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# Molecular cloning and expression profiles of nitric oxide synthase (NOS) in mud crab *Scylla paramamosain*

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ABSTRACT

The importance of the nitric oxide synthase (NOS) gene family is demonstrated by many studies in vertebrates and invertebrates in recent years. However, it keeps unknown of nitric oxide (NO) system and NOS gene family in mud crab *Scylla paramamosain*, an important cultured commercial crustacean in China and Pacific area. In this report, the cDNA of NOS containing full-length ORF was cloned from mud crab, *S. paramamosain*. It was of 4424 bp, including a 5′-terminal untranslated region (UTR) of 239 bp, a 3′-terminal UTR of 540 bp, which contained two ATTTA motifs, and an open reading frame (ORF) of 3645 bp encoding a polypeptide of 1214 amino acids. Structural analysis indicated that NOS contained a typical NO synthase domain at the N-terminal, next to a flavodoxin 1 domain, a flavin adenine dinucleotide (FAD) binding domain, respectively, and a conservative nicotinamide adenine dinucleotide (NAD) binding domain structure at the C-terminal. Quantitative real-time PCR analysis revealed *S. paramamosain* NOS (SpNOS) to be expressed in all tissues examined, with the highest expression in midintestine and the weakest level in heart and eyestalk. The expression profiles of SpNOS indicated that the NOS expression levels were significantly induced in midintestine, hepatopancrease and hemocytes after challenged with *Vibrio Parahaemolyticus,* the synthetic double-stranded RNA polyinosinic polycytidylic acid (poly I:C) and lipopolysaccharides (LPS). The NOS activity in hemocytes showed significant increase during at 24 h–48 h time period after immune challenges with *V. Parahaemolyticus,* poly I:C and LPS. Results here may suggest that the inducible NOS play an important role in mud crab's defense against pathogenic infection.



## 1. Introduction

The mud crab *Scylla paramamosain* (*S. paramamosain*), one of the most valuable shellfish and the largest crab fishery in China, is widely cultured in brackish and seawater ponds with salinities generally below 9‰ along the coast of southern China. However, infectious diseases have affected the successive development of its aquaculture in recent years. The diseases broke out mainly in the fall when the crab is near maturity, thus resulting in large economic losses [1]. Therefore, it is vital to find effective ways to combat pathogens and avoid further losses.

Research into animal's immune defense mechanisms is important for developing disease control strategies [2]. In eukaryotes, the innate immune system is a critical way for host defense against microbial infections, especially for invertebrates (such as crabs and shrimps) lacking of adaptive immunity. Now, it is well established that inducible nitric oxide synthase (iNOS) is a very important component of the innate immune system participating in the elimination of pathogens in which its oxidation-derived compounds have antibacterial and antiviral activity [3,4] in vertebrates. Nitric oxide synthase (NOS) produces nitric oxide (NO) by catalyzing the conversion of L-arginine to L-citrulline, with the concomitant oxidation of NADPH. NO is a short-lived radical and plays diverse physiological and pathophysiological roles including regulation of vascular tone, neuronal transmission, and antitumor and antimicrobial activities [5]. It has been found to be induced in macrophages, Kupffer cells, neutrophils, fibroblasts vascular smooth muscle and endothelial cells in response to pathological stimuli [6].

So far, there are few reports about the NOS function and its mechanism involved in immune system in invertebrates, most of which are in preliminary level and need further investigation. Most of the crustaceans studies on NOS and their relation to the immune system have been made at the enzymatic level, and NOS cDNA has been cloned and sequenced in only a few crustaceans, such as the

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decapods [7–11] and the cladocerans [12,13]. In *Gecarcinus lateralis*, it was speculated that the NOS expression in gills as a possible role of this enzyme in host defense [7]. In *Daphnia magna,* a challenge with its natural pathogen, *Pasteuria ramosa*, did not vary NOS expression level, but a significant increase in host resistance was found when L-arginine was added to the water [13]. In short, the function of NOS and its role in immune system defense needs further clarification.

Until now, characterization and function researches of immune related genes in mud crab are poorly understood. There is no study demonstrating changes in NOS expression after *in vitro* or *in vivo* stimulating immune system of the crustacean *S. paramamosain*. These immune related genes should be further studied for developing the disease control strategies of the mud crab. In the present study, we are aimed to clone the full-length ORF of NOS from mud crab *S. paramamosain* and characterize its expression distribution in various tissues and the temporal expression profiles of the gene by real-time qPCR and NOS enzyme activity were measured after stimulation with *Vibrio Parahaemolyticus*, the synthetic double-stranded RNA polyinosinic polycytidylic acid (poly I:C) and lipopolysaccharides (LPS). Our findings will contribute to the basic knowledge of NOS system in crustaceans and will be useful to develop strategy to control disease outbreak in mud crab farming.

## 2. Materials and methods

### 2.1. Experimental crabs, RNA extraction and immune challenges

Healthy crabs (150 g ± 20 g) purchased from a commercial crab farm of Niutianyang, Shan Tou, China, were acclimated in the laboratory for 3 days in 1 $m^3$ tanks with 3 crabs per tank in the conditions of salinity (8‰), temperature (28 °C) similar to those of the culture ponds from which the crabs were obtained. For NOS cloning and tissue specific expression, haemolymph of live healthy crab was collected from leg and mixed with an equal volume of anti-coagulant solution [14], followed by centrifugation at 800× *g* at 4 °C for 20 min. The resulting hemocyte pellets were washed twice and suspended again in anti-coagulant solution, then immediately preserved in liquid nitrogen. At the same time, different crab tissues including midintestine, hepatopancrease, muscle, brain, skin, gill, heart, eyestalk, were dissected out and immediately preserved in liquid nitrogen and stored at −80 °C prior to RNA extraction. Hemocytes and tissues were homogenized using a tissue homogenizer (Promega, USA) for RNA extraction. For expression profiles analysis, three groups of crabs were injected with *V. parahaemolyticus* ($1 \times 10^7$cfu $ml^{-1}$) at a dose of 0.5 ml per 200 g crab body weight, poly I:C from Amersham Biosciences (GE Healthcare, Piscataway, NJ) at a dose of 500 ug per 200 g crab body weight and LPS (Sigma, USA) at a dose of 0.5 mg $kg^{-1}$, respectively. Crabs injected with 50 μl of sterile physiologic saline solution (0.8%, m/V) were maintained as controls. Three crabs were used for each group, and every treatment was composed of three replicates. Hemocyte, midintestine, and hepatopancrease of each group were collected at 0, 6, 12, 24, 48 h after challenges and preserved for RNA extraction.

### 2.2. Cloning of NOS cDNA fragment and sequence analysis

Total RNA was extracted from the hemocytes of mud crab using Trizol Reagent (Invitrogen, USA) and digested by the RNase-free DNase I (TaKaRa, Dalian, China) to remove trace amounts of genomic DNA contamination. Total RNA concentration and quality were further determined by UV-absorbance at 260 nm, the A260/A280 ratio and denaturing agarose gel electrophoresis. The first-strand cDNA was synthesized from total RNA by M-MLV reverse transcriptase, following the manufacturer's protocol (TOYOBO, Japan). A partial middle fragment of NOS cDNA was initially obtained by nested RT-PCR using the four degenerate primers (NOS1F 5′-CGCCGAGTCCTTCATGAARCAYTTYG-3′, NOS1R 5′-CCACT GCTTGAAGGCYTGYTCYTG-3′, NOS2F 5′-CCCTTCAAYGGSTGGTAC ATG-3′ and NOS2R 5′-GGSACRATCCASACCCAGTC-3′) that were designed according to Kim et al. [7]and Rose et al. [15] based on highly conserved regions in a wide variety of NOS genes in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) by using bioinformatic analysis-multiple alignment [16]. The first-round PCR was performed using primers NOS1F and NOS1R with 1 cycle of denaturation at 95 °C for 3 min, 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min, followed by a 10 min extension at 72 °C. The nested PCR was then performed using primers NOS2F and NOS2R under the same conditions. All PCR products were cloned into pMD18-T Vector (TaKaRa, Dalian, China) and sequenced by BGI Shenzhen (Shenzhen, China).

### 2.3. Rapid amplification of cDNA ends (RACE)

To obtain the full-length cDNA of NOS, 5′ and 3′ RACE-PCR was performed using 5′-Full RACE Kit and 3′-Full RACE Core Set (TaKaRa). Primers for the 5′ and 3′ RACE were designed based on the partial cDNA sequences obtained above. For 5′ RACE, 2–5 μg total RNA was dephosphorylated with calf intestine phosphatase, digested by tobacco acid pyrophosphate (TAP) to remove the 5′ cap structure, and ligated to 5′ RACE adapter at 5′ end using T4 RNA ligase. The ligated RNA was transcribed into cDNA with random 9-mer primer and used as template for the subsequent outer PCR reaction with 5′ RACE Outer Primer/RP5-1 primer (5′-GCAGGA CACCCACCTCGCAGCTTCTG-3′). The inner PCR reaction was performed with 5′ RACE Inner Primer/RP5-2 (5′-CCATCCTCTTCCC CACCGTCTCCATAAT-3′) using outer PCR product as template. The resulting PCR product was cloned into pMD18-T simple vector and sequenced. For 3′ RACE, 1 μg total RNA was transcribed into cDNA with 3′ RACE adapter and used as a template for the subsequent outer PCR reaction with 3′ RACE Outer Primer/RP3-1 primer (5′-CTGAGCTTGGTGGGGAGCGGCTGAT-3′). The inner PCR reaction was performed with 3′ RACE Inner Primer/RP3-2 primer (5′-ATTTCC TGAAGGAAGTGTCACCTG-3′) using the outer PCR product as template. The resulting PCR product was cloned into pMD18-T simple vector and sequenced. All the sequences were assembled and the full-length cDNA of NOS was obtained. To confirm the integrity of the cDNA sequence, PCR was performed with primer Pf (5′-ACGCGGGGCAGTCCCGGAGCGGCGG-3′) and Pr (5′-TGCGCCCCGTCAGGGCCTCGCCGCC-3′) using the first-strand cDNA as a template and the resulted fragment was sequenced.

### 2.4. Alignments of sequences and construction of a phylogenetic tree

The nucleotide and deduced amino acid sequences of NOS cDNA from *S. paramamosain* were analyzed using BioEdit 7.0.1 and the Expasy search program (http://au.expasy.org/tools/). The sequences of NOS from various species were downloaded from NCBI database and compared by the NCBI BLAST search program. A multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and a phylogenetic tree of NOS was made using the neighbor-joining method in MEGA 4.1 package (http://www.megasoftware.net).

### 2.5. Real-time qPCR analysis of NOS mRNA expression

The expression of *S. paramamosain* NOS in the skin, muscle, stomach, eyestalk, heart, nerve, hemocyte, gill, hepatopancrease and intestine, and the temporal expression of NOS in hemocyte were detected by real-time qPCR. The house-keeping gene β-actin was