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Molecular diversity and evolution of defensins in the manila clam *Ruditapes philippinarum*

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ABSTRACT

Four types of defensins were identified in Manila clam and designated as Rpdef1, Rpdef2, Rpdef3 and Rpdef4, which encoded a polypeptide of 49, 46, 45 and 42 amino acids, respectively. Sequence alignments indicated that Rpdef1 shared 46.9% identity with Rpdef2, 40.8% with Rpdef3, and 34.7% with Rpdef4. Analysis of transcript polymorphism showed that Rpdef3 accounted for about 60% frequency of Rpdefs occurrence in clams from three geographic origins (Dalian, Qingdao and Hangzhou). By quantitative real-time RT-PCR (qRT-PCR) analysis, the transcripts of Rpdefs were mainly detected in hemocytes and they responded sensitively to bacterial challenge in hemocytes. Evolutionary analysis indicated that all Rpdefs were under positive selection with positively selected basic amino acid residues detected in the C-terminal regions, which perhaps have a functional relevance by modifying the charge distribution of Rpdefs. The results also showed some lineages with dN/dS > 1, suggesting positive selection pressures existed in some lineages of phylogeny tree constructed by mollusk defensins. Overall, our results suggest that Rpdefs perhaps played important roles in host defense and positive selection is the major driving force in generating high diversity of defensins in the Manila clam.

1. Introduction

Invertebrates exclusively depend on their innate immunity which consists of both cellular and humoral defenses [1,2]. The former includes phagocytosis or encapsulation of pathogens with subsequent pathogen destruction via enzyme activity and oxygen metabolite release, while the latter includes various reactions mediated by molecules such as antimicrobial peptides (AMPs) and proteins [3,4]. In marine invertebrates, AMPs represent the major humoral defense system against infection. The modes of action by which AMPs kill bacteria are diverse, and most of them related to plasma membrane disturbance and lethal alteration of microbial integrity [5]. In marine mollusks, several kinds of AMPs have been characterized and studied, including defensins, mytilins, myticins, mytimycin, big defensins and mytimacins [6–13].

Among the large number of AMPs, defensin is one of the most ubiquitous families [1]. Defensins are a collection of small cationic peptides with molecular weights of approximately 3–5 kDa [14]. Generally, the animal defensin molecules can be classified into four major groups according to their structure and origin: α-defensin, β-defensin, θ-defensin and invertebrate defensin [15]. These defensins display similar structural features: the presence of a signal peptide at the N-terminal region, followed by the mature peptide region which is characterized by 6–8 conserved cysteine residues forming three or four disulfide bonds, and a C-terminal extension rich in anionic residues [16]. Defensins have been found to be widely distributed in marine invertebrate animals, especially in mollusks. Presently, multiple defensin molecules have been successively identified from mussels, oysters, clam and abalone [14,17–20]. It has been shown that defensins from marine mollusks are active against Gram-positive and Gram-negative bacteria and fungi, suggesting that they play important roles in innate immune response of mollusks [6,7,17,18,21,22].

Due to their direct interaction with altered/new pathogens, AMPs exhibit an extraordinary diversity in their structure and

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function [23,24]. Molecular diversity of AMPs such as myticin and defensin has been detected in marine mussels and oysters [8,25,26]. Recently, the defensin from freshwater pearl mussel *Hyriopsis cumingii* has also been shown to contain six isoforms [27]. Sequence diversification of AMPs by gene duplication has been reported for both vertebrates and invertebrates [28,29]. Moreover, an increasing number of studies suggested that the evolution of AMPs is driven by positive selection in both vertebrates and invertebrates [26,30–32].

Although the knowledge on mollusk defensins has been much reported, the information on evolutionary pattern of mollusk defensins is still very limited. In this study, four isoforms of defensins have been characterized from the Manila clam and their biochemical properties and structures have been predicted. Moreover, the evolutionary patterns of these defensins from Manila clam and other mollusks have also been discussed.

2. Materials and methods

2.1. Animal culture and challenge

For mRNA polymorphism characterization, the clams *Ruditapes philippinarum* (shell length: ~3.0–4.0 cm) were purchased from culture farms at three different sites (Dalian, Qingdao and Hangzhou) (Fig. 1). The clams are acclimated for two week before commencement of the experiment. They were maintained in filtered seawater at 20–22 °C and 30‰ salinity throughout the whole experiment. Then sixty clams of three geographic origins (20 individuals for each location) were immersed with high density of live *Micrococcus luteus* and *Vibrio anguillarum* with a final concentration of 1×10^7 CFU mL^{-1} respectively. After 24 h of challenge, the hemocytes, digestive glands and gills of 45 individuals (15 individuals for each location) were sampled and stored in liquid nitrogen before use.

For bacterial challenge experiment, adult clams (shell-length: ~3.5–4.5 cm) were purchased from a local culturing farm (Yantai) and acclimatized for 7 days. Then the clams were exposed to *V. anguillarum* at a final concentration of 1×10^7 CFU mL^{-1}. At 12 h, 24 h and 48 h intervals following the challenge, the hemocytes of four individuals were sampled and stored in liquid nitrogen. Meanwhile, the hemocytes, gill, digestive gland, mantle and foot of four untreated clams were also sampled to determine the tissue–distribution profiles of Rpdefs.

2.2. Total RNA extraction and sequence amplification

Frozen tissues were pulverized under liquid nitrogen, and subjected to total RNA extraction using the TRIzol Reagent (Invitrogen, USA). The extracted RNA was then treated with RQ1 RNase-Free DNase (Promega, USA) to remove DNA contamination. Single-stranded cDNA was synthesized from the total RNA with M-MLV reverse transcriptase (Promega, USA).

The EST sequences from cDNA library constructed from Manila clam hemocytes (unpublished) were used to construct a blast

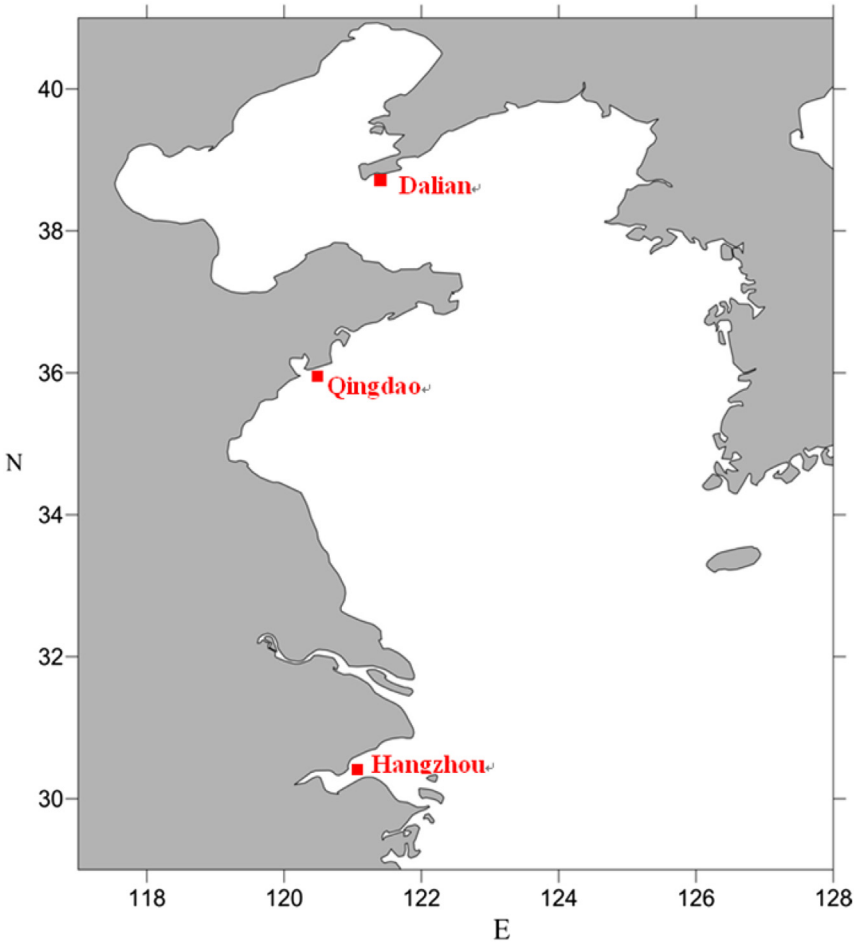

Fig. 1. The sampling sites of manila clam *Ruditapes philippinarum* along the coast of China.

Table 1
Primers used in the present study.

Primer	Sequence (5′-3′)	Sequence information
P1 (reverse)	TGGTGCTGTGATGAGTTCTAT′	5′ RACE primer
P2 (reverse)	TGCACCTCTGACGTAATGT	5′ RACE primer
P3 (forward)	GGTTTGGTTGCCCTGAAGATGA	3′ RACE primer
P4 (forward)	GAACTCATCACAGCACCAACA	3′ RACE primer
P5 (forward)	CAACAGGTTTAGCACTCAACGG	Polymorphism detection primer
P6 (reverse)	AAACTTGCTTGCGTGTTGGTGC	Polymorphism detection primer
P7 (forward)	TTGATGCCGGGTTTGGTTG	Real time primer for Rpdef1
P8 (reverse)	CAACCGTAACAAGTGCACCT	Real time primer for Rpdef1
P9 (forward)	CCGAAAATGGCTGCCCTAAT	Real time primer for Rpdef2
P10 (reverse)	GTTACACAGGCACGACAAGT	Real time primer for Rpdef2
P11 (forward)	AGGACGATGATTGCTTTTACTGT	Real time primer for Rpdef3
P12 (reverse)	CCCAGCAATCGTTACACCTG	Real time primer for Rpdef3
P13 (forward)	CGTTGATGGCTGTCGTGTAT	Real time primer for Rpdef4
P14 (reverse)	CGAGCAAGCGTAACACCTG	Real time primer for Rpdef4
P15 (forward)	CTCCCTTGAGAAGAGCTACGA	Real time primer for β-actin
P16 (reverse)	GATACCAGCAGATTCCATACCC	Real time primer for β-actin

database using the makeblastdb program available from the NCBI website. The oyster defensin sequence (GenBank accession no. CAJ19280) was used as a query sequence in a tblastn search (default parameters, version 2.2.28+) against this database to identify homologues within the database. Then a putative Manila defensin sequence was identified and subjected to blastx searches against the NCBI nucleotide database, to confirm its identity. To generate the full-length cDNA of *R. philippinarum* genes (Rpdefs), two reverse primers P1 and P2, and two forward primers P3 and P4 (Table 1), were designed based on the EST sequence. The nested PCR strategy was applied to the 3′ and 5′ RACE. For transcript polymorphism detection, two specific primers (P5 and P6, Table 1) designed in the 5′UTR and 3′UTR were employed to clone the full coding sequence of Rpdefs. The PCR profile and subsequent sequencing were conducted as described previously [33,34]. A total of 126 positive Rpdef clones were bi-directionally sequenced respectively.

2.3. Quantitative real-time PCR (qRT-PCR) assay

Quantitative real-time PCR (qRT-PCR) was carried out in an ABI 7500 Real-time Detection System by using the SYBR ExScript qPCR Kit (Takara, Japan) as described previously [33]. The PCR amplification was carried out in a total volume of 50 μL, containing 25 μL of 2 × SYBR Green PCR Master Mix, 20 μL of the diluted cDNA, 1 μL of each of primers (10 μmol/L), and 3 μL of DEPC-treated water. The thermal profile for qPCR was 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. Dissociation curve analysis of amplicons was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The expressions of Rpdefs were analyzed using the $2^{-\Delta\Delta CT}$ method with β-actin gene as the internal control. The primers used to quantify the expression of Rpdefs were listed in Table 1.

2.4. Sequence analysis, structure prediction and phylogenetic analysis

The searches for nucleotide and protein sequence similarities were performed with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). The deduced protein sequences were analyzed with ExPASy (http://www.expasy.org/). Signal peptide was predicted by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Prediction of putative disulfide bonds was performed using Scratch Protein Predictor (http://scratch.proteomics.ics.uci.edu/), DISULFIND web-server (http://cassandra.dsi.unifi.it/) and DiANNA web server (http://clavius.bc.edu/~clotelab/DiANNA/). The 3D structure of defensins was predicted with Phyre 2 server (Protein Homology/analogY Recognition Engine V 2.0), and visualized using the PyMOL software (DeLano, The PyMOL Molecular Graphics System, 2002, http://www.pymol.org). Multiple alignments were performed with the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A maximum likelihood (ML) phylogenetic tree based on the nucleotide sequences of mollusk defensins was constructed using PhyML 3.0 [35]. For phylogenetic analyses, the optimum evolutionary models were selected using the jModelTest program [36]. For ML analysis, 100 bootstraps were used to estimate the node reliability.

2.5. Testing for positive selection

The nucleotide sequences encoding amino acids of Rpdefs were used to construct a Maximum likelihood (ML) tree using appropriate nucleotide substitution model. The reliability of interior branches of each phylogeny was assessed with 1000 bootstraps. The phylogeny was used to estimate nonsynonymous to synonymous substitution rate ratio (ω = dN/dS) by the maximum likelihood (ML) method implemented in CODEML program of the PAML 4.4 software package [37]. Positive selection can be inferred from a higher proportion of nonsynonymous than synonymous substitutions per site (dN/dS > 1). Likelihood ratio tests (LRTs) were used to determine whether any codon positions were subjected to positive selection as indicated by ω > 1.

To test for heterogeneous selective pressure at amino acid sites, the site-specific models were tested: M0 (one-ratio) against M3 (discrete), M1a (nearly neutral) against M2a (positive selection), M7 (beta) against M8 (beta & ω). The assumption and parameters of each model were as describe previously [33]. The branch model was also conducted using the likelihood ratio test between the one-ratio model and the free-ratio model results to detect positive selection acting on particular lineages of the phylogenetic tree. The ω value in the one-ratio model was fixed whereas the value in the free-ratio model was estimated. The LRTs between nested models were conducted by comparing twice the difference of the log-likelihood values (2ΔL) between two models with the χ^2 distribution. The Naive Empirical Bayes (NEB) method and Bayes empirical Bayes (BEB) method were used to calculate the posterior probability that each codon was from the site class of positive selection under models M3, M2a and M8 respectively [38].

2.6. Statistical analysis

SPSS 16.0 software (SPSS Inc., USA) was used for statistical

analysis. All data were given in terms of relative mRNA expression as means ± SE (n = 4). One-way analysis of variance (ANOVA) was performed on all data and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Sequence analysis of Rpdefs

The 126 cDNA sequences of Rpdefs were deposited in GenBank under accession no. JX096678-JX096804. These sequences coded for defensins that fell in four defensin categories, which were named as Rpdef1, Rpdef2, Rpdef3 and Rpdef4, respectively. The complete coding sequence of Rpdef1, Rpdef2, Rpdef3 and Rpdef4 was of 219 bp, 210 bp, 207 bp and 195 bp in length, which encoded a polypeptide of 72, 69, 68 and 64 amino acids, respectively. The putative signal peptide of Rpdef1, Rpdef2 and Rpdef3 was identified at the N-terminal sequence with the first 24 amino acids, while the putative signal peptide of Rpdef4 comprised of the first 21 residues. The mature peptide of Rpdef1, Rpdef2, Rpdef3 and Rpdef4 consisted of 49, 46, 45 and 42 amino acids, which had a theoretical isoelectric point (*p*I) of 6.86, 8.23, 8.50 and 8.73 with an increasing predicted net charge of 0, +2, +3 and +4. The amino acid alignments indicated that Rpdef1 shared 46.9% identity with Rpdef2, 40.8% with Rpdef3, and 34.7% with Rpdef4. The transcript polymorphisms of these defensins have been shown in Supplemental figures.

3.2. Structure prediction

All of the Rpdefs have eight cysteine residues, which are predicted to form four disulfide bonds. However, the linkage patterns of the disulfide bridges predicted by different servers were not the same. Three-dimensional structure of Rpdef1 and Rpdef3 was predicted based on the template of *Crassostrea gigas* defensin (PDB ID: 2B68) with confidence = 99.9% (coverage = 86%) and confidence = 92.1% (coverage = 84%), respectively. However, 3-D structure of Rpdef2 and Rpdef4 was predicted based on the template of actinomycin (PDB ID: 2RU0) and scorpion toxin (PDB ID: 1SXM), with confidence = 63.3% (coverage = 57%) and confidence = 83.0% (coverage = 55%), respectively. All the predicted 3-D structures of Rpdefs possessed characteristic features of defensins, which comprised of one α-helix and two antiparallel β-sheets (Fig. 2). Rpdef1 and Rpdef3 had similar speculated 3-D structures as *C. gigas* defensin, while Rpdef2 and Rpdef4 displayed unique 3-D structure respectively.

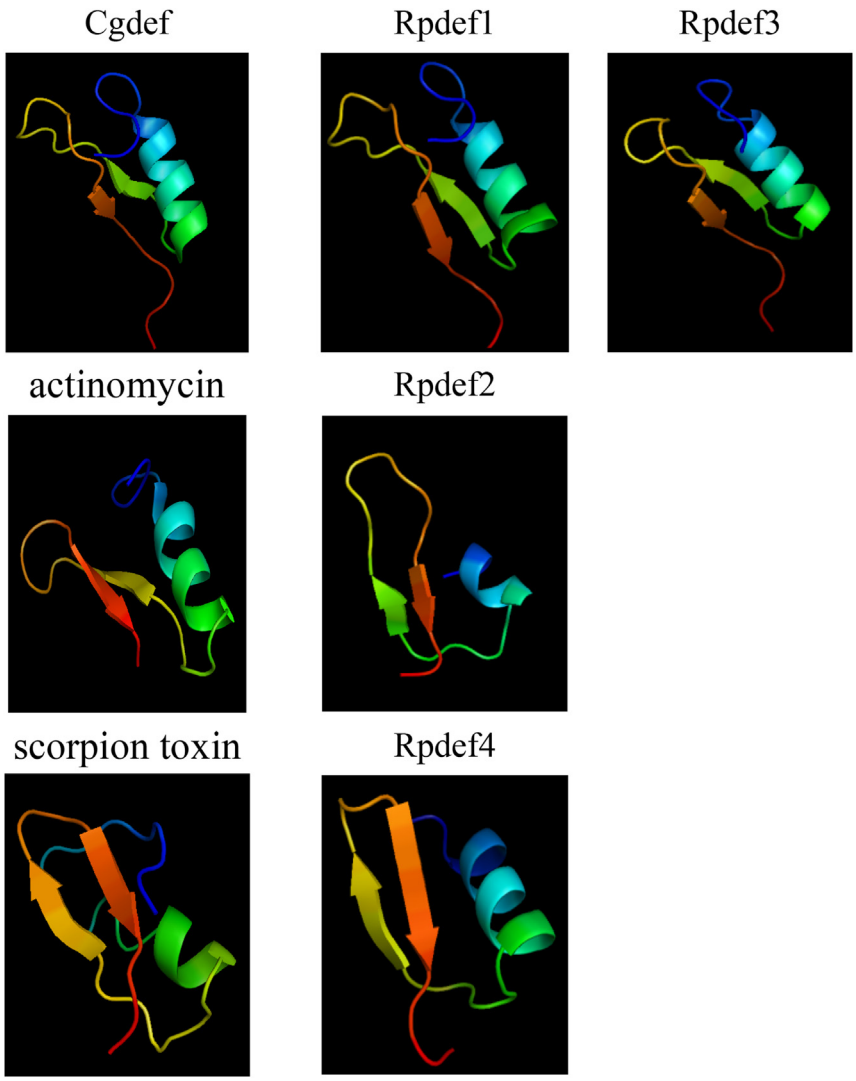

Fig. 2. Three-dimensional structures of Rpdefs and their counterparts. Cgdef (PDB ID: 2B68), actinomycin (PDB ID: 2RU0), scorpion toxin (PDB ID: 1SXM).

Table 2
Sequences used for multiple alignments and phylogenetic analysis.

Peptide	Species	MW Da	*p*I	Accession numbers
MGD1	*Mytilusgalloprovincialis*	4091	9.03	P80571
MGD2	*Mytilusgalloprovincialis*	4126	8.81	AAD52660
MedefA	*Mytilusedulis*	4151	9.15	P81610
MedefB	*Mytilusedulis*	4271	9.18	P81611
Cgdef	*Crassostreagigas*	4642	8.73	CAJ19280
Cgdefh1	*Crassostreagigas*	4763	8.50	ABD66301
Cgdefh2	*Crassostreagigas*	4677	8.51	ABD66302
Cvdef	*Crassostreavirginica*	4265	9.18	P85008
Hsdef	*Hyriopsisschlegelii*	4877	7.78	AEJ86348
Hssdef	*Haliotis discus discus*	4902	7.85	ACZ15982
Dpdef	*Dreissenapolymorpha*	5684	6.27	ACZ02692
RpdefB	*Ruditapesphilippinarum*	5287	7.79	AEK78067
MCdef	*Ruditapesphilippinarum*	4975	8.71	Adhya et al., 2012
Rpdef1	*Ruditapesphilippinarum*	5435	6.86	AFP50047
Rpdef2	*Ruditapesphilippinarum*	5430	8.23	AFP49990
Rpdef3	*Ruditapesphilippinarum*	5330	8.50	AFP49946
Rpdef4	*Ruditapesphilippinarum*	4749	8.72	AFP49977
Hcdef1	*Hyriopsiscumingii*	4264	8.50	Ren et al., 2011
Hcdef2	*Hyriopsiscumingii*	4275	8.51	Ren et al., 2011
Hcdef3	*Hyriopsiscumingii*	4906	8.32	Ren et al., 2011
Hcdef4	*Hyriopsiscumingii*	4880	8.66	Ren et al., 2011
Hcdef5	*Hyriopsiscumingii*	7112	8.48	Ren et al., 2011
Hcdef6	*Hyriopsiscumingii*	4602	8.50	Ren et al., 2011

3.3. Multiple alignment and phylogenetic relationships

The sequences used for multiple alignments and phylogenetic analysis were shown in Table 2. Multiple alignments indicated that eight cysteine residues and C-terminal motif (-RRSIQ-) were highly conserved in Rpdefs (Fig. 3a), whereas only four cysteine residues were conserved in mollusk defensins (Fig. 3b). Rpdefs had eight cysteine residues like defensins from the Manila clam (McDef), pacific oyster (Cgdef, Cgdefh1 and Cgdefh2), Mediterranean mussel (MGD1, MGD2) and triangle-shell pearl mussel (Hcdef5, Hcdef6), while some mollusk defensins contain a pattern of six conserved cysteine residues (Fig. 3b).

The phylogeny tree was generated using the models GTR+G for mollusk defensins determined by the Akaike information criterion. Phylogenetic analysis showed that Rpdef2 and Rpdef3 first clustered together, then segregated with Rpdef1, Rpdef4 and McDef successively and formed as a subclade (Fig. 4). The subclade of defensins from Manila clam first rooted with defensins from fresh water mussels *Hyriopsis schlegelii* and *Hyriopsis cumingii*, further clustered with defensins from oysters and mussels and formed a clade with RpdefB (a defensin deposited in the Genbank database) from the Manila clam. At last, these defensins grouped with defensins from the abalone and zebra mussel. These results indicated that RpdefB diverged from Rpdefs and McDef before the divergence of Manila clam with pacific oyster and Mediterranean mussel.

3.4. mRNA polymorphism and diversity of Rpdefs

A total of 126 clones were sequenced and virtually translated into amino acid sequences. Rpdef3 accounted for 60% frequency of occurrence of the clones, with Rpdef1, Rpdef2 and Rpdef4 accounting for 17%, 13% and 10% of the clones (Table 3). Multiple alignments indicated that Rpdef1 had 8 different kinds of amino acid sequences of the 22 sequences, as for Rpdef2 with 4 out of 17 sequences, Rpdef3 with 16 out of 75 sequences and Rpdef4 with 5 out of 12 sequences. The above results suggested that the transcript variation of Rpdef1 and Rpdef4 was larger than that of Rpdef2 and Rpdef3. The phylogeny trees were also constructed to compare the sequence variance between these four Rpdef genes with PhyML 3.0. The models employed for phylogeny analyses (HKY+G for Rpdef1, HKY+G+I for Rpdef2, GTR for Rpdef3, and HKY+I for Rpdef4) were determined by the Akaike information criterion. The phylogeny analysis indicated that the Rpdef1 sequences are more divergent than those of Rpdef2, Rpdef3 and Rpdef4 (Fig. 5).

With regard to clams from Dalian, frequencies of occurrence were extremely different, with Rpdef3 accounting for 82% of the

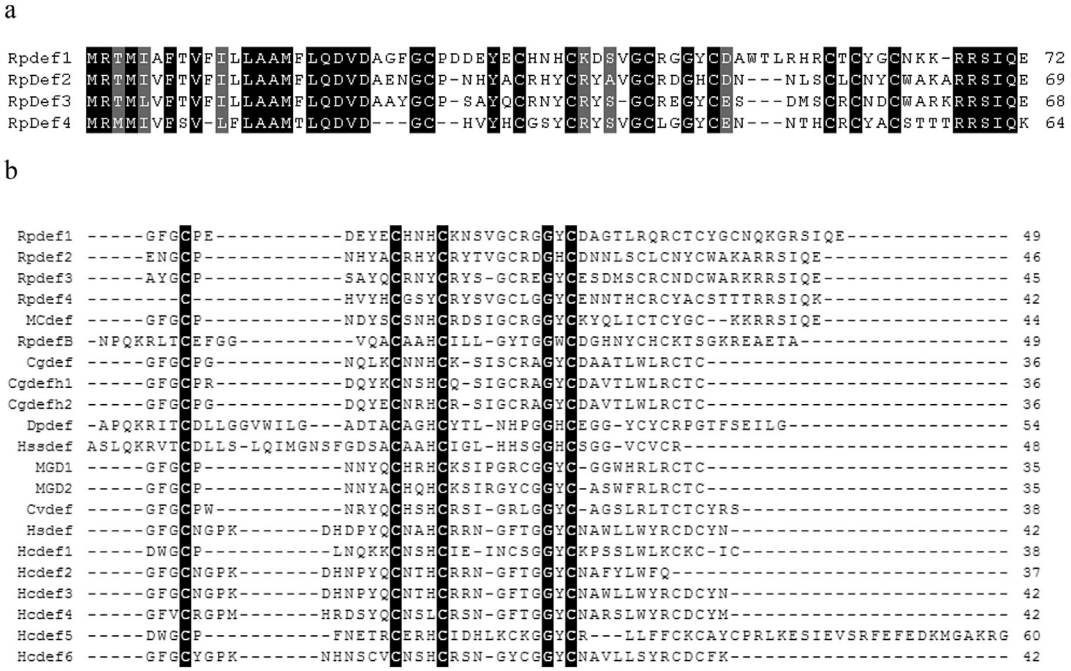

Fig. 3. (a) Multiple alignments of Rpdef1 with Rpdef2, Rpdef3 and Rpdef4. (b) Multiple alignments of Rpdefs with other mollusk defensins deposited in GenBank. The black shadow region indicates positions where all sequences share the same amino acid residue. Gaps are indicated by dashes to improve the alignment. The GenBank accession numbers and the species are shown in Table 2.

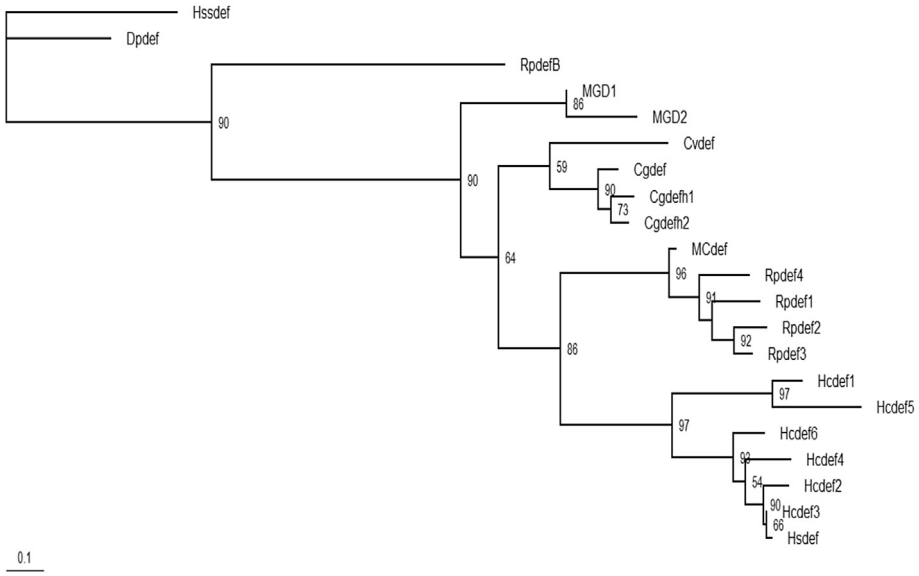

Fig. 4. Phylogenetic tree constructed by maximum likelihood method based on the nucleotide sequences of defensins from mollusks. Numbers at the forks indicate the bootstrap values (in %) out of 100 replicates. The sequences used to construct phylogeny trees of defensins are shown in Table 2.

clones and Rpdef4 present in only one clone. Other coding sequences have been found in 3–5 clones. However, as for clams from Qingdao and Hangzhou, Rpdef3 accounted for 43% and 48% of the clones; Rpdef2 and Rpdef4 showed the least frequency of occurrence (Table 3).

As concerned to tissue distribution of the different Rpdef transcripts, 52 clones were present in gills, whereas 36 and 38 clones were present in hemocytes and digestive gland respectively. As for Rpdef1, 50% clones were present in hemocytes, while for Rpdef2 and Rpdef3, the clones were almost averagely present in three tissues. The clones of Rpdef4 were predominantly present in gills and digestive gland (Table 3).

3.5. Tissue-specific expressions profiles of Rpdefs mRNAs

The tissue distribution of Repdef mRNAs was investigated by qRT-PCR with β-actin as internal control. During the qRT-PCR assays, only one peak was detected at the corresponding melting temperature in the dissociation curve analysis, suggesting that the PCR was specifically amplified. The transcripts of Rpdefs were detected in all the tissues examined, including gills, digestive gland, hemocytes, mantle and foot. The transcripts of Rpdefs with the exception of Rpdef4 were dominantly expressed in hemocytes, moderately expressed in gills, mantle and foot (Fig. 6A, B, C), and least detected in digestive gland. However, the expression level of Rpdef4 mRNA was high in gills and hemocytes, moderate in mantle and digestive gland, and least in foot (Fig. 6D).

Table 3
Number of clones of Rpdefs in different location and different tissues.

Genes	Number of clones					
	Location			Tissue		
	Dalian	Qingdao	Hangzhou	Hemcoytes	Gill	Digestive gland
Rpdef1	5	11	6	11	8	3
Rpdef2	3	6	8	6	6	5
Rpdef3	40	20	15	18	32	25
Rpdef4	1	9	2	1	6	5

3.6. Temporal expression profiles of Repdefs mRNAs in hemocytes post bacterial challenge

Following bacterial challenge, the expression levels of all Rpdefs in hemocytes (Fig. 7) increased significantly at 12 h post challenge ($P < 0.05$). For Rpdef1, the expression level returned to the original level at 24 h and 48 h post challenge (Fig. 7A). However, the transcripts of Rpdef2 and Rpdef3 were significantly inhibited at 24 h and 48 h following challenge ($P < 0.05$) (Fig. 7B, C). The expression level of Rpdef4 was significantly up-regulated at 12 h and 24 h, and down-regulated at 48 h post challenge ($P < 0.05$) (Fig. 7D).

3.7. Evolutionary analysis of Rpdefs and other mollusk defensin genes

Phylogeny-based codon substitution models were used to identify the codons under positive selection. For all Rpdefs, the M0–M3 comparison revealed that M3 was not a better fit to the data than M0 ($2\Delta l = 130$; $P > 0.05$). The comparison of null models (M1a and M7) with their corresponding alternative models (M2a and M8) rejected the null models (Table 4), thus indicated that the variants of Rpdefs have evolved under positive selection with the positively selected sites R^{63} and R^{64} falling in the C-terminus. For Rpdef1, Rpdef2 and Rpdef4, the comparison of null models with their corresponding alternative models agreed the null models ($P > 0.05$), suggesting no amino acid residue under positive selection. However, the amino acid residues R^{63}, R^{64}, S^{65}, I^{66} and Q^{67} in Rpdef3 were detected under positive selection (Table 5).

The M1–M0 comparison revealed that model M1 was better fit to the data than M0 ($P < 0.05$), indicating the ω ratios were different among lineages of the phylogeny tree. The lineage-specific selection test showed that the ω values along most examined lineages (28 out of 39) were less than 1, whereas there were also some branches and internal branches with high ω values (Table 6). As shown in Fig. 6, the Rpdef3 lineage and Rpdefs branch (formed by Rpdef1, Rpdef2, Rpdef3 and Rpdef4) had ω ratios >1, indicating Rpdef3 lineage and Rpdefs branch undergo positive selection. In the clade of oysters, the ω values for the Cgdefh2 lineage and Cgdefs branch (formed with Cgdefh1 and Cgdefh2) were more than 1. There were also three lineages with

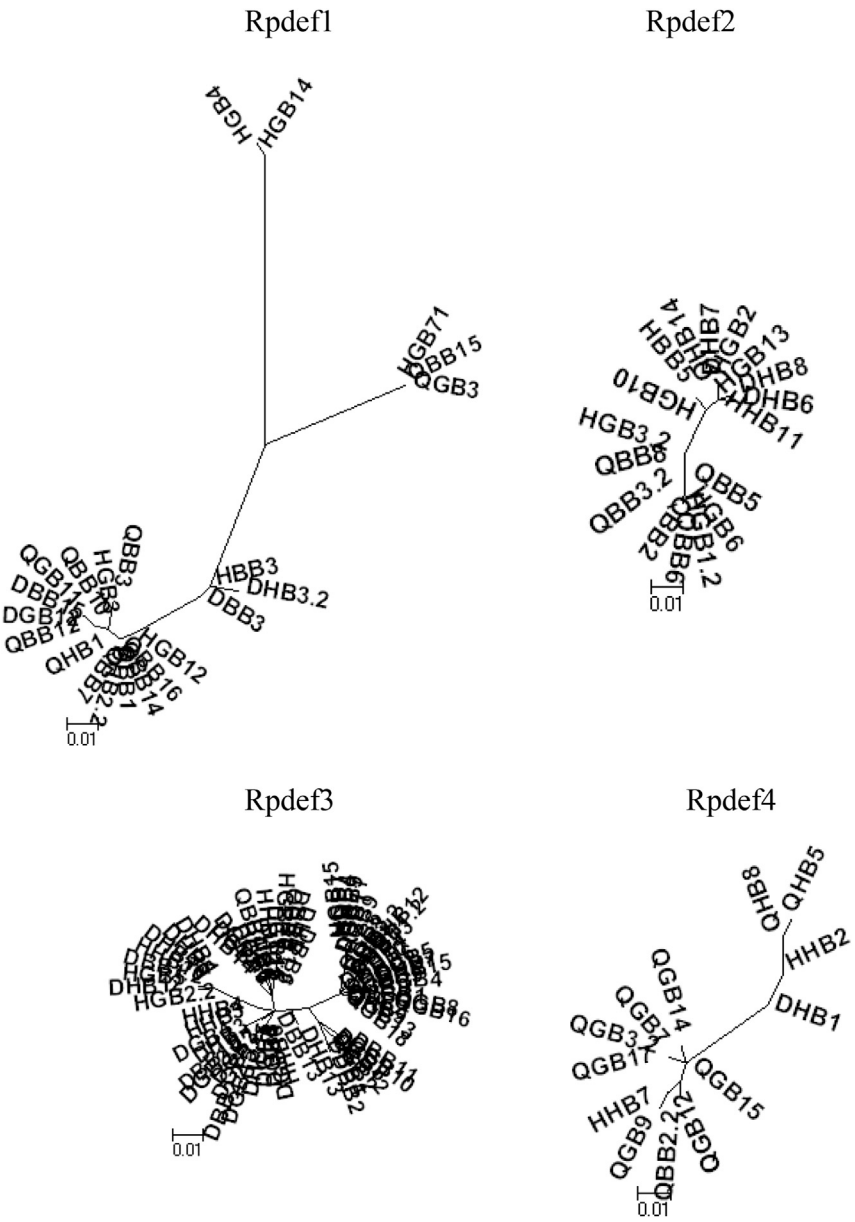

Fig. 5. Phylogenetic tree constructed by maximum likelihood method based on the nucleotide sequences of Rpdefs.

very high ω ratios in the clade of fresh water pearl mussel, implying positive selection on these lineages. However, the ω ratio value for the Mediterranean mussel branch was <1, implying no positive selection on this branch. The Manila clam branch (without RpdefB) had high ω ratios >1, revealing that this branch is under positive selection pressure during evolution (Fig. 8). In addition, there were also some clades from different taxon exhibited high ω ratios. The site-specific models were used to test for heterogeneous selective pressure at amino acid sites. The M1a–M2a comparison revealed that M2a was better fit to the data ($P < 0.01$). LRTs also gave significantly better results for M8 ($P < 0.01$). Under the M2a model, 23 amino acids within the mature peptide regions are under positive selection, while 17 amino acid residues are positively selected under the M8 model. We considered a site under positive selection if the BEB posterior probability is > 0.95. Under M2a and M8 model, 14 and 6 positively selected sites were detected with BEB posterior probability >0.95 respectively (Table 6).

4. Discussion

Marine mollusks account for a large quantity of current global aquaculture output. With the rapid development of intensive mariculture, some cultured species have been seriously affected by diseases and mortalities in recent years [39,40]. Therefore, basic knowledge on the innate immunity of commercially important mollusks is urgently needed, especially characterization of the immune-associated molecules and their functions. AMPs constitute an important first-line defense of the immune system in mollusks. Among these naturally occurring antibiotic peptides, defensins form a unique family of cysteine-rich cationic and structured polypeptides, serving as effector molecules of innate immunity [41]. Although Mollusca are the largest and most diverse phylum of animals next to arthropods, AMPs such as defensins have been characterized only in a few farmed species, such as oysters, mussels and abalones. Therefore, there is still a great potential to unveil new AMP molecules in this phylum [20].

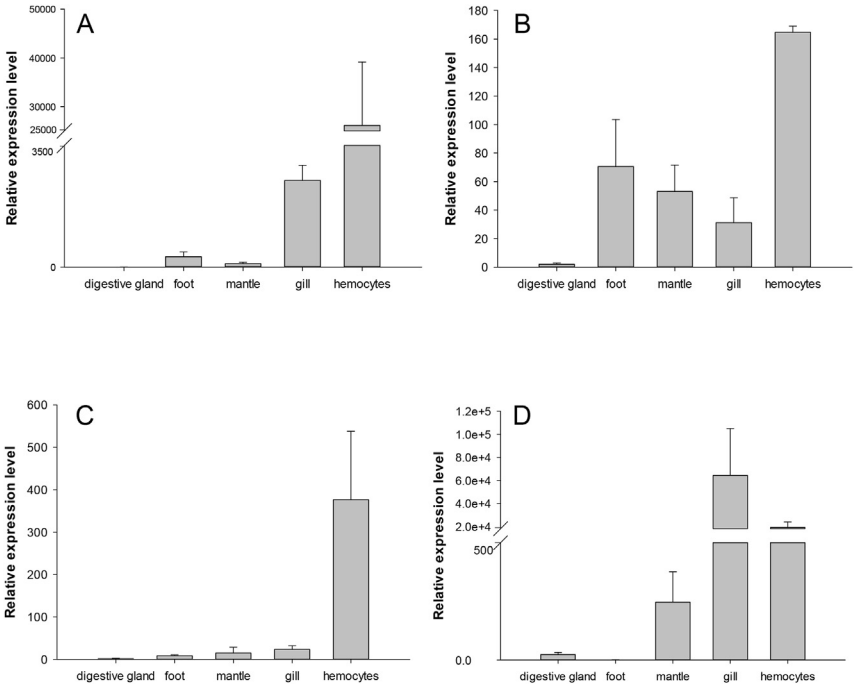

Fig. 6. Tissue-specific expression profiles of Rpdefs mRNAs measured by qRT-PCR. The mRNA expression level is calculated relative to β-actin expression. Each symbol and vertical bar represents the mean ± SE (n = 4). A-Rpdef1, B-Rpdef2, C-Rpdef3, D-Rpdef4.

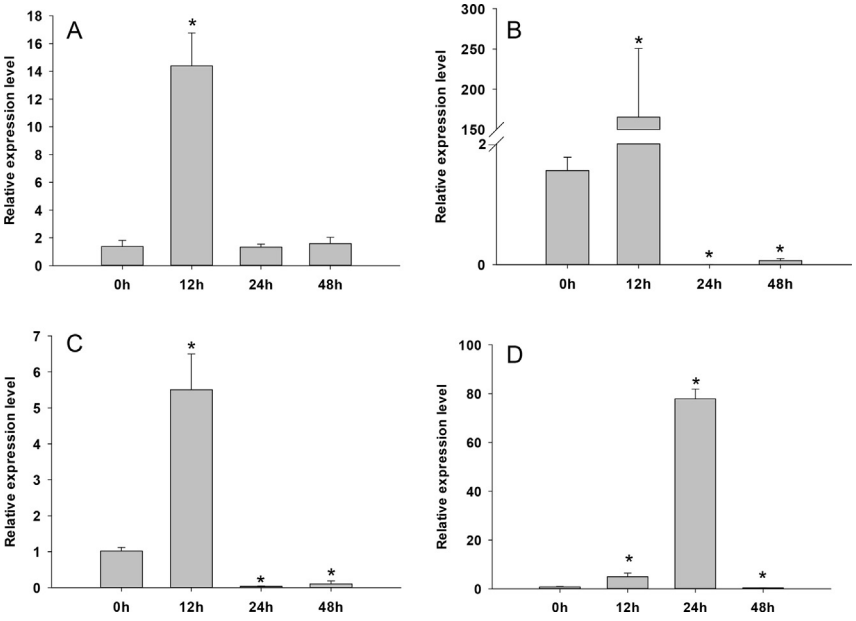

Fig. 7. Temporal expression profiles of Rpdefs (A-Rpdef1, B-Rpdef2, C-Rpdef3, D-Rpdef4) in hemocytes post *V. anguillarum* challenge. The mRNA expression level is calculated relative to β-actin expression. Each symbol and vertical bar represents the mean ± SE (n = 4). Significant difference from control is indicated with an asterisk at $P < 0.05$.

Table 4
Comparison among different nested models to test for positive selection among codons of all Rpdef sequences.

Model	lnL	Estimates of parameters	2ΔI	Positively selected sites
M1a (nearly neutral)	−1702	P0 = 0.25618, (p1 = 0.74382)	46	Not allowed
M2a (positively selection)	−1679	p0 = 0.22939, p1 = 0.73902, (p2 = 0.03159), ω0 = 0.08282, (ω1 = 1), ω2 = 14.61681	$P < 0.01$	63R*, 64R*
M7 (beta)	−1703	p = 0.43916, q = 0.33694	46	Not allowed
M8 (beta & w > 1)	−1680	p0 = 0.96839, (p1 = 0.03161), p = 0.14181, q = 0.04183, ωs = 14.21207	$P < 0.01$	63R*, 64R*

Table 5
Comparison among different nested models to test for positive selection among codons of Rpdef1, Rpdef2, Rpdef3 and Rpdef4.

	Model	lnL	Estimates of parameters	2ΔI	Positively selected sites
Rpdef1	M1a (nearly neutral)	−700.29	p0 = 0.25658, (p1 = 0.74342)	4.62	Not allowed
	M2a (positively selection)	−697.98	p0 = 0.08753, p1 = 0.69312, (p2 = 0.21935), ω0 = 0.00000, (ω1 = 1), ω2 = 7.59516	$P > 0.05$	4M, 10F, 28P, 29D, 32Y, 40D, 50D, 51A, 52W, 53T, 55R, 56H, 65K, 67R
	M7 (beta)	−700.34	p = 0.14734, q = 0.04084	4.82	Not allowed
	M8 (beta & w > 1)	−697.93	p0 = 0.78255, (p1 = 0.21745), p = 0.10482, q = 0.02625, ωs = 6.67735	$P > 0.05$	4M, 10F, 28P, 29D*, 30D, 32Y, 40D, 50D, 51A, 52W, 53T, 55R, 56H, 65K, 67R
Rpdef2	M1a (nearly neutral)	−319.19	p0 = 0.00001, (p1 = 0.99999)	5.00	Not allowed
	M2a (positively selection)	−316.69	p0 = 0.00000, p1 = 0.67091, (p2 = 0.32909), ω0 = 1.00000, (ω1 = 1), ω2 = 999.00000	$P > 0.05$	all amino acids
	M7 (beta)	−319.19	p = 2.02546, q = 0.00500	5.00	Not allowed
	M8 (beta & w > 1)	−316.69	p0 = 0.66973, (p1 = 0.3302), p = 0.00500, q = 1.57842, ωs = 999.00000	$P > 0.05$	all amino acids
Rpdef3	M1a (nearly neutral)	−733.46	p0 = 0.78832, (p1 = 0.21168)	63.74	Not allowed
	M2a (positively selection)	−701.59	p0 = 0.56707, p1 = 0.35845, (p2 = 0.07449), ω0 = 0.07666, (ω1 = 1), ω2 = 21.89229	$P < 0.01$	63R*, 64R*, 65S*, 66I*, 67Q*
	M7 (beta)	−734.99	p = 0.05393, q = 0.06892	66.66	Not allowed
	M8 (beta & w > 1)	−701.69	p0 = 0.92554, (p1 = 0.07446), p = 0.06396, q = 0.07578, ωs = 20.39602	$P < 0.01$	63R*, 64R*, 65S*, 66I*, 67Q*
Rpdef4	M1a (nearly neutral)	−320.09	p0 = 0.00001, (p1 = 0.99999)	0.50	Not allowed
	M2a (positively selection)	−319.84	p0 = 0.40603, p1 = 0.00000, (p2 = 0.59397), ω0 = 0.00000, (ω1 = 1), ω2 = 2.45542	$P > 0.05$	14A, 19D, 46N
	M7 (beta)	−320.09	p = 1.18224, q = 0.00500	0.50	Not allowed
	M8 (beta & w > 1)	−319.84	p0 = 0.40603, (p1 = 0.59397), p = 0.00845, q = 1.78436, ωs = 2.45543	$P > 0.05$	14A, 19D, 24H, 26Y, 27H, 34Y, 42Y, 46N

Table 6
Parameter estimates and log-likelihood values under different models of variable ω ratios among sites. Site numbers and amino acids refer to the Rpdef1 sequence.

Model	Model code	lnL	Estimates of parameters	2Δ*l*	*P* Value	Positively selected sites
Branch model	One ratio model	−3427.1	ω = 0.39891	57.2 (df = 38)	$P < 0.05$	NA
	Free-ratio model	−3398.5	ω estimated independently for each branch (see Fig. 4)			
Site model	M1a	−3426.9	P0 = 0.01754 (p1 = 0.98246)	51.2	$P < 0.01$	Not allowed
	M2a	−3401.3	p0 = 0.01756, p1 = 0.63873, (p2 = 0.34371), ω0 = 0.00000, (ω1 = 1), ω2 = 4.82950			28P*, 31E*, 32Y**, 33E, 34C, 35H**, 37H, 38C*, 40D**, 41S, 43G, 44C, 47G, 48Y**, 49C*, 50D, 51A**, 52W*, 53T**, 54L**, 55R**,56H**, 57R
	M7	−3363.9	p = 0.06961, q = 0.09195	126	$P < 0.01$	Not allowed
	M8	−3426.9	p0 = 0.01754, p = 0.00500, q = 0.00500, (p1 = 0.00500), w = 1.00000			28P, 31E, 32Y**, 33E, 35H**, 38C, 40D*, 43G, 48Y, 49C, 50D, 51A**, 52W, 53T, 54L** 55R,56H**

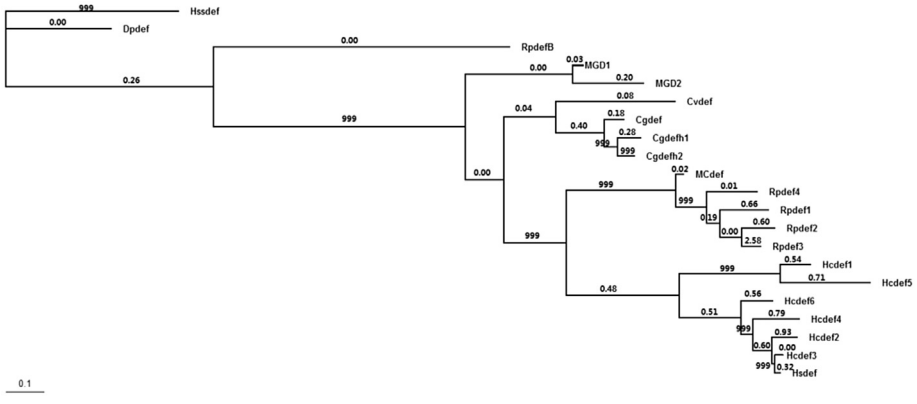

Fig. 8. Positive selection analyses of defensins across the mollusk phylogeny. The numbers shown along each branch are the dN/dS values for the entire gene along that branch.

In this study, we have characterized four types of defensins from the commercially important Manila clam. Presently, six defensin isoforms including McDef and RpdefB (deposited in the Genbank database) have been isolated from this species. The amino acid alignments indicated that Rpdef1 shared 65.3% with McDef, 46.9% identity with Rpdef2, 40.8% with Rpdef3, and 34.7% with Rpdef4, whereas it had only 18.4% identity with RpdefB. Like other mollusk defensins, all of these defensins from Manila clam with the exception of Rpdef1 had a net positive charge, and their theoretical *p*Is were more than 8.0 (Table 2). However, Rpdef1 and defensin from *Dreissena*

polymorpha (Dpdef) had a theoretical *p*I of less than 7, and possessed a net charge of 0 and −1, respectively. Although the vast majority of AMPs are cationic in nature, a significant number of anionic AMPs have also been reported. As for the non-cationic defensins, perhaps they can dock to the bacterial membrane via the positively charged and hydrophobic loop near their C-terminus [42].

Rpdef3 sequences accounted for the most percentage of the Rpdefs sequences, suggesting this defensin might play a more important role than other Rpdefs in this clam. The occurrence frequencies of different Rpdefs varied in clams from different geographical locations. The transcript variation was perhaps related to pathogen load in clams and other possible environmental factors of different sites, which may have forced the defensin genes to diversify [43]. The transcript sequences of Rpdef2 and Rpdef3 were almost averagely present in three tissues, while the transcript sequences of Rpdef1 were mainly present in hemocytes and Rpdef4 were mainly detected in gills and digestive gland. These above results indicated different Rpdefs perhaps played different immune functions in specific tissues. In addition, multiple alignment and phylogeny analysis showed that Rpdef1 had a higher diversity than other Rpdefs, while Rpdef3 had the lowest diversity. The divergence of Rpdef1 was likely due to purifying selection acting in a long term manner, while the polymorphism reduction of Rpdef3 was probably caused by a recent selective pressure [26], which has been detected by evolutionary analysis in the present study.

In this study, the constitutive expression of all Rpdefs was mainly detected in hemocytes, which are primarily responsible for the defense against pathogens in bivalves. Similarly, it has been reported that oyster defensin (Cgdefh2) and mussel defensins (MGD1 and MGD2) were abundantly expressed in hemocytes [14,16,21]. As for Rpdef4, the highest expression was found in gills, which is continuously exposed to environmental stress factors such as toxic substances and pathogens. These types of tissue–distribution profiles suggested these defensins could respond promptly to bacterial challenge. However, it was reported that MCdef transcript was expressed at the highest level in adductor muscle of the Manila clam [32]; and the abalone defensin transcript was highly expressed in the mantle and hepatopancreas [19]. Thus, these results demonstrated that these defensins may play different immune roles in different tissues of these bivalves.

In order to further understand the possible biological functions of the Rpdefs, their mRNA expression patterns post bacterial challenge were examined at different time intervals in hemocytes. The expression of all Rpdefs was up-regulated remarkably after 12 h bacterial challenge compared to that of the control, indicating that the transcripts of Rpdefs were induced to fight against the bacteria. The up-regulation of defensin transcripts was also observed in disk abalone [19], Manila clam (MCdef) [32] and freshwater pearl mussel *Hyriopsis schlegelii* [44]. Then the expression levels of all Rpdefs except Rpdef4 returned to original level or were inhibited at 24 h and 48 h post challenge. The possible reason of down-regulation was that there was no need to produce large amount of defensins with the progressive clearance of the invasive bacteria. In fact, the expression of oyster defensin (Cgdefh2) and mussel defensin (MGD2) was also found to be significantly down-regulated in hemocytes after bacterial infection [14,16]. Above all, the transcriptional up-regulation of Rpdefs against bacterial challenge as well as their highly constitutive expression in hemocytes indicated that they may play important roles in innate immunity.

It has been shown that genes involved in the immune system of various animals typically show a faster rate of amino acid substitutions and have evolved under positive selection [45,46]. Due to their direct interaction with altered/new pathogens, AMPs exhibit an extraordinary diversity in their biochemical and biological functions. It has been reported that positive Darwinian selection was the major driving force in the generation of diverse AMPs [47].

Consistent with earlier studies on molecular evolution of several AMPs [25–31], our results suggested that all Rpdefs evolved through positive selection. Positively selected codons R^{63} and R^{64} had been detected in the C-terminal regions of all these Rpdefs. In addition, the amino acid residues S^{65}, I^{66} and Q^{67} in the C-terminal region of Rpdef3 were also detected under positive selection. Similarly, the majority of positively selected sites are located in the C-terminal region of myticin-C [25,48]. Based on the predicted tertiary structure of Rpdefs, the positively selected amino acids fall in the predicted C-terminal coils (data not shown), which are highly exposed at the surface of Rpdefs. Moreover, the positively charged R^{63} and R^{64} perhaps increase the binding ability of peptide to the bacteria. It had been demonstrated that positively charged amino acids on the surface of the defensin could greatly improve their antibacterial activity, probably by promoting a better binding to the cell wall or membrane of target bacteria [49,50]. Therefore, the positively selected amino acids might have a functional relevance by modifying the charge distribution of Rpdefs. Further investigation on the functional roles of C-terminal domain in defensins will be crucial to interpret these observations [48].

The results of the present study provide strong evidence that positive selection is the major driving force in generating high diversity in Rpdefs. Zhu et al. [47] also reported that many members of the CS-αβ superfamily exhibited molecular diversity and diverse biological functions, and suggested that positive Darwinian selection is the major driving force in generating such diversity. The direct involvement of these peptides with the altered pathogens in a changing environment is probably the cause of such adaptive molecular evolution of these AMPs [25].

5. Conclusions

We characterized the complete coding sequences and predicted the 3-D structures of four defensins from the Manila clam. Next, we studied the diversity of defensins from three geographical clam populations and found that Rpdef3 accounted for about 60% frequency of Rpdefs occurrence in these clams. The expression profiles post bacterial challenge suggested that Rpdefs were involved in the host defense in this clam. Furthermore, it was found that Rpdef3 and all Rpdefs were under positive selection with positively selected amino acid residues detected in the C-terminal regions, which perhaps have a functional relevance by modifying the charge distribution of Rpdefs. Therefore, positive selection could be the major driving force in generating high diversity of defensins in the Manila clam.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.09.008

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