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Characterize a typically Dscam with alternative splicing in mud crab *Scylla paramamosain*

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ABSTRACT

As a member of the immunoglobulin superfamily, Down syndrome cell adhesion molecule (Dscam) could function in the innate immunity of invertebrates. Recently, it is shown that arthropod Dscams play similar functions as antibodies in the adaptive immune system. Dscam could produce thousands of isoforms by alternative splicing and specifically bind to various pathogens. In the present study, we cloned the first Dscam from mud crab *Scylla paramamosain* (*Sp*Dscam), with full-length cDNA 7363 bp containing an open reading frame (ORF) of 6069bp and encoding 2022 amino acids, which had typical domain architecture as other arthropods, i.e., 10 immunoglobulin domains (Ig), 6 fibronectin type 3 domains (FN III), transmembrane and cytoplasmic tail. Quantitative real-time PCR revealed that *Sp*Dscam was highly expressed in brain, skin, muscle, intestine and hepatopancreas, but weakly expressed in hemolymph, heart and gill. *Sp*Dscam had three alternative splicing regions, located at the N-terminal of Ig2 and Ig3 as well as on the whole Ig7. In these regions, 32, 41 and 14 exons were detected, together with the two exon types of transmembrane domain, indicating *Sp*Dscam could potentially encode at least 36,736 unique isoforms. *Sp*Dscam induced by *Vibrio parahaemolyticus* challenge had strong binding ability to *V. parahaemolyticus*. Further, *Sp*Dscam induced by *V. parahaemolyticus* possessed a clearance of *V. parahaemolyticus* in *S. paramamosain*. Collectively, the results indicated *Sp*Dscam was a hypervariable pattern-recognition receptor (PRR) by alternative splicing in innate immunity system of mud crab *S. paramamosain*.

1. Introduction

It is generally believed that invertebrates lack an adaptive immune and mainly rely on the innate immunity for body defense. As an invertebrate, mud crab *Scylla paramamosain* have many physical barriers, such as chitinous exoskeleton and peritrophic membrane of digestive tract, as well as cellular and humoral immunity [1]. In recent years, there has been increasing information that invertebrates have specific immunity similar to adaptive immune response in vertebrates. Pattern recognition receptors (PRRs) which are a kind of immune factors in humoral immune, have been shown to exhibit specificity by several studies. In amphioxus which lacks an adaptive immunity, multigenic families encoding diversified immunoglobulin-like variable (V) domains were identified [2,3]. Similarly, fibrinogen-related proteins (FREP) in *Biomphalaria glabrata* are reported having nine ancestor sequences which generates at least 314 variant sequences through genetic recombination and mutation [4]. Furthermore, in order to recognize the pathogen-associated molecular patterns (PAMPs), the 185/333 family of *Sea urchins* could reconstruct the internal 23 elements to generate varied molecules which ranges from 0.16 kb to 1.5 kb [5].

Besides the molecular polymorphism of PRRs in studies above, some form of immune memory in invertebrates, namely “specific immune priming”, would provide the host protection from antigens after previously encountered, which was similar to that in vertebrates [6–8]. Scientists have demonstrated the existence of immunological specification and even memory in invertebrates, which was shown a stronger self-protection upon a second challenge [7,9,10]. Investigators were wondering how immune memory in invertebrates would be like, hence, the wide studies of Dscams in most invertebrate might shed some insights on a possible explanation about the immune priming.

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Dscam, which was first found in the human chromosome 21q22 was related to Down syndrome, and exhibited extraordinary variants through alternative splicing [11–13]. It has been revealed that the extracellular domain of Dscam from *Drosophila melanogaster* may in theory yield 38,000 isoforms by alternative splicing on the N-terminal halves of the Ig2, Ig3, the entire Ig7 and transmembrane domain [14,15]. In arthropods, studies have demonstrated that the diversity of Dscams played an essential role in specific recognition, with each kind of isoforms recognizing a specific pathogen, which was synanomous with the acquired immune system in vertebrates [16–18]. For instance, *Anopheles gambiae* Dscam (*Ag*Dscam) isoforms could specifically response against various pathogen challenges [19]. Dscam in two insect species, *D. melanogaster* and *Tribolium castaneum*, could play a delayed role in immunity and strongly influence behavior and fecundity [20]. When induced by bacteria, fungi and plasmodium, *A. gambiae* would produce variable Ig2-spliced isoforms [21]. The depletion of the *Ag*Dscam protein results in a greater increase in microbial infection and lower survival rate. *Ag*Dscam splice-form repertoires induced by bacteria would have a higher affinity to the bacteria and therefore provide a better self-protection to *A. gambiae*. When Dscam binds to the homologuos structure of pathogens, the selective pressure from the host-parasite interaction would act on Dscam to generate diversified isoforms by alternative splicing [14]. However, the individual diversification affected by Dscam differed from the immune receptors encoded by germline, which mainly came from somatic diversification [22]. By testing the genome SNPs of *Apis mellifera*, it was found that Dscam had a lower genomic differentiation rate, within only one intron balancing the selection [23]. Although many researches about Dscams in various animals have been done, no explanation has been offered about the mechanism of Dscams of the pathogen-specific binding.

Currently, studies about Dscam in arthropod are approached diversely. For example, various bacterial-induced Dscam isoforms and their roles played in the immune system are studied in the assay of gene silencing, bacterial binding assay and pathogen clearance [21,24–26]. On the other hand, there are many studies about the phenomenon of specific immune priming, including the rapidity and strength of Dscam responding to pathogens after previous encounter [27–29]. Although there are many researches on Dscam and its involvement in immune priming in invertebrates [25,30–32], the range of species employed is limited and the results can not be extended to all invertebrates.

Mud crab *S. paramamosain* is a commercially important crustacean species, which is widely distributed along the coast of southern China [33]. Based on our previous transcriptome database of *S. paramamosain* [34], we cloned the full-length cDNA of *Sp*Dscam. Furthermore, the hypervariable regions were identified and the potential isoforms were calculated. The binding and clearance ability of each pathogen-specific isoform was confirmed by various pathogenic bacteria. This study highlights the molecular diversity of Dscam and shows that it is widely found in arthropods. Most importantly, our data would help deepen our knowledge of the molecular mechanism of immune defense in *S. paramamosain,* which would ultimately provide a better insight for disease control in mud crab aquaculture.

2. Materials and methods

2.1. Experimental animals and immune challenges

Healthy mud crabs (approximately 100 g each) were bought from Niutianyang (Shantou, Guangdong, China), and acclimated in tanks maintained at 8‰ salinity and 25 °C for a week before they were used for experiments. Four healthy crabs were used to investigate the tissue distribution of *Sp*Dscam. For challenge experiments, 200 μL *V. parahemolyticus* suspension (1×10^7 *cfu* mL^{-1}), or 200 μL white spot syndrome virus (WSSV) suspension (containing 10^6 *pfu* mL^{-1}) were injected into the base of the fourth leg of each crab [35]. Other immune stimulants including LPS (0.5 mg mL^{-1}) and Poly I:C (1 mg mL^{-1}) were injected into mud crabs as previously described [36], 200 μL normal saline 0.8% NaCl (NS) was used as blank control. Each challenged group had eighteen crabs.

2.2. Sample collection and RNA isolation

For tissue distribution, eight tissues from each mud crab (including brain, hepatopancreas, hemolymph, gill, muscle, heart, mid-intestine and skin) were collected. For challenge experiments, the tissues were collected at six time points (i.e., 0, 6, 12, 24, 48, and 96 h after the immune challenges). All the tissue samples except hemolymph were stored in −80 °C for subsequent total RNA extraction.

Hemolymph was withdrawn using a syringe and mixed with an equal volume of anticoagulant (450 mM NaCl, 100 mM glucose, 2.6 mM citric acid, 30 mM trisodium citrate, pH 4.6), and then centrifuged at 800 × *g* at 4 °C for 20 min. Sediments were harvested and added in 1 mL TRIzol® Reagent (Ambion, USA) for subsequent RNA extraction.

RNA samples were extracted from all tissues by the TRIzol® Reagent and measured by a Nanodrop® ND-1000 spectrophotometer at the absorbance on 260 nm/280 nm (A260/A280) (LabTech, Holliston, MA), and the integrity was detected using 1% (w/v) agarose gel electrophoresis. The first-strand cDNA was synthesized using the PrimeScript RT reagent Kit according to the manufacturer's protocol (Takara, Dalian, China).

2.3. Cloning of full length SpDscam cDNA

Total RNA was extracted from hemocytes of the healthy mud crabs using TRIzol® Reagent. The cDNA of 5′RACE (Rapid Amplification of cDNA Ends, RACE) and 3′RACE were synthesized by BD SMART™ RACE cDNA Amplification Kit (Clontech) and M-MLV First-Strand cDNA Synthesis Kit (Invitrogen, USA), respectively. The primers were designed according to the partial sequence of *Sp*Dscam. The RACE PCR was carried out by touchdown PCR and nested PCR strategy according to the manufacturer's protocol. The PCR reaction volume contained 2.5 μL of the first strand cDNA as the temple, 5 μL Universal Primer A Mix (UPM), 1 μL (10 μM) gene-specific primer, 0.5 μL LA Taq (TaKaRa, Dalian, China), 8 μL dNTP mixture, 5 μL 10 × GC Buffer, and 28 μL sterile distilled water up to a total volume of 50 μL. Due to the large size of the Dscam in arthropods, three rounds primers for 5′RACE and one round for 3′RACE were employed with Oligo (dG)-adaptor (AP-dT) and Oligo (dG)-adaptor (AP-dG) to obtain the ends of *Sp*Dscam. PCR amplification conditions for both 3′RACE and 5′RACE were 5 cycles at 94 °C 30 s, 72 °C 3 min; 5 cycles at 94 °C 30 s, 68 °C 3 min; 25 cycles at 94 °C 30 s, 64 °C 3 min; and finally 72 °C for 10 min. The DNA fragment was cleaned using the SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China). Purified DNA fragment was cloned into pMD®19-T vector (TaKaRa, Dalian, China) and then transformed into *E. coli*. Positive recombinant clones were identified by PCR screening with M13R and M13F primers, and subsequently sequenced by a commercial company (BGI, Shenzhen, China).

2.4. SpDscam sequence analysis and phylogenetic analysis

*Sp*Dscam full-length cDNA and deduced amino acid sequences were compared with other Dscams using the BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequence was obtained with the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi), then analyzed with the Expert Protein Analysis System (http://www.expasy.org) and Signal P 3.0 program was used to predict the presence and location of signal peptide (http://www.cbs.dtu.dk/services/SignalP). The transmembrane domain was predicted by the TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Multiple protein sequences alignment was performed using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/