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Chandan Haldar
PhD Research Scholar, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

Raju Ram
PhD student, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

Suchismita Nath
DBT-JRF, ICAR-CIFE, Rohtak Center, Rohtak, Haryana, India

Aparna Chaudhary
PS, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

A Pavan Kumar
Senior Scientist, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

Gireesh Babu P
Senior Scientist, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

Correspondence
Chandan Haldar
PhD Research Scholar, Department of Fish Genetics & Biotechnology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra, India

Genetic diversity estimation in Kerala wild population of *Macrobrachium rosenbergii* (De Man, 1879) using SNP markers

Chandan Haldar, Raju Ram, Suchismita Nath, A Chaudhari, A Pavan Kumar and Gireesh Babu P

Abstract
Global aquaculture has expanded rapidly to address increasing demand for aquatic protein needs and an uncertain future for wild fisheries. To date, however, most farmed aquatic stocks are mostly wild and little is known about their genomes or the genes that affect important economic traits in culture. Many factors can influence individual disease resistance in target species but of particular importance in agriculture and aquaculture will be identification and characterization of the specific gene loci that contribute essential phenotypic variation to immunity because the information can be applied to speed up genetic improvement programs and increase productivity via marker-assisted selection (MAS). Genetic variation in a species improves the capability of organisms to adapt to changing environment and is vital for the survival of the species. The genetic diversity data have varied application in research on evolution, conservation, and management of natural resources and genetic improvement programs, etc. In our current study, we sequenced the different types of lectin genes using Sanger sequencing method to discover genetic diversity in Kerala wild population of *Macrobrachium rosenbergii*. We collected 23 individuals from Vembanad lake Kerala, and after sequencing the 23 gDNA, we found that a total of eleven SNPs in the three immune genes (Lectin3, lectin4, and tachylectin). We also reported three and two haplotypes from the LEC3 and LEC4 gene respectively, which shows the genetic diversity within the Kerala shock. The SNPs have considered as validated because all of them is detected through Sanger sequencing method. These SNPs could be used as a molecular marker in marker-assisted selection in the development of the QLTs for this species.

Keywords: Lectin gene, sanger sequencing, marker assisted selection, disease resistance, single nucleotide polymorphism (SNPs), selective breeding

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. More than 200 aquatic taxa (including crustaceans, fish, molluscs and some algae) are now considered resources to be exploited for human consumption and profit. If current fishing practices and rates of wild fish population exploitation continue, the future of most marine ecosystems and wild fisheries will be uncertain due mainly to overfishing and habitat degradation (Worm *et al*. 2006) [12]. As a consequence, to meet rising global demand for aquatic animal food, aquaculture has developed rapidly and is now practiced widely around the world. In a resource-constrained world where the redirection of edible foods as feedstock for animal production is coming under increased scrutiny, achieving maximum productivity in aquaculture systems will be an imperative as the global community strives to achieve sustainable food security. Some decapod crustacean taxa constitute important aquaculture species including; crabs, crayfish, lobsters, shrimps and prawns that contribute substantially to the modern US$60 billion global aquaculture industry (FAO 2009) [3]. Most cultured crustaceans are high value species, and research is now directed at stock enhancement programs to improve their culture performance (New, 2000) [8]. To date, still, a lack of genomic resources for most aquatic species, and related poor understanding of biochemical

and molecular processes underpinning growth and other economically-important traits has hindered efforts to increase the productivity of crustacean species in aquaculture. Genomics is a new science that analyses the whole or partial genomes of target species, and genomic approaches are now being applied in a wide range of species to elucidate genetic factors that contribute to economically important traits and phenotypes and to manage genetic diversity in crop and livestock species (Mather, 2003) [7]. Giant freshwater prawn (GFP) culture is also complicated by some other issues including the differential growth of males due to social hierarchy in ponds. In this scenario, the ease of cultivating *L. vannamei* has attracted the shrimp farmers. It is likely that GFP, which is a completely domesticated species, can recover its former preferred status if it is genetically improved for disease resistance. A major focus of this growth will be identification and characterization of genes and markers that affect variation in economically important traits. Among different types of molecular marker single nucleotide polymorphism (SNPs) are mostly used for trait association study. 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 (Haldar, 2018a) [4]. These markers, including those affecting growth or immunity related traits, can then be applied strategically to speed up genetic improvement programs for cultured crustacean species via marker-assisted selection (MAS) programs. Despite the limited genetic information that is available, a number of putative candidate genes related to growth and muscle development have already been identified in crustacean species. Now it is time to find the association between putative SNPs with the immune response traits. Protein-carbohydrate relations mediated by lectins have been accepted as key components of innate immunity in vertebrates and invertebrates, not only for recognition of possible pathogens, but also for contributing in downstream effector functions, such as their immobilization, agglutination, and complement-mediated opsonization and killing. More recently, lectins have been identified as crucial regulators of mammalian adaptive immune responses. Fish are endowed with virtually all components of the mammalian adaptive immunity and are equipped with a complex lectin repertoire (Casadevall, 2000) [2]. Some lectins show biological activities that suggest their involvement in immune regulation and homeostasis. The turbot CTL SmLec1 stimulates kidney lymphocyte proliferation and enhance the killing of the bacterial pathogen by macrophages, thereby suggesting that SmLec1 has immunomodulatory activity (Zhang *et al*., 2010) [13]. In an era when almost any study organism can 'go genomic,' understanding gene function and genetic effects on expressed phenotypes will be fundamental to future production systems. Modern DNA-sequencing technologies have replaced the once painstaking hunt for individual genes associated with particular phenotypes, and this development will likely revolutionize genetic improvement programs for cultured crustaceans in the future (Jung *et al*. 2011) [6]. Application of genomic approaches to stock improvement in aquaculture species has already resulted in some significant production gains in some aquatic organisms including carp, rainbow trout, Atlantic salmon, tilapia, and catfish. The progress of molecular genetic markers has powerful capacity to detect genetic studies of individuals, populations or species. These molecular markers combined with new statistical developments have revolutionized the analytical power, necessary to explore the genetic diversity. Molecular markers and their statistical analysis revolutionized the analytical power, necessary to explore the genetic diversity.

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. We used the sample to estimate the genetic diversity in the Kerala wild population using SNP marker through Sanger sequencing method. 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 ºC until nucleic acids were extracted.

2.1 Primer designing for target gDNA sequences

Sequences of *M. rosenbergii* lectin gene transcripts (LEC3, LEC4 & TLEC) available on GenBank, NCBI were downloaded in FASTA format. Lectin 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) [1]. Gene Runner software (v. 3.05) was used for primer designing to amplify gene fragments of sizes ~175 to 235 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 for all the genes. 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) [10].

3. Result and discussion

Three immune genes (Lectin 3, lectin 4 and tachylectin) were selected for estimation of genetic diversity in this gene within the Kerala wild stock population. The genetic diversity was estimated by sequenced each gene in 23 individuals for using Sanger sequencing method. The genes have a role in defense function of crustaceans including scampi. The GenBank, 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 amplicon sizes were 175 to 235 bp 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 ℃ was used for PCR amplification of the fragments and all the fragments were amplified successfully to obtain sa ingle 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 (Fig. 2). All the 23 wild individuals' gDNA sequences were aligned with the reference gene from GenBank (Accession numbers mentioned in Table 1). A total of 11 SNPs were identified from 3 gene fragments, 8 from LEC3, 3 in LEC4 and no SNPs found in TLEC. The reason for getting more number of SNPs in LEC3 and LEC4 may be because of the presence of the selected gene fragments in the 3' UTR region of the genes. As the selected fragment for TLEC gene is mainly present in the CDS region so no SNPs detected from this region. At all the SNP loci only 1 altered nucleotide was observed. These eleven SNPs are considered as validated SNPs because as they are confirmed by Sanger sequencing of 23 individuals gene.

Previously, Agarwal *et al*. (2016) [1] reported eight putative SNPs using the amplicon approach and high-throughput sequencing platforms in lectin genes of *M. rosenbergii* from 4 wild stocks of India. Jung *et al*. (2011) [6] 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 6, 66,517 ESTs assembled, but could finally validate only 342 by Sanger sequencing. Out of the eleven SNPs detected in our study 6 shows transition and other five 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 a significant role in the immune response and help in the disease resistance. So the changes in the nucleotide could affect the immune response of the individuals. Out of the 11 detected SNPs 5 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 disease resistance body (Haldar, 2018b) [5]. Salem *et al*. (2012) [9] conducted a study on SNPs using RNA-seq to identify markers and genes associated with genetic variation for growth in rainbow trout. SNPs from expressed sequences of rainbow trout were genotyped in populations selected for improved growth and unselected genetic cohorts (10 fish from 1 full-sib family each). A subset of SNPs (n = 54) was validated and evaluated for association with growth traits in 778 individuals of a three-generation parent/offspring panel representing 40 families. Twenty two SNP markers and one mitochondrial haplotype showed significant association with growth trait. A study on SNPs in actin and CHH genes of M. rosenbergii was carried out in Vietnam by Thanh *et al*. (2010) [11]. SNPs identified in actin gene amplified from three different stocks obtained from Dong Nai, Mekong and Hawaii, were 7, 8 & 4, respectively. SNPs identified in the CHH gene were found to be present in intronic, exonic and 5' untranslated regions. Twenty two SNPs (including 15 in introns, 4 in exons and 3 in 5' UTR) were identified in Dong Nai stock, while the Mekong and Hawaii stocks had 16 and 9 SNPs, respectively. Among the SNPs identified in both the genes, the SNPs of actin gene are found to be unrelated to growth, but the SNP located in the third intron of the CHH gene had a positive correlation with growth.

Table 1: Details of the genes used in the study

Gene ID	Amplicon length (bp)	Sequenced region	Primers	Annealing temp (℃)	Source
Lectin 3 (LEC3) (JQ349149)	216	993 to 1209	F- ACTTCTTCGACGACGAGGACTG R- TAGCAATGAAATGTCACAACGG	58	China
Lectin 4 (LEC4) (JQ349150)	235	999 to 1233	F- CAGAATTTGGACAATGGGAAC R- AAACTGGATGATTCAGGGAAAG	58	China
Tachylectin (TLEC) (JQ413341)	175	886 to 1060	F- AGTTCCACCTTTATGGCATTTC R- CCCACACATGCAATATTTCC	58	Malaysia

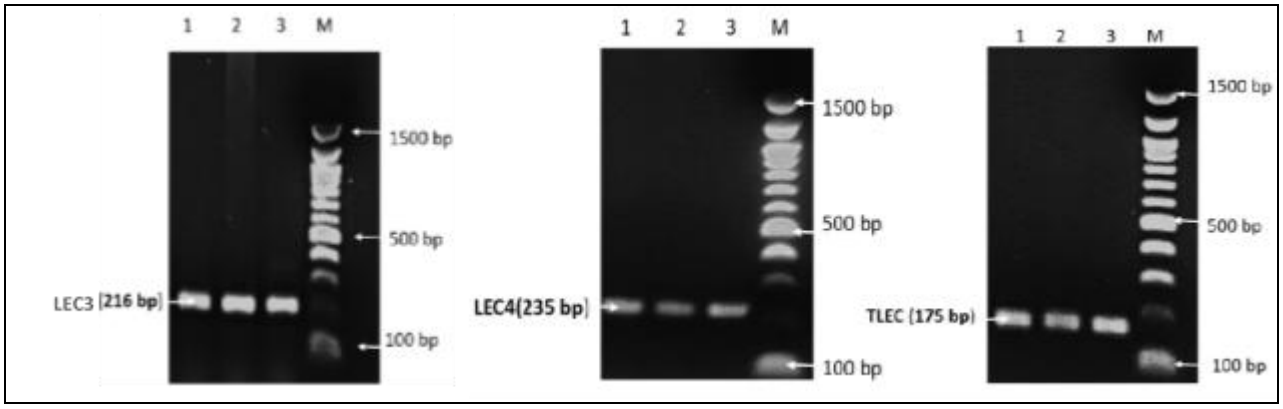

Fig 1: Fragments of LEC3 (216 bp), LEC4(235 bp) and TLEC (175 bp) on 1.5% agarose gel with 100bp ladder (Merck, USA)

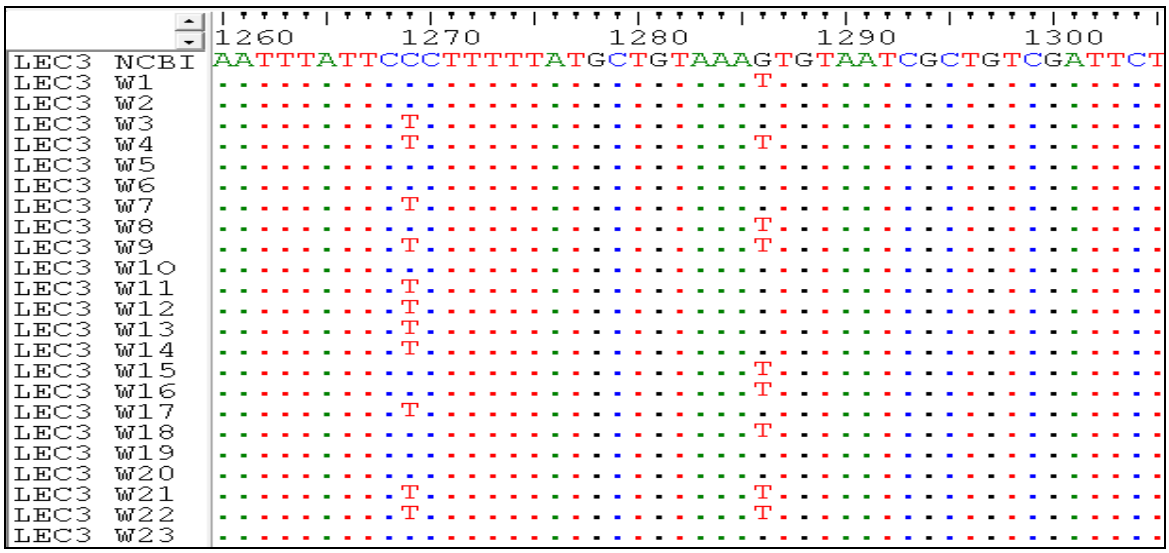

Fig 2: Clustal W multiple alignments of LEC3 gene in BioEdit software: Two SNPs in 1269 (C/T) and 1286 (G/T) detected

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