1 × TAE, voltage: 100 V, time: 20 min). One PCR reaction system consisted of 0.5 μL forward primer (5 μmol/L), 0.5 μL reverse primer (5 μmol/L), 10 μL TB Green Premix Ex Taq (TaKaRa Bio), 1 μL cDNA template, and 8 μL ddH_2O. PCR reactions were conducted on an Eppendorf RealPlex Mastercycler with the following conditions: 95 °C for 2 min; 95 °C for 15 s (40 cycles), 60 °C for 30 s. Transcription elongation factor 1 alpha (*EF1α*) was used as the reference gene to normalize the expression levels of the positively selected genes (Wang et al., 2016). The $2^{-\Delta\Delta Ct}$ method was employed to acsess the relative alterations in gene expression levels (Livak and Schmittgen, 2001).

2.9. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 25 (IBM Corp., USA). After comfirming the homogeneity of variance and normal distribution of qRT-PCR data, we employed the Student's *t*-test

to assess the difference between the salinity stress groups and the control group. Statistical significance was considere when $P < 0.05$.

3. Results

3.1. Gene family clustering and phylogenetic tree construction of the twelve bivalve species

A total of 71,837 gene families were identified in 12 bivalve species, of which 209 single-copy sequences were utilized for the construction of a phylogenetic tree (Fig. 1). The tree comprises two primary branches, namely Heteroconchia and Pteriomorphia, respectively. Three euryhaline species (*C. sinensis*, *S. constricta* and *M. mercenaria*) were clustered with their stenohaline relatives (*D. polymorpha*) within the Heteroconchia branch. The three scallop species (euryhaline *A. irradians*, and stenohaline *M. yessoensis* and *P. maximus*) and three oyster species (euryhaline *C. gigas*, *C. hongkongensis*, and *C. virginica*) were closely clustered within the same clade, respectively. The stenohaline *M. philippinarum* and *P. fucata* were grouped together with scallops and oysters within the Pteriomorphia branch. The clustering relationships of these species in this phylogenetic tree are consistent with their taxonomic status.

3.2. Significantly expanded and contracted gene families in euryhaline bivalves

In euryhaline bivalves, a total of 24 and 45 gene families were under significant ($P < 0.05$) expansion and contraction, respectively. Among the 24 significantly expanded gene families, we observed a considerably high abundance of solute carrier family 23 (*SLC23*), 7 transmembrane receptor (rhodopsin family), and ankyrin repeats family members in euryhaline species (Fig. 2A). Since the significance of *SLC* in transmembrane transport and osmoregulation has been widely acknowledged across various aquatic organisms, we further selected *SLC23* for subsequent analyses. Fig. 2B shows the phylogenetic relationship of *SLC23* gene family members in 12 bivalve species. Among the 45 significantly contracted gene families in euryhaline species, the abundance of BTB/POZ domain-containing protein and TatD related DNase family members is considerably lower compared to those in stenohaline species (Fig. S1).

3.3. Physicochemical properties, structural characteristics, and tissue expression profiles of solute carrier family 23 (SLC23)

Given our research team's dedicated focus on elucidating the molecular mechanisms underlying salinity adaptation in hard clams, we have chosen this species as a model to investigate the structural characteristics and gene expression patterns of *SLC23* family members (*MmSLC23*). Except for one member located on chromosome 9, other *MmSLC23* are arranged in clusters on chromosome 3 (Fig. 2C). As shown in Table 1, the predicted MWs and pI of MmSLC23 ranged from 25.07 to 72.22 kDa, and 5.13 to 7.01, respectively. All MmSLC23 were located on the cell membrane. The GRAVY values of MmSLC23 ranged from 0.26 to 0.64, indicating that they are hydrophobic proteins. Moreover, the conserved Xan_ur_permease domain was observed in each MmSLC23, including the shortest sequence (Mme.03g00414) (Fig. 2D). The motif analysis revealed a high degree of conservation (motifs 9, 7, 2, 3, 1, 6, 10) in five longest MmSLC23. Additionally, a tissue-specific expression of *MmSLC23* was observed in hard clams. Specifically, four *MmSLC23* (Mme.09g01999, Mme.03g00414, Mme.03g00413, and Mme.03g00609) exhibited high expression levels in the mantles, while two genes (Mme.03g00016 and Mme.03g00609) demonstrated pronounced expression in the gills. Three *MmSLC23* (Mme.09g01999, Mme.03g00412, and Mme.03g00415) displayed elevated expression levels in the hemolymph. We observed that the tissue expression patterns of two *MmSLC23* (Mm.03g00412 and Mm.03g00415) exhibited remarkable similarity, as they were closely clustered together in the same clade within the phylogenetic tree. Additionally, all *MmSLC23* exhibited low expression in feet, adductor muscles, liver, stomach, intestines, testes, and ovaries (Fig. 2D).

3.4. Functional enrichment analysis of the positively selected genes

A total of 4879 OGs (one gene per species) were identified. They were subjected to branch-site model testing using PAML software. Of these, 659 OGs were under positive selection in seven euryhaline bivalves. The probability values of the positively selected sites in these OGs exceeded 0.95. KEGG enrichment analysis revealed that a total of 24 pathways were significantly ($P < 0.05$) enriched by the positively selected genes. These pathways primarily encompassed nine pathways of amino acids metabolism, including cysteine, methionine, histidine, valine, leucine, isoleucine, lysine, arginine, tryptophan, alanine, aspartate, glutamate; four pathways of carbohydrates metabolism, including

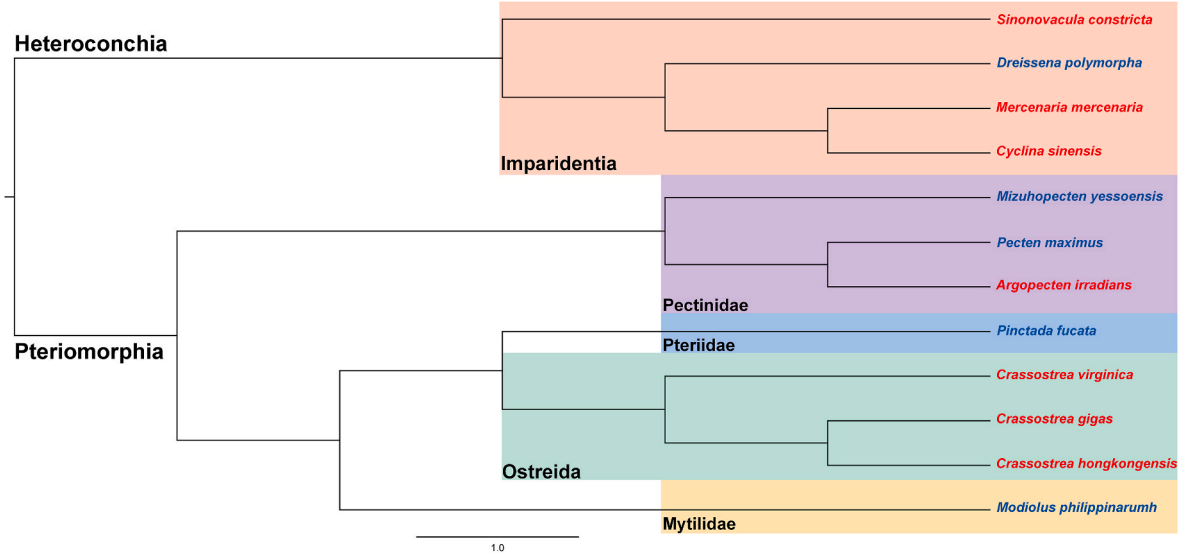

Fig. 1. Phylogenetic tree of the 12 bivalves species. The phylogenetic tree was constructed using maximumlikelihood (ML) analysis based on 209 single-copy genes. Euryhaline and stenohaline species are marked in red and blue, respectively.

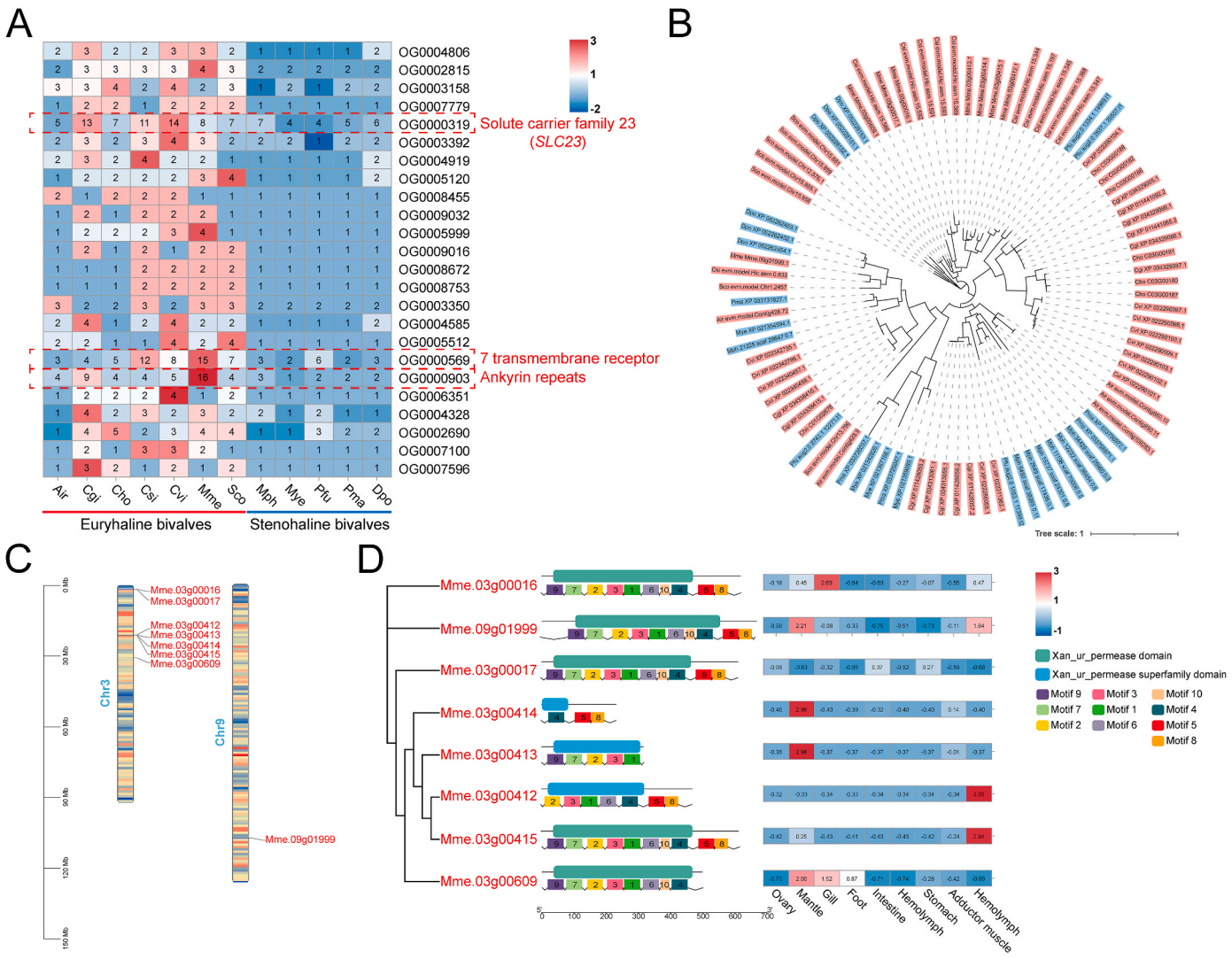

Fig. 2. The significantly expanded gene families in euryhaline bivalves. (A) Distribution pattern of the significantly expanded gene families in euryhaline bivalves. Gene number is shown in each box of the heatmap. The species used for comparison included *Argopecten irradians* (Air), *Crassostrea gigas* (Cgi), *Crassostrea hongkongensis* (Cho), *Crassostrea virginica* (Cvi), *Cyclina sinensis* (Csi), *Sinonovacula constricta* (Sco), *Mercenaria* (Mme), *Dreissena polymorpha* (Dpo), *Mizuhopecten yessoensis* (Mye), *Modiolus philippinarum* (Mph), *Pecten maximus* (Pma), and *Pinctada fucata* (Pfu). (B) Phylogenetic tree of the solute carrier family 23 (*SLC23*) gene family. Members in euryhaline and stenohaline species are marked in red and blue, respectively. (C) Chromosomal distribution of *MmSLC23*. (D) Phylogenetic tree, conserved domains, motifs distribution, and tissue expression patterns of *MmSLC23*. Red indicates a high level of gene expression, and blue indicates low expression.

Table 1
Physicochemical properties of *MmSLC23*. MWs: molecular weights; pI: isoelectric points; GRAVY: grand averages of hydropathicity.

Protein id	Number of amino acids	MWs (kDa)	pI	GRAVY	Subcellular localization
Mme.03g00016	616	66.75	6.13	0.37	plasma membrane
Mme.03g00017	607	65.16	5.79	0.46	plasma membrane
Mme.03g00412	466	50.54	6.49	0.41	plasma membrane
Mme.03g00413	315	35.14	5.13	0.40	plasma membrane
Mme.03g00414	230	25.07	5.26	0.26	plasma membrane
Mme.03g00415	611	66.08	5.55	0.35	plasma membrane
Mme.03g00609	496	53.36	6.22	0.64	plasma membrane
Mme.09g01999	665	72.22	7.01	0.35	plasma membrane

pyruvate, ascorbate, glycolysis/gluconeogenesis, fructose, mannose; and three pathways of lipids metabolism comprising fatty acid, sphingolipid, and linoleic acid (Fig. 3A). The aldehyde dehydrogenase (NAD^+) (*ALDH*) and alcohol dehydrogenase (*ADH*) were found to participate in diverse pathways of carbohydrates and lipids metabolism. The positively selected genes in euryhaline bivalves were also significantly ($P < 0.05$) enriched in lysosome pathway (ko04142). These genes included V-type H^+-transporting ATPase (*V-ATPase*) and multiple acid hydrolase (comprising glycosidases, lysophospholipases, and acid phosphatases). Additionally, GO enrichment analysis revealed a significant ($P < 0.05$) enrichment of these positively selected genes in 77 GO terms, including 44 in biological process (BP), 20 in molecular function (MF), and 3 in cellular component (CC). For BP, tryptophan and other amino acids metabolic process were most significantly ($P < 0.05$) enriched GO terms (Fig. 3B). We observed that the positively selected genes were significantly enriched in numerous osmoregulation-related GO terms ($P < 0.05$) such as transmembrane transport (GO:0055085), transport (GO:0006810), and inorganic anion transport (GO:0015698). Regarding MF, the positively selected genes showed significant enrichment in catalytic activity (GO:0003824), oxidoreductase activity

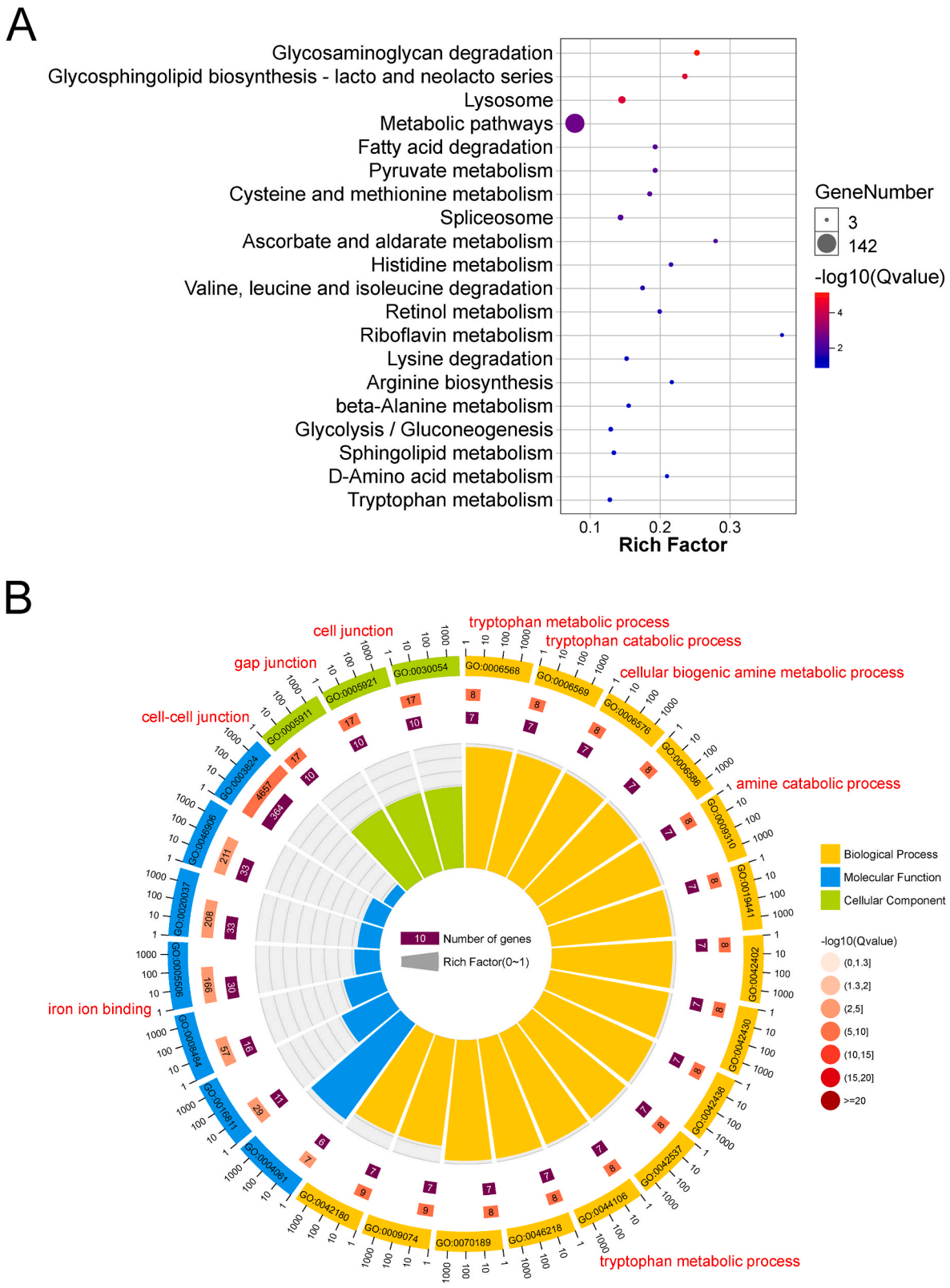

Fig. 3. The representative KEGG pathways (A) and GO terms (B) enriched by the positively selected genes in euryhaline bivalves.

(GO:0016705), hydrolase activity (GO:0016787), galactosyltransferase activity (GO:0008378), and NAD^+ ADP-ribosyltransferase activity (GO:0003950) (Supplemental file 1).

3.5. Variation in expression levels of eight positively selected genes under salinity stress

We selected eight important positively selected genes for further investigation of their gene expression levels in hard clams subjected to

short-term acute and long-term chronic salinity stress using qRT-PCR analysis (Fig. 4). The primer sequences of these genes are presented in Table S2. The gene expression level of *SLC23A1* was significantly ($P < 0.05$) up-regulated in the AL, AH, and CL groups (Fig. 4A). Conversely, *SLC23A2* expression exhibited a significant increase in the CH group (Fig. 4B). The expression of glutaminase (*GLS*) was significantly up-regulated in the AH group, showing approximately a 2-fold increase compared to the control group (Fig. 4C). The gene expression of thioredoxin reductases (*TXNRD*) was significantly inhibited in the AH and CL groups ($P < 0.05$), whereas it was enhanced in the CH group (Fig. 4D). A similarity in the gene expression patterns was observed between *ALDH* and *ADH*, both of which specifically responded to short-term acute salinity stress (Fig. 4E and F). A significant up-regulation in the gene expression level of *V-ATPase* was observed in the AL, CL, and CH groups (Fig. 4G). Notably, phospholipase A2 (*PLA2*) was found to specifically responded to long-term chronic salinity stress (Fig. 4H).

3.6. Adaptive evolutionary characteristics in euryhaline bivalves

As shown in Fig. 5, we concluded the adaptive evolutionary characteristics in 7 euryhaline bivalves based on the aforementioned findings. The *SLC23* has undergone significant expansion and positive selection during long-term evolution, thereby facilitating the transmembrane transport of ascorbic acids in euryhaline bivalves. As a ROS scavenger, ascorbic acid plays a pivotal role in mitigating oxidative damage. Moreover, the antioxidant genes, including *TXNRD* and glutathione S-transferase (*GST*), have undergone positive selection in euryhaline bivalves. These adaptive evolutionary characteristics might enhance the capacity of ROS scavenging in euryhaline bivalves. Additionally, positive selection of the genes enriched in carbohydrates and lipids metabolism pathways might play a crucial role in providing energy for osmoregulation and maintenance of homeostasis. Positive selection of *V-ATPase* might facilitate the transmembrane of H^+, thereby maintaining an acidic environment within the lysosomes lumen. Multiple acid hydrolase involved in the metabolism of carbohydrates and lipids were found to be under positive selection. Positive selection was also observed in numerous genes involved in amino acids metabolism, inorganic anion transport, and transmembrane transport. This contributes to the rapid regulation of inorganic ions and FAAs during osmoregulation in euryhaline bivalves. The *PLA2* experienced positive selection in euryhaline bivalves, indicating the enhanced capacity to efficiently eliminate the damaged membrane lipids resulted from salinity stress. We also found that the positively selected genes were significantly enriched in sphingolipid metabolism and glycosphingolipid biosynthesis pathways. This indicates that adaptive evolution has occurred in membrane lipids adjustments in euryhaline bivalves (Fig. 5).

4. Discussion

Marine bivalves inhabiting intertidal and estuarine areas frequently encounter salinity stress resulting from persistent rainfall and drought (Gagnaire et al., 2006; Bussell et al., 2008). Euryhaline bivalves exhibit the ability to tolerate a wide range of salinity changes through sophisticated regulation of multiple physiological processes (Zhang et al., 2012). In this study, we conducted comparative genomics analyses in order to elucidate the adaptive evolutionary characteristics in euryhaline bivalves.

4.1. Adaptive evolution in osmoregulation

Osmoconformers lack the ability to regulate osmolarity as their body fluids maintain isotonicity with the surrounding environments (Sokolov and Sokolova, 2019). However, through prolonged periods of evolution, numerous bivalves inhibiting intertidal and estuarine regions have evolved euryhalinity. Accordingly, adaptive evolution might has occurred in osmoregulation in euryhaline bivalves, encompassing the regulation of transmembrane transport of inorganic ions and amino acids. The *SLC* superfamily, comprising over 65 families, represents a prominent sub-group of membrane proteins (Aykac and Sehirli, 2020). The *SLC* plays a pivotal role in osmoregulation by governing the transmembrane transport of diverse substrates, including inorganic ions, sugars, amino acids, lipids, neurotransmitters and hormones (Higuchi et al., 2018). A recently published transcriptomics research has revealed that *SLC* plays a crucial role in the adaption of oysters to hyper- and hypo-salinity habitats (Zhang et al., 2023). In this study, we identified several positively selected genes that were significantly enriched in the GO terms related to transmembrane transport and inorganic anion transport, including *SLC22*, chloride channel protein D (*CLCND*), and voltage-dependent L-type calcium channel (*VDCC*). In marine organisms, Na^+, Ca^{2+}, Mg^{2+}, and Cl^- are the primary ions responsible for maintaining ionic gradients (Faria et al., 2011). The *SLC22* family members exhibit specific transport functions for a diverse range of organic ions (Jacobsson et al., 2007). The *CLCND* and *VDCC* promote the transport of Cl^- and s Ca^{2+} across the cell membrane. These channels have been identified as crucial contributors to membrane potential regulation and cellular volume maintenance (Kim et al., 2022). Our previous research has demonstrated a significant down-regulation of *VDCC* gene expression in hard clams under acute hyposalinity stress, which effectively prevents the influx of ambient Ca^{2+} into cells (Zhou et al., 2022). The positive selection of these genes indicates an enhanced capacity for ions transport during osmoregulation in euryhaline bivalves.

In addition to inorganic ions, osmoconforming bivalves primarily rely on the rapid accumulation or release of intracellular organic osmolytes for osmoregulation (Kube et al., 2006). FAAs constitute approximately 30 % of the total osmotically active substances in bivalves (Pourmozaffar et al., 2020). Bivalves that possess a larger reservoir of FAAs exhibit enhanced resilience against salinity stress (Pierce, 1971). Osmoregulation in different bivalves involves a diverse array of free amino acids (FAAs) and their derivatives, with alanine, glycine, and proline being the predominant FAAs (Sokolowski et al., 2003; Huo et al., 2014). In this study, we found that the positively selected genes in euryhaline bivalves were significantly enriched in the metabolism pathways of alanine, glycine, and proline. Similarly, a previous comparative genomics analysis revealed that the expansion of FAAs metabolism pathways served as the molecular basis for the euryhalinity of *C. gigas* (Zhang et al., 2016). We hypothesize that positive selection of the genes involved in the metabolism of alanine, glycine and proline might facilitate the rapid regulation of intracellular FAAs in euryhaline bivalves under salinity stress. The *GLS*, which plays a crucial role in catalyzing the hydrolysis of glutamine to glutamate, exhibited positive selection in euryhaline bivalves. Moreover, we observed that *GLS* was highly expressed in hard clams under short-term acute hypersalinity stress. These findings are consistent with those in a previous report which demonstrated that nitrogenous waste resulting from glutamine catabolism was essential for maintaining ionic homeostasis in euryhaline teleosts (*Oryzias latipes*) during hypersalinity stress (Huang et al., 2020). Taken together, these results suggest that the positive selection of genes involved in inorganic ion transport and amino acid metabolism might enhance the capacity of osmoregulation and confer the remarkable salinity resistance of euryhaline bivalves.

4.2. Adaptive evolution in ROS scavenging

The ROS, including superoxide radicals, hydroxyl radicals, and H_2O_2, are continually generated through redox reactions in the mitochondrial electron transport chain (Birnie-Gauvin et al., 2017). Salinity stress has been reported to disrupt the equilibrium between ROS production and scavenging in various bivalves, such as *Mytilus galloprovincialis* (Freitas et al., 2017), *Ruditapes philippinarum* (Nie et al., 2017), and *M. mercenaria* (Zhou et al., 2022). The excessive accumulation of intracellular ROS can attack the surrounding biological molecules,

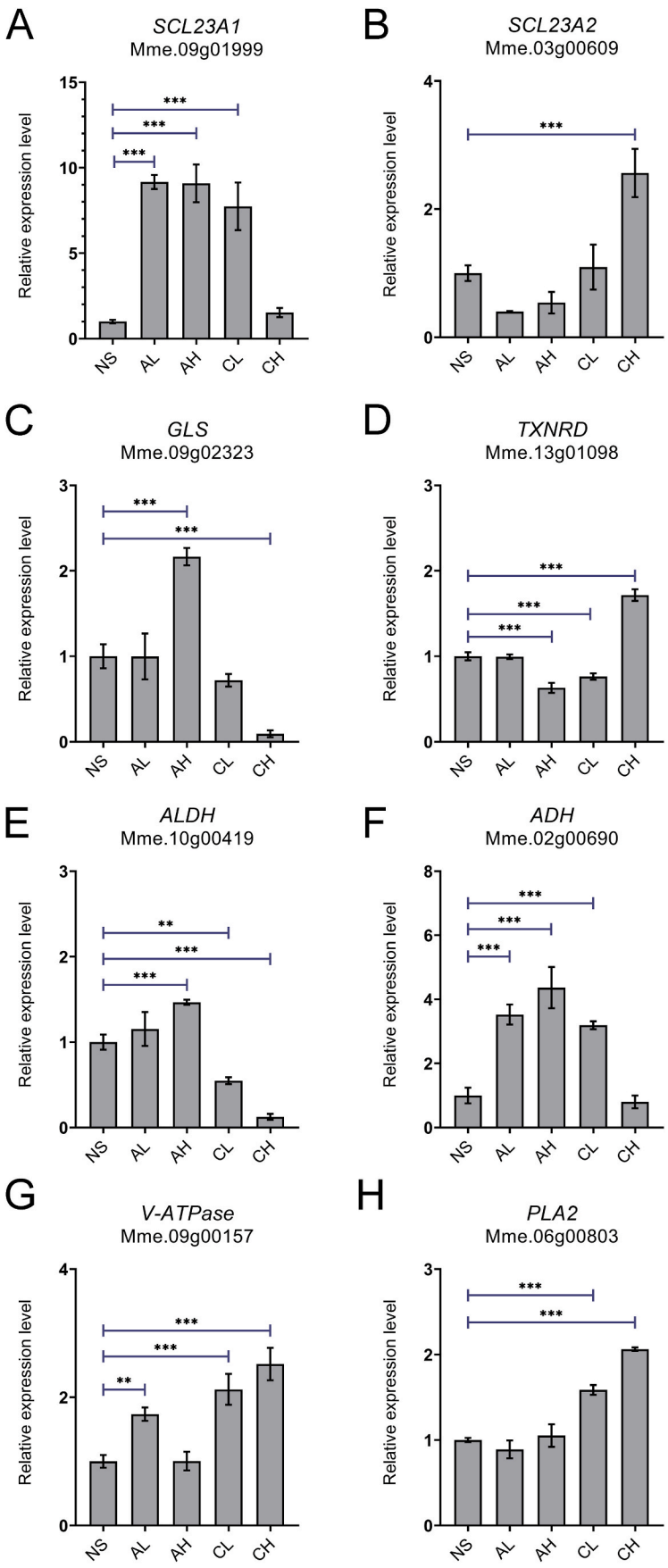

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Fig. 4. Variation in expression levels of eight crucial positively selected genes, including solute carrier family 23 member 1 (*SLC23A1*) (A), solute carrier family 23 member 2 (*SLC23A2*) (B), glutaminase (*GLS*) (C), thioredoxin reductases (*TXNRD*) (D), aldehyde dehydrogenase (NAD$^+$) (*ALDH*) (E), alcohol dehydrogenase (*ADH*) (F), V-type H$^+$-transporting ATPase (*V-ATPase*) (G), and phospholipase A2 (*PLA2*) (H), in hard clams under short-term acute hyposalinity (AL) and hypersalinity (AH), and long-term chronic hyposalinity (CL) and hypersalinity (CH) stress.

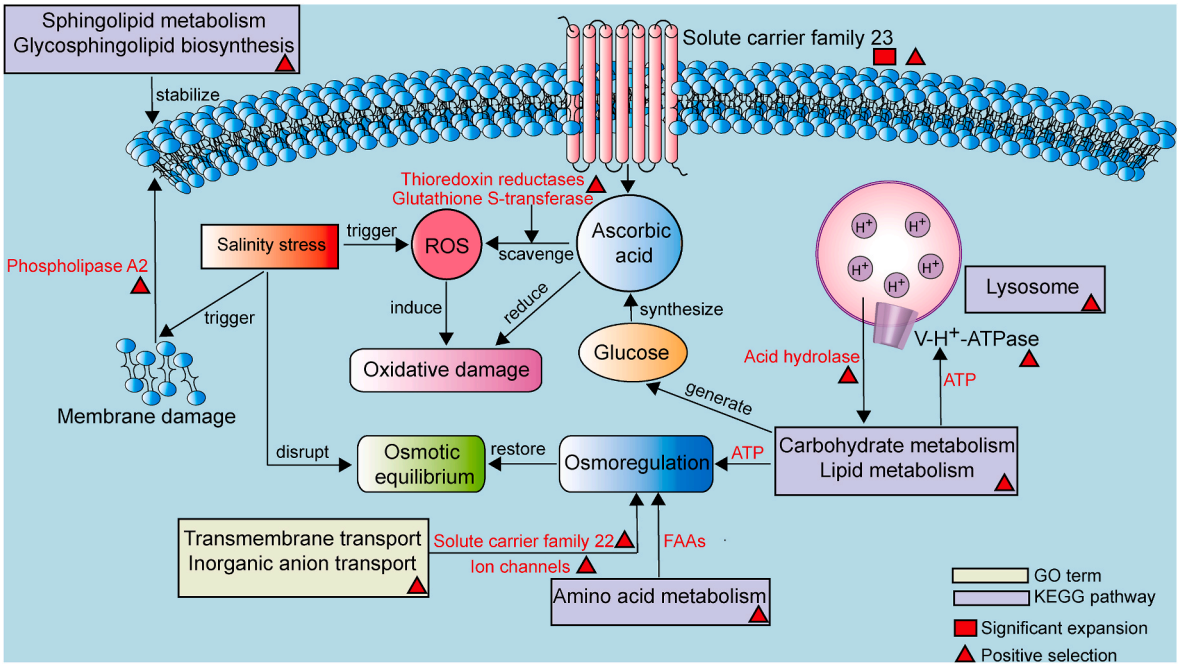

Fig. 5. A schematic of the adaptive evolutionary characteristics in euryhaline bivalves. The significantly expanded gene families are marked by red rectangles, and the positively selected genes are marked by red triangles.

resulting in oxidative damage in aquatic animals (Paital and Chainy, 2012). For instance, a significant increase in the content of oxidized lipids was observed in hard clams under acute hyposalinity stress (Zhou et al., 2022). Therefore, the activation of antioxidant response is crucial for bivalves to mitigate oxidative stress and maintain redox homeostasis. In this study, we observed a significant expansion and positive selection of *SLC23* gene family in euryhaline bivalves. The *SLC23* is specifically responsible for the transmembrane transport of ascorbic acid (Bürzle and Hediger, 2012). Ascorbic acid serves as an effective antioxidant and free-radical scavenger, playing a crucial role in protecting metabolically active cells from oxidative stress (Bürzle and Hediger, 2012). Additionally, ascorbic acid acts as an essential cofactor for numerous enzymatic reactions involved in oxidation-reduction process (Takanaga et al., 2004). Similarly, a significant increase in the levels of ascorbic acid and other representative antioxidants such as allantoin, cinnamic acid, and trigonelline, was observed in hard clams under acute hypersalinity stress (Zhou et al., 2023). We hypothesize that the significant expansion and positive selection of *SLC23* gene family might contribute to the efficient transport of ascorbic acid and elimination of ROS in euryhaline bivalves during exposure to salinity stress. In addition to antioxidants, bivalves could enhance the activities and gene expression levels of antioxidant enzymes to scavenge excessive intracellular ROS (Freitas et al., 2017). The glutathione and thioredoxin systems are the two primary antioxidant defence mechanisms in eukaryotic cells (Rahlfs et al., 2002). Glutathione synthesis is primarily regulated by *GST*, which plays a crucial role in mitigating the damage caused by ROS in living organisms (Yan et al., 2008). Additionally, *TXNRD* facilitates the transfer of reducing power to the thioredoxin/peroxiredoxin system for rapid ROS scavenging (Cha et al., 2015). In this study, both *GST* and *TXNRD* have undergone positive selection in euryhaline bivalves. Furthermore, the expression level of *TXNRD* was significantly up-regulated in hard clams subjected to long-term chronic hypersalinity stress. Collectively, these findings provide compelling evidence that adaptive evolution has occurred in ROS scavenging system of euryhaline bivalves. Augmentation of ROS scavenging properties contributes to a rapid mitigation of oxidative damage induced by salinity stress.

4.3. Adaptive evolution in energy metabolism

Adequate and immediate replenishment of energy are essential for ions transport and maintenance of homeostasis in aquatic organisms under salinity stress (Huang et al., 2020). Carbohydrates and lipids metabolism are the primary pathways for energy production (Romano and Conway, 1996; Toprak, 2020). In this study, the positively selected genes in euryhaline bivalves were significantly enriched in KEGG pathways related to carbohydrates and lipids metabolism, including glycolysis, pyruvate metabolism, fructose and mannose metabolism, as well as fatty acid degradation. Augmentations of glycolysis and β-oxidation of long-chain fatty acids have been demonstrated to provide substantial energy for hard clams to resist salinity stress (Zhou et al., 2023). Among these positively selected genes enriched in carbohydrates and lipids metabolism pathways, a substantial number were annotated as *ALDH* and *ADH*. We further found that the gene expression levels of *ALDH* and *ADH* exhibited significant up-regulations in hard clams under different salinity stressors. Additionally, the positively selected genes were also enriched in lysosomes pathway. These genes included *V-ATPase* and acid hydrolases. Lysosomes maintain pH gradients of acid hydrolases by *V-ATPase*, which utilizes the metabolic energy in the form of adenosine triphosphate (ATP) to pump H$^+$ into the lysosome lumen (Mindell, 2012). Positive selection of *V-ATPase* and the acid hydrolases might enhance the efficiency of energy supply through carbohydrates and lipids metabolism. These findings suggest that adaptive evolution has occurred in energy metabolism of euryhaline bivalves. During exposure to salinity stress, bivalves tend to initiate anaerobic

metabolism as a result of tissues hypoxia caused by prolonged shell closure (Zhou et al., 2022). A significant elevation in the content of lactic acid, the characteristic product of anaerobic metabolism, was observed in hard clams during exposure to hypersalinity stress (Zhou et al., 2023). Moreover, the gene expression levels of monocarboxylate transporters (*MCT*) were found to be significantly up-regulated in this species during anaerobic metabolism (Zhou et al., 2021). The *MCT* was responsible for mediating the transport of monocarboxylic acids, such as lactic acid and pyruvate, across cell membranes (Bosshart et al., 2021). In this study, we observed that numerous members in *MCT* gene family were under positive selection in euryhaline bivalves. Consequently, we assume that the positive selection of *MCT* might contribute to prevent the excessive accumulation of intracellular lactic acids during anaerobic metabolism in euryhaline bivalves.

4.4. Adaptive evolution in membrane lipids adjustments

Phospholipids, including glycerol phospholipids and sphingomyelin, constitute the fundamental components of cell membranes. In this study, *PLA2* was found to be under positive selection in euryhaline bivalves and demonstrated notably elevated expression levels under chronic salinity stress. The *PLA2* are capable of catalyzing the hydrolysis of the fatty acyl bond at the sn-2 position of glycerol phospholipids, resulting in the generation of free fatty acids and lysophospholipids (Murakami et al., 2011). The presence of accumulated lysophospholipids serves as an indicator for cell membrane rupture. Our previous research has documented the occurrence of hyposalinity-induced membrane damage and a significant accumulation of lysophospholipids in hard clams (Zhou et al., 2022). Positive selection of *PLA2* potentially contributes to the removal of damaged membrane lipids and maintenance of overall membrane stability in euryhaline bivalves under salinity stress. Moreover, lysophospholipids can be converted into phospholipids. This process facilitates the repair and synthesis of cell membrane (Murakami et al., 2011). We also found that the positive selected genes in euryhaline bivalves were significantly enriched in sphingolipid metabolism and glycosphingolipid biosynthesis pathways. Sphingolipids and their metabolites play pivotal roles in cellular function as both structural components of membranes and signaling molecules that mediate responses to physiological stresses (Breslow and Weissman, 2010). Glycosphingolipids are essential constituents of cell membranes involved in diverse biological events and capable of regulating enzymes and receptors within the plasma membrane. Our findings suggest that adaptive evolution has occurred in membrane lipids adjustments in euryhaline bivalves.

Collectively, we elucidated the adaptive evolutionary characteristics of seven euryhaline bivalve species in this study. Our findings suggest that adaptive evolution has occurred in osmoregulation, ROS scavenging, energy metabolism, and membrane lipids adjustments in euryhaline bivalves. This study provides novel insights into the molecular mechanisms underlying the remarkable salinity adaptation in euryhaline bivalves.

Authorship

Cong Zhou: Investigation, Data curation, Methodology, Writing – original draft. **Mei-jie Yang:** Methodology, Investigation. **Zhi Hu:** Methodology. **Pu Shi:** Investigation, Data curation. **Yong-ren Li:** Investigation. **Yong-jun Guo:** Investigation, Methodology. **Hao Song, Tao Zhang:** Conceptualization, Supervision, Funding acquisition, Writing–review & editing.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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