Indian J. Fish., 73 (3): 122-132, 2026 doi:10.21077/ijf.2026.73.3.179597-13

Available online at: epubs.icar.org.in/index.php/IJF

Molecular cloning and characterisation of integrin gene in the mud crab *Scylla serrata* (Forskal, 1775)

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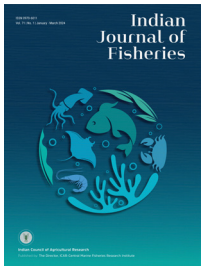

Abstract

The present study reports the first full length sequence of the integrin gene of the mud crab *Scylla serrata* (*Ss*-integrin). *Ss*-integrin cDNA comprises an open reading frame (ORF) of 2349 bp encoding a putative protein of 782 amino acids (aa). The sequence showed high similarity with the integrin gene of *Scylla paramamosain* (97.06% nucleotide identity and 95.78% amino acid identity). mRNA expression of *Ss*-integrin was detected in haemocytes, heart, gills, hepatopancreas, gut and muscle tissues. The response of the gene to white spot syndrome virus (WSSV) was investigated in experimentally infected *S. serrata*. The virus challenge resulted in varying patterns of immune response of the gene in different tissues. *Ss*-integrin was significantly upregulated in haemocytes, heart, gill, and hepatopancreas post-WSSV challenge, showing a tissue-specific temporal correlation with WSSV infection. The study indicated the role of *Ss*-Integrin in the immune response against WSSV infection in the mud crab, a relatively resistant host of the virus.

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Keywords:

Host defence mechanism, *Scylla paramamosain*, White spot syndrome virus

Received : 30.05.2026

Accepted : 09.09.2026

Introduction

Crustaceans primarily exhibit innate immune responses (Zhang *et al*., 2019), relying on cellular and humoral mechanisms such as encapsulation, phagocytosis, autophagy, melanisation, coagulation, and antimicrobial peptide (AMPs) synthesis (Chen *et al*., 2019; Tran *et al*., 2020; Kulkarni *et al*., 2021). Pattern recognition receptors (PRRs), which are essential in identifying foreign pathogens, stimulate immunological responses in crustaceans (Zhou *et al*., 2021). Among these receptors, integrin, a transmembrane receptor linking the extracellular matrix to the cytoskeleton, plays a crucial role and was first characterised by Tamkun *et al*. (1986). Integrin can signal bidirectionally across cell membranes, influencing cellular processes through "inside-out" and "outside-in" signaling (Faull and Ginsberg, 1996; Hynes, 2002; Zhu *et al*., 2007). Studies have revealed the presence of integrin in various crustacean tissues, with varying expression levels (Tang *et al*., 2012; Lin *et al*., 2013; Zhong *et al*., 2013; Shi *et al*., 2020).

The first invertebrate integrin gene was discovered in the haemocytes of the freshwater crayfish *Pacifastacus leniusculus* (Holmblad *et al*., 1997). Notably, the integrin subunit *β*-integrin has been implicated in pathogen infections, including viral infections. Research on the Chinese white shrimp *Fenneropenaeus chinensis*, demonstrated that white spot syndrome virus (WSSV) induces the expression of *Fc*-Int (Tang *et al*., 2012; Zhong *et al*., 2013). Furthermore, integrin-related signaling pathways during WSSV stimulation may facilitate virus spread (Tang *et al*., 2017). Shrimp integrin gene silencing was reported to prevent WSSV infection, highlighting the role of integrin as a WSSV receptor (Tang *et al*., 2017). Lin *et al*. (2013) reported the downregulation of integrin in *Penaeus* (*Litopenaeus*) *vannamei* challenged with WSSV, and integrin characterised in *P. monodon* also showed responses against bacterial pathogens (Shi *et al*., 2020).

Scylla serrata (Forskal, 1775), also known as the Indo-Pacific swamp crab or giant mud crab, is a commercially important species, contributing 2.2% of the total crustacean production (FAO, 2022). Among the many challenges in mud crab farming, viral infections, particularly WSSV, pose a significant threat to commercial success and crab survival (Lo *et al*., 1996; Kanchanaphum *et al*., 1998; Kou *et al*., 1998;

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Supamattaya *et al*., 1998; Rajendran *et al*., 1999; Chen *et al*., 2000; Chakraborty *et al*., 2002; Lavilla-Pitogo and de la Pena, 2004). As mud crabs rely on innate immune components to combat pathogens, understanding these innate immune mechanisms is crucial for managing their health. However, studies on innate immune responses, especially pathogen recognition receptors in mud crabs, are limited (Cao *et al*., 2013; Vidya *et al*., 2016; Chen *et al*., 2018; Kong *et al*., 2018; Yang *et al*., 2023). While the *β*-integrin gene has been sequenced in *Scylla paramamosain*, the characterisation of integrin in *S. serrata* is yet to be completed. Furthermore, since *S. serrata* has been reported to be comparatively resistant to WSSV (Somboonna *et al*., 2010; Pratapa *et al*., 2023), elucidating the role of integrin in responding to WSSV infection becomes significant. Against this background, the present study aims to characterise the integrin gene in *S. serrata* and to understand the gene's expression in response to WSSV infection. We hypothesise that *Ss*-integrin contributes to modulating antiviral immune responses in *S. serrata*, a species comparatively resistant to WSSV.

Materials and methods

Experimental animals

Healthy, juvenile wild caught mud crabs (*S. serrata*), weighing 110-145 g were collected from Versova, Mumbai, India and transported to the Aquatic Animal Health Laboratory of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai. A total of 81 crabs were used in this study, of which 9 individuals were used for gene sequencing, while the remaining 72 were allocated to the treatment and control groups. Three animals were pooled into a single biological replicate. The animals were reared at an ambient temperature of 28–30°C in rectangular plastic crates (24 l capacity) holding seawater at a salinity of 33‰). Hideouts were provided to minimise cannibalism. The crabs were fed chopped fish meat daily *ad libitum* and were monitored regularly throughout the experimental period.

Identification and characterisation of *S. serrata* integrin gene (Ss-integrin)

cDNA synthesis, PCR amplification and sequencing

The animals were first immobilised on ice, and haemolymph was drawn from the coxa of the swimming legs using a sterile syringe fitted with a 24-gauge needle in sodium citrate solution (4% w/v) at a 1:1 ratio. Haemocytes were collected from pooled haemolymph by centrifugation at 10000 *g* for 5 min at 4°C and were stored at -80°C until RNA isolation. Total RNA was isolated from the haemocytes of *S. serrata* using TRIzol® Reagent (Invitrogen, USA) following the manufacturer's protocol. The quality and quantity of extracted RNA were estimated using a Nanodrop spectrophotometer (Thermo Scientific, USA). The cDNA was synthesised using the First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's protocol.

The primers were designed based on the conserved sequences of the *β*-integrin mRNA of *S. paramamosain* (Accession no. HQ260916.1). PCR was performed using the following cycling conditions: 1 cycle of initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the primer-specific temperature as mentioned in Table 1 (Tm °C) for 1 min, and extension at 72°C for 5 min. DNA concentration was standardised to 100 ng per reaction. The primers used to generate the integrin gene sequences, amplicon size and the annealing temperature are summarised in Table 1. The amplified PCR products were electrophoresed on a 1.2% agarose gel and visualised under a gel documentation system (Vilber Lourmat, Germany). The PCR products were purified using a gel extraction kit (Thermo Scientific, USA). The eluted DNA was sequenced through Eurofins Genomics India Pvt Ltd., Bengaluru, India.

Table 1. Details of primers, amplicon size and annealing temperature (T_A) used in the study

Primer	Sequence 5' –3'	Amplicon size	T_A
Ss-Integrin -1	F-GGAGACCATGAGGAACCTTACAA R-GCACGGCGAAGATGATGTT	500	58
Ss-Integrin -2	F-GACGACGAGGTAACCTTCAACCT R-GACAGTGGACGCCAGAGTATCC	496	60
Ss-Integrin -3	F-CGATCGCCGGGTGTGT R-TGGAGTTGGAGAGGTCCATGA	401	59
Ss-Integrin -4	F- ATGAGGAGGAGAGGCATG R-CTGAATTATGGAGCTGGGTG	595	56
Ss-Integrin -5	F-ACAACGAGTACACGGCCTTT R-GCCGTGATCTCCAGGTTGAA	367	56
Ss-Integrin -6	F-ACAACGAGTACACGGCCTTT R-TCAGTGTTTCTGGCCCTCA	1200	60
Ss-Integrin -7	F-CCTACACGGGCCGCTTC R-TCAGTGTTTCTGGCCCTCA	518	56

Bioinformatic analysis

Sequences were first assembled using Finch TV 1.4.0 Software, followed by a homology search utilising the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequence alignments were performed using the Clustal Omega multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Open reading frame (ORF) was predicted using ORF Finder (http:// www.ncbi.nlm.nih.gov/gorf/gorf.html). Translation was performed by the ExPaSy tool (http://www.expasy.org/tools/). Simple modular architecture research tool SMART (http:// smart.embl-heidelberg.de/) was used to analyse the protein domain topology of the deduced amino acid sequence, and NetNGlyc predicted N-glycosylation sites in the coding region of *Ss*-integrin. The molecular weight and isoelectric point of the deduced *Ss*-integrin protein were predicted using the ExPASy ProtParam tool. The 3D structure of *Ss*-integrin was predicted using the SWISS-MODEL tool. Similar gene sequences from various crustacean species were downloaded from NCBI GenBank for phylogenetic tree construction using MEGA 11 software (Tamura *et al*., 2021).

Functional characterization of mud crab integrin gene

Virus inoculum and experimental infection

White spot syndrome virus (WSSV) inoculum was prepared from WSSV-positive *P. (L.) vannamei* as per the procedure described by

Rajendran *et al*. (1999). Briefly, infected tissues (gills, stomach, and epidermal layer) were homogenised in sterile marine phosphate buffered saline (PBS) at a ratio of 1 g of tissue to 1 ml of PBS. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C, and the resulting supernatant was passed through a 0.22 µm membrane filter to obtain the viral filtrate. Healthy mud crabs (*S. serrata*) that tested negative for WSSV by PCR were selected for the experimental infection. Crabs were intramuscularly injected with 100 µl of WSSV inoculum (1.22 × 10^3 copies per µl) into the coxa of the swimming legs. A control group received an equivalent volume (100 µl) of sterile PBS *via* identical route.

WSSV infection in the experimental animals was confirmed using WSSV-specific PCR with 146F2/146R2 primers (146F2: 5'-GTA ACT GCC CCT TCC ATC TCC A-3'; 146R2: 5'-TAC GGC AGC TGC TGC ACC TTG T-3'), and for quality verification of extracted DNA, PCR was conducted using decapod-specific primers, 143F (5'-TGC-CTT-ATC-AGC-TAC-GTT-CGA-TTG-TAG-3') and 143R (5'-TTC-AGA-CGT-TTT-GCA-ACC-ATACTT-CCC-3') (Lo *et al*., 1996). Thermal cycling parameters for both conventional PCR assays comprised an initial denaturation at 95°C for 5 min; 30 cycles including annealing at 55°C for 1 min; and a final extension at 72°C for 1 min.

Viral load was then quantified using real-time PCR, as described by Deepika *et al*. (2014). Reactions were conducted in a total volume of 25 µl containing 12.5 µl of 2x SYBR Green master mix (Takara Bio Inc., Japan), 2 µl of template DNA (50 ng $µl^{-1}$), and 0.5 µl of each WSSV-gene-specific primer (wssv366F1 and wssv366R1; stock concentration of 10 µM). Amplification was carried out in 96-well plates using an ABI 7500 Real-Time PCR System (Applied Biosystems, USA) set at its default thermal profile. Viral copy numbers were calculated by extrapolating values from a standard curve generated using a 10-fold serially diluted wssv366 plasmid DNA standard.

Expression profile of integrin gene in healthy and experimentally infected mud crab

To study the normal expression of the gene, haemolymph and tissues such as heart, gill, hepatopancreas, gut and muscle were collected from nine healthy mud crabs, and samples from three animals were pooled into one biological replicate. Similarly, for post-infection gene expression analysis, crabs were divided into treatment (WSSV-injected) and control (PBS-injected) groups, and samples were collected at 0, 24, 48, and 72 h post-infection. A total of 72 crabs (36 control, 36 treatment) were sacrificed, with nine crabs per group at each time point, and tissues including haemolymph, heart, gill, hepatopancreas, gut, and muscle were collected. Samples from three crabs were pooled to form one biological replicate, resulting in three pooled samples per group per time point. Subsequently, RNA was isolated from these samples using TRIzol® Reagent (Invitrogen, USA), and cDNA was prepared as described in section 2.2.1. Primers for the real-time PCR were designed to target the conserved region of the integrin gene sequence obtained from *S. serrata*. Similarly, primers for the housekeeping gene EF-1α were also designed (Table 2). Integrin mRNA expression profile was determined using Light cycler® Real-time PCR system. The comparative Ct method was used to estimate the relative expression of mRNA. The difference in the Ct value between the integrin gene and the internal reference EF-1α gene (ΔCt beta integrin- ΔCt EF-1α) was calculated, and the relative expression level of the gene to EF α was determined using $2^{-\Delta\Delta Ct}$ method proposed by Livak (2001).

Table 2. Details of primers used in the gene expression studies employing real time PCR

Primer	Sequence 5' –3'	Amplicon size	T_A
EF-1α	F-GGTGCTGGACAAGCTGAAGGC R-GGTTCCGGTGATCATGTTCTTGATG	138	58
Ss-Integrin -qPCR	F-CCACTCAAGGACAACTGCGA R-CCTGTGTACCTTCGAGGACG	101	58

Statistical analysis

Statistical processing of gene expression data was executed using SPSS software (v16.0), with differences considered statistically significant at $p<0.05$. To obtain high quality RNA and account for individual biological variation, tissue and haemolymph samples from three crabs were pooled into a single biological replicate (N=3 pooled biological replicates per treatment group per time point, totalling 9 crabs per group). Relative mRNA levels were quantified using the 2−ΔΔCt method. Statistical comparisons were performed on normalised ΔCt values, while 2−ΔΔCt values were utilised for graphical presentation. Differences in transcript levels between control and WSSV-infected groups across post-infection time points (24, 48, and 72 hpi) were determined independently for each tissue using one-way analysis of variance (ANOVA) and Duncan's new multiple-range *post-hoc* test. Results are presented as mean±standard error (SE) of three biological replicates, with distinct superscript letters (a–d) denoting significant inter-group differences.

Results

Sequence information of *S. serrata* integrin (Ss-integrin)

cDNA of *S. serrata* integrin (*Ss*-integrin) was amplified with different sets of primers, resulting in the amplification of 500, 496, 401, 367, 1200 and 518 bp amplicons, and the PCR products were then purified and sequenced. The partial sequences obtained from each amplicon were verified and assembled to obtain the full-length *Ss*-integrin cDNA sequence. The open reading frame (ORF) of *Ss*-integrin cDNA was 2349 nucleotides (nt) long and encodes a putative protein of 782 amino acids (AA) (Fig. 1). The BLAST analysis revealed that the *Ss*-integrin nucleotide sequence shares 97.06% identity with *S. paramamosain* integrin cDNA. The identified cDNA sequence of *Ss*-integrin was deposited in GenBank under the Accession no, OR360538.

The protein domain topology prediction of the *Ss*-integrin deduced amino acid sequence showed a signal peptide domain (1 to 27), a Von Willebrand adhesion factor (136 to 364 aa) and an integrin beta subunit (N terminal portion of extracellular region) (35 to 450 aa) in the extracellular domain, an EGF-like domain (450 to 504 aa), an integrin B tail domain (628 to 710 aa), a single transmembrane domain (711 to 733 aa) and an intracellular cytoplasmic domain (734 to 780 aa) (Fig. 2a).

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   1 ATGAGGAGGAGAGGCATGGTGGCGGCGGTGGTGCTGGGGGTGGTGATCGCCGCGGTGTGTGAGGAGCAGCAGAGCCAGGGGCAGGAGTGT   90
      M  R  R  R  G  M  V  A  A  V  V  L  G  V  V  I  A  A  V  C  E  E  Q  Q  S  Q  G  Q  E  C   30

  91 AGGGCGCAGAGGACGTGTGACAAATGCATCCAATCGCAAGGGTGCTCGTGGTGCATCAATCCCCAGAGCGGGGATCACTGCCAACTTGCC  180
      R  A  Q  R  T  C  D  K  C  I  Q  S  Q  G  C  S  W  C  I  N  P  Q  S  G  D  H  C  Q  L  A   60

 181 GCCAACTTTAATGCATCCTTATGTAACCCCAGTGACATCAGGAACCCACACAACACCGTGACACTGGTACAGAATGAGGAGCTAACAGGC  270
      A  N  F  N  A  S  L  C  N  P  S  D  I  R  N  P  H  N  T  V  T  L  V  Q  N  E  E  L  T  G   90

 271 ACAATCCAGAAGAAAAGGAAAGAATGGGACAAAAACGCGGAGATCTACCAACTAAAGCCACAAAGAGTGAAGCTCCAGCTGCGCAAGGGT  315
      T  I  Q  K  K  R  K  E  W  D  K  N  A  E  I  Y  Q  L  K  P  Q  R  V  K  L  Q  L  R  K  G  120

 316 TACCAACTAAAGCCACAAAGAGTGAAGCTCCAGCTGCGCAAGGGTATGCCACAGAAACTGGACATCAAGTACCGGCGCGCCCTTGACTAC  405
      Y  Q  L  K  P  Q  R  V  K  L  Q  L  R  K  G  M  P  Q  K  L  D  I  K  Y  R  R  A  L  D  Y  150

 406 CCCGTCGACCTGTACTACCTCATGGACCTCTCCAACTCCATGAAGGACGACAAGGAGCATCTGGCCAACGCGGGCGCTATTCTGGCAGAG  495
      P  V  D  L  Y  Y  L  M  D  L  S  N  S  M  K  D  D  K  E  H  L  A  N  A  G  A  I  L  A  E  180

 496 ACCATGAGGAACCTTACAACGCAGTTTACCCTCGGCTTCGGCTCCTTCGTGGACAAGGTCATGATGCCTTTCACCAACACCACCCAGCTC  585
      T  M  R  N  L  T  T  Q  F  T  L  G  F  G  S  F  V  D  K  V  M  M  P  F  T  N  T  T  Q  L  210

 586 CATAATTCACCTCCGTGCGCCGGCTGCGCCCCCCCTTACTCCTTCAGGAATGACATGAAGCTTAGTCAGGACCACGCAGCCTTCACGGAG  675
      H  N  S  P  P  C  A  G  C  A  P  P  Y  S  F  R  N  D  M  K  L  S  Q  D  H  A  A  F  T  E  240

 676 CAAGTGTCCAACGCCCAGATCTCTGGCAACCTGGACCATCCCGAGGGCGGGTTTGATGCACTGATGCAGGCCATGGTGTGCACAGATCAG  765
      Q  V  S  N  A  Q  I  S  G  N  L  D  H  P  E  G  G  F  D  A  L  M  Q  A  M  V  C  T  D  Q  270

 766 ATTGGTTGGCGGGACAAGGTGCGCCACATACTCGTCTTCTCCACTGACGCGAAGTTTCATCACGCTGGCGACGGACGGCTGGCAGGAGTG  855
      I  G  W  R  D  K  V  R  H  I  L  V  F  S  T  D  A  K  F  H  H  A  G  D  G  R  L  A  G  V  300

 856 GTGGAGCCCAATGATGAGAAGTGTCACCTGAACGAAAAAAACGAGTACACGGCGTTTGACAAGTACGACTACCCATCCATTGCACAGATC  945
      V  E  P  N  D  E  K  C  H  L  N  E  K  N  E  Y  T  A  F  D  K  Y  D  Y  P  S  I  A  Q  I  330

 946 AACAAAGTGGCAGAGGAAAAGAACATCAACATCATCTTCGCCGTGCTGCAGAACGAGAGACTCTACCGCGAACTTCAGAAACAGATCAAA 1035
      N  K  V  A  E  E  K  N  I  N  I  I  F  A  V  L  Q  N  E  R  L  Y  R  E  L  Q  K  Q  I  K  360

1036 ACTTCTTCCTTTGGCTTGCTGGAGGAAGGATCGGCGAACGTGGTGGACCTCATCAAGGATCAGTACAATAGCATCTCGAGCAGCGTGTCC 1125
      T  S  S  F  G  L  L  E  E  G  S  A  N  V  V  D  L  I  K  D  Q  Y  N  S  I  S  S  S  V  S  390

1126 CTCACAGACAATAGCACCAGCGCCATCACCATCAAGTACAGCTCCTCCTGCAAGGGGAGTGAGGTCAGGGAGACCAGGGACTGTGACGGC 1215
      L  T  D  N  S  T  S  A  I  T  I  K  Y  S  S  S  C  K  G  S  E  V  R  E  T  R  D  C  D  G  420

1216 ATTAGCGAGGACGACGAGGTAACCTTCAACCTGGAGATCACGGCCAACACGTGTTCGGATGGAGAGCAATCCATTGTGGAGGTGAAGACT 1305
      I  S  E  D  D  E  V  T  F  N  L  E  I  T  A  N  T  C  S  D  G  E  Q  S  I  V  E  V  K  T  450

1306 CTGGAGGCGAACGTGATTCTTGAGATTGAACTCCTGTGTACCTGTGACTGCCCCCATGATGTTTCCAGCTTACCGTGCAAAAACGGCGGC 1395
      L  E  A  N  V  I  L  E  I  E  L  L  C  T  C  D  C  P  H  D  V  S  S  L  P  C  K  N  G  G  480

1396 TCCATGATATGCGGCGTGTGCGAGTGTCCGAGTGGCTTTTACGGAGAGGAGTGTCAGTGTGCGAGTGGCAGCGTGTCCCAAGATATAAGC 1485
      S  M  I  C  G  V  C  E  C  P  S  G  F  Y  G  E  E  C  Q  C  A  S  G  S  V  S  Q  D  I  S  510

1486 GAGAACCAGGACCAGACCAGCAAGTGCATGGCCAGCGAGAAGGACACAAGGCCTTGCTCGGGGCGCGGCGCCTGCAAGTGTGGCGTCTGC 1575
      E  N  Q  D  Q  T  S  K  C  M  A  S  E  K  D  T  R  P  C  S  G  R  G  A  C  K  C  G  V  C  540

1576 AACTGTTTCAAGCCTGAGGAGGTGTCCGGGACTTACTGCCAGTGTAATAGGCGGAAATGCATGAGCTCCAACAAGCTGGAGTGCTCGGGT 1665
      N  C  F  K  P  E  E  V  S  G  T  Y  C  Q  C  N  R  R  K  C  M  S  S  N  K  L  E  C  S  G  570

1666 CATGGCACATGTGACTGCAACGAGTGTAGATGTAATGAGGGATACTCTGGCGTCCACTGTCATTGTAACGACCAAGACTGCATCCCGACA 1755
      H  G  T  C  D  C  N  E  C  R  C  N  E  G  Y  S  G  V  H  C  H  C  N  D  Q  D  C  I  P  T  600

1756 GGGTCTGAAGAGGTGTGCTCGGGCCACGGCAAGTGTGATTGTGGCCGTTGCATCTGTGACCAGCAGAAGAACGTCACCTACACGGGCCGC 1845
      G  S  E  E  V  C  S  G  H  G  K  C  D  C  G  R  C  I  C  D  Q  Q  K  N  V  T  Y  T  G  R  630

1846 TTCTGTGAGGACTGCCTGAGTTGCGGTGGAGGGAAGTGCAAAAAGTTCAGAGACTGTGTGCAGTGTTTCCACTTTGGGATTGGCCCACTC 1935
      F  C  E  D  C  L  S  C  G  G  G  K  C  K  K  F  R  D  C  V  Q  C  F  H  F  G  I  G  P  L  660

1936 AAGGACAACTGCGAACAGTGTGAATTCTCCACAGCTGTGGAGAATCTGGATGACTACATGTACGAAAACTCCCGCCTGTGTACCTTCGAG 2025
      K  D  N  C  E  Q  C  E  F  S  T  A  V  E  N  L  D  D  Y  M  Y  E  N  S  R  L  C  T  F  E  690

2026 GACGAGGACGGCTGCTACCTGAATTTCACCTACCGCTACCTGGAGGCCACCAACAAGGACGAGGTGCACGTGCAGGCAGAGAGGAAGTGC 2115
      D  E  D  G  C  Y  L  N  F  T  Y  R  Y  L  E  A  T  N  K  D  E  V  H  V  Q  A  E  R  K  C  720

2116 CGTGAAAAGCCACCCATTGCAGCCATCGTGTTCGCCCTGATCGGCACCATCGTGGGCATCGGCGTGCTCTCCCTCATTCTGTGGAAGATC 2160
      R  E  K  P  P  I  A  A  I  V  F  A  L  I  G  T  I  V  G  I  G  V  L  S  L  I  L  W  K  I

2161 ACCATCGTGGGCATCGGCGTGCTCTCCCTCATTCTGTGGAAGATCTTCACCACCATCCACGACAAGCGGGAGTACGCCAAGTTCCTGGAG 2250
      T  I  V  G  I  G  V  L  S  L  I  L  W  K  I  F  T  T  I  H  D  K  R  E  Y  A  K  F  L  E  750

2251 GAGACCAAGCGACCTCAGTTCAGCGCTGAGCAAAACCCGCTGTTTATTGACCCCAACACCACCACGCAGAACCCGATGTTTGAGGGCCAG 2340
      E  T  K  R  P  Q  F  S  A  E  Q  N  P  L  F  I  D  P  N  T  T  T  Q  N  P  M  F  E  G  Q  780

2341 AAACACTGA   2349
      K  H  *   782
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Fig. 1. The nucleotide and deduced amino acid sequence of *Ss*-Integrin. Nucleotides and amino acids are numbered from left margin to right margin. The start codon (ATG) and stop codon (TGA) are marked in bold; Integrin beta subunit is in light grey, Integrin_B_tail is highlighted in dark grey, transmembrane domain is underlined and Integrin_B_cyt domain in deep green

Sequence homology and phylogenetic analysis

BLASTp analysis of the 782 deduced amino acid sequence of *Ss*-integrin showed the highest sequence similarity with the *β*-integrin gene of *S. paramamosain* (95.78 % identity), followed by *Portunus trituberculatus* (74.46%), *Eriocheir sinensis* (57.31%), *Pacifastacus leniusculus* (52.81%), *Penaeus chinensis* (51.01%) and *Penaeus japonicus* (50.69%). Eight N-linked glycosylation sites were identified in the coding region of the deduced amino acid sequence (Fig. 2b). The predicted molecular weight of the protein was 87.22 kDa, with an isoelectric point of 5.25. The 3-D structure of *Ss*-integrin is shown in Fig. 2c. Phylogenetic analysis revealed the closest relationship between *Ss*-integrin and *S. paramamosain* integrin (Fig. 3).

Expression pattern of Ss-integrin gene in different tissues

Initially, *Ss*-integrin mRNA expression was examined in cDNA synthesised from RNA isolated from haemocytes and various tissues such as the heart, gill, hepatopancreas, intestine and muscles of healthy *S. serrata*. Semi-quantitative RT-PCR was

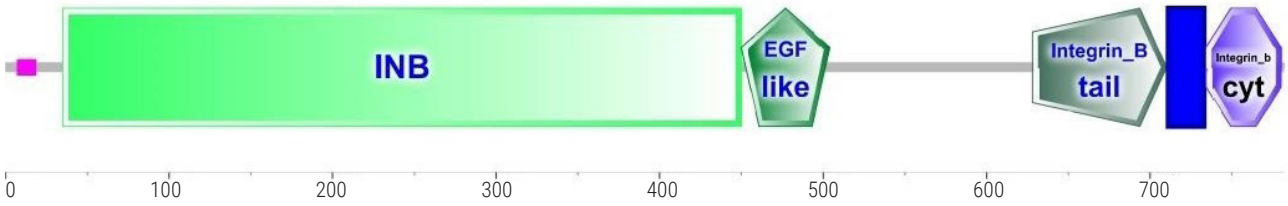

Fig. 2a. Schematic representation of the domain architecture of *Ss*-Integrin protein

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MRRRGMVAAVVLGVVIAAVCEEQQSQGQECRAQRTCDKCIQSQGCSWCINPQSGDHCQLAANFNASLCNPSDIRNPHNTV   80
TLVQNEELTGTIQKKRKEWDKNAEIYQLKPQRVKLQLRKGMPQKLDIKYRRALDYPVDLYYLMDLSNSMKDDKEHLANAG  160
AILAETMRNLTTQFTLGFGSFVDKVMMPFTNTTQLHNSPPCAGCAPPYSFRNDMKLSQDHAAFTEQVSNAQISGNLDHPE  240
GGFDALMQAMVCTDQIGWRDKVRHILVFSTDAKFHHAGDGRLAGVVEPNDEKCHLNEKNEYTAFDKYDYPSIAQINKVAE  320
EKNINIIFAVLQNERLYRELQKQIKTSSFGLLEEGSANVVDLIKDQYNSISSSVSLTDNSTSAITIKYSSSCKGSEVRET  400
RDCDGISEDDEVTFNLEITANTCSDGEQSIVEVKTLEANVILEIELLCTCDCPHDVSSLPCKNGGSMICGVCECPSGFYG  480
EECQCASGSVSQDISENQDQTSKCMASEKDTRPCSGRGACKCGVCNCFKPEEVSGTYCQCNRRKCMSSNKLECSGHGTCD  560
CNECRCNEGYSGVHCHCNDQDCIPTGSEEVCSGHGKCDCGRCICDQQKNVTYTGRFCEDCLSCGGGKCKKFRDCVQCFHF  640
GIGPLKDNCEQCEFSTAVENLDDYMYENSRLCTFEDEDGCYLNFTYRYLEATNKDEVHVQAERKCREKPPIAAIVFALIG  720
TIVGIGVLSLILWKIFTTIHDKREYAKFLEETKRPQFSAEQNPLFIDPNTTTQNPMFEGQKH
.............................................................N....N...........   80
.............................................................................   160
........N.....................N...............................................   240
.............................................................................   320
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.............................................................................   480
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...............................................N.............................   640
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...............................................N.............                 800
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Fig. 2b. Schematic representation of the domain architecture of *Ss*-Integrin protein

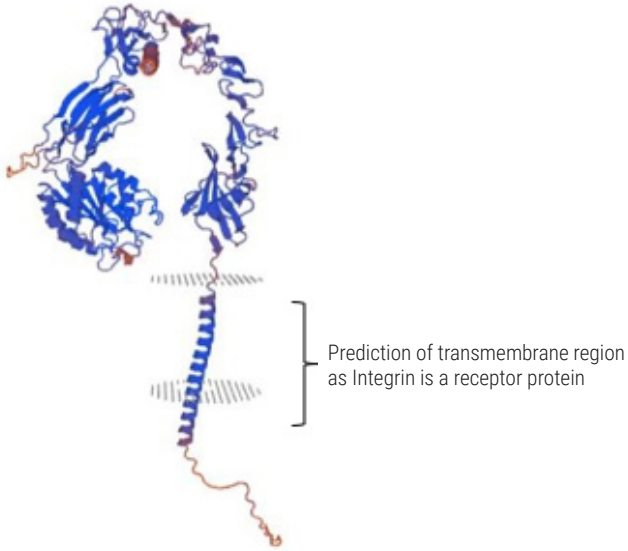

Fig. 2c. Predicted 3-D structure of *Ss*-Integrin protein by SWISS-MODEL

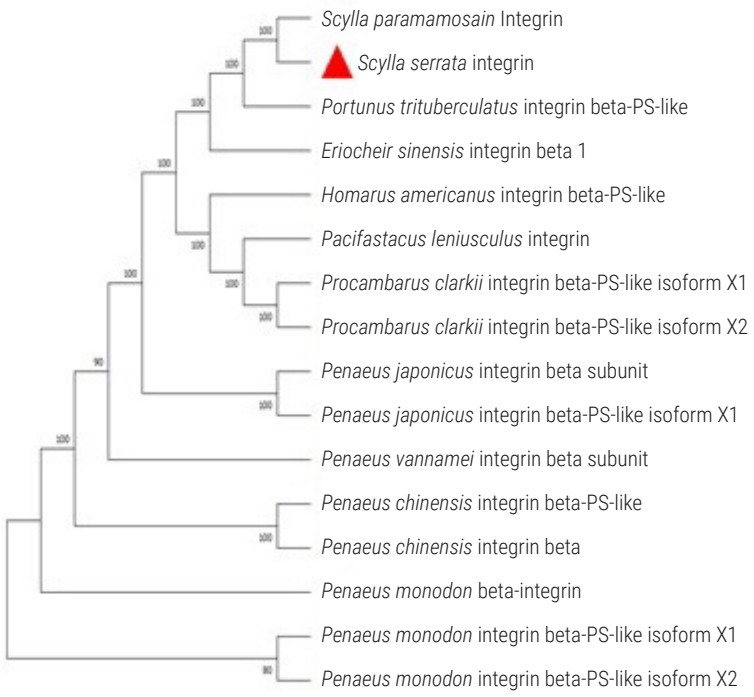

Fig. 3. Phylogenetic tree of deduced amino acid sequences of *Ss*-Integrin with other crustaceans

performed using *Ss*-integrin specific primers, with EF-1α as the internal control. *Ss*-integrin transcripts were detected in all the tissues tested (Fig. 4). Subsequently, the transcript levels were estimated using real-time PCR. For this, three pooled samples were examined, and each pool comprised tissues collected from three individual animals. The highest expression of *Ss*-integrin was noticed in haemocytes, followed by the heart, gills, hepatopancreas, gut and muscle (Fig. 5).

Response of Ss-integrin gene to WSSV infection

Before determining the *Ss*-integrin gene expression in the WSSV-injected crab, the infection was confirmed in the experimentally infected crab using WSSV-specific PCR (Fig. 6a). Specific PCR amplification was detected in the gill tissue of crabs collected at 24, 48 and 72 hpi. Further, real-time PCR assay showed the highest viral load (1.64 x 10^9 copies per100 ng DNA) noted at 48 hpi compared to 1.6 x 10^7 and 8.75 x10^7 copies per 100 ng DNA) observed at 24 and 72 hpi, respectively (Fig. 6b).

Ss-integrin gene expression kinetics following WSSV infection exhibited distinct tissue-specific patterns. Haemocytes displayed a classic rapid induction of *Ss*-integrin expression, reaching a maximum of ~8-fold at 24 hpi, followed by a decline of ~6-fold at 72 hpi (Fig. 7a). A similar early response was observed in the heart, where expression peaked at 5.5-fold at 24 hpi, dropped sharply to 2-fold at 48 hpi and remained at a similar level at 72 hpi (Fig. 7b). In contrast, the gill, hepatopancreas, and gut exhibited a delayed response, with *Ss*-integrin expression peaking at 48 hpi, followed by a decline at 72 hpi (Fig. 7c, d, e). The magnitude of upregulation in these tissues was comparatively lower with maximum increases of approximately 2.7, 1.75, and 2.25-fold in the gill, hepatopancreas and gut, respectively. Among the various tissues examined, no

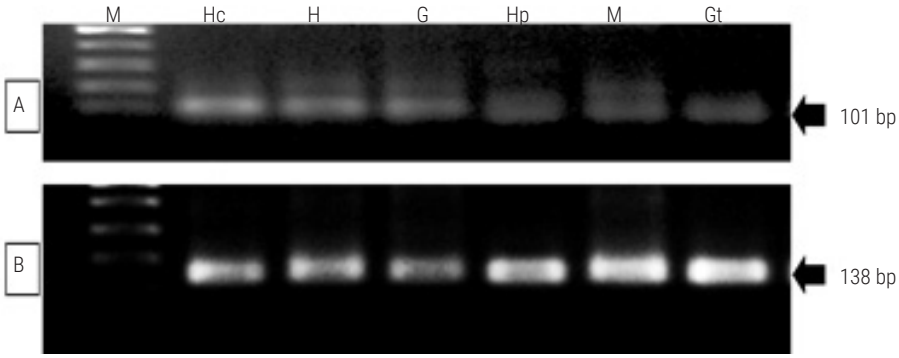

Fig. 4. Semi-quantitative expression of *Ss-Integrin* in different tissues of healthy *S. serrata*. (a) *Ss*-Integrin; (b) EF-1α. M - 100 bp plus molecular weight marker, Hc – Haemocytes, H – Heart, G – Gill, Hp – Hepatopancreas, M – Muscle, Gt – Gut

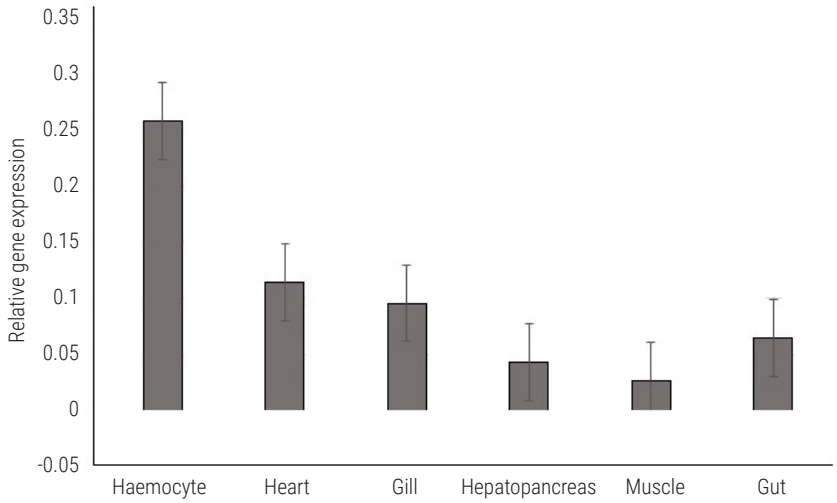

Fig. 5. Normal expression profile of *Ss*-Integrin in different tissues of healthy *S. serrata* estimated using qPCR

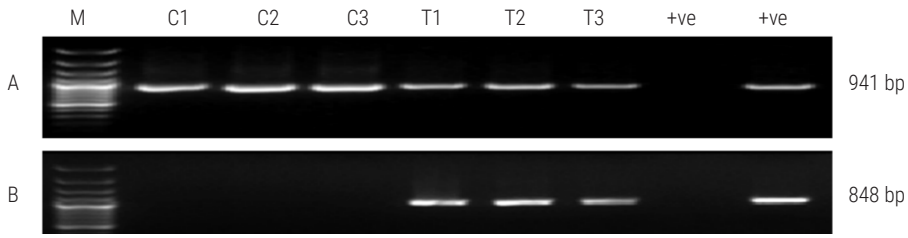

Fig. 6a. WSSV infection confirmed by WSSV-specific PCR. (a) WSSV-specifec pirmer, (b) Decapod- specific primer. M - 100 bp plus molecular weight marker; C1-Control 24 hpi, C2-Control 48 hpi, C3-Control 72 hpi, T1-Treatment 24 hpi, T2-Treatment 48 hpi and T3-Treatment 72 hpi

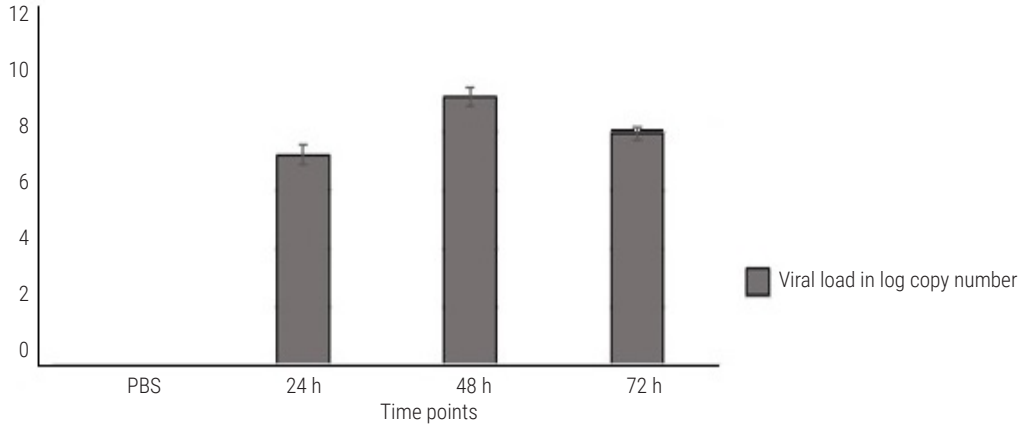

Fig. 6b. Viral load determined in the gill tissue of WSSV-challenged mud crab *S. serrata* at different time points post-challenge

significant changes in *Ss*-integrin expression were observed in the muscle tissue at any time point following WSSV infection (Fig. 7f).

Discussion

Integrins are transmembrane receptor proteins that primarily recognize ligands found in the extracellular matrix, on cell surfaces, and in some soluble forms. Several researchers have indicated that the integrin in invertebrates could participate in the regulation of cellular immune reactions such as melanisation, phagocytosis, nodulation and encapsulation in response to infection or injury (Lavine and Strand, 2003; Mamali *et al*., 2009; Zhuang *et al*., 2008). Despite this understanding, comprehensive research providing evidence regarding the involvement of invertebrate integrins, especially crustaceans, in innate immunity is yet to be undertaken. Against this background, identifying and characterising the integrin

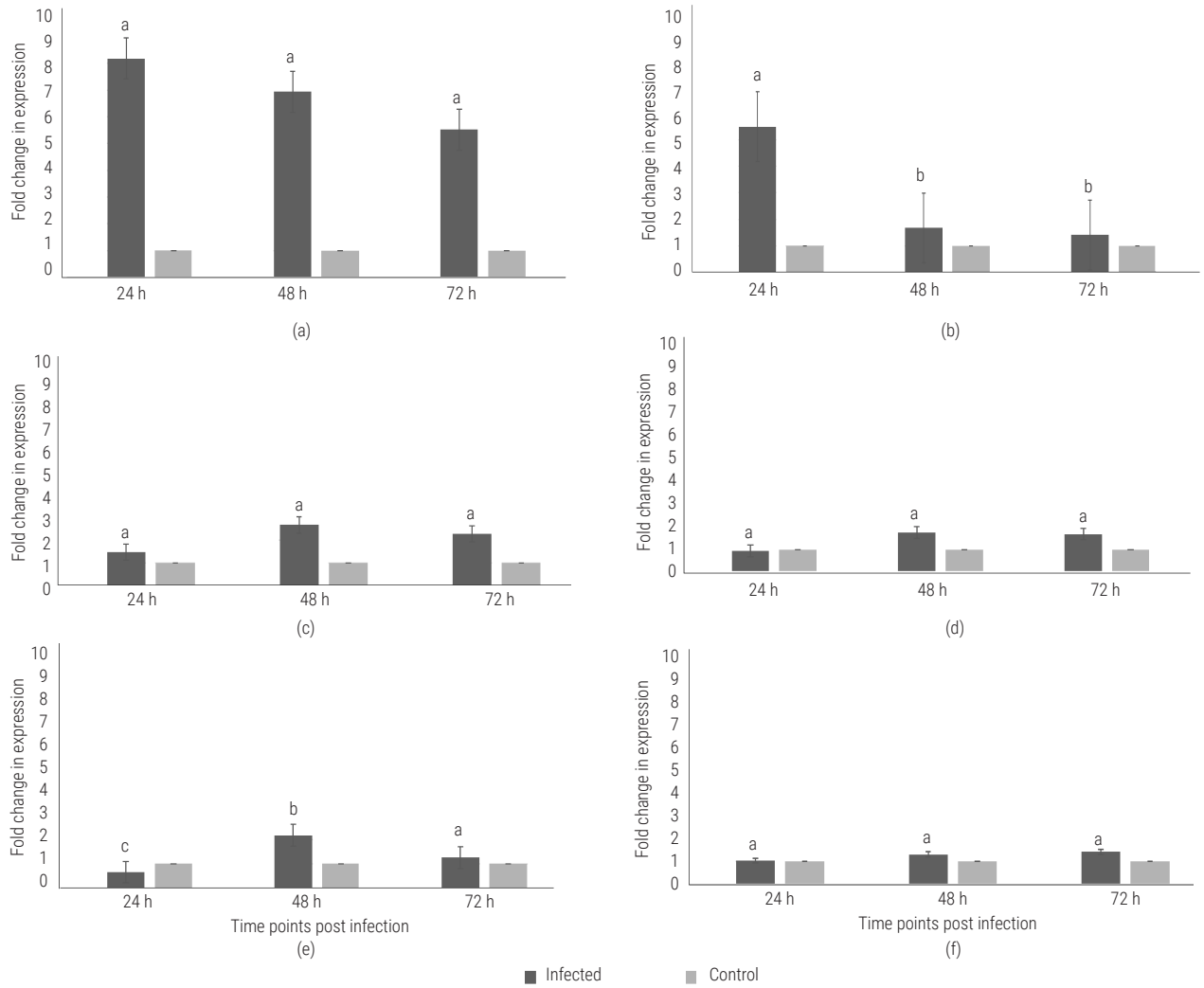

Fig. 7. Expression profile of *Ss*-Integrin in different tissues of mud crab *S. serrata* at different time-points following viral challenge with WSSV. (a) Haemocytes; (b) Heart; (c) Gill; (d) Hepatopancreas; (e) Gut and (f) Muscle. The expression level in the control group was set as the baseline (1) in the bar graphs. Different letters indicate significant differences ($p \leq 0.05$) between different time-points within a treatment group compared to the control. Error bars represent ±SD of three independent PCR amplifications

gene of mud crab *S. serrata*, which is comparatively more resistant to WSSV than other crustaceans like shrimp (Somboonna *et al*., 2010; Pratapa *et al*., 2023), and further elucidating the response of the gene to WSSV is significant. Sequencing revealed that the *Ss*-integrin cDNA has a 2349 nt ORF encoding a 782 aa polypeptide. The ORF recorded for *Ss*-integrin is found to be shorter than that reported for the other crab species, *Eriocheir sinensis* (ORF of 2457 nt coding 818 aa) (Huang *et al*., 2015). However, the domain architecture determined for *Ss*-integrin is similar to that reported for the integrin from *E. sinensis* by Huang *et al*. (2015). At the amino acid level, *Ss*-integrin showed 95.78 % identity, and the phylogenetic tree revealed a close evolutionary relationship between *Ss*-integrin and *S. paramamosain*. However, integrin genes from other crab species, crayfish and shrimp showed lower levels of sequence identity ranging from 50.69 to 74.46 %. The conserved domain architecture and relatively high amino acid sequence identity between *Ss*-integrin and integrin of other species indicate that, *Ss*-integrin may share functional characteristics with other members of the integrin family, particularly in processes related to signal transduction and immune responses.

Integrins have been reported to be distributed throughout various tissues in crustaceans with varying degrees of expression (Tang *et al*., 2012; Huang *et al*., 2015). Analysis of transcript abundance using conventional and qPCR assays showed that *Ss*-integrin was expressed in haemocytes and a range of tissues such as heart, gill, hepatopancreas, intestine and muscle of healthy mud crabs. A similar tissue distribution has been reported in *Fenneropenaeus chinensis* (Tang *et al*., 2017), suggesting *Ss*-integrin may have multiple biological functions. *Ss*-integrin was found to be highly expressed in haemocytes, consistent with observations in *E. sinensis* (Huang *et al*., 2015), *P. vannamei* (Lin *et al*., 2013; Zhang *et al*., 2012), *P. chinensis* (Tang *et al*., 2017), *P. monodon* (Shi *et al*., 2020), the insect *Ostrinia furnacalis* (Hu *et al*., 2010) and the tobacco hornworm, *Manduca sexta* (Levin *et al*., 2005). The predominant expression of *Ss*-integrin in haemocytes suggests a potential role in cell-mediated immune

responses in *S. serrata*, given that haemocytes are key effector cells involved in crustacean immunity.

Integrin receptors on host cells have been identified as important targets of WSSV, as several WSSV proteins, including VP187, VP31, VP110, VP33, VP36A, VP36B, and VP281, contain the RGD motif and have been reported to interact with shrimp integrins during infection (Huang *et al*., 2002; Tsai *et al*., 2004; Li *et al*., 2006; Xie *et al*., 2006). Studies by Tang *et al*. (2012, 2017) in *F. chinensis* and Li *et al*. (2007) in *Marsupenaeus japonicus* showed that *β*-integrin can specifically bind and interact with WSSV, suggesting a possible role as a cellular receptor or post-attachment receptor or co-receptor during WSSV infection. In the present study, *Ss*-integrin expression following WSSV challenge, was investigated across various tissues, with the most pronounced and significant upregulation observed in haemocytes at all time points. In contrast, *β*-integrin was reported to be downregulated at 24 and 96 h post-WSSV challenge in *P. (L.) vannamei* (Lin *et al*., 2013), indicating species specific differences in the regulation of integrin mediated responses to WSSV infection. The marked upregulation of *Ss*-integrin in haemocytes may indicate its involvement in the host response to WSSV, potentially through modulation of cell adhesion signal transduction and immune processes. However, whether the observed increase directly contributes to antiviral defence or influences WSSV attachment and entry remains to be experimentally established.

The high expression of *Ss*-integrin in haemocytes suggests that integrin might play a pivotal role in cell-mediated immune regulation in invertebrates (Zhuang *et al*., 2007). Haemocytes are considered crucial target cells for WSSVin shrimp and exhibit high expression of immune related genes (Zhan *et al*., 1999; Wang *et al*., 2002; Wen *et al*., 2013). In crustaceans, haemocytes and hepatopancreas are vital tissues involved in humoral immune responses, while the gills are also recognised as an important tissue associated with immune functions (Ji *et al*., 2009; Garcia-Orozco *et al*., 2012; Sun *et al*., 2015). In the present study, *Ss*-integrin expression in the gills was significantly modulated at all time points following WSSV challenge, whereas the changes observed in the hepatopancreas were not statistically significant. This pattern differs from the observations made in *F. chinensis,* where *Fcβ*Int mRNA was significantly upregulated in the gills post-WSSV challenge (Tang *et al*., 2012; Zhong *et al*., 2013). Previous studies have suggested that activation of integrin-related signalling pathways following WSSV exposure may facilitate viral infection in hosts by upregulating *β*-integrin expression (Tang *et al*., 2017). Silencing of shrimp integrin gene has been reported to reduce WSSV infection and increase host survival following WSSV exposure (Tang *et al*., 2017). However, whether *Ss*-integrin plays a similar role in WSSV infection *S. serrata* remains unclear. As *S. serrata* is a comparatively resistant host to WSSV, further functional studies, including integrin knockdown or blocking experiments, are needed to determine whether *Ss*-integrin contributes to WSSV attachment, entry, immune signalling or host defense.

In conclusion, the present study identified and characterised the integrin gene in *S. serrata* (*Ss-integrin*) for the first time. Our findings revealed baseline expression of *Ss*-integrin across multiple tissues with primary abundance in haemocytes and demonstrated significant transcriptional modulation following WSSV infection. While these results highlight *Ss*-integrin as a key virus-responsive component of host immune dynamics, transcriptomic observations primarily reflect cellular reactivity rather than direct mechanistic involvement. Further investigations employing targeted functional approaches, such as RNA interference and protein–protein interaction studies, will be essential to elucidate its precise role as a cellular receptor, diagnostic biomarker, or therapeutic target in mud crab antiviral strategies.

Acknowledgements

The first author sincerely thanks ICAR-CIFE, Mumbai, for awarding the Institutional Fellowship during the study period. The authors also express their gratitude to the Director/Vice-Chancellor of ICAR-CIFE for providing the necessary facilities and support to carry out this research.

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