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Isolation, characterization and activity analysis of selected promoters of mud crab, *Scylla serrata*

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Abstract
Promoters viz. penaeidin (PEN), heat shock cognate 70 (HSC70), single whey acidic domain protein (SWD) and histone3 (H3) were PCR amplified and cloned from the genomic DNA of mud crab, *Scylla serrata*. The nucleotide sequences of the promoters when compared in *silico* with the sequences from other crustaceans, from the data base indicated the presence of several cis-acting regulatory elements like TATA, CAAT, SP1, NF-κB motifs etc. Each of the promoters was cloned into a promoter-less EGFP vector and their efficacy was analysed in mammalian cell lines. The histone3 promoter (pH3-EGFP) showed higher expression levels among the four promoters tested. These promoters can be useful for gene expression and transgenic animal model studies in crustaceans.

Keywords: *Scylla serrata*, promoter, enhanced green fluorescent protein (EGFP), Chinese hamster ovary (CHO) cell line

1. Introduction
Aquaculture is considered the world's fastest growing industry since it provides high quality protein for human consumption thus boosts nutritional security, but disease outbreaks have caused serious economic losses to this industry in many South-east Asian countries [1, 2]. The outbreaks of viral, fungal and bacterial infections threaten the sustainability and profitability of crustacean aquaculture is becoming a major global concern [3]. One such pathogen, which has a great impact on the shrimp propagation, is the white spot syndrome virus (WSSV), which infects a wide range of aquatic crustaceans including mud crabs, and other arthropods such as copepods, insects, pest prawns etc. [4]. Mud crab, *Scylla serrata*, is an economically important aquaculture species, which has been cultured throughout the Indo-Pacific regions for more than 50 years [5]. The mud crabs have been considered a very dangerous threat to shrimps since they are believed to be highly tolerant to WSSV, as they remain infected without the manifestation of the disease for a long period of time [6, 7]. In the present study, an attempt was made to analyse the promoters viz. penaeidin (PEN), heat shock cognate 70 (HSC70), single whey acidic domain protein (SWD) and histone3 (H3) from *S. serrata*. Promoters are transcriptional regulatory sequences in complex genomes and are located immediately upstream of transcription start sites. They are composed of two separate segments such as core and extended promoter regions. The core promoter region encompasses the site of transcription initiation, the site for pre-initiation complex and the assembly of the general transcription machinery while the extended promoter contains specific regulatory sequences for spatial and temporal expression of the downstream gene [8, 9]. The promoter sequences were isolated and cloned from genomic DNA and their efficiency was examined by green fluorescent protein (GFP) expression driven by them in Chinese hamster ovary (CHO) cell lines. To our knowledge, the reports regarding the promoters of *S. serrata* are little explored, and our findings using the mud crab promoters would be valuable for the gene expression studies or transgenic model studies of crustaceans since it can replace the commonly using viral promoters.

2. Materials and Methods
2.1 Genomic DNA extraction from crab hemolymph
The genomic DNA (gDNA) was isolated by phenol-chloroform method [10]. Briefly,

hemolymph was collected from the mud crab using a one milliliter insulin syringe containing anticoagulant (0.5 M EDTA), and centrifuged at 5000× *g* for 10 min at 4 ºC. The resulting hemocyte pellet was treated with lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl), and incubated for 3 h at 50 ºC along with SDS and Proteinase K (final conc. of 1% and 100 µg/ml, respectively). Followed by phenol-chloroform extractions, DNA was precipitated with 3M sodium acetate. After washing with 70% ethanol, DNA was allowed to air dry and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was quantified in a Nanodrop (ND – 1000, USA) and stored at 4 ºC for further use.

2.2 PCR amplification and cloning of the promoters

The primers for PCR amplification of the promoters from the gDNA of mud crab were designed from the nucleotide sequence available in GenBank (Table 1). PCR reactions were carried out in 25 µl final volume of hot start PCR master mix (Thermo Scientific) containing 1 µl of 100 ng templates DNA, 2.5 µM of each forward and reverse primers etc. The thermal profile consisted of an initial denaturation at 94 °C for 30 sec, annealing at respective temperatures (Table 1) and a final extension at 68 °C for 10 min for 35 cycles. The resulting PCR product was analysed on a 1.5% agarose gel followed by the cloning of the purified product into a TA cloning vector, pTZ57R/T (Fermentas). The recombinant vector was transformed into competent *E. coli* DH5α cells by heat shock at 42 ºC for 90 sec, and the colonies were screened on Luria-Bertani (LB) agar plate containing ampicillin (100 µg/ml). The positive clones were determined by PCR using sequence specific primers, and the plasmids were extracted by alkali lysis method. The clones were sequenced using fluorescently labeled dye terminator cycle sequencing kit in an automated capillary array based DNA Sequencer (Model 3730, Applied Biosystems) at Sequencing Facility Centre, CCMB, Hyderabad.

Table 1: Primers used for the amplification of *S. serrata* promoters

Promoter	Primer sequence (5' to 3')	Tm (ºC)	Amplicon size (bp)	Reference
Penaeidin -F	ACCCCCTTTGAGAACTCTCCT	56	456	FJ418753
Penaeidin -R	ACCGGGGGCTGTGGTGCTTTTATA			
HSC70 -F	TATGCAATACTGTCAAGGACATT	56	1099	EF472918
HSC70 -R	AATAGGTTATTGGCTTACCTTAT			
SWD -F	AGGATCATATTCCTAATAGAGATTGAG	56	1013	Chen *et al*. 2006
SWD -R	TTTGGGAATCAGATCCTGTAAACAAAG			
Histone3 -F	GAGAAGGCCGTCAAAGCCAAGT	60	496	GU066313
Histone3 -R	GGCGCTAGCTAGCTTCCTTCTT			

2.3 Promoter nucleotide sequences analysis

The sequences thus obtained were analysed for potential transcription factor binding sites specific for promoters using TSSW (http://linux1.softberry.com/berry.phtml) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) programme. Similarity analysis was performed by BLAST (http://www.ncbi.nlm.nih.gov/) and a neighbor-joining phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA5) software [11] based on the nucleotide sequences of H3 promoters of crustaceans and fishes. Bootstrap sampling was reiterated 1,000 times.

2.4 Transfection of promoter-EGFP vector

The plasmid vectors having promoter sequence were digested with *Sac* I and *Bam* HI and the purified product was ligated into the upstream of a green fluorescence protein reporter vector, pEGFP-1 (Clontech). After transformation in DH5α cells, the colonies were picked up from LB agar plates containing kanamycin (50 µg/ml), and confirmed by colony PCR using gene specific primers. The resulting recombinant plasmids were designated as pPEN-EGFP, pHSC-EGFP, pSWD-EGFP, and pH3-EGFP. Transfection was done in a 24-well plate, 1.5×10^5 CHO cells were seeded in 0.5 ml of growth medium with serum, and incubated at 37°C in a CO_2 incubator until the cells reached 50-80% confluent. The diluted plasmid DNA (1 µg) and 2 µl of lipofectamine (Invitrogen) mixed gently and incubated at room temperature for 15 min to allow the formation of DNA-liposome complexes. The medium was replaced with 0.2 ml of transfection medium without serum. The cells were overlaid with the diluted lipofectamine-DNA complex solution and incubated for 24 h. After incubation, 0.4 ml of growth medium, which contains twice the normal concentration of serum, was added to it. The pEGFP vector carrying CMV promoter and without promoter were used as positive and negative controls, respectively. The GFP expression was observed using an inverted fluorescence microscope after 24 h of transfection.

3. Results and Discussion

3.1 Sequence analysis of PEN promoter

The promoter sequence of PEN showed characteristic putative TATA box, but no consensus CAAT box (Fig. 1). TATA box is essential for the formation of transcription machinery [12] and is found in many genes, especially in penaeidin promoters [13, 14]. A continuous stretch of 22 nucleotide TC repeats (GAGA or GA/TC) was visible in the upstream of TATA box, and this repeat has been reported to be associated especially with the transcriptional regulation [15]. The PEN sequence of mud crab showed 99 and 94% homology with the *P. monodon* isolates having GenBank numbers GU086168 and FJ418753, respectively.

ACCCCCTTTGAGAACTCTCCTGACAACGCTTACTAACAGC
TTGCCTGTTGTATAGTGTCAGTTGGTCTTCATTACGTGTGG
TATATGTTTAAAAAAAAGGGAGGTTTAAAAGTTAAAATT
GACGATGATGATGATGAATAATGATGAAACTGAAAAATC
TTATATTTTTTCCATGTTTTTTATCTGTCTGTCTGTCAATCT
AATAGTTAGCTATCTATCTATTTACCATTCTGTTTAGTTTT
GAATCTCTTTTTCTGTTTGTCTCTATGCCTATTTATATTTCT
TTCTTTCCCTCTTACCCCCCTTCTCTCTCTCTCTCTCTCTT
CCTTTCTCTATCTATCTTTACCTTCCTGTCTTTCTCTCCCCC
CGTCTCTCTCTTTAAAACTGCTTGCACAACCGCGTGGCGT
CTCTATAAAAGCACCACAGCCCCCGGT

Fig 1: Nucleotide sequence of penaeidin promoter region (435 bp) amplified from the gDNA of *S. serrata.* The TATA-box and GAGA (GA/TC) repeat are bold and underlined.

3.2 Sequence analysis of HSC70 promoter

A 1,093 bp fragment of HSC70 promoter sequence analysis (Fig. 2) revealed the presence of a core promoter element with a perfect heat shock element (HSE) and an imperfect HSE. The core promoter is believed to be essential for the assembling of pre-initiation complex [16]. Heat shock factors will get activated by heat or stress stimulation and bind to the HSEs then rapidly start the transcription [17]. A single perfect HSE (GAAGGTTCTAGAA) was detected in the mud crab HSC70. Instead of a putative TATA box a GATAAA box was visible, which functions as weak TATA box. It was reported that many promoters do not have any TATA elements [18]. But canonical TATA box is reported in the Hsp70 promoter region of tilapia (*Oreochromis mossambicus*), mosquito (*Anopheles gambiae*), and a parasite, *Brugia malayi* [19-21] etc. Transcription enhancers like SP1 (GGGCGG), NF-κB (GTAAATTTCC), GC boxes (GCGGCG, GGCGGC) were also present in the promoter region. NF-κB transcription factor responds to stresses and is a hallmark of most viral infections [22]. SP1 is a ubiquitous transcription factor which involves in the regulation of various house-keeping genes [23], and also responsible for cellular activities such as metabolism, cell growth, differentiation, angiogenesis and apoptosis [24].

TATGCAATACGTTCAGGACATTTACTGAGTGATGTG
TGATCGGGTCCTTCTCGACATGACTGATAAATGAGG
CGAGGGCAGCTGATTAGGATTCGTAGATACCAGAC
ATATTTCAACGCATTAAAAGTGTGCGGCGTTTTAAA
CGTGGGCTTTATATTTATTAATATGAGTGTTCCACTT
TCATGAAATAAATTGATATGGAAATGTTAGCTTATT
CATTTCATTTTTGATTGCTCATTATACTTTCTATTTA
AAAAGTACTTTCTCTTCTAAATTTTATGATCACTTTT
AAGTCGAAGTTTAAATCTAACTTCGTATTTTATTCA
CGCTCCCGTCATAATGTATAATATATCAGTAAAAAC
AGAAACAATTTGTAATAATATTGTCACTGCGTCTTG
TAAGCCTGAAATAACTTGAGGGTGTACTTGCAGCTA
TTATATGAATTCAGTTAGTGTAACCGTGTTATTATA
TTACATTAACATAATAACTTTAACGTTATTGTTATG
GCTAGTAGCTATAGACATTCTAGATAATACTAGTTA
TCACAATCGTCCAACATATCAATAAATGATTTGATG
TTCATCAGTGTTTCTAAAGAAGTTATTTATACCACG
GGCTACGGTTGCATAAAGCCGGGCCACGTGATATTC
TCGCAGACGGTCTCCCGCGTCTGGACCTCGTTGGCT
GGCGGCTTTGCCATGAGGTCGCTCCCCACGTGGCGA
AGGAACCGCTGTCATGTTGGCACGCCTGGTACGCCG
AGCTGTTCTGCAGTGACGCGCGTAAATTTCCCGCCA
AATACCGTCCAATCAGCATCGACCATTCCGGCATAG
TTGACCAATAAGAACGTTTCACTTACCACGTCACGG
TTATGAAGCCCTACGATTGGAACCAGAAGTGACGT
CATAAACTAGGAACTCTGTGAATGGCCAACGTTCCG
AAGAAGGTTCTAGAAGGTTTACAAAGGGGTCGATG
TCGCCGCTCTATGGATTTGCCGAAGTCATACCAGCC
GGAGACCGAGAGAAGAGCGGACGTGTTACAATTAG
CTCTTAGGACTATTTAAAAATATCTAAAATAAGGTA
AGCCAATAACCTAT

Fig 2: Nucleotide sequence of the HSC70 promoter region (1093 bp) amplified from the gDNA of *S. serrata*. The perfect HSC (GAAGGTTCTAGAA), core promoter region are underlined, whereas imperfect HSC (GAAATAAATTG), GAF elements (GAGAG), CAAT-box (CCAAT), SP1 (GGGCAG), GATA-box (GATAAA), NF-κB (GTAAATTTCC) are marked as bold.

There were three more predicted CAAT boxes (CCAAT) in the region and are reported to be common in HSC70 promoters of human, Xenopus, rat etc. [25-27]. GAF elements, a pentamer GAGAG with a single base pair substitution (GAGGG), were seen in the promoter region. It is considered to be involved in chromatin remodelling and facilitate the binding of transcription factors to the core promoter, and seemed to be critical in the regulation of Hsp70 gene [28]. The HSC70 promoter sequence exhibited 98 and 97% similarity with the HSC70 of *P. monodon* available in the GenBank sequences of JF707774 and EF472918, respectively, whereas 86% similarity was seen with *Litopenaeus vannamei* (JQ736788) sequence.

3.3 Sequence analysis of SWD promoter

The single whey acidic domain protein (SWD) promoter region showed two GATAAA boxes instead of any canonical TATA box (Fig. 3). It has been reported that the GATAAA motif functions as a weak TATA box, since most point mutations of the consensus TATAAA site lead to reduce *in vitro* activity [29]. Multiple numbers of transcription activators like GAF elements (GAGAG) were visible in the SWD promoter region of the mud crab. Three more GATA boxes were present in the promoter sequence (T/A-GATA-G/A), which were transcriptional regulators and play an important role in cell development, differentiation and proliferation [30]. The SWD is synthesised in hemocytes and secreted to hemolymphs both as the antimicrobial peptide and the proteinase inhibitor to protect shrimps from infections [31]. Besides, it is believed to functions as immune regulators in recovering from wounds, traumas, or physiological stresses [32]. The SWD promoter sequence of *S. serrata* showed 97% similarity with the *P. monodon* sequence available in GenBank (JF707775).

AGGATCATATTCCTAATAGAGATTGAGATAGAAGT
AGGAAGGCGAACGGATAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGGAGAAAGAGAGAGAGAGT
TGGATTTCCCTACTCTGCAGGAAAATTTCCAAACGG
GTTGCAGATGCTTCTTGTTACCACATTTTATCCCTCA
AGTTATCTTCGAGTAATACATTTATGACACATGAGT
GTCTCAGTATAGATGTTAATAATAATTTCCATAACA
CATGTGACAAATTAGAGCTATATTCTTTCCAGGTAA
GAACAGGTGATAAACTTTAAAATTTCTTCATTCGAT
GTCAGTCGGTGACATAAATATCCTGCGCAGTGCCCT
TGAGCTTCACACACCTCTGTCGCGCTCAGCAAAACA
CAGGTGAGACGCGTTTAACTTACTTATTCGTTTAGA
TTTTGCAAGATATGTATACGTGGTGTAGTGTGTAAG
TGAGAGCTAGTATATATATATGTAGGAATGCAAGT
ATGTATGTGTGTGAGTGCGCGCACATCCACATCCAC
ACCCACACACACACGCACAAACTCAAACACATACA
CACTCATATATACATAGATATTATACATGTATATAT
GTATATCCATATACATATATACATTTCTATATATAT
ACATATATATTTATTTTTAGTTCTGTGTGTTTGTACA
ATAAACAAAGGGTATTAAACCATCTGCTACAAAAT
GGAAAACCATCATAGGTAAACAAACCTTTTTTGAG
ATCATCATGGTCACGTTTTCCTTAATCCGTGAGAAC
GATACAGGGAATAAAACTACCATTAAGGTGTGTAT
AGACGGACAGATAGGTAGAAAGATAGATGAATAAA
GAGAAGAAGAGAGGAAGAAAGACGAGGACGGGAT
GAAAGTGAGCAAATAAGAAAATCAAGAGAAGAGA
AGAATGAGATAAATAACAGACAATAGATGATTAGA
TATCCAGGCAAGTGAAAAGATCAACGTTTCATCCG
ACCCTTTGTTTACAGGATCTGATTCCCAAA

Fig 3: Nucleotide sequence of the SWD promoter region (1023 bp) amplified from the gDNA of *S. serrata*. The GATAAA and GATA boxes are bold and underlined, where GAF elements (GAGAG) are in bold.

3.4 Sequence analysis of H3 promoter

The nucleotide sequence of H3 promoter region amplified from the gDNA exhibited putative TATA and CAAT boxes (Fig. 4). A family-specific sequence, GACTTC, is reported in many histone promoters [33], and one base change of such sequence (GACTAC) was reported in the Atlantic salmon [34] found in this mud crab H3 too. Transcription regulating factors such as SP1 (GGGCGG), GAF elements (GAGAG) were also present in H3 promoter region of *S. serrata*. There are reports that multiple repeats of GC boxes and CAAT boxes are common in H3.3A and H3.3B, respectively [35, 36]. BLAST analysis of H3 promoter was performed to identify similarity with H3 of other crustaceans and fishes. A neighbor-joining phylogenetic tree was constructed with twenty five H3 sequences, with 1000 bootstraps using MEGA 5.2 (Fig. 5). The tree indicated that these sequences may be grouped into three groups where *S. serrata* came in group I, which was dominated with fishes viz. carp and also of shrimp, *P. monodon* (JF707776). *Oncorhynchus* sp. and *Salmo* sp. were in group II and III, respectively.

GGCCGTCAAAGCCAAGTAAAATCGCTGGTGCGGCT
GCAACTTGACTACTCAACCCCCAAAGGCTCTTTTAA
GAGCCAACCACCTAGCTCAGCAAAAGCGCAAAGTG
TCCTTTCTATGGCTGGCCAACTATTTGGCGTGTTTGT
TAGATACATATACACGGCACAGTATCAAGTGCCCTC
ATGAGGCCTACATGAAGAATAACAACTACTAGGCT
AAAATGAGAGCATTATTGCGCGTAAAGTGTAACGT
TGCTCGCGGCCCTAACAAAAGACCCAAGCGCGCCT
CGGCGAGGGGGGGGGTTGCGTTTTGGGGTGGCACG
GAGAGGCCGAGCGTCCCGTCCAACGGGTGGCGGAG
GAGGCCTCCGCAACGGGCCAATCAGGGCGGTGCGG
AGATGGTGACCAATCAGCAGACGCCGCTGCCGGCT
TTATAAACTTCACATAGGCATTTGGAGGCTATACTC
CGACTGTGAAGACGAGCTAGCTAGCGCC

Fig 4: Nucleotide sequence of the histone3 promoter region (488 bp) amplified from the gDNA of *S. serrata*. The TATA box (TATAAA) and CAAT box (CCAAT) are bold and underlined, whereas SP1 (GGGCGG), GAF elements (GAGAG), histone promoter specific sequence (GACTAC) are bold.

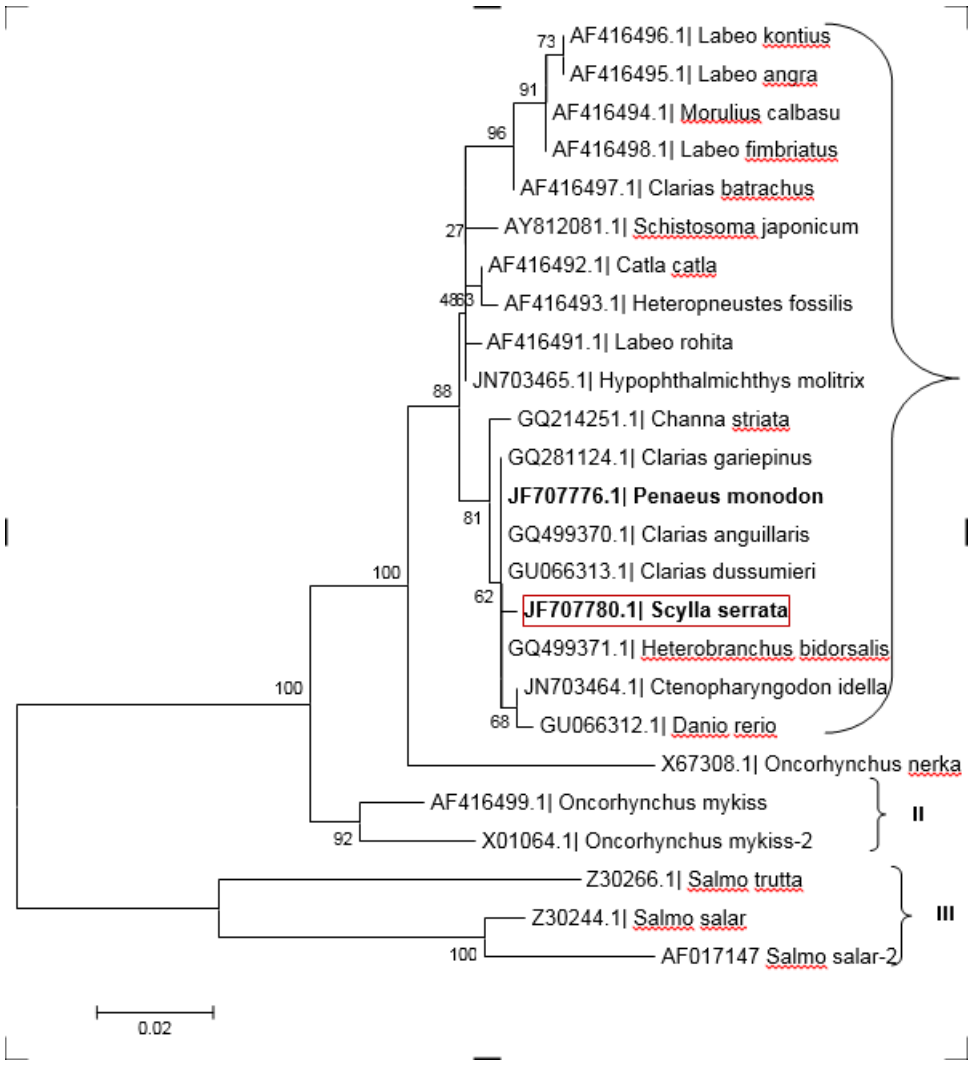

Fig. 5: Neighbor-joining phylogenetic tree of Histone3 promoter of *S. serrata* generated using MEGA 5.2. The numbers at the nodes indicate bootstrap values and were reiterated 1000 times. The analysis involved 25 nucleotide sequences available in GenBank, NCBI. H3 promoter sequence of *S. serrata* is boxed, and H3 promoter sequence of shrimp *P. monodon* is highlighted in bold (I – group I, II – group II, III – group III).

3.5 Promoter activity in CHO cell lines

The promoters in the pTZ vector were digested with corresponding restriction enzymes and ligated upstream of a GFP vector, pEGFP-1. It is a promoter-less vector which can be used to monitor promoter activity of an unknown sequence. Green fluorescence protein is an excellent tool for

gene expression and protein localization studies and does not require any exogenous substrates or cofactors for emission of the fluorescence [37]. Even though hundreds of cell lines from insects and mammals have developed, not a single cell line from shrimp and other marine invertebrates reported so far [38]. Hence, the mammalian CHO cell line was used for the expression of GFP in transfection experiments. The GFP expression was induced under the control of the constitutive promoters from mud crab, and pH3-EGFP showed higher fluorescence among the four followed by pSWD-EGFP (Fig. 6). There was no trace of fluorescence in the negative control, which was the pEGFP vector without any promoters.

In conclusion, the present study has dealt with the cloning and sequencing of four constitutive promoters such as penaeidin, HSC70, SWD and histone3, from mud crab *Scylla serrata*, and identified some of the putative transcription binding sites in the nucleotide sequences *in silico*. The promoter sequences have been deposited in GenBank under accession numbers JF707777, JF707778, JF707779, and JF707780 for PEN, HSC70, SWD and H3, respectively. This study could provide the information regarding the promoters of *S. serrata*, which is a carrier of white spot syndrome virus (WSSV) in shrimp ponds. This information may help in the future application of the use of these promoters in studies involving gene expression, DNA vaccine preparation or transgenic animal models instead of any viral promoters.

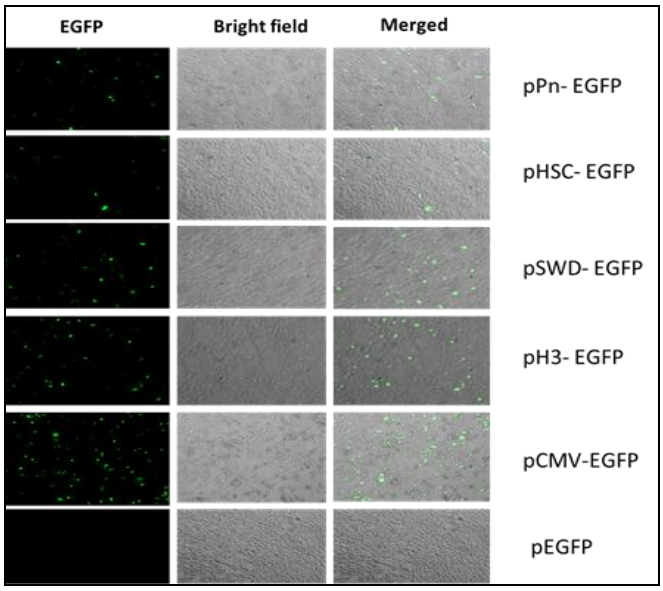

Fig 6: Promoter efficiency analyses in CHO cell lines by transfecting promoter-less EGFP vector contains respective promoters in the upstream of EGFP coding region using lipofectamine along with positive (pCMV- EGFP) and negative controls (pEGFP). The fluorescence was checked after 24 h of transfection by an inverted fluorescence microscope.

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