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# Characterization of hemocyanin-like subunits in giant freshwater prawn *Macrobrachium rosenbergii*

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**Abstract**. *Macrobrachium rosenbergii*, a freshwater prawn, is one of the leading cultured and exported species of prawn in the world. Disease outbreaks such as the White Spot Syndrome Virus (WSSV) led to the decline of shrimp production. Shrimps and prawns only rely in the innate immune system against viruses and there is little knowledge about the genome of *M. rosenbergii.* Contig 13 (C13) and Contig 37 (C37), originally found in the genome of *Marsupenaeus japonicus* and of unknown identity and function, were predicted to be homologous to corresponding WSSV ORFs suggesting interactions between the virus and its host. The study aims to confirm the presence of C13 and C37 in *M. rosenbergii* and characterize the two contigs in the prawn. Through Rapid amplification of cDNA ends-Polymerase Chain Reaction (RACE–PCR), 1,256 bp for C13 and 1,118 bp for C37 were obtained from the sequences. Both contigs are predicted to be a hemocyanin gene and has a role in the innate host defense in shrimps and prawns. Basic Local Alignment Search Tool (BLAST) coupled with homologous sequences in NCBI-GenBank revealed the homology of each contig to hemocyanin. Multiple sequence alignment revealed several conserved regions of C13 and C37 with other hemocyanin of shrimp and prawn species. Interestingly, a maximum parsimony tree created a clade with C13 grouping with freshwater shrimp species, closely related to *Macrobrachium nipponense* with 81% bootstrap support; and C37 with saltwater shrimp species with a bootstrap support of 95%. Gene expression revealed that C37 is found to be expressed in the heart, hepatopancreas, muscle, intestine, hemocyte, and pleopods of healthy *M. rosenbergii* while the gene is found to be upregulated at the early stages of its WSSV infection.
**Key Words**: contig 37, contig 13, hemocyanin, *Macrobrachium rosenbergii*, WSSV.

**Introduction**. Prawn is an important commodity in most Southeast Asian countries where in *Macrobrachium rosenbergii* is one of the major exports. Cultivation of *M. rosenbergii* was pioneered by Thailand and Taiwan, and was later expanded to other neighboring countries such as the Philippines (FAO 2014). It proved beneficial to the country's economy as it can be an alternative to tilapia (Rosario & Tayamen 2004). However, production of cultured prawn declined as a result of pathogen infection, especially the white spot syndrome virus (WSSV) (Van Duijn et al 2012).

Infection of WSSV primarily targets almost all organs of the host including the gills, heart, hepatopancreas, muscle, intestine, hemocyte, lymphoid organ, and cells associated with the nervous system (Escobedo-Bonilla et al 2008; Reddy et al 2013). The susceptibility of the prawns to WSSV is because they only rely in their exoskeleton, hemolymph, and innate immune system as defense mechanisms (Supamattaya et al 2006; Sanchez-Paz 2010). Due to the lack of adaptive immune response of the shrimp and limited knowledge regarding the genome composition of *M. rosenbergii* (Jung et al 2011), genomic studies have recently been used to better understand the crustacean immune system. Use of genomic approaches, such as modern DNA-sequencing

technologies, can determine specific regions of a genome for a specific trait of interest (Stillman et al 2008).

In a study by Koyama et al (2010), a bacterial artificial chromosome (BAC) library was constructed to provide genetic information for the kuruma shrimp, *Marsupenaeus japonicus*. There were several identified open reading frames (ORFs) in *M. japonicus*, which were predicted to be homologous to WSSV ORFs. Two of the genes identified in kuruma shrimp, having an unknown identity and function, are Contig 13 (C13) and Contig 37 (C37). The homologies of C13 to WSSVORF5 and C37 to WSSVORF72 can have an involvement in host-WSSV interactions of the shrimp. Characterization of C37 in the study specifically aims to assess the expression levels through Reverse Transcription Polymerase Chain Reaction (RT-PCR); identifying the gene through Basic Local Alignment Search Tool (BLAST), and to align the sequence acquired through Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) were compared to other homologous genes for both contigs. The study contributes to the limited knowledge on host-virus interactions particularly in the freshwater shrimp, *M. rosenbergii*. It is also the first to report on the function of hemocyanin in a fresh water shrimp.

**Material and Method**. Shrimp sampling and subsequent experiments were done from August 2014 until March 2015.

***Sample collection***. Healthy juvenile *Macrobrachium rosenbergii* of 2-3 grams were collected from Southeast Asian Fisheries Development Center (SEAFDEC) Binangonan, Rizal, Philippines. The prawns were placed in a plastic container with 100 liters of aerated pond water and were transported to Thomas Aquinas Research Complex (TARC) of University of Santo Tomas, España, Manila. Subsequently, the prawns were acclimated in a re-circulating tank system in the laboratory maintained at about 25$^{o}$C and 0 ppt salinity suitable for freshwater prawn growth. Commercial shrimp feeds were used in feeding the prawn.

***RNA isolation and cDNA synthesis***. Heart, hepatopancreas, muscle, intestine, hemocyte, and pleopod were extracted from a healthy *M. rosenbergii* and Trizol reagent (Life technologies, USA) was used according to the manufacturer's protocol. Gel electrophoresis and nanospectrophotometry were used to determine the quality and concentration of the isolated RNA. SuperScript$^{TM}$ III Reverse Transcriptase (Invitrogen, USA) was used to synthesize first strand cDNA from corresponding amounts of total isolated RNA.

***Rapid amplification of cDNA ends-Polymerase Chain Reaction (RACE-PCR)***. Purelink RNA Mini Kit (Life Technologies, USA) was used to extract purified RNA from hemocytes in healthy shrimps. SMART$^{TM}$ RACE cDNA Amplification Kit (Clontech, USA) was used to synthesize 5' and 3' RACE-ready cDNA from the extracted RNA. A master mix from Advantage® PCR kit (Clontech, USA) was prepared as follows: 34.5 µL PCR-Grade water, 5 µL 10x Advantage 2 PCR buffer, 1 µL dNTP mix (10mM) and 1 µL 50x Advantage 2 polymerase mix. Subsequently, 41.5 µL of the master mix was added to the 5'- and 3'-RACE PCR Reactions with 2.5 µL RACE-ready cDNA, 5 µL 10x Universal primer, and 1 µL Gene-specific primer in Table 1 (10uM) (Reverse primer for 5'- and Forward primer for 3'-RACE PCR Reaction) for a total of 50 µL. Thermal cycler profile for the target genes were optimized as indicated: initial denaturation at 95$^{o}$C for five (5) minutes, denaturation 95$^{o}$C for thirty (30) seconds, annealing at 55$^{o}$C for thirty (30) seconds, extension at 72$^{o}$C for three (3) minutes for 30 cycles and final extension at 72$^{o}$C for five (5) minutes.

Table 1

Primers used in the study

| *Primer name* | *Oligonucleotide sequence* |
|---|---|
| Beta-actin F | 5'-AACTCCCATGACATGGAGAACATC-3' |
| Beta-actin R | 5'-TCTTCTCACGGTTGGCCTTG-3' |
| C13 F | 5'-GTCGATTCGGGATCACAGAC-3' |
| C13 R | 5'-CACGAGTCTCTTCCTCTTCG-3' |
| C37 F | 5'-GTAACGGGCTCAAAAGCAAC-3' |
| C37 R | 5'-GCATTCCGCTTTCCTTGTAG-3' |

***Bioinformatics analyses***. The RACE-PCR products were sent to Macrogen, South Korea for sequencing. The resulting gene sequence was analyzed using the Basic Local Alignment Search Tool (BLAST). The nucleotide sequences from varied organisms were translated into amino acid sequences and the longest ORF of C13 and C37 was determined using ExPASy. Parallel analysis for the Expasy/nucleotide BLAST results and determination of conserved sequences was done using Clustal Omega. Molecular Evolutionary Genetics Analysis (MEGA 6) was used to align the sequences and create a Maximum Likelihood phylogenetic tree. The organisms included in the tree were the ones displayed in the BLAST search.

***WSSV inoculum preparation and WSSV infection***. WSSV viral inoculum was isolated from infected shrimps. DNA was extracted from three infected specimens and was homogenized in PBS (24.3 mL, Phosphate Buffered Saline, pH 7.2, GIBCO, Life Technologies), pooled, and were centrifuged at 8,000 rpm for 10 minutes at 4$^{o}$C. The final supernatant solution obtained was filtered through a 0.45 µm Nylon Filter Media (Millipore Corp., USA). The filtrate (approximately 25 mL), containing the virus, was placed in an aliquot solution and stored at -80°C. This served as the experimental virus stock.

In vivo titration assay was done to determine the appropriate dose of WSSV for the mortality assay. WSSV stock was diluted with 1x PBS buffer ranging from $10^{-1}$ to $10^{-3}$ concentration for the experimental viral inoculum. $LD_{50}$ was performed to determine the proper dilution utilized for the expression of C37, establishing a serial dilution of $10^{-2}$ as the median lethal dosage. A total of twenty (20) healthy prawns each with a weight of 2-3 grams were intramuscularly injected in the 3rd abdominal segment with 100 µL of viral inocula.

***Gene expression analysis of WSSV-infected shrimp***. Three sampling days was done: day 0, 1, and 7. Day 0 post infection corresponded to the day two hours post-injection of WSSV. For each sampling day, tissues from the heart, hepatopancreas, muscle, intestine, hemocyte, and pleopod were collected from three randomly sampled shrimps. Expression of C37 in WSSV-infected shrimp was determined by RT-PCR and the products were visualized and analyzed through gel electrophoresis.

**Results and Discussion**. The nucleotide sequence of the target genes from *M. rosenbergii* were obtained through RACE-PCR (Figure 1). C13 comprised of 1,256 base pairs while C37 consisted of 1,118 base pairs. BLAST results show that the contigs are highly similar to the hemocyanin. According to Burmester (2002), there is much more variability in the subunit organization of crustaceans as compared to the structure of Chelicerate hemocyanin. In line with the results of nucleotide BLAST, a study by Zhao et al (2012) indicated a similar nucleotide identity of 67.0-86.0% in *Litopenaeus vannamei* hemocyanin with other hemocyanins (hemocyanin from *Penaeus monodon, Fenneropenaeus chinensis, Callinectes sapidus,* pseudo-hemocyanin of *Homarus americanus,* hemocyanin sub-units L and Y of *M. japonicus,* cryptocyanin from *Metacarcinus magister*) stating that the region was variable in different hemocyanin species.

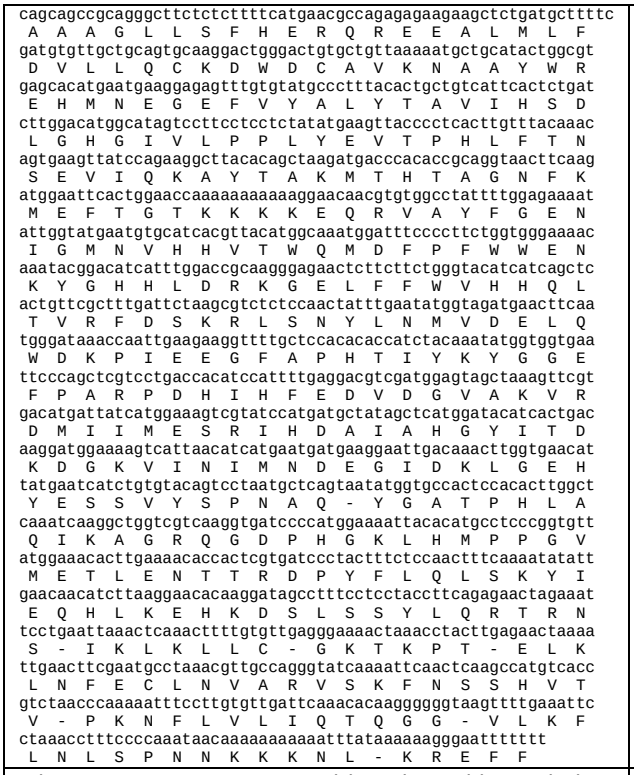


Figure 1a. cDNA sequence with amino acid translation of C13.

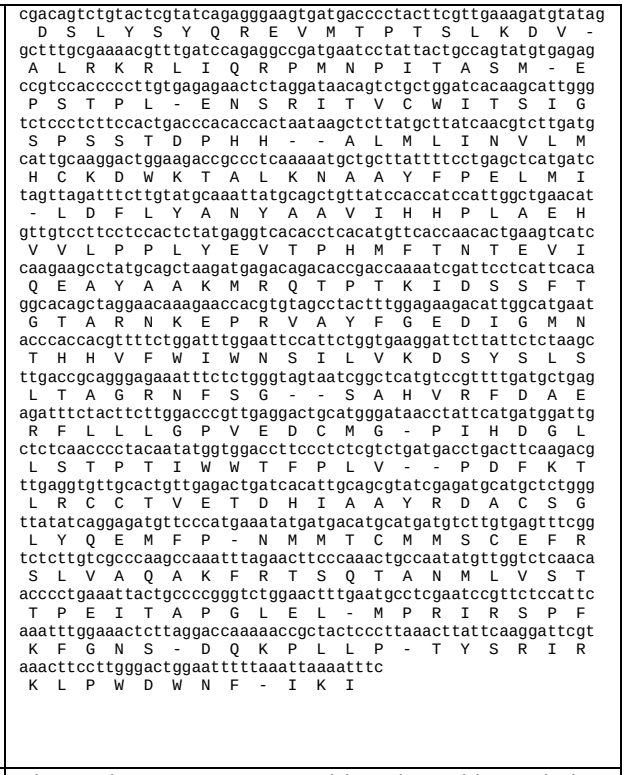


Figure 1b. cDNA sequence with amino acid translation of C37.

Nucleotide sequence obtained from *M. rosenbergii* was aligned with ten (10) other hemocyanin genes, shown in Figure 2. The alignment shows C13 and C37 to be highly similar to the other crustacean hemocyanin genes and yielded several conserved sequence motifs with the other sequences, which suggests that both contigs could possibly be the hemocyanin gene of *M. rosenbergii.* Hemocyanin is the main protein component of hemolymph and occurs as two oligomer, hexamers and dodecamers containing copper-binding proteins which primarily functions as an oxygen carrier (Marchler-Bauer et al 2013). It is made up of monomers of about 75 kDa (Sellos et al 1997; van Holde et al 2001). The range of 75 kDa is conserved within all arthropod hemocyanin, with cases that may vary due to association of hexamers (Markl & Decker 1992). From the multiple sequence alignment of C13 and C37 with the hemocyanin of the other species, there have been regions that were conserved. The phylogenetic tree showed that C13 clustered with *M. nipponense* with a 81% bootstrap support of 500 replicates; C37 clustered with *M. japonicus* with 95% bootstrap support. Based on the conserved regions and the phylogenetic tree, it suggests that C13 in *M. rosenbergii* is closely related to the hemocyanin of *M. nipponense* instead of *M. japonicus* while C37 is closely related to *M. japonicus*.

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C37              VERCIGFAKTFDPEADESYYCQYVRAVHPLVREL*DNSLLDHKHWVSLFH*PTPLISSYA  73
F. chinensis     DPNLKGKADSFDPEADLSHYSDSGEAVHKLIRDLKDHRLLEQNHWFSLLSPRXAS*STYA  113
M. japonicus     DDALKAKADSFDPEADLSHYSDDGEAVHTLIRDLKDDRLLQQKHWFSLFNSRXAS*STYA  114
P. monodon       DSNLKGKADSFDPEANLSHYSDGGKAVQKLMRDLKDNRLLQQRHWFSLFNPRXTRRSTYA  116
L. vannamei      DSDLKAKADSFDPAADLSHYSDGGEAVQKLVRDLKDDRLLQQRHWFSLFNPRXAS*STHA  105
P. leniusculus   YTDLKGIAGTFSPEADTSIYTDDGAAAHVLMEELRDGRLLEQHHWFSLFNTRXT*RSYHA  115
H. americanus    YSDLKQISETFSPEADTSMYTDGGTAAHHLMEELNDHRFLEQHHWFSLFNPRXA*RSSHA  104
C. multidentata  DGELKTIAGSFTPEADKSIYTDGGEAVHHLVQELNDHRLLEQHHWFSLFNERXERGSSNV  118
A. moluccensis   DGELKSIAGSFNPEGDKSIYSDGGEAVHHLVQELNDHRLLEQHHWFSLFNSRXTRGGTHV  117
E. carinicauda   FDDLKGYAASFDPVADKSQYKDGGEAAEHLVQEYKDHRLLEQHHWFSLFNERXKRRGPYA  106
C13              ----------------------------------------XQPQGFSLFXERXERRSSDA  20
M. nipponense    FDDLKGYASSFNPEGDTSMYKDGGEAVHHLAKEYKDHRLLEQHHWFSLFNERXEGRSSDA  102
                                                         : : .**:      .  .

C37              YQRLDALQGLEDRPQKCCLFS*AHDLVRFLVCKLCSCYPPSIG*TCCPSSTL*GHTSHVH  130
F. chinensis     LRCSHSLQGLGYICQQCSLLPSAYERGRVCLRLVCCSHPLSSG*TRCTSSTL*GRSSSLH  171
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M. japonicus    IRCPHSLQGLGYIRQQCCLLPPTYE*GRVCQCLVCCGHPLILG*VCCTSTSV*GYPSPLY  171
P. monodon      F*CSHSLQGLGYICQQCCLLPPNYE*GRICLCLVCCGHPLTSV*TRCTSSTL*SHATSLH  172
L. vannamei     FRCSHSLQGLEYICQQRCLLPSAYE*RRVCLCSVCCSHPLTSD*ARCTSSTL*GHASPLH  162
P. leniusculus  L*SSDSL*VLGVLC*KRCLLP*AHE*RRVRVCTLCGCHPH*PGTWHCTSSTLRSHSSPVH  169
H. americanus   F*CPDALQVLGVLC*QCRLLP*TYE*RRIRLRTLYSSYPL*VGTRDCITSTLRNHSPYVH  159
C. multidentata I*RLYALQELDLFCQQCCLLARKDERRRICICPLHSSHTF*SG*GSRLASSL*SYSTLVH  174
A. moluccensis  V*CSHALQELELLCQQCCLLA*KNE*GRICLRTLRSCHSF*RG*GSRLTSSL*SYSPPVH  171
E. carinicauda  LRCLHAMQDLGLCCS*CCILA*TYE*RRVCLCFVHSCYSLRPWTWHCSSPTL*SYPSYVH  162
C13             FRCVAAVQGLGLCC*KCCILA*AHE*RRVCVCPLHCCHSL*SWTWHSPSSSI*SYPSLVY  75
M. nipponense   FRCIHAVQGLGLCC*KCCILA*AHE*RRVCVCPLHCCHSL*SWTWHCSPPTL*SYSSHVH  157
                     ::  *       ::    :  *.    :   :           ::      ::

C37             QH*SHPRSLCS*DETDTDQNRFLIHRHS*EQRTTCSLLWRRHWHEYPPRFLXFGIXILVX  187
F. chinensis    *Q*RH*SSLSCQADTDPW*IPVFLYWNKEEP*TKSSLFRRGHWNEHSPRYMAYGIPILVA  226
M. japonicus    QQ*GYRGSLSC*ADTKAR*IQVVLYWN*EEP*TKSCLLWRGHWNEHSPRDLAYGIPILVA  226
P. monodon      QQRSH*SSLSCQANTKTW*I*V*LHWN*EES*TKSSLFRRGHWNEHSSRHLAYGIPILVG  226
L. vannamei     *QRSH*ISLSCQADTKTW*I*V*FHWNQEES*TESSLFRRGHWNEHSPCYLAHGIPILVG  216
P. leniusculus  QQ*SHQ*GLLSQDDPDTRQIQHGFHWNKKEQGTACGLLW*GYWHEHSPRYLAYGLSLLVE  226
H. americanus   QQ*NHPQGLHCQDDPDTRQV*DEIHRDEKEQGTKSGLLR*GHWPQYSPRYLAYGLPLLVE  216
C. multidentata QQ*NYPESIYC*DDTHSWKIPHGIHWNQEK*GTACSILWRRYWYEYSPRHLAYGLPILVE  231
A. moluccensis  QQ*NHPASLYC*DD*HSWKIPHGIHRNQKK*GTTRGLLWRRYWYEYSPRHLAYGLSILVE  227
E. carinicauda  KQ*NYPKGLHSKDD*STR*I*DGVHWYKEK*GTTCGLLWRRYWNESPSRHMAYGLSLLVG  217
C13             KQ*SYPEGLHS*DDPHRR*LQDGIHWNQKKKGTTCGLFWRKYWYECASRYMANGFPLLVG  132
M. nipponense   KQ*SYPESLHRQDDQYPR*LLYGVHWYQKEQGTTCGLLWRGHWHECAPRHLAYGLPLLVG  215
                 :  :  .:    :         .:    :  *   ::   :* :    :  *: :**

C37             GFLFX*A*PQGEXXSLGX*SAXCPF*C*EISXLLGPX*XTAXG*XYS*WIXS-QPLQXWW  238
F. chinensis    R*IQSSSGSQRRELLLGTSSSCRSL*C*TSLQLFGSRRRTSLGEAHRTRFCSPHHLQVWR  283
M. japonicus    GQIQPSSGSQRRELLLGSSSTYRPF*CRTSFQLLGSCR*ASLGKAHRTRLCSPHHLQVWR  284
P. monodon      RQIQPSSGSQRREFLLGTSSTYRSF*C*TSLQLFGSRRRTSLGEANRTRFCSPHHLQVWR  284
L. vannamei     RQI*PSSGSQRRELLLGTSSTYRSL*CRTSLQLLRSRRGT*LG*THRTRFCSSHHLQVWR  272
P. leniusculus  GLLRLPSGPQGRALLLGSSPTYCSL*L*ASLQLAGCC**TTLGRRYPRRICPTY*LQVWW  281
H. americanus   GLLWLPS*SQGRAILLGPSSTHCTL*R*APL*LARPS*RASLGAYYS*RFCTTYQLQVWW  270
C. multidentata GFLWLSS*S*GRALLLGPSSTYNSL*C*AFIQLARPCR*TSLGKSH*RRFRSTYHL*VRR  284
A. moluccensis  GFLWLPSGSQGRTLLLGSSSTYCSF*C*TSLQLS*PS**TSLGETH*RRFCSTYHLQVWR  281
E. carinicauda  RQIWRTLGSQGRTFLLGSSSVDCSL*FRASLQPFRYGG*TPVGETSRRRLCPTHYLQVWW  275
C13             KQIRTSFGPQGRTLLLGTSSAHCSL*F*ASLQLFEYGR*TSMG*TN*RRFCSTHHLQIWW  187
M. nipponense   RQVRTSLGPQGRTLLLGTSSAHCAL*F*ASLQLFGHGR*TSMGETS*GRICPSHYLQIWW  271
                  :        .   **       :*            :  *      :     *

C37             XPSLSX**PXLXRR*XCCTXLRXDHIAXXYXRCXCSGLYXXRCSHEI**HA*XXSCEFRS  292
F. chinensis    SVPLSSR*CKLRGCGWCCSNSRSAHC*EPNS*CYCPRLYY*QTRQSH*HHE*AWH*HSWR  336
M. japonicus    SVPLSSR*CTIRGCGRRCPNSRLAHC*EPNP*CYCPRLHCRQGRQTH*YHE*AWY*RPWR  338
P. monodon      SVPLSSR*CRLRGCGWCCSYSRLAYCREPNPRCYCTWLYRRQGW*SY*YHE*AWN*RSWR  339
L. vannamei     SVPLSSR*CRLRGCGRCCSHSRLAYYREPNSRCYCPRLYCR*GWQSH*HHE*AWNRRPWR  328
P. leniusculus  RVPSTS*QCTLRRRGWCS*SA*HGYLRESYSRRHCSWLHH*PQWQQNRHQKRAWHRHPW*  336
H. americanus   *VPCTS*QRTL*GCGRCSSRA*HDYHRESHS*RHCSRLRDRQSW**HQYQE*SWH*RPRG  321
C. multidentata *VPSSS*SHSF*RCRWSS*SS*HDHHGEPYSRRHCTRIH*G**RLSH*HHE*SWH**TGR  332
A. moluccensis  RIPCSS*QHSF*RRRWSSKSSRHDHHREPYS*CHCSRIH*S**WFSH*HRK*AWY*QTR*  331
E. carinicauda  RIPCPS*PYSL*RC*WSSKSS*HGYYRKSHP*CYSSWIHHRQRWQSY*YHE*PRR*QTW*  326
C13             *IPSSS*PHPF*GRRWSS*SS*HDYHGKSYP*CYSSWIHH*QGWKSH*HHE**RN*QTW*  235
M. nipponense   RIPSPS*PHPL*GR*WRSSSS*HDYHGKSYP*CYISWIHH*QGWKSHRHHE*RGH*QTW*  322
                         :     .     :           :          :

C37             LVAQXQI*NFPNCQYVXSQQPX*NYCPGSGTLNASNPFSIQXXWKLLGPKTATX-XLNLF  349
F. chinensis    XIIESSMYSPNVQYYGXFA*YCSYXYSVDRPIHMENTLYHLVY*NILKLPHVIXSFFRLH  394
M. japonicus    XIIESSLYSPNVQYYGXLA*YCSHXCLVDRVIPMANTICPQVC*NTSKLPPVTXSFFRLH  396
P. monodon      XVIESSLYSPNVQYYGXLAQYCSHXYLVDRVIHMENMIYPLVCWNTLKLPPVIXSFFRLH  399
L. vannamei     XVIESSLYSPNVQYYGXFAQYCSHXYLVDRVILMESTLYPLVY*NTLKLPPVIXSFFRLH  387
P. leniusculus  XIIESSVYSPNVQYYGXPAQHSSHXCLVAKVILTASSTCLQV*WNTSRLPPATXSFFRLH  395
H. americanus   XIIESSVYSPNAQYYGXTAQHCSHXCLVDKVIPMANSTCLPV*WNTLRLPPATXSFFRLH  380
C. multidentata XIIESSLYSPNRQYYGXPPQSCSHXCLAARAILMENTTCLLVSWNILKQLHVIXSFFRLH  392
A. moluccensis  XIIESSLYSPNIQYYGXASQLSSHXCLVARAILMENTTYLLVSWNTLKLPPAIXSFFRLH  391
E. carinicauda  XIIESSMYSPNVQYYGXSPQHGSHXCLAVKEILMENTICLQV*WNILKQLLVIXTFFRLH  385
C13             XIXESSVYSPNAQ*YGATPHXGSNXRLVVKVIPMENYTCLPVLWKHLKTPLVIPTFSNFX  294
M. nipponense   XIIESSMYSPNAQYYGXTP*YCSRXCLVVKVIPMENTTCLQVLWNTLKQPPVIXTFFRLH  381
                 : : .: .     *              :   .       :      .     .:
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C37              KDXX*TSLGLEFLN*NF----------------------------------------  364
F. chinensis     KYXG*YLQRT*GFSSPILKGRINFHRC*C*KSIR*W*IRDLL*GL*VQSY*CC*RH*RNC  443
M. japonicus     KYXG*HLQGTQGHSPSLYCRRTDICWC*C*QYCNRGCTRNVLRGL*IQSY*CC*RH*TNS  449
P. monodon       KYXG*HLQGTQGQSPSIHRGRTNICRCKCRQCSN*RRTRDLLRGFRIQSH*RC**H*TNS  453
L. vannamei      KYXG*HLQGTQGQSPSLHSGRTNICRCKCRQRSN*RRTRDLLRGLRIQSY*RRR*H*TDC  442
P. leniusculus   KYXGQHL*RTQRHPSSLHQRGHRFPWSGLGQCYH*WGTQDIL*HFRV*SC*CCRPV*QGG  449
H. americanus    KYXG*HL*GTQGLTSSLHQS*H*VYWCGG*RS*NCWAAKYIL**I*IQSRQCCG*IRKSS  430
C. multidentata  KYXG*YF*RT*GLTTPIHKRGTRIPRN*RRKHWC*GRTQNIL*RL*IRPS*CCRQC*RY*  442
A. moluccensis   KYXG*HLQRT*GFTSSLHKRRLGFPWS*R*EHWCSGRTQDLL*RL*I*PS*CC*QC*RYC  441
E. carinicauda   KYXG*HL*GT*I*SSSIHS*RPRLPWCQH**P*H*GRA*NIL*GL*I*PSKCCRLC*RYC  431
C13              KYIEQHLXGTQG*PFLLPSXELEIPXN*TXKLLC*GKTKXLLXELXN*TSXCXKRCXGYQ  350
M. nipponense    KYXGQHLQGTQG*PSSLHS*RLGFPWR*HRKCWC*WRAKNLL*AL*IRSAQCSRQCRRHR  435
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Figure 2. Conserved regions observed in Contigs 13 and 37 with hemocyanin amino acid sequences of related shrimp and prawn species. An asterisk denotes identity in all sequences of the alignment; colons denote conserved substitutions; full stops denote semi-conserved substitutions. Accession numbers include *Macrobrahium nipponense* (JF683437.1), *Exopalaemon carinicauda* (JF939200.1), *Caridina multidentata* (HE650712.1), *Atyopsis moluccensis* (HE650707.1), *Marsupenaeus japonicus* (EF375711.1), *Fenneropenaeus chinensis* (FJ594414.1), *Penaeus monodon* (JF357966.1), *Litopenaeus vannamei* (HQ709161.1), *Pacifastacus leniusculus* (AY193781.1) and *Homarus americanus* (AJ272095.1).

The nucleotide sequence of C13 and C37, together with the sequences of the ten (10) shrimp and prawn species from nucleotide BLAST results, was used to construct a maximum parsimony tree. As shown in Figure 3, *M. rosenbergii* C37 clustered with *M. japonicus*, supported with a 95% bootstrap value. It is also shown that the hemocyanin of *M. rosenbergii* and *M. nipponense* is related to the hemocyanin of the group containing *Exopalaemon carinicauda, Caridina multidentata, Atyopsis moluccensis, F. chinensis, P. monodon* and *L. vannamei*.

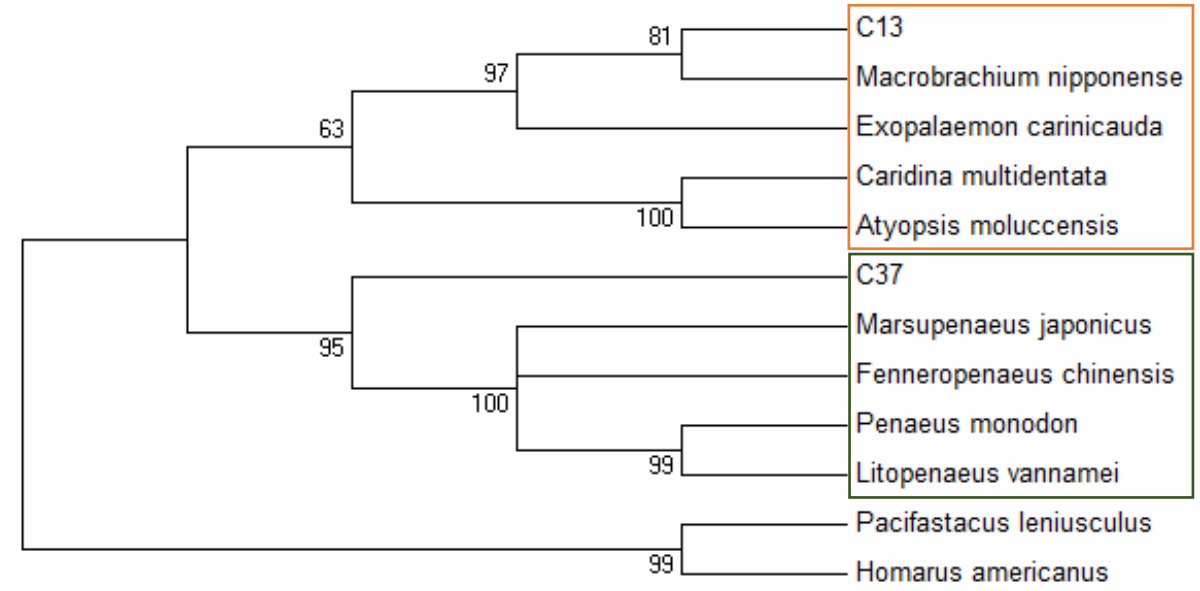


Figure 3. Maximum Parsimony tree of *Macrobrahium rosenbergii* C13 and C37 with ten other hemocyanin of shrimps and prawns and relative species, crayfish and lobster. The values in the branches represent a bootstrap value of 500 replicates. Accession numbers include *Macrobrahium nipponense* (JF683437.1), *Exopalaemon carinicauda* (JF939200.1), *Caridina multidentata* (HE650712.1), *Atyopsis moluccensis* (HE650707.1), *Marsupenaeus japonicus* (EF375711.1), *Fenneropenaeus chinensis* (FJ594414.1), *Penaeus monodon* (JF357966.1), *Litopenaeus vannamei* (HQ709161.1), *Pacifastacus leniusculus* (AY193781.1) and *Homarus americanus* (AJ272095.1).

Gene expression analysis through Reverse-transcription Polymerase Chain Reaction (RT-PCR) shows the expression of C37 in various organs of the prawn namely heart, hepatopancreas, muscle, intestines, hemocyte and pleopods as shown in Figure 4. Contig 37 is ubiquitously expressed in the vital organs of a healthy prawn which suggests an importance in the physiological processes involved in the survival of the prawn.

Furthermore, gene expression analysis of C37 in infected prawns shows an up-regulation of the hemocyanin gene in the hepatopancreas during the early periods of infection (Figure 5).

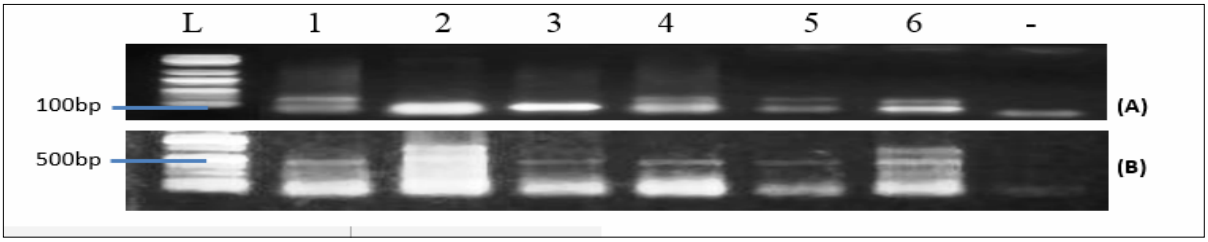


Figure 4. Gene expression of C37 in healthy *M. rosenbergii.* (A) Beta-actin served as positive control at 150 base pairs. (B) Gene expression of C37 from various organs at 500 base pairs (1-heart, 2-hepatopancreas, 3-muscle, 4-intestine, 5-hemocyte, 6-pleopod).

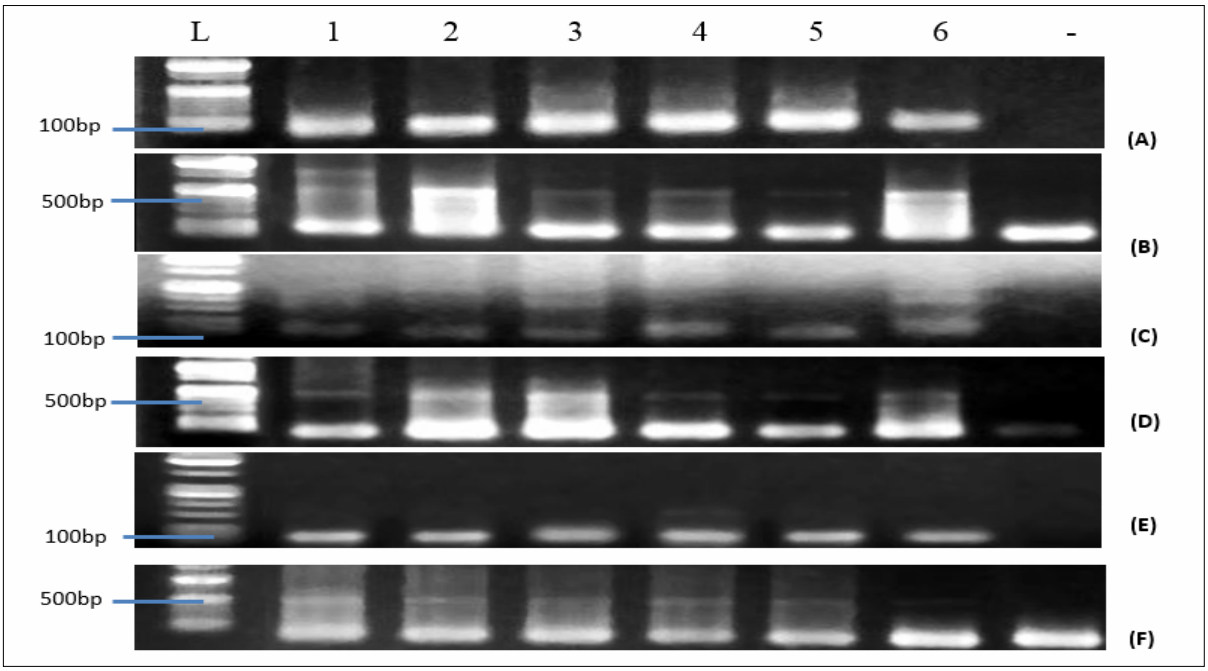


Figure 5. Gene expression of C37 in WSSV-infected shrimps. Beta-actin served as positive control at 150 base pairs in A, C, and E for days 0,1, and 7 respectively Gene expression of C37 from various organs at 500 base pairs in B, D, and F for days 0, 1, and 7 respectively (1-heart, 2-hepatopancreas, 3-muscle, 4-intestine, 5-hemocyte, 6-pleopod).

Since WSSV may induce the aggregation of hemocytes at the sites of infection (van de Braak et al 2002), hemocyanin is upregulated at its early stages. Gene expression of WSSV-infected shrimps at day 0 and day 1 had more distinct and solid bands as compared to the expression in the healthy shrimp. Strong expression of the gene in the hepatopancreas at day 0 supports its physiological function in the hemocyanin synthesis (Spindler et al 1992; Sellos et al 1997). Upregulated clottable protein at the early infection stage may indicate an innate immune response of the host (Robalino et al 2011; Lei et al 2008). RT-PCR results for gene expression on day 7 samples still showed consistent bands at 500 base pairs but not as distinctly expressed as in day 0 and day 1. The faint bands signify its downregulation at the late infection stage. This explains why hemolymph from shrimp at this stage of infection does not coagulate anymore (Robalino et al 2011; Yoganandhan et al 2003). According to Aoki et al (2011), the expression of hemocyanin was indeed suppressed in WSSV-treated shrimps, which is consistent with the results of this study. Moreover, Pan et al (2005) founded that the antiviral activity of WSSV-resistant shrimp may be due to strong expression of hemocyanin in the hepatopancreas. WSSV may suppress the expression of hemocyanin in order to complete its viral replication in WSSV-susceptible shrimps (Aoki et al 2011). Day 7 was considered

to be at the late stage of infection because based on the study by Wu et al (2005), WSSV can already cause 100% accumulative mortality two to ten days post-infection. A research by Zhang et al (2004) revealed that hemocyanin was unable to inhibit virus replication completely, but it is still a potent virus inhibitor in virus-infected shrimps.

**Conclusions**. Contig 13 and Contig 37 in *M. rosenbergii* is predicted to be the subunits of hemocyanin gene. Together with the presence of phenoloxidase in the hemocyanin, these can indeed function as an innate host defense in shrimps and prawns. To further support the results of this study, silencing of the two contigs is recommended. On the other hand, RNA interference can be employed to support its identification as a hemocyanin and determine if shrimp mortality will increase if C13 and C37 will be silenced.

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