


Ancestral Physical Stress and Later Immune Gene Family Expansions Shaped Bivalve Mollusc Evolution

Tim Regan ^{1,*}, Lewis Stevens², Carolina Peñaloza¹, Ross D. Houston¹, Diego Robledo¹, and Tim P. Bean¹

*Corresponding author: E-mail: tim.regan@roslin.ed.ac.uk.

Accepted: 28 July 2021

Abstract

Bivalve molluscs comprise 20,000 species occupying a wide diversity of marine habitats. As filter feeders and detritivores they act as ecosystem engineers clarifying water, creating reefs, and protecting coastlines. The global decline of natural oyster reefs has led to increased restoration efforts in recent years. Bivalves also play an important role in global food security contributing to >20% of worldwide aquaculture production. Despite this importance, relatively little is known about bivalve evolutionary adaptation strategies. Difficulties previously associated with highly heterozygous and repetitive regions of bivalve genomes have been overcome by long-read sequencing, enabling the generation of accurate bivalve assemblies. With these resources we have analyzed the genomes of 32 species representing each molluscan class, including 15 bivalve species, to identify gene families that have undergone expansion during bivalve evolution. Gene family expansions across bivalve genomes occur at the point of evolutionary pressures. We uncovered two key factors that shape bivalve evolutionary history: expansion of bivalvia into environmental niches with high stress followed by later exposure to specific pathogenic pressures. The conserved expansion of protein recycling gene families we found across bivalvia is mirrored by adaptations to a sedentary lifestyle seen in plants. These results reflect the ability of bivalves to tolerate high levels of environmental stress and constant exposure to pathogens as filter feeders. The increasing availability of accurate genome assemblies will provide greater resolution to these analyses allowing further points of evolutionary pressure to become clear in other understudied taxa and potentially different populations of a single species.

Key words: bivalve, mollusc, orthology, evolution.

Significance

Despite the important roles bivalve molluscs play as ecosystem engineers and in global food supply, we know relatively little about the evolutionary history that has allowed them to thrive in diverse habitats and cope with the constant physical and pathogenic challenges they face as sedentary filter feeders. To explore these adaptations, we identified gene families that were expanded throughout bivalve evolutionary history by comparing the genomes of 15 bivalve species against 17 nonbivalve molluscs. We found conserved expansion from the last common ancestor of bivalves in gene families associated with a sedentary lifestyle in addition to physical, chemical, and temperature stressors and more recent expansions in innate immune response gene families reflecting adaptations to constant pathogen exposure as filter feeders.

Main Text

Bivalves are ecosystem engineers. Through filter-feeding they recycle nutrients, clarify water, and can protect coastlines from extreme weather by reef formation (Helmer et al. 2019; van der Schatte Olivier et al. 2020; Ray and Fulweiler 2021). The ongoing global decline of wild oyster reefs has led to an interest in applying restorative aquaculture to recover these vital ecosystem services (Vaughn and Hoellein 2018).

They also comprise >20% of global aquaculture production (FAO 2020; Houston et al. 2020). To establish a community for restocking or aquaculture, robust stocks are crucial, underscoring the importance of effective breeding strategies (Gutierrez et al. 2017; Potts et al. 2021) which in turn require better understanding of immunity and resilience mechanisms employed by bivalves (Tan et al. 2020).

© The Author(s) 2021. Published by Oxford University Press on behalf of the Society for Molecular Biology and Evolution.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

Since diverging from other molluscs approximately 530 Ma (Kocot et al. 2020), bivalves have adapted to a diverse range of niches including freshwater, intertidal zones, abyssal plains, and deep sea hydrothermal vents (Vaughn and Hoellein 2018), with over 20,000 known species globally. However, we know relatively little about what has allowed bivalves to thrive in these diverse habitats. Due to their largely sedentary or sessile lifestyle (Vaughn and Hoellein 2018), parallels have been drawn between bivalves and long-lived, highly fecund plants (Williams 1975; Plough 2016). Sessility requires adaptation to local environmental stressors such as air exposure and variations in temperature, pH, and salinity, whereas filter-feeding exposes bivalves to a wide range of chemical and pathogenic stressors in the water column (Burge et al. 2016). This constant exposure requires robust adaptation mechanisms, and the molecular basis of bivalve stress responses and the gene families involved deserves further attention.

Exploring the evolution of these gene families requires high-quality bivalve reference genomes. Generating such genomes has been impeded by high levels of heterozygosity (Plough 2016; Takeuchi 2017; Hollenbeck and Johnston 2018), repetitive regions (Davison and Neiman 2021), and structural variations (Calcino et al. 2021). A pan-genome based on gene presence–absence variation has recently been suggested for the Mediterranean mussel (Gerdol et al. 2020). However, long-read sequencing (Sun et al. 2021) has somewhat overcome these issues, leading to an increasing number of improved assemblies (Caurcel et al. 2021; Peñaloza et al. 2021). These resources present a new opportunity; here, using the genomes of 32 species representing each molluscan class, including 15 bivalve species, we identify gene families which have expanded during early and recent bivalve evolution.

Using OrthoFinder (Emms and Kelly 2019), we clustered the longest isoform of each protein-coding gene from each species into orthogroups (OGs), that is, orthologous groups of genes sharing a common ancestor. This resulted in >90% of all proteins being assigned to an OG (supplementary fig. 2, Supplementary Material online). We inferred the molluscan phylogeny using a concatenated alignment of 813 single-copy orthologs present in at least 28 of the 32 species and maximum likelihood under the general-time reversible substitution model with gamma-distributed rate variation among sites (GTR + Γ). The phylogeny is rooted on the branch separating Aculifera (a clade comprising Caudofoveata, Polyplacophora, and Solenogastres) from Conchifera (a clade comprising Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, and Scaphopoda). The resulting topology was congruent with previously published phylogenies and all molluscan classes were recovered as monophyletic (Kocot et al. 2020) (fig. 1).

We used two complementary approaches to identify gene families that have undergone expansion in bivalves. First,

using the molluscan species tree and OrthoFinder, we inferred gene trees and gene duplication events. Of these, we looked at OGs with an arbitrarily designated minimum of 5-fold genes per species in bivalves relative to other molluscs which also contained duplications that occurred in the last common ancestor (LCA) of all bivalve taxa and that have been retained by >70% of all bivalve species in our study. The aim of this analysis was to focus on OGs with more genes in bivalves relative to other molluscs which also contained conserved duplication events. Second, we used KinFin (Laetsch and Blaxter 2017) to identify gene families that were significantly (P value $< 4 \times 10^{-5}$) overrepresented in bivalves relative to other molluscan taxa (referred to as “bivalve-enriched”), regardless of presence in LCA. This latter approach allowed us to identify gene families that have undergone expansion in bivalves over ancient and also more recent evolutionary time scales (i.e., clade- or species-specific expansions). In total, we identified 16 gene families with ancestrally conserved duplications across all bivalves (table 1; examples shown in fig. 2a–c and 15 that were significantly bivalve-enriched (table 2; examples shown in fig. 2d–f). Two gene families, OG10 and OG53, were found in both analyses. We also analyzed the genome-wide distribution of these expanded gene families by comparing their positions across five chromosomally resolved molluscan reference genomes (supplementary fig. 4, Supplementary Material online).

The largest heat shock protein (HSP) family identified from our analysis (OG10, the tenth largest molluscan OG overall) (fig. 2a) was the second most significantly bivalve-enriched OG ($P = 1.27 \times 10^{-6}$, table 2). This is consistent with the previous genomics and molecular biology studies highlighting the significance of HSPs in bivalves for maintaining cellular function during exogenous redox/chemical/pH stress. Bivalves may also induce intracellular stress to handle constant exposure to intracellular pathogens, requiring chaperone and HSPs to prevent apoptosis and retain cellular function (Venier et al. 2011; Gust et al. 2013; Leite et al. 2013; Al-Khalaifah and Afaf 2018; Smits et al. 2020). In bivalves, members of this gene family are distributed throughout the genome (e.g., in *Crassostrea gigas*, they are present on six of the ten chromosomes; supplementary fig. 4a and b, Supplementary Material online), whereas they are restricted to two chromosomes in the gastropod *Pomacea canaliculata* (supplementary fig. 4c, Supplementary Material online) and one chromosome in the cephalopod *Octopus vulgaris* (supplementary fig. 4d, Supplementary Material online). Mitochondria-eating protein (*Mieap*) families are responsible for maintaining healthy mitochondria after intracellular damage (Kitamura et al. 2011; Nakamura and Arakawa 2017). The largest *Mieap* OG identified in our analysis was OG24 (fig. 2b). These OGs (fig. 2a–c) each had three duplications in the LCA conserved across all bivalves used in this analysis (table 1). Mitophagy results in high levels of intracellular reactive oxygen species, requiring chaperone proteins to maintain cellular functioning (Rose et

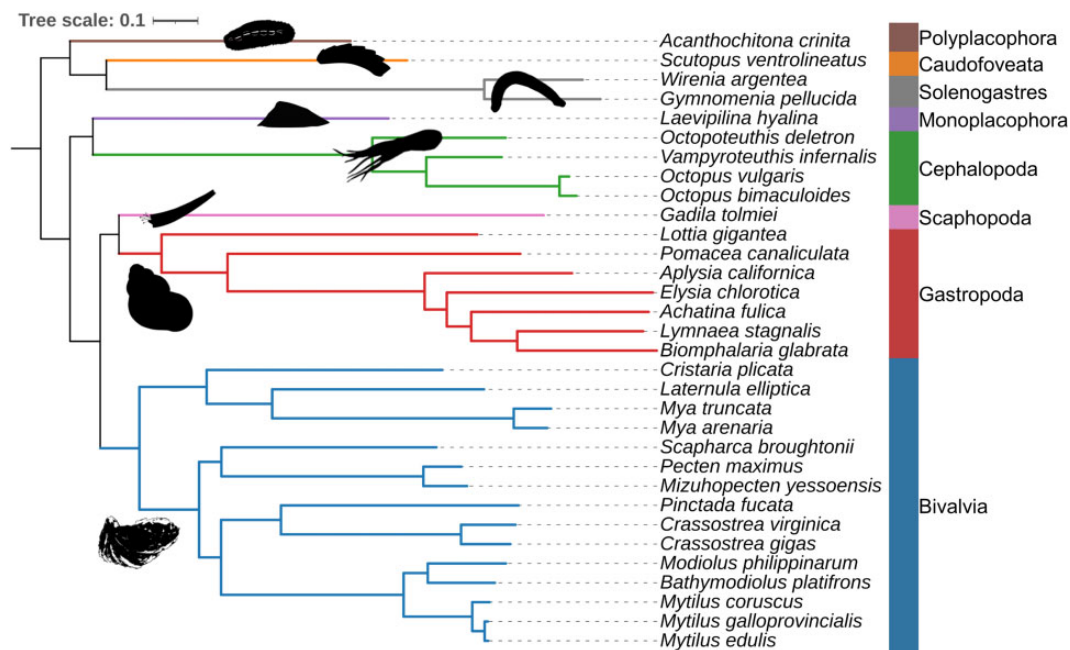


Fig. 1.—The molluscan phylogeny. Branch lengths are in amino acid substitutions per site; scale is shown. Colors on tree branches represent each of the molluscan classes as indicated. Silhouettes are reproduced from <http://phylopic.org>.

al. 2011). The fact that both of these large gene families operate to regulate intracellular damage suggests that this was an important function during early bivalve evolution.

Hypoxic stress is inextricable from bivalve life history in the intertidal environment where access to oxygenated water is dependent on tide. It is also a secondary effect of the primary defence mechanism of shell clamping (Long et al. 2008) and is induced by bivalves during infection (Al-Khalaifah and Afaf 2018; Smits et al. 2020; Steffen et al. 2020). OG53, the second most significantly bivalve-enriched gene family, consists of oxidoreductase and tyrosinase proteins ($P=7.87 \times 10^{-6}$, table 2). This OG contained one duplication event in the LCA of all bivalves that has since been retained by all bivalve species we sampled (fig. 2c). In addition to hypoxic stress, this gene family may also be involved in melanization (Kobayashi et al. 1994), a well described invertebrate wound response (Bilandzija et al. 2017) and another physical stress response.

As bivalves lack an adaptive immune system, constant pathogen exposure necessitates specialized tolerance mechanisms (Wang et al. 2013). Protein recycling pathways, chaperone proteins, and apoptotic inhibitors can be employed to maintain cellular function during infection by inducing high levels of oxidative stress to remove intracellular pathogens (Sunila and LaBanca 2003; Donaghy et al. 2009; Hughes et al. 2010; Zhang et al. 2011; Brulle et al. 2012; McDowell et al. 2014; Smits et al. 2020). This is reflected in the high diversity of inhibitor of apoptosis proteins (IAPs) shared by bivalves (Song et al. 2021; Vogeler et al. 2021). The largest IAP gene family we identified was OG17. Although this OG contained 3-fold more duplications in bivalves compared with

other molluscan classes (table 2), these duplications were not ancestrally retained across Bivalvia (no LCA duplications retained by >50% of bivalves used in this study), suggesting more recent expansion and diversification (fig. 2d). This may reflect selective pressure from certain pathogens that can take advantage of these pathways, for example, during *Bonamia ostreae* and OsHV-1 infections (de Lorgeril et al. 2018; Cocci et al. 2020).

Central to initiation of the innate immune transcriptional response is inflammasome formation, largely regulated by IAP and CARD protein families (Latz et al. 2013). The fourth most significantly bivalve-enriched OG ($P=5.01 \times 10^{-6}$) was a CARD protein family (OG863, table 2). Similar to the IAP gene family, no LCA duplication events were found in this OG suggesting more recent expansion and diversification (fig. 2e). The earliest phase of TLR signal transduction following pathogen detection is regulated by Toll/interleukin-1 receptor homology (TIR) domain-containing proteins (O'Neill and Bowie 2007). OG1241, a TIR domain protein family, was the third most significantly bivalve-enriched OG ($P=2.17 \times 10^{-6}$) with an average of 3-fold more copies in bivalve species compared with nonbivalve molluscs (table 2). Again, this gene family contained no LCA duplication events across bivalves (fig. 2f).

Unlike other OGs with associated immune function, the *C1q* gene family OG92 contained a LCA duplication event retained by >70% of bivalves. OG92 also consisted almost entirely of bivalve genes and is likely to have expanded greatly over time following the ancestral duplication event. Although the innate immune complement system is best described role

Table 1

OGs with Conserved Ancestral Duplications and a Relatively High Number of Genes per Species in Bivalves

Orthogroup		Avg. Genes/Species		No. of Duplications in LCA Retained by >70% of Bivalves	Functional Annotation	Source
Name	Size	Bivalvia (15)	Others (17)			
OG643	143	9.5	0	1	<ul style="list-style-type: none"> • Domain ATPase, dynein-related, AAA domain • Negative regulation of noncanonical Wnt signaling pathway 	<ul style="list-style-type: none"> • IPR, GO, Pfam • eggNOG
OG950	110	7.3	0	1	—	—
OG1538	82	5.5	0	1	<ul style="list-style-type: none"> • OTU-like cysteine protease • Thiol-dependent ubiquitin-specific protease activity 	<ul style="list-style-type: none"> • IPR, Pfam • eggNOG
OG2450	63	4.2	0	1	—	—
OG3835	50	3.3	0	1	Domain of unknown function (DUF1772)	Pfam, eggNOG
OG92	441	29.3	0.1	1	Complement C1q domain	IPR, Pfam, eggNOG
OG24	807	53.1	0.6	3 (3 that are conserved in 100% of bivalves)	Mitochondria-eating protein	IPR, Pfam
OG708	135	8.9	0.1	1	<ul style="list-style-type: none"> • Domain B-box-type zinc finger • Interferon-beta production 	<ul style="list-style-type: none"> • IPR, GO, Pfam • eggNOG
OG314	234	15.1	0.4	1	<ul style="list-style-type: none"> • Mib-herc2 • Protein ubiquitination 	<ul style="list-style-type: none"> • IPR, Pfam • GO, eggNOG
OG1139	99	6.4	0.2	2	Zinc finger, C3HC4 type (RING finger)	IPR, GO, Pfam, eggNOG
OG913	113	7.1	0.4	1	Repeat leucine-rich repeat	IPR, Pfam, eggNOG
OG10	1,096	68.1	4.4	8 (3 that are conserved in 100% of bivalves)	<ul style="list-style-type: none"> • Heat shock protein 70 family • Regulation of apoptotic process 	<ul style="list-style-type: none"> • Pfam, IPR, eggNOG • GO
OG3669	51	2.9	0.4	1	—	—
OG888	115	6.5	1.1	1	Rho GTPase	IPR, GO, Pfam, eggNOG
OG53	552	30.6	5.5	3 (1 that is conserved in 100% of bivalves)	<ul style="list-style-type: none"> • Tyrosinase copper-binding domain • Oxidoreductase activity 	<ul style="list-style-type: none"> • IPR, Pfam, eggNOG • GO
OG29	767	41.8	8.2	1	Neuronal acetylcholine receptor subunit	IPR, GO, Pfam, eggNOG

NOTE.—OGs with at least five times as many bivalve genes per species compared with other molluscan classes, and at least one duplication event in LCA of bivalves retained by >70% of bivalve species used in this study.

for *C1q*, its function has diversified over time with recently described shell formation proteins described in bivalves for this protein family (Xiong et al. 2021). This presents an example of LCA duplications in an OG retained across bivalves followed by further expansions over time and potential caveats of ascribing single functions to entire gene families based on orthology.

Similar to plants, bivalves are mostly sessile and require the ability to respond to highly transient environmental conditions in a rapid and efficient manner. Protein recycling gene family expansion in plants is thought to be an adaptation to sessile lifestyle with protein modifications enabling a fast and easily reversible modulation of protein function (Hua and Vierstra 2011; Park and Seo 2015; Chi et al. 2019). It is possible that

bivalves have evolved similar mechanisms to adapt to sessility. We identified multiple protein recycling gene families that were overrepresented in bivalves (tables 1 and 2) including two OGs unique to Bivalvia (OG643, OG1538). In addition to informing lifestyle adaptation, this may underscore the importance of proteomics in experiments investigating bivalve resilience/immune mechanisms. For instance, proteomics revealed the importance of redox homeostasis for resistance to brown ring disease in a Manila clam infection challenge (Smits et al. 2020).

Expansion of complement, redox, chaperone, and protein recycling enzyme families across bivalves is thought to have occurred in a species-specific manner (Wang et al. 2010, 2016; Fleury and Huvet 2012; Takeuchi et al. 2016). We

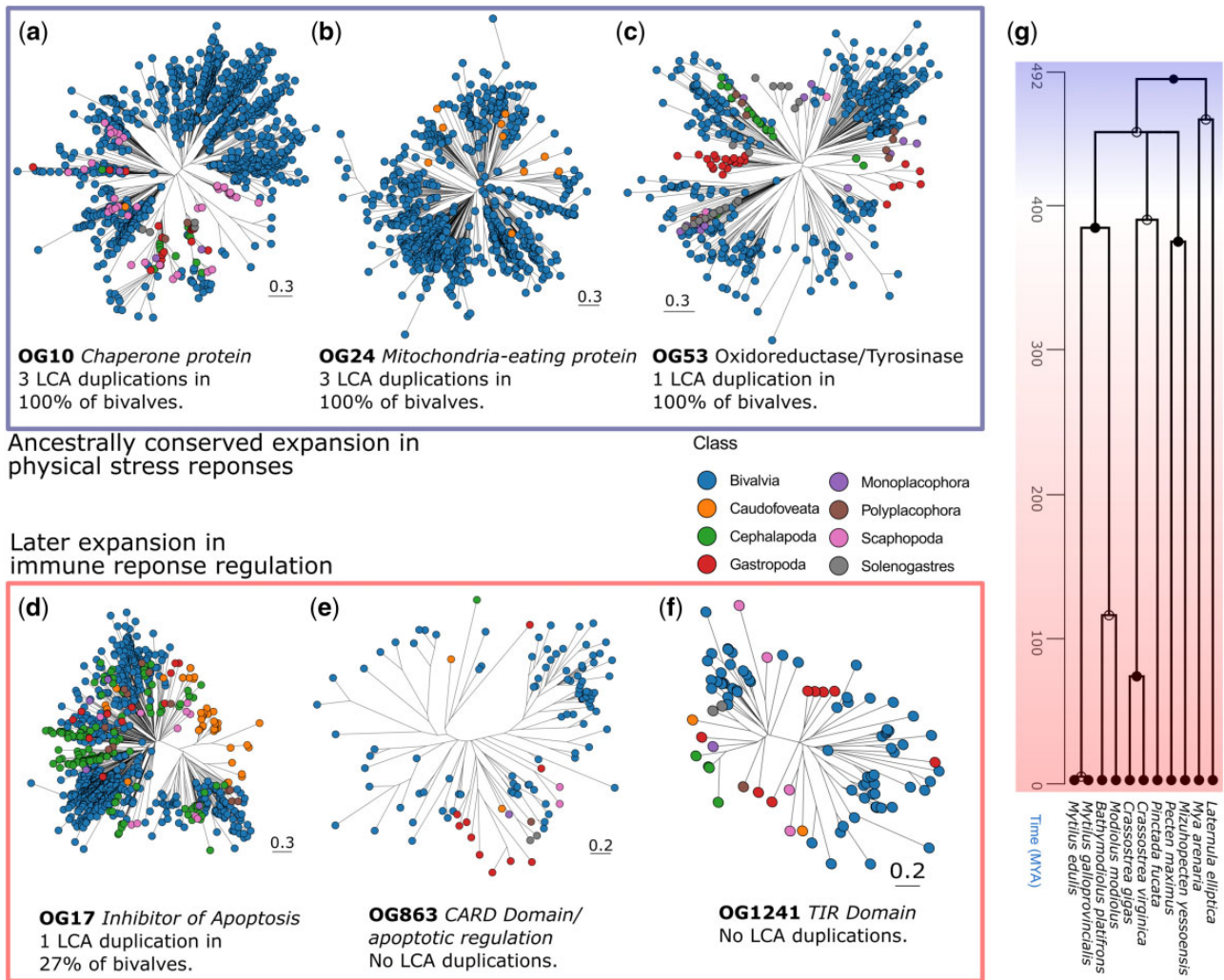


FIG. 2.—Gene families that have undergone expansion in bivalves. Trees of gene families with duplication events conserved in 100% of bivalves since LCA (a–c) and more recent expansion (d–f). Timescale phylogeny tree for bivalves (g) produced using TimeTree (Kumar et al. 2017). Gene family name and function are displayed with number of LCA duplications conserved across all bivalves. Gene trees were inferred using FastTree (Price et al. 2010) within OrthoFinder and plotted using ggtree (Yu et al. 2017). Nodes representing genes are colored according to taxonomic class.

found evidence of conserved ancestral duplication (retained by >66% of bivalves) among four gene families (OG92, OG10, OG53, OG314, and OG1538) (table 1). In the context of innate immunity, pattern recognition receptors are described as having undergone a broad expansion across molluscan evolution (Vasta and Wang 2020). We found gene families with immune function, that is, TLR, CLR, and inflammasome regulation (OG2, OG17, OG863, and OG1241) underwent more recent expansion with little or no conserved LCA duplications (table 2). This could reflect the fact that all or most bivalves experience similar physiological stressors from fluctuations in temperature, pH, tides, and oxygen availability. Although these stressors have remained constant since the LCA of bivalves, pathogenic challenges continue to evolve over time requiring more recent adaptations (Obbard et al. 2009; Webb et al. 2015). This is reflected in the clade- and

species-specific diversification among gene families regulating innate immunity.

Using genome biology to examine evolutionary history of gene families, we have revealed and differentiated both recent and ancestral adaptation strategies. Future studies using different populations of bivalves should further explore the gene families described here to identify their redundancies and functional diversity. This improved understanding of basic biology will provide fundamental knowledge which can aid bivalve aquaculture or restoration efforts. We have primarily focused on OGs containing more genes in bivalves relative to other molluscs on the assumption that expansion of gene families across this class correlate with natural selection. It should be noted that neutral forces as well as selective forces may lead to such expansion and these forces should also be analyzed further in future studies (Hahn et al. 2005).

Table 2Functional Annotation of Bivalve-Enriched Orthogroups (each bivalve species used has at least one gene, cut-off P value $<4 \times 10^{-5}$)

Orthogroup		Avg. Genes/Species		No. of Duplications in LCA Retained by >50% of Bivalves	P Value (bivalvia vs. others)	Functional Annotation	Source
Name	Size	Bivalves (15)	Others (17)				
OG3213	54	2.4	1.1	1	4.48E-07	<ul style="list-style-type: none"> • Domain F-box domain • Regulation of muscle adaptation 	<ul style="list-style-type: none"> • IPR, Pfam • eggNOG
OG10	1,096	68.1	4.4	11	1.27E-06	<ul style="list-style-type: none"> • Heat shock protein 70 family • Regulation of apoptotic process 	<ul style="list-style-type: none"> • Pfam, IPR, eggNOG • GO
OG1241	93	4.7	1.3	0	2.17E-06	Toll/interleukin-1 receptor homology (TIR) domain	Pfam, IPR, eggNOG
OG863	117	6.5	1.1	0	5.01E-06	<ul style="list-style-type: none"> • CARD domain • Regulation of apoptotic process 	<ul style="list-style-type: none"> • IPR, Pfam, eggNOG • GO
OG13	949	56.5	5.9	1	7.07E-06	Complement C1q domain	IPR, Pfam, eggNOG
OG53	552	30.6	5.5	3	7.87E-06	<ul style="list-style-type: none"> • Tyrosinase copper-binding domain • Oxidoreductase activity 	<ul style="list-style-type: none"> • IPR, Pfam, eggNOG • GO
OG268	257	13.1	3.6	0	9.12E-06	<ul style="list-style-type: none"> • Domain Deltex, C-terminal • E3 ubiquitin-protein ligase 	<ul style="list-style-type: none"> • IPR, Pfam • eggNOG
OG272	255	15.1	1.7	1	9.54E-06	<ul style="list-style-type: none"> • Repeat LDLR class B repeat • Endocytosis 	IPR, Pfam, eggNOG
OG298	240	13.7	2.0	0	1.12E-05	<ul style="list-style-type: none"> • Galactose-binding lectin domain • Autophagy and apoptosis 	<ul style="list-style-type: none"> • IPR, Pfam • eggNOG
OG4321	47	2.0	1.0	0	1.37E-05	Phosphatase activity	Pfam, GO, IPR
OG17	919	46.4	13.1	0	1.76E-05	Inhibitor of Apoptosis domain	Pfam, IPR, eggNOG
OG498	171	9.2	1.9	0	2.78E-05	<ul style="list-style-type: none"> • TROVE domain • U2 snRNA binding 	<ul style="list-style-type: none"> • IPR, Pfam • eggNOG
OG18	905	38.2	19.5	0	3.11E-05	<ul style="list-style-type: none"> • EF-hand domain • Calmodulin 	<ul style="list-style-type: none"> • IPR, Pfam • GO, eggNOG
OG857	117	5.7	1.8	0	3.33E-05	Acyl-CoA oxidase, C-terminal	Pfam, IPR, GO, eggNOG
OG2	2,007	102.7	27.4	0	3.82E-05	C-type lectin-like	Pfam, IPR, eggNOG

With the rapidly increasing availability and improvement of genome assemblies, the methods described here will allow for more specific biological analyses, including higher taxonomic resolution. For instance, by adopting a similar approach, studies examining adaptation of different populations across a single species could focus on gene families across the taxonomic class with and without conserved duplications, using sister species to define the LCA, rather than species from a different taxonomic class. This demonstrates the utility of genome biology to better understand the evolutionary history of understudied species.

Materials and Methods

Orthology Clustering

Details of the proteomes used in our analyses are detailed in [supplementary table 1](#), [Supplementary Material](#) online. Briefly, we selected the longest isoform for each protein-coding gene in each species using AGAT (version 0.4.0) (Jacques Dainat 2020). We assessed the completeness and level of duplication in each of the isoform-filtered proteomes using BUSCO (version 4.06 against mollusca_odb10 database) (Seppey et al. 2019) and CD-hit (version 4.6.8) (Fu et

al. 2012) using 90% sequence identity threshold and a word length of 5 (Supplementary fig. 1, Supplementary Material online). We clustered the isoform-filtered proteomes into OGs using OrthoFinder (version 2.3.11) (Emms and Kelly 2019).

Species Tree Inference

A very small number of OGs (3 in total) were single copy across all 32 species, likely as result of incomplete assemblies and/or annotations or as a result of haplotypic duplication. To circumvent this, we selected 2,933 OGs that were present in at least 75% of species and had an average count of 1 using KinFin (v1.0; Laetsch and Blaxter 2017). To remove clusters containing paralogous sequences, we aligned the protein sequences of each selected OG using MAFFT (v7.455; Katoh and Standley 2013) and generated a maximum likelihood tree along with 1,000 ultrafast bootstraps (Hoang et al. 2018) using IQ-TREE (v2.0.3; Nguyen et al. 2015), allowing the best-fitting substitution model to be selected automatically (Kalyaanamoorthy et al. 2017). We screened each gene tree for evidence of paralogy using PhyloTreePruner (v1.0; Kocot et al. 2013) and retained 813 OGs containing orthologous sequences from at least 28 of the 32 species. If two representative sequences were present for any species (i.e., in-paralogs) after this paralog screening step, the longest of the two sequences was retained and the other discarded. We realigned the protein sequences of each filtered OG using MAFFT and trimmed spuriously aligned regions from each alignment using trimAl. The trimmed alignments were concatenated using catfasta2phyml (available from: <https://github.com/nylander/catfasta2phyml>) to form a supermatrix. We inferred the species tree using maximum likelihood using IQ-TREE, with the general-time reversible (GTR) substitution model with gamma-distributed rate variation among sites (+ Γ) along with 1,000 ultrafast bootstraps. As per Kocot et al. (2013), we rooted the resulting phylogeny on the branch separating the aculiferans (a clade comprising Caudofoveata, Polyplacophora, and Solenogastres) from the conchiferans (a clade comprising Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, and Scaphopoda). The phylogeny was visualized using iTOL (available at <https://itol.embl.de/>).

Identification of Expanded Gene Families

To identify gene families that underwent expansion during early bivalve evolution, we provided the orthology clustering and the inferred species tree to OrthoFinder, which inferred gene duplication events. Briefly, OrthoFinder infers gene trees for each OG using FastTree and uses the rooted species tree to infer gene duplication events using duplication-loss-coalescent model. We identified OGs that underwent gene duplication events in the LCA of all bivalve species and

selected only those that had been retained by 11 of 15 bivalve species in our study, as an arbitrarily designated cutoff. Of these, we kept OGs where bivalves had an average of five times as many genes than species from other molluscan classes (table 1). To identify gene families that have undergone expansion during more recent bivalve evolution, we used KinFin (version 1.0) to identify OGs that were significantly overrepresented in bivalves compared with nonbivalve molluscs. We selected OGs that had a P value $< 4 \times 10^{-5}$ (table 2). For both sets of expanded OGs, we visualized the gene tree used ggtree R package (Yu et al. 2017). We also functionally annotated each expanded OG by searching each protein against the Pfam (Mistry et al. 2021) database and eukaryotic SignalP database (Petersen et al. 2011) using InterProScan (version 5.47-82.0) (Jones et al. 2014) and provided the resulting annotations to KinFin (Laetsch and Blaxter 2017) or using the eggNOG mapper (<http://eggno-mapper.embl.de/>) (Huerta-Cepas et al. 2017).

Synteny

To analyze chromosome rearrangements and synteny between bivalves and other molluscs, we identified all one-to-one orthologs between *C. gigas* and each of the four assemblies used *Crassostrea virginica*, *Pecten maximus*, *Pomacea canaliculata*, or *Octopus vulgaris*. We then mapped the locations of each gene from selected bivalve paralog-rich OGs with recent, or ancestrally conserved duplications (OG10 and OG1241). Circos plots were generated using Circos v0.69–8 (Krzywinski et al. 2009).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

This work was supported by funding from the Biotechnology and Biological Sciences Research Council (BBS/E/D/10002070, BBS/E/D/30002275, BBS/E/D/10002071) and the Wellcome Trust (206194, 218328).

Data Availability

Data relevant to this study including the genome assemblies used, supermatrix file of the concatenated alignments of 813 single-copy orthologs used to infer the species tree, orthology clustering file, the species tree used to infer duplications, orthology clustering file of protein sequences and gene duplications from Orthofinder and KinFin results are available in Zenodo with the identifier "doi:10.5281/zenodo.4697197."

Literature Cited

- Al-Khalaifah H, Al-Nasser A. 2018. Immune response of molluscs. In: Diarte-Plata G, Escamilla-Montes R, editors. *Molluscs*. IntechOpen. doi:10.5772/intechopen.81778.
- Bilandzija H, Laslo M, Porter ML, Fong DW. 2017. Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. *Sci Rep*. 7(1):17148.
- Brulle F, Jeffroy F, Madec S, Nicolas JL, Paillard C. 2012. Transcriptomic analysis of *Ruditapes philippinarum* hemocytes reveals cytoskeleton disruption after in vitro *Vibrio tapetis* challenge. *Dev Comp Immunol*. 38(2):368–376.
- Burge CA, et al. 2016. The use of filter-feeders to manage disease in a changing world. *Integr Comp Biol*. 56(4):573–587.
- Calcino AD, Kenny NJ, Gerdol M. 2021. Single individual structural variant detection uncovers widespread hemizyosity in molluscs. *Philos Trans R Soc Lond B Biol Sci*. 376(1825):20200153.
- Caurcel C, et al. 2021. MolluscDB: a genome and transcriptome database for molluscs. *Philos Trans R Soc Lond B Biol Sci*. 376(1825):20200157.
- Chi YH, et al. 2019. The physiological functions of universal stress proteins and their molecular mechanism to protect plants from environmental stresses. *Front Plant Sci*. 10:750.
- Cocci P, Roncarati A, Capriotti M, Mosconi G, Palermo FA. 2020. Transcriptional alteration of gene biomarkers in hemocytes of wild. *Pathogens* 9(5):323.
- Davison A, Neiman M. 2021. Pearls of wisdom—a Theo Murphy issue on molluscan genomics. *Philos Trans R Soc Lond B Biol Sci*. 376(1825):20200151.
- de Lorgeril J, et al. 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat Commun*. 9(1):4215.
- Donaghy L, Lambert C, Choi K-S, Soudant P. 2009. Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): current knowledge and future prospects. *Aquaculture* 297(1–4):10–24.
- Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biol*. 20(1):238.
- FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome (Italy): FAO.
- Fleury E, Huvet A. 2012. Microarray analysis highlights immune response of pacific oysters as a determinant of resistance to summer mortality. *Mar Biotechnol (NY)*. 14(2):203–217.
- Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28(23):3150–3152.
- Gerdol M, et al. 2020. Massive gene presence-absence variation shapes an open pan-genome in the Mediterranean mussel. *Genome Biol*. 21(1):275.
- Gust M, Fortier M, Garric J, Fournier M, Gagné F. 2013. Effects of short-term exposure to environmentally relevant concentrations of different pharmaceutical mixtures on the immune response of the pond snail *Lymnaea stagnalis*. *Sci Total Environ*. 445–446:210–218.
- Gutierrez AP, et al. 2017. Development of a medium density combined-species SNP array for Pacific and European oysters (*Crassostrea gigas* and *Ostrea edulis*). *G3 (Bethesda)* 7(7):2209–2218.
- Hahn MW, De Bie T, Stajich JE, Nguyen C, Cristianini N. 2005. Estimating the tempo and mode of gene family evolution from comparative genomic data. *Genome Res*. 15(8):1153–1160.
- Helmer L, et al. 2019. Active management is required to turn the tide for depleted *Ostrea edulis* stocks from the effects of overfishing, disease and invasive species. *PeerJ* 7:e6431.
- Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. 2018. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol*. 35(2):518–522.
- Hollenbeck CM, Johnston IA. 2018. Genomic tools and selective breeding in molluscs. *Front Genet*. 9:253.
- Houston RD, et al. 2020. Harnessing genomics to fast-track genetic improvement in aquaculture. *Nat Rev Genet*. 21(7):389–409.
- Hua Z, Vierstra RD. 2011. The cullin-RING ubiquitin-protein ligases. *Annu Rev Plant Biol*. 62:299–334.
- Huerta-Cepas J, et al. 2017. Fast genome-wide functional annotation through orthology assignment by eggNOG-Mapper. *Mol Biol Evol*. 34(8):2115–2122.
- Hughes FM, Foster B, Grewal S, Sokolova IM. 2010. Apoptosis as a host defense mechanism in *Crassostrea virginica* and its modulation by *Perkinsus marinus*. *Fish Shellfish Immunol*. 29(2):247–257.
- Jacques Dainat DH. 2020. AGAT-v0.4.0 (version v0.4.0). doi:http://doi.org/10.5281/zenodo.3877441.
- Jones P, et al. 2014. InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30(9):1236–1240.
- Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermin LS. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*. 14(6):587–589.
- Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*. 30(4):772–780.
- Kitamura N, et al. 2011. Mieap, a p53-inducible protein, controls mitochondrial quality by repairing or eliminating unhealthy mitochondria. *PLoS One* 6(1):e16060.
- Kobayashi T, et al. 1994. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *EMBO J*. 13(24):5818–5825.
- Kocot KM, Citarella MR, Moroz LL, Halanych KM. 2013. PhyloTreePruner: a phylogenetic tree-based approach for selection of orthologous sequences for phylogenomics. *Evol Bioinform Online*. 9:429–435.
- Kocot KM, Poustka AJ, Stöger I, Halanych KM, Schrödl M. 2020. New data from Monoplacophora and a carefully-curated dataset resolve molluscan relationships. *Sci Rep*. 10(1):101.
- Krzywinski M, et al. 2009. Circos: an information aesthetic for comparative genomics. *Genome Res*. 19(9):1639–1645.
- Kumar S, Stecher G, Suleski M, Hedges SB. 2017. TimeTree: a resource for timelines, timetrees, and divergence times. *Mol Biol Evol*. 34(7):1812–1819.
- Laetsch DR, Blaxter ML. 2017. KinFin: software for taxon-aware analysis of clustered protein sequences. *G3 (Bethesda)* 7(10):3349–3357.
- Latz E, Xiao TS, Stutz A. 2013. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. 13(6):397–411.
- Leite RB, et al. 2013. mRNA-Seq and microarray development for the Grooved Carpet shell clam, *Ruditapes decussatus*: a functional approach to unravel host-parasite interaction. *BMC Genomics* 14:741.
- Long WC, Brylawski BJ, Seitz RD. 2008. Behavioral effects of low dissolved oxygen on the bivalve *Macoma balthica*. *J Exp Mar Biol Ecol*. 359(1):34–39.
- McDowell IC, et al. 2014. Transcriptome of American oysters, *Crassostrea virginica*, in response to bacterial challenge: insights into potential mechanisms of disease resistance. *PLoS One* 9(8):e105097.
- Mistry J, et al. 2021. Pfam: the protein families database in 2021. *Nucleic Acids Res*. 49(D1):D412–D419.
- Nakamura Y, Arakawa H. 2017. Discovery of Mieap-regulated mitochondrial quality control as a new function of tumor suppressor p53. *Cancer Sci*. 108(5):809–817.
- Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 32(1):268–274.
- O'Neill LA, Bowie AG. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol*. 7(5):353–364.
- Obbard DJ, Welch JJ, Kim K-W, Jiggins FM. 2009. Quantifying adaptive evolution in the *Drosophila* immune system. *PLoS Genet*. 5(10):e1000698.

- Park C-J, Seo Y-S. 2015. Heat shock proteins: a review of the molecular chaperones for plant immunity. *Plant Pathol J.* 31(4):323–333.
- Peñalosa C, et al. 2021. A chromosome-level genome assembly for the Pacific oyster *Crassostrea gigas*. *GigaScience* 10(3):giab020.
- Petersen TN, Brunak S, Von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods.* 8(10):785–786.
- Plough LV. 2016. Genetic load in marine animals: a review. *Curr Zool.* 62(6):567–579.
- Potts RWA, Gutierrez AP, Penalosa CS, Regan T, Bean TP, Houston RD. 2021. Potential of genomic technologies to improve disease resistance in molluscan aquaculture. *Philos Trans R Soc Lond B Biol Sci* 376(1825):20200168.
- Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS One* 5(3):e9490.
- Ray NE, Fulweiler RW. 2021. Meta-analysis of oyster impacts on coastal biogeochemistry. *Nat Sustain.* 4(3):261–269.
- Rose JM, Novoselov SS, Robinson PA, Cheetham ME. 2011. Molecular chaperone-mediated rescue of mitophagy by a Parkin RING1 domain mutant. *Hum Mol Genet.* 20(1):16–27.
- Seppey M, Manni M, Zdobnov EM. 2019. BUSCO: assessing genome assembly and annotation completeness. *Methods Mol Biol.* 1962:227–245.
- Smits M, et al. 2020. A proteomic study of resistance to Brown Ring disease in the Manila clam, *Ruditapes philippinarum*. *Fish Shellfish Immunol.* 99:641–653.
- Song H, et al. 2021. The hard clam genome reveals massive expansion and diversification of inhibitors of apoptosis in *Bivalvia*. *BMC Biol.* 19(1):15.
- Steffen JBM, Falfushynska HI, Piontkivska H, Sokolova IM. 2020. Molecular biomarkers of the mitochondrial quality control are differently affected by hypoxia-reoxygenation stress in marine bivalves *Crassostrea gigas* and *Mytilus edulis*. *Front Mar Sci.* 7:1048.
- Sun J, Li R, Chen C, Sigwart JD, Kocot KM. 2021. Benchmarking Oxford Nanopore read assemblers for high-quality molluscan genomes. *Philos Trans R Soc Lond B Biol Sci.* 376(1825):20200160.
- Sunila I, LaBanca J. 2003. Apoptosis in the pathogenesis of infectious diseases of the eastern oyster *Crassostrea virginica*. *Dis Aquat Organ.* 56(2):163–170.
- Takeuchi T. 2017. Molluscan genomics: implications for biology and aquaculture. *Curr Mol Bio Rep.* 3(4):297–305.
- Takeuchi T, et al. 2016. Bivalve-specific gene expansion in the pearl oyster genome: implications of adaptation to a sessile lifestyle. *Zool Lett.* 2:3.
- Tan K, Zhang H, Zheng H. 2020. Selective breeding of edible bivalves and its implication of global climate change. *Rev Aquacult.* 12(4):2559–2572.
- van der Schatte Olivier A, et al. 2020. A global review of the ecosystem services provided by bivalve aquaculture. *Rev Aquacult.* 12(1):3–25.
- Vasta GR, Wang JX. 2020. Galectin-mediated immune recognition: opsonic roles with contrasting outcomes in selected shrimp and bivalve mollusk species. *Dev Comp Immunol.* 110:103721.
- Vaughn CC, Hoellein TJ. 2018. Bivalve impacts in freshwater and marine ecosystems. *Annu Rev Ecol Evol Syst.* 49(1):183–208.
- Venier P, et al. 2011. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. *BMC Genomics* 12:69.
- Vogeler S, Carboni S, Li X, Joyce A. 2021. Phylogenetic analysis of the caspase family in bivalves: implications for programmed cell death, immune response and development. *BMC Genomics* 22(1):80.
- Wang K, Pales Espinosa E, Tanguy A, Allam B. 2016. Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature. *Fish Shellfish Immunol.* 49:163–176.
- Wang L, Qiu L, Zhou Z, Song L. 2013. Research progress on the mollusc immunity in China. *Dev Comp Immunol.* 39(1–2):2–10.
- Wang SP, et al. 2010. Microarray analysis of gene expression in eastern oyster (*Crassostrea virginica*) reveals a novel combination of antimicrobial and oxidative stress host responses after dermo (*Perkinsus marinus*) challenge. *Fish Shellfish Immunol.* 29(6):921–929.
- Webb AE, et al. 2015. Adaptive evolution as a predictor of species-specific innate immune response. *Mol Biol Evol.* 32(7):1717–1729.
- Williams G. 1975. Sex and evolution. *Monogr Popul Biol.* (8):3–200.
- Xiong X, Li C, Zheng Z, Du X. 2021. Novel globular C1q domain-containing protein (PmC1qDC-1) participates in shell formation and responses to pathogen-associated molecular patterns stimulation in *Pinctada fucata martensii*. *Sci Rep.* 11(1):1105.
- Yu G, Smith DK, Zhu H, Guan Y, Lam TT-Y. 2017. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol Evol.* 8(1):28–36.
- Zhang L, Li L, Zhang G. 2011. Gene discovery, comparative analysis and expression profile reveal the complexity of the *Crassostrea gigas* apoptosis system. *Dev Comp Immunol.* 35(5):603–610.

Associate editor: Helen Piontkivska