Fish & Shellfish Immunology 41 (2014) 428–436

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

# Polymorphisms of heat shock protein 90 (Hsp90) in the sea cucumber *Apostichopus japonicus* and their association with heat-resistance

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ARTICLE INFO

*Article history:*
Received 24 July 2014
Received in revised form
9 September 2014
Accepted 20 September 2014
Available online 28 September 2014

*Keywords:*
Heat shock protein 90
Heat-resistance
SNPs
Association analysis
*Apostichopus japonicus*

ABSTRACT

Heat shock protein 90 (Hsp90) functions as a molecular chaperone and plays an important role in the resistance of organisms to stress, particularly heat-stress. In our study, 12 exons and 11 introns of *hsp90* were identified in the sea cucumber *Apostichopus japonicus*. Twenty-two single nucleotide polymorphisms (SNPs), including three non-synonymous mutations, were detected in the exons. Susceptible and resistant individuals were distinguished using a high-temperature (32 °C) challenge experiment. Three blocks with high linkage disequilibrium were detected among these SNPs. Five of the twenty-two SNPs were shown to be significantly associated with susceptibility/resistance to high temperature by correlation analysis (chi-square test, $P < 0.05$). To confirm the importance of these five SNPs, a heat-resistance strain (HRS) was selected through three generations. Using the common population as the control group, it was shown that the distributions of genotypes and alleles of SNP e10-1 and e11-6 were significantly different between the two groups ($P < 0.05$). SNP e10-1 was trimorphic, with three alleles (A, C and T) and five genotypes (AA, CC, AT, CT and AC). The allele frequency of SNP e2-3 was also significantly associated with this trait ($P < 0.05$). This is the first demonstration of SNPs related to heat-resistance in *A. japonicus* and supports the use of SNP markers in the selective breeding of sea cucumbers.


## 1. Introduction

The sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea) is an important species for commercial fisheries in some Asia countries, including China, Japan, Korea and Russia [1]. Developments in culture facilities and improvements to culture techniques have led to large increases in the scale and the yield of *A. japonicus* aquaculture in China [2,3]. When the temperature of the seawater increases to a threshold (ranging from 20 °C to 26 °C), the sea cucumber *A. japonicus* enters aestivation, which is a kind of ecological adaptation for the environment [4,5]. However, in recent years, mass mortalities of *A. japonicus* in summer have been reported and have caused heavy economic losses, and it appears that extremely high temperatures and the consequent deterioration of water quality were the main reasons for the deaths [6–8]. Rapid expansion of intensive culture systems and increasing culture density have aggravated the situation. Thus, breeding programs for improved domesticated strains of sea cucumber are urgently needed for aquaculture. Marker assisted selection (MAS), which integrates molecular genetic technologies into selective breeding programs, enables more efficient and cost-effective selection [9]. Compared with traditional selection methods, MAS relies on easily measured genotypic criteria and minimizes relatively unreliable evaluation of phenotypes. In particular, MAS facilitates breeding programs for traits of low heritability, sex-limited traits, and traits that are hard to measure [9]. However, little attention has been paid to molecular approaches to assist breeding for heat-resistance in *A. japonicus*.

DNA markers, including microsatellites, amplified fragment length polymorphisms (AFLPs), and single nucleotide polymorphisms (SNPs), have been successfully applied in MAS breeding programs of many domestic animals and a few aquatic species [10–14]. SNP markers, usually with only one alternative nucleotide at a given position, have come into focus in recent decades [11]. Although SNPs are usually simple bi-allelic co-dominant markers, which provide little genetic information, they are becoming increasingly popular [11,15]. One reason for this is their abundant distribution in genomic sequences; a number of potential SNPs can

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http://dx.doi.org/10.1016/j.fsi.2014.09.025
1050-4648/

usually be found near or within a locus of interest [16]. In marine animals, the nucleotide polymorphism rates are remarkably high; e.g., SNP rates are >1.0% in the sea urchin *Strongylocentrotus purpuratus* [17], about 1.1% in the chordate *Ciona intestinalis* [18], and 1.3% in the Pacific oyster *Crassostrea gigas* [19]. More importantly, certain SNPs occurring in the coding regions may affect phenotypic properties directly by changing the structure and function of corresponding proteins [16,20,21]. In this regard, SNPs, particularly those in exons, have received growing attention. Further advantages of SNPs include their ease of detection and the use of high throughput sequencing technologies, which may revolutionize animal breeding in the near future [14,16].

Heat shock proteins (HSPs) function as molecular chaperones and play essential roles in the immunity of organisms, particularly in relation to heat-resistance [22–26]. Therefore, searches for SNP markers associated with heat-resistance have mainly concentrated on HSPs as the most suitable candidate genes [27–31]. The gene *hsp90* has been identified and characterized in diverse species and is involved in a variety of cellular processes, including protein regulation, control of apoptosis, and signal transduction in the stress response [32–35]. In *A. japonicus*, impressive achievements have been achieved in understanding of the protein structures, functions and patterns of expression of *hsp90* [36–38]. These achievements encouraged us to choose *hsp90* as the candidate gene for this study on the SNPs associated with heat-resistance in *A. japonicus*.

In this paper, we identify specific SNP markers linked to heat-resistance of *A. japonicus* in the exons of *hsp90*. The significant SNPs were also confirmed in our selected heat-resistance enhanced strain which selected by traditional method. To our knowledge, this is the first demonstration of SNP markers associated with this important trait in this species. These linked SNPs potentially could be applied in heat-resistant breeding programs for *A. japonicus*.

## 2. Materials and methods

### 2.1. Animals, high temperature challenge and sampling

More than 250 sea cucumbers, each weighing about 10 g, were randomly collected from three farms in Weihai (Shandong, China) in April 2013. Sea cucumbers were acclimated in seawater tanks (30 ppt salinity, 16 °C) for 1 week before processing. They were divided into five groups of each with about 50 sea cucumbers in separate tanks (volume 0.1 $m^3$; 50 cm × 50 cm × 40 cm). The water temperatures of four tanks were simultaneously increased to 32 °C, using a heating rod (1 kW), and then maintained at that temperature during the whole experiment. The fifth (control) seawater tank was unheated and remained at about 16 °C. All groups were monitored every 3 h until the high-temperature challenge ended (5 days). The 'susceptible group' and 'resistant group' were defined according to their survival time. The first 50 individuals that died in the experiment would be considered as the susceptible group while the 50 individuals with longest survival time would be regarded as the resistant group. The body-wall tissues of these two groups were sampled and kept at −80 °C for isolation of DNA.

### 2.2. DNA isolation, characterization of gene structure and identification of polymorphisms in the coding region

The genomic DNAs of the body-wall tissues from each individual in the susceptible and resistant groups were extracted following the protocol of the TIANamp Marine Animals DNA Kit (Tiangen, China). The same method was used for the samples from the HRS and the control groups.

**Table 1**
Primers used in the genomic sequence amplification.

| Primer names | Sequence (5′-3′) |
|---|---|
| F1 | ACATGGGGACCACTGAACAAG |
| R1 | AGGTGAGCCTTGGTCATA |
| F2 | GTATGACCAAGGCTCACCT |
| R2 | CTCCCACCTGTAGCATTCGT |
| F3 | ACGAATGCTACAGGTGGGAG |
| R3 | TTTGTTCAGCTCCTCCTCGT |
| F4 | ACGAGGAGGAGCTGAACAAA |
| R4 | GTTGAGAGGCAAGTCTTCTG |
| F5 | CAGAAGACTTGCCTCTCAAC |
| R5 | GCAGAGTTGGCTACCTGGTC |
| F6 | GACCAGGTAGCCAACTCTGC |
| R6 | CGAGACTGAATCCAGAAGAG |
| F7 | AGAAGTCAACCCAGATCATCC |
| R7 | GTAGCTTGTTCCATGGGATTT |
| F8 | AAATCCCATGGAACAAGCTAC |
| R8 | CAAGGAACCAGCAAGGATT |

To amplify genomic DNA fragments, eight pairs of primers were designed based on the sequences of cDNA of *hsp90* in the sea cucumber *A. japonicus* (GenBank accession no. HQ689677.1) (Table 1). PCR amplification was performed with the extracted pooled gDNA (20 sea cucumbers from the susceptible group and 20 sea cucumbers from the resistant group) as the template, using the following conditions: (1) 1 min at 94 °C for 1 cycle, (2) 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C for 40 cycles, (3) 10 min at 72 °C as the final extension (Mastercycler Pro, Eppendorf, Germany). PCR products of the expected-size were detected by electrophoresis on 1% agarose gels and purified by a PCR gel purification kit (Takara, Japan). Objective fragments were then ligated into pMD19-T simple vector (Takara, Japan) and transformed into *Escherichia coli* JM109 competent cells (Takara, Japan). At least 15 positive recombinant clones were identified by screening with M13 forward and reverse primers and sequenced by Beijing Genomics Institute (Huada Corp., Beijing, China). The sequences were analyzed and assembled using DNAStar software (DNAStar Inc., Madison, WI). Polymorphisms in exonic regions were detected by sequence alignment of different clones.

### 2.3. Genotyping and analyses of SNPs between the susceptible and resistant groups

Sequences in SNP sites were detected and analyzed in all individuals of the susceptible group (50 individuals) and resistant group (50 individuals). Specific primers were designed and used for amplification of fragments where the SNPs were located (Table 2). The PCR program was 1 cycle of 94 °C for 4 min for denaturation, followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. The PCR products were sequenced by Beijing Genomics Institute (Huada Corp., Beijing, China). SNP screening was performed by reading the sequencing maps using BioEdit version 7.0.5.2. Because of the poor efficiency of the primers for some sequences, we detected genotypes of 10 SNPs in exon1, exon8 and exon11 using the Multiplex SNaPshot system (Applied Biosystems, Foster City, CA, USA) with a genetic analyzer (ABI 3730XL; Applied Biosystems). Genotypes of these SNPs were identified using the software of Genemapper 4.0 (Applied Biosystems). The SNaPshot primers are listed in Table 3.

### 2.4. Statistical analysis

SPSS 16.0 software was used to analyze the association between allele and genotype frequencies and individual susceptibility/