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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

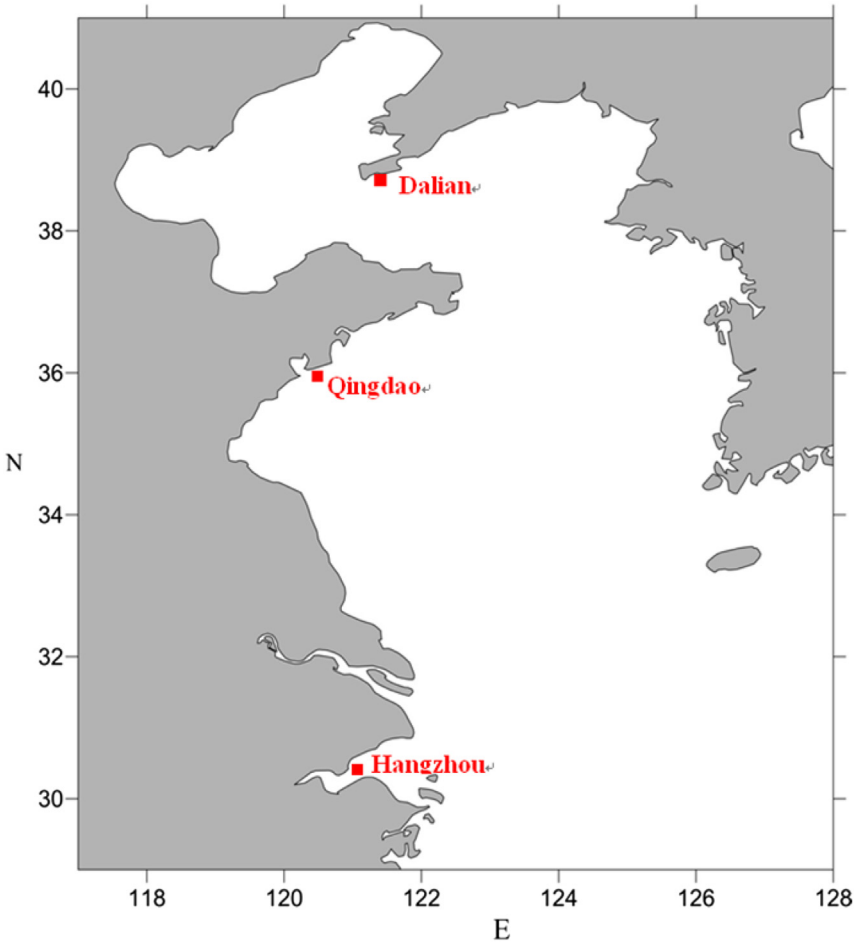

Fig. 1. The sampling sites of manila clam *Ruditapes philippinarum* along the coast of China.