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Validation of SNPs in heat shock protein gene of *Macrobrachium rosenbergii* (De Man, 1879) using Sanger sequencing

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Abstract

Macrobrachium rosenbergii also known as giant freshwater prawn, is a commercially important species of crustacean cultured extensively throughout Southeast Asia. Giant freshwater prawn has always been concerned as a suitable species for aquaculture because it can be grown in both fresh and low salinity waters. However, now the wild stocks of *M. rosenbergii* is declining due to the over-harvesting, habitat loss and increased pollution and these cause the loss of genetic diversity due to the small population size. So it is essential now to discover the genetic diversity present among the different stock of *Macrobrachium rosenbergii* for proper maintenance of the stock and to decrease the inbreeding depression in the stocks by cross breeding of genetically different stocks. With these aim in a previous study, we develop a total of eight single nucleotide polymorphism in heat shock gene among the four different stock from Gujarat, Maharashtra, Kolkata and Kerala using next-generation sequencing approach (Ion Torrent). In our current study we resequenced the heat shock protein gene using Sanger sequencing method to validate the SNPs generated from the previous study. We collected 23 individuals from Kerala and after sequencing the 23 gDNA, we found that five SNPs are common to the previous study. These SNPs are considered as the validated SNPs and only one novel SNPs was discovered from the current study. We also reported three and two haplotypes from the HSP21 and HSP70 gene respectively, which shows the genetic diversity within the Kerala shock. The validate SNPs could be used as a molecular marker in marker-assisted selection in the development of the QLTs for this species.

Keywords: Next generation sequencing, Single nucleotide polymorphism, Heat shock protein, Sanger sequencing, Marker assisted selection, Expressed sequence tag

1. Introduction

Macrobrachium rosenbergii, or giant freshwater prawn (De Man) is an economically important species belonging to the genus *Macrobrachium* (Crustacea: Palaemonidae). Among the cultivable prawns, *M. rosenbergii* dominated entirely the commercial freshwater prawn culture in all producing countries except China owing to its superior cultivable attributes such as very fast growth rate, high market demand, hardiness, euryhaline nature and its compatibility to grow with the other cultivable fin fishes such as Indian major carp, catfishes and tilapia. About 200 species of the genus have been described, and by far, this species is the most widely cultured species among all (Nandlal & Pickering, 2005) ^[1]. The natural distribution of *M. rosenbergii* extends from Pakistan in the west to southern Vietnam in the east, across South East Asia, south to northern Australia, Papua New Guinea, and some Pacific and Indian Ocean Islands (Mather & de Bruyn, 2003) ^[2]. In the late 1960s, the modern aquaculture of the species began with the discovery that larval survival mandatory brackishwater conditions. Commercial development of this species was possible because of the research directed by Takuji Fujimura which allow the ready availability of postlarvae of *M. rosenbergii* (New, 2008) ^[3]. Giant freshwater prawn is of high value among cultured species due to its dainty taste and high protein content, both attributes that are favourable to the consumer. Further, other factors such as ease of culture and global export potential also contribute to the popularity of *M. rosenbergii* commercial culture (Whangchai *et al.*, 2007) ^[4]. Single nucleotide polymorphism (SNP) is a substitution of one nucleotide with another, and both versions are observed with the frequency higher than 1% in a population. SNPs may be present within and/or outside genic regions and the majority of them do not produce physical changes in the individual with affected DNA. SNPs found within a coding sequence are of particular interest because they are more likely to alter the biological function of a protein

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(Haldar.2018a) ^[5]. Though, only 3 to 5 percent of total DNA codes for the production of proteins, it is observed that the SNPs occur ~1% in case of human and most of the SNPs found outside of coding regions. Some wild stocks of *M. rosenbergii* have been seen to decline rapidly in recent years; which is largely as a result of over-harvesting, habitat loss and increased pollution, particularly in South East Asia. Declines have been recorded in Bangladesh, India, Indonesia, Malaysia, Philippines and Thailand (New *et al.*, 2000) ^[6]. Wild stocks can provide an immediate resource for addressing genetic diversity problems in cultured stocks due to inbreeding depression etc., and consequently require conservation. To protect natural resources and to maintain a sustainable prawn aquaculture industry, the key strategy is to prevent the loss of genetic diversity of wild prawns and to increase the genetic variability of cultured. To this end, the patterns and extent of genetic diversity that are present in wild stocks need to be adequately documented so that we can identify which stocks may carry unique genetic attributes and prioritize conservation efforts. To measure the genetic diversity different types of molecular marker are used among those the single nucleotide polymorphisms (SNPs) marker is very useful due to its abundance throughout the genome and shows high polymorphism. To discover the genetic diversity, Agarwal *et al.*, (2016) ^[7] used next-generation sequencing approach in the selected transcripts and discovered 320 SNPs among four Indian Stocks of *Macrobrachium rosenbergii* (Scampi). The present study deals with validation of the previously identified SNPs in heat shock protein gene through Sanger sequencing method in Scampi.

2. Materials and methods

Live *M. rosenbergii* specimens were collected from Vembanad Lake, Kerala (Lat: 9° 34' 59.9988" N, Long: 76° 25' 0.0012" E) which was one of the four stock from where Agarwal *et al.*, (2016) ^[7] collected the samples for SNPs mining. We used the sample to validate the previously identified SNPs using Sanger sequencing methods. The pleopods were collected from 23 adult individuals (including male and female) from the wild stock. The tissues were preserved in RNA later (Life Technologies, USA). The tissue samples were stored at -20°C until nucleic acids were extracted.

2.1 Primer designing for target gDNA sequences

Sequences of *M. rosenbergii* heat shock protein transcripts (HSP21 & HSP70) available on GenBank, NCBI were downloaded in FASTA format. Heat shock protein gene fragments with a high number of SNPs were selected for primer designing. The frequency of the SNPs in the selected gene fragments was reported in earlier work from our lab by Agarwal *et al.* (2016). Gene Runner software (v. 3.05) was used for primer designing to amplify gene fragments of sizes ~535–668 bp. A total of two primer pairs were designed and commercially synthesized by Xcelris Genomic Lab, Ahmedabad, India.

2.2 Amplification of gene fragments from *M. rosenbergii* stocks

Total genomic was isolated from pleopods from 23 individuals each from wild stock. PCR was performed in 25µl reactions containing, 0.5µl dNTP mix (10mM; Merck), 2.5µl (1x) Taq buffer and 0.25µl (3U/µl) Taq DNA Polymerase

(Thermo Scientific, USA), 1µl (10 pmoles) of each primer and 1µl gDNA (~25 ng/µl) and rest volume make up by Milli-Q water. The program included Initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 30s), annealing (58°C for 30s), extension (72°C for 45s). A final extension step was carried out at 72°C for 7 min. The PCR products were separated on 1.5% agarose gels and the desired DNA fragments were sliced carefully from the agarose gel with a coverslip avoiding excess agarose. DNA was eluted using QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions and DNA was collected in 1.5 ml microfuge tubes in volumes ranging from 15 to 20 µl depending on the band volume. The eluted product was quantified on Nanodrop spectrophotometer (Thermo Scientific, USA). The PCR products were sent for Sanger sequencing to the Xcelris Genomics Lab, India. Forward and reverse sequencing was done only for both genes HSP21 and HSP70 because of their fragment size is 535 and 668 respectively. Seqman software was used to assemble the forward and reverse fragments to get the full length of the amplified gene fragment.

2.3 Sequence analysis, alignment and SNP calling

Sequences were edited and aligned using BioEdit sequence alignment software. All filtered sequence reads were mapped (aligned) to reference gene sequences downloaded from NCBI GenBank using ClustalW multiple alignment tools in BioEdit software to detect SNPs and insertions/deletions (indels). Total number of non-synonymous (Ka), synonymous (Ks), transitions (Ti), and transversions (Tv) were calculated in MEGA5 (Tamura *et al.* 2011) ^[8].

3. Result and discussion

Two heat shock protein genes (HSP21 & HSP70) were selected for validation of the previously reported SNPs. The genes have a role in heat shock (stress) response and the heat shock protein acts as a molecular chaperone for proper folding of other proteins. The Gen Bank, NCBI Accession numbers, gene names and country of origin is mentioned in Table 1. Specific primers designed to amplify selected regions of *M. rosenbergii* transcribed sequences are listed in Table 1. The primers were designed such that it amplifies the particular gene fragment where the most numbers of SNPs were detected by Agarwal *et al.*, 2016 ^[7]. The amplicon sizes were 530 to 650 so that they could be sequenced by Sanger sequencing method.

Genomic DNA was isolated from the pleopod tissue of *M. rosenbergii* using Standard phenol-chloroform extraction method. After DNA extraction the quality and quantity of DNA were checked using nanodrop and 0.8% agarose gel. The annealing temperature 58 °C was used for PCR amplification of the fragments and all the fragments were amplified successfully to obtain single band of expected sizes. After amplification, all the fragments were eluted on 1.5% agarose gel to check the single band of desirable size for gene fragments. Fig. 1 shows the eluted bands for the HSP gene fragments on 1.5% agarose gel.

SNP calling in *M. rosenbergii* was done in BioEdit by ClustalW multiple alignment. All the 23 wild individuals' gDNA sequences were aligned with the reference gene from GenBank (Accession numbers mentioned in Table 1). A total of 6 SNPs were identified from 2 gene fragments, 4 from HSP21 and 2 from HSP70. The reason for getting less number

of SNPs may be because of the presence of the selected gene fragments in the coding region of the gene. At all the SNP loci only 1 altered nucleotide was observed. Here, we found 5 common SNPs out of 8 SNPs previously reported from Vembanad lake wild stock of *M. rosenbergii* from heat shock genes. These five SNPs are considered as validated SNPs because as they are confirmed by Sanger sequencing of 23 individuals gene.

The putative SNPs were identified using the amplicon approach and high-throughput sequencing platforms in heat shock protein genes of *M. rosenbergii* from 4 wild stocks of India (Agarwal *et al.*, 2016) [7]. Jung *et al.* (2011) [9] sequenced the transcriptome from muscle, ovary and testis tissues of *M. rosenbergii* using the 454GS-FLX. They produced 244.37 Mb of sequence data comprising 787,731 reads with mean length 310 bp. They could detect a total of 834 SNPs from 666517 ESTs assembled, but could finally validate only 342 by Sanger sequencing. Out of the six SNPs detected in our study 3 shows transition and other 3 transversion changes. The validated SNPs are useful in estimating the genetic diversity of the scampi stock in all the

different location of India. Here we have found total 3 and 2 haplotypes in HSP21 and HSP70 genes respectively. These genes have an important role in the stress response and help in the folding of other proteins. So the changes in the nucleotide could affect the biological performance of the individuals. Out of the 6 detected SNPs 3 shows non-synonymous changes and affect the protein sequences. These SNPs have good potential to be used as a molecular marker for the marker-assisted selection if they could be associated with the performance traits like body growth and disease resistance (Haldar, 2018b) [10]. Thanh *et al.* (2010) [11] assessed correlations between single nucleotide polymorphisms (SNPs) in the actin and crustacean hyperglycemic hormone (CHH) genes with individual growth performance in giant freshwater prawn *Macrobrachium rosenbergii*. Thirty SNPs were detected in the actin and CHH genes in broodstock of three *M. rosenbergii* strains (Dong Nai, Mekong, and Hawaiian). Although SNPs in actin genes were not related to growth, the one located in the third intron of CHH gene had a positive correlation.

Table 1: Details of the genes used in study:

Gene ID	Amplicon length (bp)	Sequenced region	Primers	Annealing temp (°C)	Source
Heat shock protein 21 (HQ668088)	535	7 - 541	F- CTGTTAGTCGCCAAGAAGTGTC R- CGCATGGAAGTTCTTGCTCC	58	Malaysia
Heat shock protein 70 (AY466445)	668	1408 -2075	F-GAGTGCCACAAATTGAAGTGAC R- TCATTCAATAATTTGGCAGAC	58	China

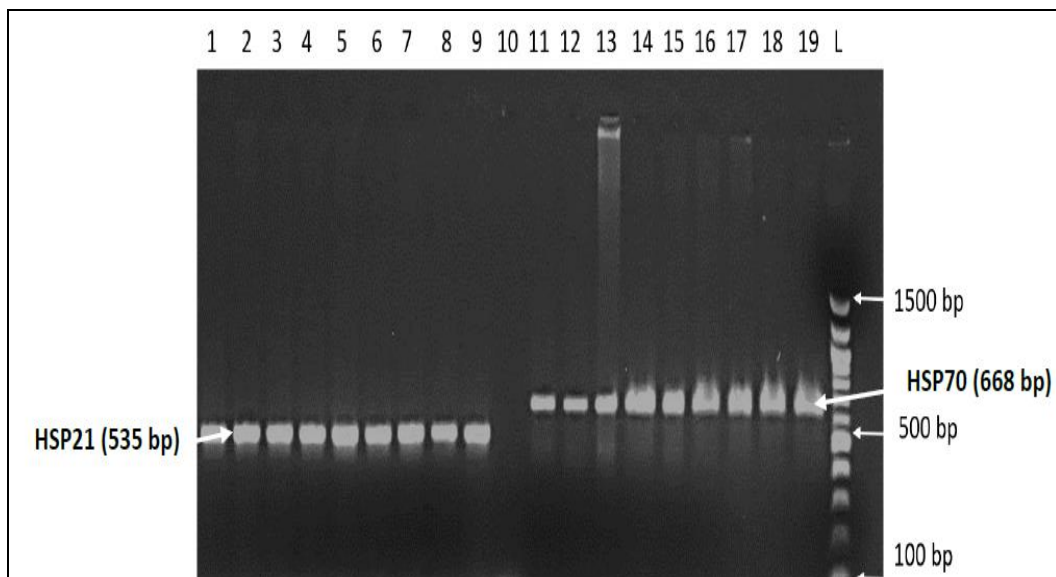


Fig 1: Fragments of HSP21 (535bp) and HSP70 (668bp) on 1.5% agarose gel with 100bp ladder (Merck, USA).

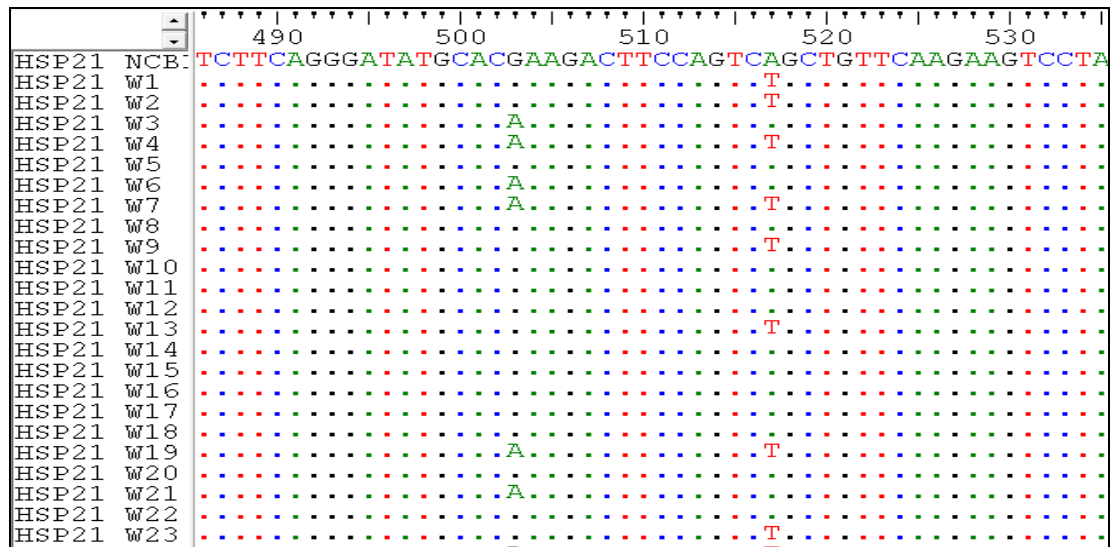


Fig 2: Clustal W multiple alignment of HSP21 gene in BioEdit software: Two SNPs in 503 (G/A) and 517 (A/T) detected.

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