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Identification and *in silico* characterization of hemocyanin γ-type subunit protein in *Vibrio harveyi* infected freshwater prawn, *Macrobrachium rosenbergii*

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Abstract

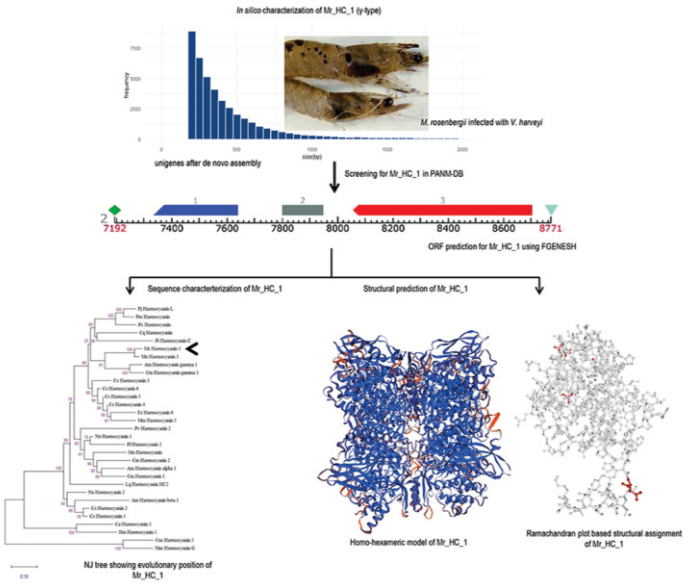
Aim: Identification of full-length ORF of hemocyanin subunit-1 (*Mr_HC_1*) from the hepatopancreas transcriptome of freshwater prawn, *Macrobrachium rosenbergii* infected with *Vibrio harveyi* and characterization of its sequence and structure by *in silico* tools and softwares.

Methodology: Illumina HiSeq and de novo assembled unigenes were scanned against PANM-DB to screen *Mr_HC_1*. FGENESH gene prediction and SMART programs were used to predict the ORF region. Subsequently, Clustal X2 and MEGA *in-silico* tools were used to understand the sequence relatedness and evolutionary status of *Mr_HC_1*. Structural prediction was performed by SWISS-MODEL and Ramachandran plot modeling programs.

Results: The full-length ORF was 1983 bp in length encoding a polypeptide of 661 amino acid residues. Mr_HC_1 showed a putative signal peptide of 21 amino acid residues at the N-terminus and three hemocyanin domains. Homology analysis of Mr_HC_1 amino acid sequence confirms maximum identity to *M. nipponense* hemocyanin subunit-1 (Mn_HC_1). Phylogenetic analysis showed that Mr_HC_1 is more closely related to the hemocyanin γ-type subunit of freshwater shrimps. Homology modeling of Mr_HC_1 showed homo-hexameric protein containing 12 copper ions. With a QMEAN score of -3.33 and model-template sequence identity of 59.15%, the predicted model of Mr_HC_1 is convincing.

Interpretation: This study characterizes the hemocyanin γ-type subunit protein of freshwater prawn, *M. rosenbergii* for future studies on host defense mechanisms.

Key words: Hemocyanin subunit-1, *In silico* studies, *Macrobrachium rosenbergii*, Structural prediction, Transcriptome



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Introduction

Crustaceans, like other invertebrates solely depend on the innate immune mechanisms for defense against pathogens. The innate immune cascade recognizes the exogenous pathogens (signal) as non-self and activates the downstream signaling components (either humoral or cell-mediated) to eliminate the pathogens (either by transcriptional regulation of antimicrobial proteins or phagocytosis). Hemocyanin is a copper-containing protein in the hemolymph of crustaceans, which turns blue in color when oxygenated. Hemocyanin protein families have been found distributed within the 'Malacostraca', 'Remipedia', and 'Ostracoda' classes of Crustacea (Marxen *et al.,* 2014). The multidimensional role of hemocyanin is well known encompassing oxygen transport, osmoregulation, melanin synthesis, exoskeleton formation, agglutination property and antiviral functions (Zanjani *et al.,* 2016; Coates and Decker, 2017; Booncheun *et al.,* 2018). The C-terminal sequence of shrimp hemocyanin under microbial challenge has been extensively used to derive antifungal, antibacterial and antiviral peptides (Yang *et al.,* 2018; Zhan *et al.,* 2019).

Another hemocyanin derived peptide from *Litopenaeus vannamei* (B11) inhibits cancer cell proliferation causing mitochondrial dysfunction and induced apoptotic cell death (Liu *et al.,* 2018). Further in crabs, the oxygen transport functions of hemocyanin is converted to phenoloxidase during bacterial challenge and/or molting when there is a rapid need for sclerotization (Terwilliger, 2007). The common ancestral node for prophenoloxidase sequences and arthropod hemocyanin gene family explains the redundancy of immune functions between the gene families (Terwilliger and Ryan, 2006). Further, understanding the direct correlation of degrees of tolerance to hypoxia or other environmental perturbations to the differences in the regulation and function of hemocyanin is critical for suggesting physiological plasticity in crustaceans (Wang *et al.,* 2019). Hemocyanin genes have been identified, cloned, and functionally characterized in crustacean species like white-leg shrimp, *L. vannamei* (Wang *et al.,* 2019), oriental river prawn, *Macrobrachium nipponense* (Kong *et al.,* 2016), banana shrimp, *Fenneropenaeus merguiensis* (Jiewkok *et al.,* 2015), Chinese mitten crab, *Eriocheir sinensis* (Huang *et al.,* 2014), and wood shrimp, *Atyopsis moluccensis* (Marxen *et al.,* 2013).

Hemocyanin protein families have been identified and characterized from the transcriptome of some of the crustacean species such as the fish louse, *Argulus foliaceus* (Pinnow *et al.,* 2016), penaeid shrimps *L. vannamei, Farfantepenaeus aztecus* (Johnson *et al.,* 2016), and *Cleidogona* species (Scherbaum *et al.,* 2018). Most of the available literature have understood the phylogeny of hemocyanin protein from different species under α, β, and γ categories and some of them have elaborated on the direct role of copper and hemocyanin to mount an enhanced immune response against pathogens (Sun *et al.,* 2013). It is suggested that copper supplementation in the diet can lead to enhanced immune response of shrimps and other shellfishes against pathogenic load widely accustomed due to intensive farming practices. This is a case for disease control through dietary management in shrimp farming (Lall, 2002). *Macrobrachium rosenbergii* is a leading cultured freshwater prawn with substantial export potential. Due to faster growth rate, better defense against pathogens, and tolerance to wide range of temperatures, the production of this species has increased substantially with greater distributional ranges (Banu and Christianus, 2016). The post-larval prawns have been found susceptible to bacterial pathogen *Vibrio harveyi* and *M. rosenbergii* nodavirus (MrNV) due to semi-intensive and intensive farming in most developing countries (Sahul Hameed *et al.,* 2004; Murwatonko *et al.,* 2016; Ajadi *et al.,* 2019). A transcriptome analysis of *M. rosenbergii* hematopoietic tissue (hepatopancreas) was conducted before and after *V. harveyi* challenge using Illumina HiSeq platform to study the differential expression of genes and screen the immunity-related transcripts (Baliarsingh *et al.*, 2020). A hemocyanin subunit-1 protein was discovered from the transcriptome study of *M. rosenbergii*.

The protein was analyzed at the level of sequence and structure for future studies on the role of this protein in innate immunity. Earlier, hemocyanin protein has been purified from hemolymph of *M. rosenbergii* by column chromatography using different matrices (Ramasamy *et al.,* 2017). A transcriptome study of *M. rosenbergii* hepatopancreas in response to *V. parahaemolyticus* infection also characterized hemocyanin among other immune-related genes with a positive fold-change of 1.21 (Rao *et al.,* 2015). This study therefore reported a full-length open reading frame (ORF) of hemocyanin subunit-1 (Mr_HC_1) from the hepatopancreas transcriptome of freshwater prawn, *M. rosenbergii* infected with *V. harveyi* and characterizes its sequence and structure using *in silico* tools and softwares.

Materials and Methods

Screening of *V. harveyi* challenged *M. rosenbergii* hepatopancreas transcriptome: The non-redundant unigenes derived from *V. harveyi* challenged *M. rosenbergii* hepatopancreas transcriptome were annotated against the locally curated Protostome database (PANM-DB) using BLASTx at an E-value threshold of 1.0E-5. The PANM-DB annotations were screened for identification of unigenes showing homology to hemocyanin subunit-1 protein. The unigenes having id as MR_VH_Uni_04607 and MR_VH_Uni_04608 were annotated as hemocyanin subunit-1-like protein from *M. rosenbergii*. Only MR_VH_Uni_04608 was found to contain the full-length ORF of hemocyanin subunit-1 protein from *M. rosenbergii* and was considered for *in silico* analysis.

Sequence features and phylogenetic analysis: The FGENESH eukaryotic gene prediction software was used to predict ORF region of MR_VH_Uni_04608 (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). The predicted coding region with deduced amino acid sequence of polypeptide was analyzed using BLASTn and

BLASTp suite to confirm the prediction of ORF. The complete cDNA sequence was formatted using UltraEdit64-bit text-editor. For Expert Protein Analysis and reverse complement of nucleotide sequence ExPASy Translate tool was used (http://www.expasy.org). Prediction of conserved protein domains was accomplished by Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/). The Multiple sequence alignment (MSA), percent identity and distance matrix were analyzed by ClustalX 2.1 program for Windows (http://www. clustal. org/ clustal2/).

The deduced amino acid sequence of Mr_Hc_1 from *M. rosenbergii* and other invertebrate hemocyanin sequences acquired from NCBI database were aligned by software ClustalX. Phylogenetic tree was constructed based on the amino acid sequences of hemocyanin from the representative species using Neighbor-joining method (Poisson correction technique) with the bootstrap trial set to 1000, and with Molecular Evolutionary Genetics Analysis (MEGAX) (http://www.megasoftware.net/). Homology protein model was predicted using SWISS-MODEL interface (https:// swissmodel. expasy. org/) based upon the reference template (PDB id: 1hc1.1A, Arthropod hemocyanin) and that was continued for Ramachandran plot assessment of the predicted model (https://swissmodel.expasy.org/).

Results and Discussion

The hemocyanin subunit-1 like protein (Mr_HC_1) was identified from the annotated unigenes of *V. harveyi* infected *M. rosenbergii* hepatopancreas transcriptome. The unigene (MR_VH_Uni_04608) showed 100% identity with the hemocyanin subunit-1 protein from *M. rosenbergii* (GenBank Accession: AJG06859.1). Four cDNAs (of 2281, 2002, 2184, and 2069 bp) encoding distinct hemocyanin subunits have been identified from EST library of *Scylla paramamosain* and characterized by cloning and RACE-PCR (Wang *et al.,* 2015). Analysis of Crustacean annotated transcriptome (CAT) database released in the form of a graphical user interface at http://cat.sls.cuhk.edu.hk/ identifies hemocyanin subunits from a total of 71 transcriptome assemblies in the coral shrimp *Stenopus hispidus*, the cherry shrimp *Neocaridina davidi*, the redclaw crayfish *Cherax quadricarinatus*, the spiny lobster *Panulirus ornatus*, the red king crab *Paralithodes camtschaticus*, the coconut crab *Birgus latro*, and zebra mantis shrimp *Lysiosquillina maculata* (Nong *et al.,* 2020). Further, the transcriptome sequencing approach has characterized the differential regulation of hemocyanins from ~6 to 25,000 reads per kilobase per million reads (Scherbaum *et al.,* 2018). Although Mr_HC_1 was not screened as a differentially expressed transcript after *V.*

Table 1: Accession number of hemocyanin genes used for percent identity matrix, percent distance matrix and phylogenetic analysis

Species	Gene name	GenBank accession number
Macrobrachium nipponense	Hemocyanin subunit-1	AGA17871.1
Atyopsis moluccensis	Hemocyanin gamma subunit-1	CCF55383.1
Caridina multidentata	Hemocyanin gamma subunit-1	CCF55387.1
Penaeus chinensis	Hemocyanin	ACM61982.1
Penaeus monodon	Hemocyanin	AEB77775.1
Cherax quadricarinatus	Hemocyanin	AFP23115.1
Panulirus interruptus	Hemocyanin subunit c	AAB22190.1
Eriocheir sinensis	Hemocyanin subunit 6	AEG64817.1
Metacarcinus magister	Hemocyanin subunit 5	AAW57893.1
Palincurus vulgaris	Hemocyanin subunit 2	CAC69244.1
Pacifastacus leniusculus	Hemocyanin 2	AAO47336.1
Macrobrachium nipponense	Hemocyanin	AEC46861.1
Caridina multidentata	Hemocyanin alpha subunit-2	CCF55385.1
Atyopsis moluccensis	Hemocyanin alpha subunit-1	CCF55379.1
Limnoria quadripunctata	Hemocyanin HC-2	ADE58571.1
Atyopsis moluccensis	Hemocyanin beta subunit 1	CCF55382.1
Chelidura acanthopygia	Hemocyanin subunit-1 precursor	CAR85694.1
Cherax quadricarinatus	Hemocyanin	AFP23115.1
Cupiennius solei	Hemocyanin subunit-5	CAC44753.1
Trichonephila inaurata madagascariensis	Hemocyanin subunit-G	CAD68057.1
Nephrops norvegicus	Hemocyanin subunit-I	AAF04148.1
Nephrops norvegicus	Hemocyanin subunit-II	AAF04149.1
Callinectes sapidus	Hemocyanin subunit-6	QIB03062.1
Callinectes sapidus	Hemocyanin subunit-5	QIB03061.1
Callinectes sapidus	Hemocyanin subunit-4	QIB03060.1
Callinectes sapidus	Hemocyanin subunit-3	QIB03059.1
Callinectes sapidus	Hemocyanin subunit-2	QIB03058.1
Callinectes sapidus	Hemocyanin subunit-1	QIB03057.1

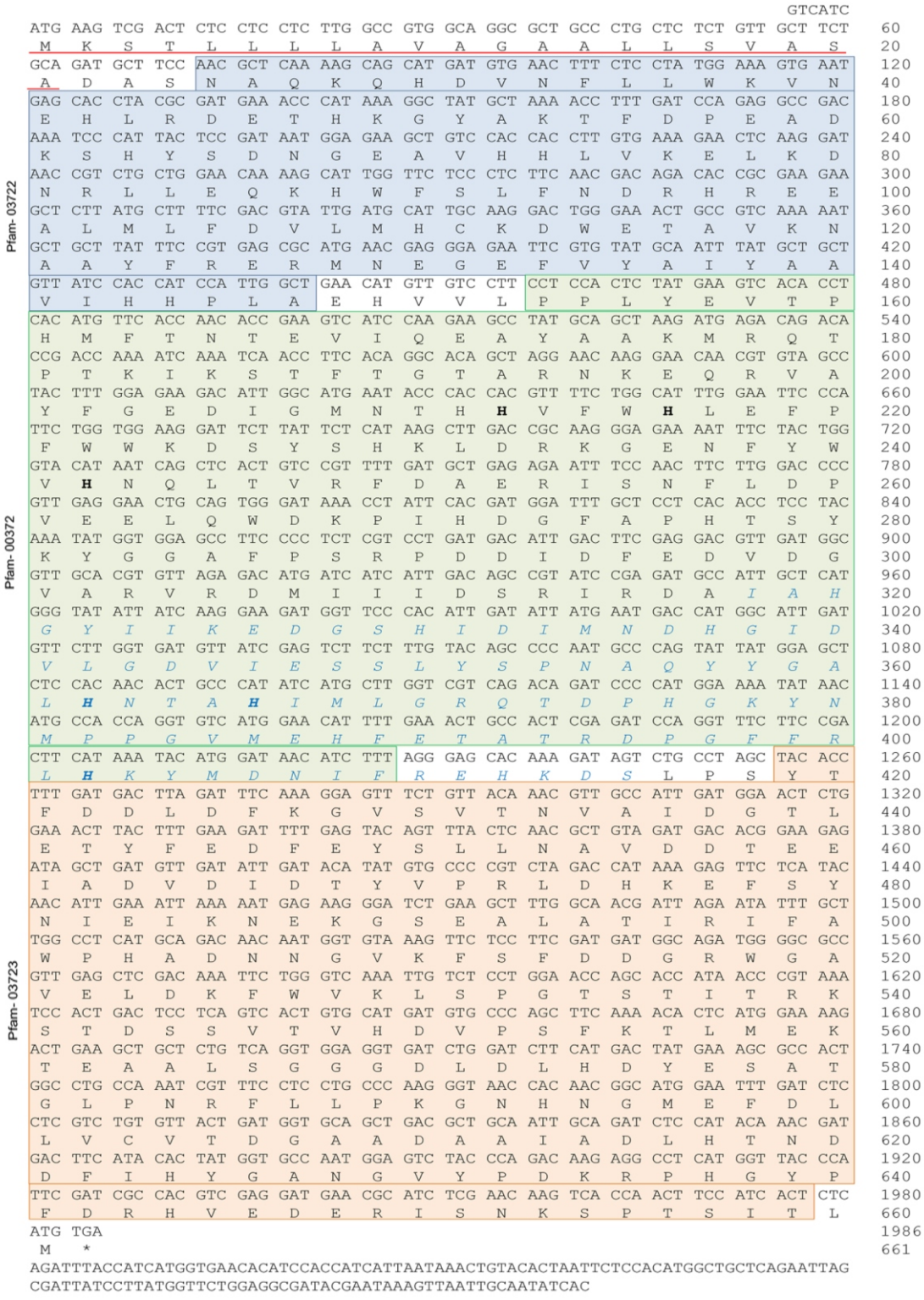

Fig. 1: cDNA and deduced amino acid sequence of *M. rosenbergii* hemocyanin subunit-1 gene. The putative signal peptide sequence of 21 amino acids is underlined. The stop codon (TGA) is marked by an asterisk. The hemocyanin-N (pfam 03722; all alpha), hemocyanin-M (pfam00372; copper containing), and hemocyanin-C (pfam 03723; Ig-like) domains are boxed and shaded blue, green, and orange, respectively. The residues belonging to the 'tyrosinase' domain within hemocyanin-M are colored blue and italicized. The six histidine residues within the hemocyanin-M domain have been marked in bold letters.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	Ca_HC_1	100	74	59	48	54	55	48	58	47	44	45	45	47	45	45	46	44	44	45	45	46	45	43	53	43	52	45	54	54	36	35
2	Hm_HC_1		100	58	48	48	54	54	48	59	46	45	44	45	44	45	44	44	44	44	44	44	43	43	52	43	51	44	53	52	37	37
3	Nn_HC_2			100	77	81	81	77	75	76	75	76	73	75	73	71	72	72	71	73	73	71	72	67	71	72	69	70	71	68	39	38
4	Lq_HC_2				100	75	77	57	81	64	65	66	64	64	63	62	60	61	61	61	62	61	63	58	74	62	72	61	74	70	33	34
5	Cs_HC_2					100	96	76	74	73	75	74	73	73	71	68	68	71	72	71	71	70	72	69	72	71	70	71	72	68	39	39
6	Cs_HC_1						100	77	74	72	74	74	73	73	71	69	69	71	71	71	72	70	73	69	72	72	71	72	73	68	38	38
7	Am_HC_β1							100	75	60	59	59	58	60	57	55	57	55	56	57	58	58	55	54	69	54	68	56	70	68	35	35
8	Nn_HC_1								100	86	82	84	81	83	80	75	75	76	75	77	77	77	75	73	74	76	73	76	76	71	39	40
9	Pl_HC_2									100	69	69	69	73	69	62	62	62	62	65	65	64	63	59	74	60	74	61	75	72	35	35
10	Am_HC_α1										100	83	80	79	66	64	64	65	63	67	66	67	64	61	75	61	73	62	75	70	35	35
11	Cm_HC_1											100	77	78	66	64	64	64	64	66	66	66	64	61	75	61	74	61	75	72	35	35
12	Cm_HC_2												100	75	65	62	62	62	61	65	65	64	61	60	73	60	72	60	74	71	34	34
13	Mn_HC													100	67	64	64	63	63	65	64	65	63	59	72	61	71	60	74	71	33	34
14	Pv_HC_2														100	60	60	59	59	63	63	62	60	61	72	60	70	61	73	70	34	34
15	Mr_HC_1															100	91	75	74	71	71	70	68	65	78	67	76	67	76	73	33	33
16	Mn_HC_1																100	75	74	72	72	72	69	64	78	68	76	68	76	74	34	34
17	Am_HC_γ1																	100	90	69	69	69	67	64	77	65	74	62	75	71	34	33
18	Cm_HC_γ1																		100	69	69	70	67	63	74	66	72	63	73	70	33	33
19	Pj_HC_L																			100	91	85	73	68	76	69	75	68	76	74	32	33
20	Pm_HC																				100	86	74	67	79	69	78	69	79	76	32	32
21	Pc_HC																					100	73	66	77	68	76	66	78	75	33	33
22	Cq_HC																						100	69	79	71	78	70	79	74	32	33
23	Pi_HC_C																							100	74	67	72	66	74	72	32	33
24	Cs_HC_5																								100	91	96	91	95	87	40	38
25	Es_HC_6																									100	89	82	89	81	33	33
26	Cs_HC_4																										100	90	94	85	40	38
27	Mm_HC_5																											100	90	83	33	33
28	Cs_HC_6																												100	90	40	39
29	Cs_HC_3																													100	40	38
30	Cus_HC_5																														100	79
31	Nim HC 5																															100

Fig. 2: Percent identity matrix of hemocyanin sequences from different species using Clustal X ver 2.1. A maximum identity of 91% was noticed in between the amino acid sequences of *M. rosenbergii* hemocyanin subunit-1 and *M. nipponense* hemocyanin subunit-1.

harveyi challenge, it was hypothesized that the transcript might putatively function as a multidimensional protein in the immune defense of host against the pathogens. Hemocyanin was identified and classified as an immune-related transcript (fold change: 1.21) in *V. parahaemolyticus* challenged *M. rosenbergii* hepatopancreas transcriptome (12 hours post-infection) (Rao *et al.,* 2015). Further, the plasma hemocyanin levels and its mRNA expression was activated after *V. harveyi* exposure to the white shrimp, *L. vannamei* suggesting hemocyanin synthesis after the invasion of pathogen (Pan *et al.,* 2019).

The absence in up- or down-expression (differential expression) of Mr_HC_1 after *V. harveyi* challenge might be due to the potential dose and period of infection (6 hours post-infection). A hemocyanin-L subunit protein from *L. vannamei* was found upregulated after white-spot syndrome virus (WSSV) challenge in WSSV-resistant shrimp and downregulated in WSSV-susceptible shrimp (Xu *et al.,* 2015). The *M. nipponense* hemocyanin subunit-1 is a protein of 675 amino acids and has been found to putatively participate in antibacterial defense of the host (Kong *et al.,* 2016). In another related study, the *F. merguiensis* hemocyanin (polypeptide of 661 amino acids) has been found to be upregulated in the hepatopancreas after *V. harveyi* exposure (Jiewkok *et al.,* 2015). Recently, the hemocyanin polypeptides purified and characterized from *M. rosenbergii* has been found to possess lectin-like activity (Mohanty *et al.,* 2020).

The Mr_HC_1 ORF sequence comprises of 1986 bases (including the stop codon) encoding a polypeptide of 661 amino acid residues (Fig. 1). The polypeptide has a calculated molecular weight of 75.531 kDa and an isoelectric point of 5.25. A putative signal peptide of 21 amino acid residues was also found at the N-terminus of Mr_HC_1. The Ala-X-Ala motif is a frequent occurrence before the signal peptide cleavage site as observed in this case, and that signifies cleavage after 21 amino acid residues (Petersen *et al.,* 2011). Domain analysis indicated that Mr_HC_1 belongs to hemocyanin family bearing three hemocyanin domains such as hemocyanin_N (all helix; N25-A147), hemocyanin_M (copper-containing; P154-F409), and hemocyanin_C (Ig-like; Y419-T659)-like domains. While hemocyanin_N and hemocyanin_C domains are variable, the hemocyanin_M domain containing six histidine residues coordinating with two copper atoms to bind oxygen is found

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Ca_HC_1	0																							
2	Hm_HC_1	0.262	0																						
3	Nn_HC_2	0.410	0.421	0																					
4	Lq_HC_2	0.520	0.525	0.233	0																				
5	Cs_HC_2	0.461	0.461	0.195	0.251	0																			
6	Cs_HC_1	0.448	0.457	0.190	0.234	0.043	0																		
7	Am_HC_β1	0.521	0.517	0.233	0.431	0.242	0.229	0																	
8	Nn_HC_1	0.419	0.408	0.248	0.188	0.264	0.255	0.251	0																
9	Pl_HC_1	0.529	0.544	0.244	0.357	0.273	0.277	0.402	0.139	0															
10	Am_HC_α1	0.556	0.552	0.252	0.346	0.251	0.255	0.412	0.180	0.306	0														
11	Cm_HC_1	0.548	0.561	0.237	0.345	0.255	0.255	0.405	0.162	0.309	0.170	0													
12	Cm_HC_2	0.546	0.553	0.271	0.362	0.268	0.268	0.419	0.188	0.309	0.197	0.234	0												
13	Mn_HC	0.534	0.553	0.252	0.361	0.273	0.268	0.396	0.165	0.268	0.211	0.223	0.246	0											
14	Pv_HC_2	0.552	0.559	0.275	0.375	0.294	0.294	0.433	0.199	0.306	0.344	0.341	0.347	0.327	0										
15	Mr_HC_1	0.548	0.553	0.286	0.385	0.316	0.307	0.450	0.252	0.375	0.357	0.365	0.383	0.356	0.399	0									
16	Mn_HC_1	0.539	0.564	0.282	0.399	0.316	0.307	0.432	0.252	0.382	0.356	0.362	0.380	0.363	0.404	0.088	0								
17	Am_HC_γ1	0.564	0.560	0.279	0.390	0.286	0.290	0.452	0.241	0.381	0.352	0.364	0.384	0.369	0.407	0.247	0.252	0							
18	Cm_HC_γ1	0.561	0.561	0.286	0.388	0.277	0.286	0.444	0.252	0.381	0.369	0.364	0.394	0.368	0.408	0.257	0.264	0.102	0						
19	Pj_HC_L	0.546	0.559	0.267	0.389	0.290	0.286	0.428	0.229	0.353	0.330	0.341	0.351	0.354	0.372	0.291	0.284	0.311	0.308	0					
20	Pm_HC	0.546	0.564	0.267	0.376	0.286	0.281	0.425	0.229	0.351	0.336	0.341	0.347	0.356	0.372	0.287	0.284	0.307	0.307	0.086	0				
21	Pc_HC	0.545	0.564	0.286	0.386	0.299	0.299	0.425	0.229	0.360	0.333	0.344	0.357	0.347	0.381	0.300	0.284	0.307	0.302	0.149	0.143	0			
22	Cq_HC	0.549	0.569	0.282	0.374	0.277	0.268	0.449	0.248	0.375	0.363	0.362	0.385	0.367	0.398	0.320	0.308	0.334	0.329	0.265	0.261	0.273	0		
23	Pi_HC_C	0.573	0.573	0.328	0.418	0.307	0.307	0.461	0.271	0.409	0.389	0.389	0.400	0.405	0.385	0.351	0.360	0.359	0.371	0.323	0.326	0.336	0.313	0	
24	Cs_HC_5	0.474	0.483	0.294	0.261	0.281	0.277	0.307	0.261	0.256	0.252	0.252	0.269	0.278	0.282	0.222	0.222	0.231	0.256	0.239	0.214	0.226	0.209	0.256	0
25	Es_HC_6	0.567	0.573	0.282	0.383	0.286	0.277	0.455	0.244	0.402	0.386	0.387	0.403	0.387	0.404	0.326	0.320	0.349	0.344	0.310	0.315	0.323	0.287	0.328	0.094
26	Cs_HC_4	0.479	0.487	0.307	0.282	0.299	0.294	0.325	0.269	0.261	0.269	0.265	0.282	0.286	0.299	0.244	0.244	0.261	0.282	0.248	0.222	0.244	0.217	0.278	0.038
27	Mm_HC_5	0.547	0.559	0.302	0.393	0.290	0.277	0.443	0.241	0.387	0.383	0.387	0.401	0.400	0.386	0.327	0.324	0.375	0.370	0.322	0.310	0.340	0.297	0.337	0.089
28	Cs_HC_6	0.457	0.470	0.286	0.261	0.281	0.273	0.303	0.244	0.252	0.252	0.248	0.265	0.261	0.269	0.235	0.235	0.248	0.269	0.239	0.214	0.218	0.209	0.261	0.047
29	Cs_HC_3	0.462	0.479	0.325	0.303	0.325	0.316	0.316	0.286	0.278	0.299	0.282	0.295	0.295	0.299	0.269	0.265	0.286	0.299	0.265	0.244	0.248	0.260	0.278	0.132
30	Cus_HC_5	0.637	0.633	0.613	0.669	0.613	0.617	0.653	0.611	0.649	0.651	0.651	0.661	0.665	0.660	0.665	0.662	0.660	0.666	0.675	0.683	0.674	0.680	0.683	0.598
31	Nim_HC_5	0.647	0.626	0.621	0.658	0.613	0.617	0.651	0.604	0.646	0.653	0.651	0.656	0.657	0.660	0.667	0.664	0.665	0.667	0.666	0.675	0.667	0.672	0.674	0.620

Fig. 3: Percent distance matrix of hemocyanin sequences from different species using Clustal X ver 2.1. A minimum distance of 0.088 was observed in between the amino acid sequences of *M. rosenbergii* hemocyanin subunit-1 and *M. nipponense* hemocyanin subunit-1.

conserved (Linzen *et al.,* 1985). The molecular diversity of hemocyanin_N domain was noticed in *L. vannamei* wherein three variants (LvHMC-Nr1, LvHMC-Nr2, and LvHMC-Nr3) were identified post *V. parahaemolyticus* challenge out of 25 variants screened through bioinformatics analysis (Fan *et al.,* 2019). Further, a tyrosinase family domain (I317-S415) was found overlapping the hemocyanin_M domain. Six histidine residues (H212, H216, H242, H362, H366, and H402) of copper binding sites were identified in Mr_HC_1.This hemocyanin_M domain with copper-binding sites and six histidine residues has been confirmed in other crustaceans (Sun *et al.,* 2012).

The sequence analysis of Mr_HC_1 was found to be closely related to the sequence features of Mn_HC_1. However, the molluscan *Sepiella maindroni* hemocyanin comprised of an ORF of 10,032 bp encoding a polypeptide of 3,343 amino acids with eight functional units (Li *et al.,* 2017). Further, a 653 amino acid polypeptide was deduced for the hemocyanin of giant African millipede, *Archispirostreptus gigas* showing a predicted molecular mass of 73.5 kDa with putative N-glycosylation sites (Daamsgaard *et al.,* 2013). The multiple sequence alignment of hemocyanin family sequences also depicts the conservation of sequences at domain level, especially at the hemocyanin_M domain (not shown). At the hindsight, evolutionary view-points suggest that the copper-containing hemocyanins have evolved from prophenoloxidases and have been found in Remipedia, Ostracoda and Branchiura and have been lost in dragonflies, mayflies and Eumetabola (Burmester 2015). Mr_HC_1 is most identical to Mn_HC_1, with which it shares 91% sequence identity followed by Cs_HC_5 (78%), Cs_HC_4 and Cs_HC_6 (76% each). The amino acid sequence identity of MR_HC_1 was least (33%) with *Cupiennius salei* (tiger wandering spider) hemocyanin_5 (Cus_HC_5) and *Nephilia inaurata* (golden orb-weaver spider) hemocyanin_5 (Nim_HC_5) (Fig. 2). Further, while the percentage identity of Mr_HC_1 was in the range of 70-75% with the hemocyanin γ-subunit proteins, it was less in the range of 60-64% with hemocyanin α-subunit proteins and about 55% with *A. moluccensis* β1 subunit. The distance scores computed also relates Mr_HC_1 with Mn_HC_1 (0.088) followed by Cs_HC_5 (0.222), Cs_HC_6 (0.235), and Cs_HC_4 (0.244) (Fig. 3). The distance scores also suggest greater distance of Mr_HC_1 with the β-subunit hemocyanin proteins and lesser distance with the γ-subunit hemocyanin proteins.

A phylogenetic tree constructed with 31 amino acid sequences of hemocyanin proteins using Neighbor joining method clearly classifies the sequences to three major clusters originating from a common ancestor (Fig. 4). The spider hemocyanin proteins (Cus_HC_5 and Nim_HC_5) forms an outgroup cluster well diverged from other hemocyanin proteins. Further, γ-type, α-type and β-type subunit hemocyanin proteins occurred as separate clades. Quite clearly, Mr_HC_1 show close relationship with Mn_HC_1 and both have been placed under the hemocyanin γ-type subunit clade that may have evolved

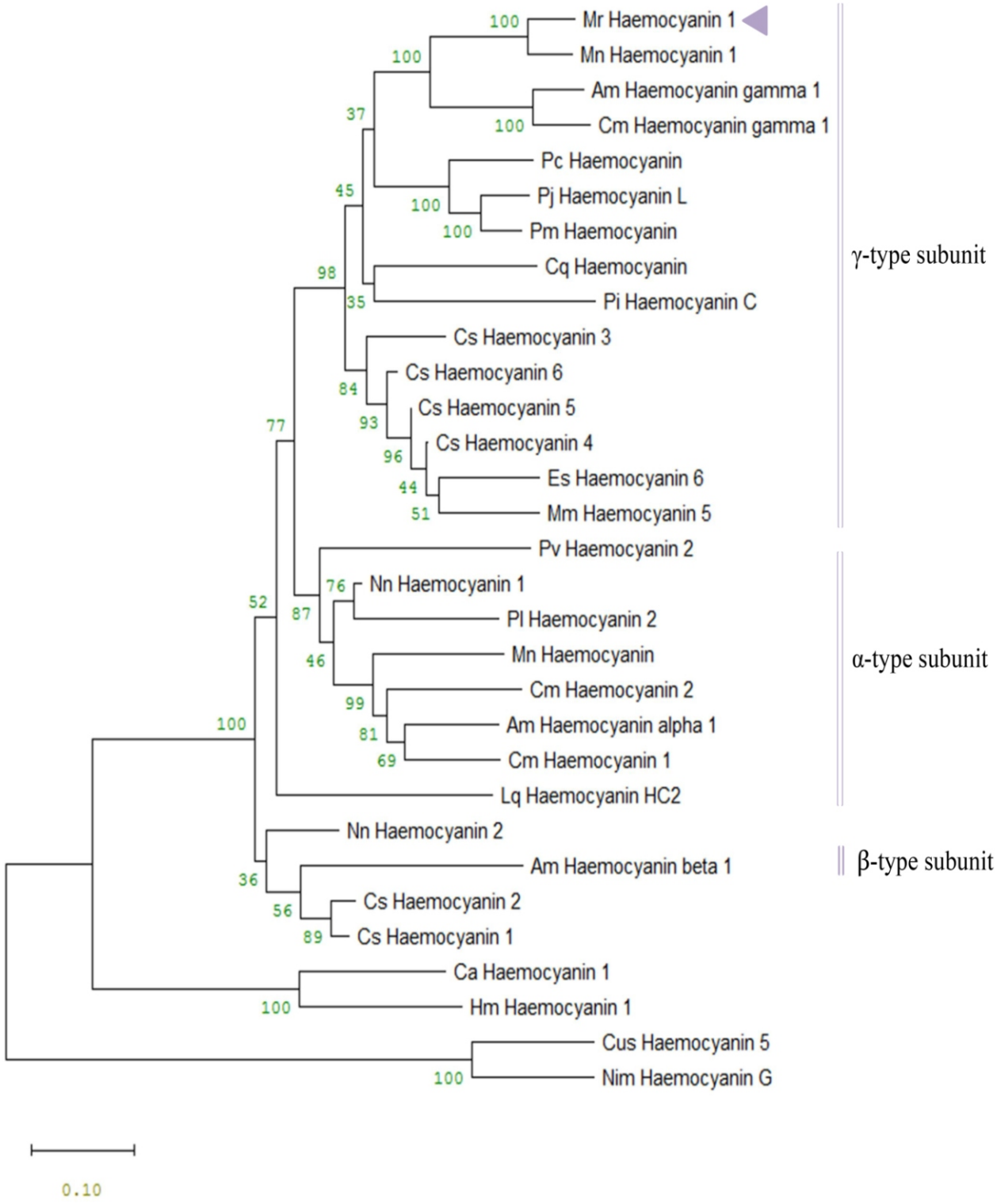

Fig. 4: Phylogenetic tree based on the hemocyanin sequences from different species conducted in MEGAX. The evolutionary tree was inferred using the Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replications) is shown next to branches. The evolutionary distances were corrected using the Poisson correction method and are in the units of number of amino acid substitutions per site.

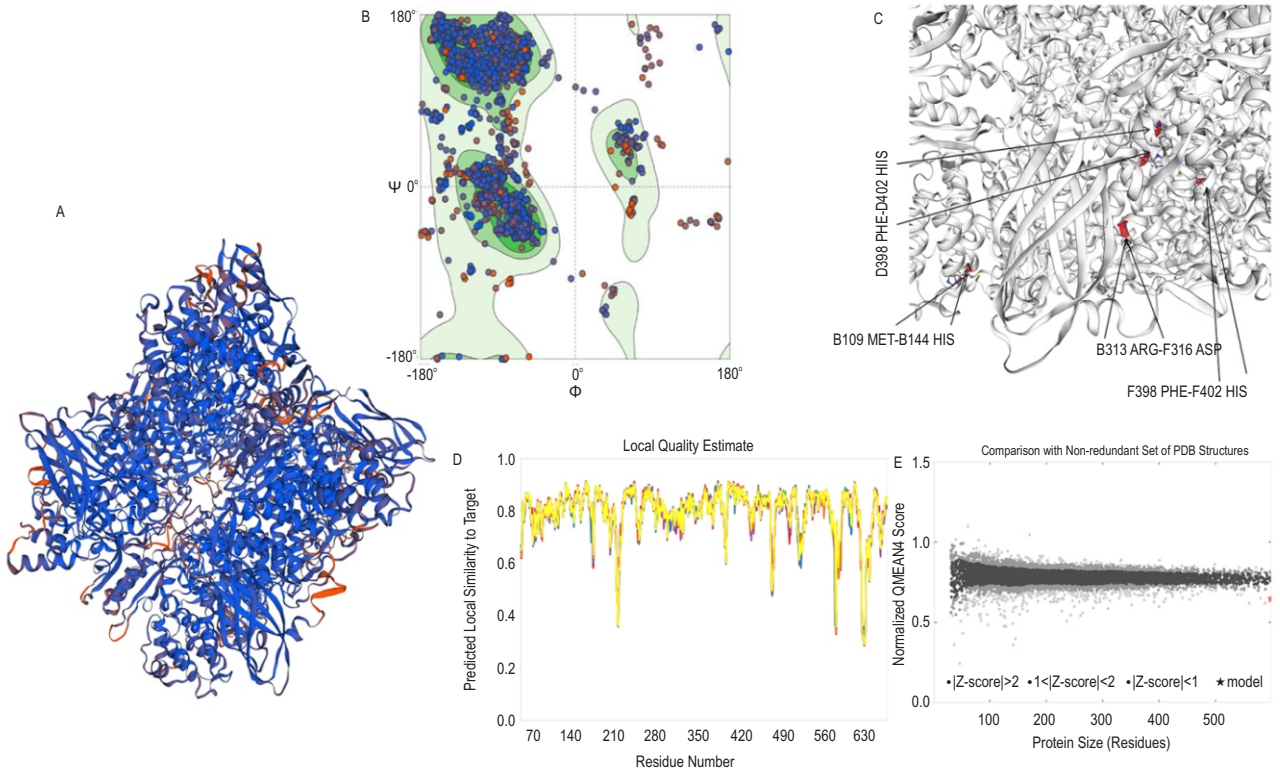

Fig. 5: Structural prediction of hemocyanin subunit-1 protein from *M. rosenbergii* using Swiss-Model workspace at ExPASY server. (A) The predicted homo-hexameric model of hemocyanin subunit-1 protein. (B) The Ramachandran plot of all residues of six chains of hemocyanin subunit-1 protein from *M. rosenbergii*. (C) Predicted structure defining the clashes of residues (clash score 0.60; Ramachandran favored 91.42%). (D) The predicted local similarity of each residue of the six chains of hemocyanin subunit-1 protein from *M. rosenbergii*. (E) The normalized QMEAN4 scores of the predicted model after comparison with a non-redundant set of PDB structures.

later as compared to β- and α-subunit clades (Marxen *et al.,* 2013). The present studies confirm earlier observations showing that Mn_HC_1 was more closely related to hemocyanin γ-type subunit cluster of freshwater shrimps *A. moluccensis* and *C. multidentata* (Kong *et al.,* 2016). The observations relate to well-known fact that the respiratory protein hemocyanin from arthropods have been classified to α-, β-, and γ-type subunits (Markl, 1986). The predicted structure of Mr_HC_1 was studied in greater details after homology modeling using SWISS-MODEL server.

The predicted structure showed 59.15% sequence identity with the crystal structure of hexameric hemocyanin from *P. interruptus* refined at 3.2 Angstrom resolution (reference structure as the template; PDB id- 1hc1.1A) (Volbeda and Hol, 1989). A homo-hexameric structure (six chains A-F) unfolds for Mr_HC_1 with a QMEAN value of -3.33 (Fig. 5A). A 2 x 6-meric hemocyanin nearly identical to hemocyanin found in decapod crustaceans has been studied from springtail taxa (Collembola) (Schmidt *et al.,* 2019). Further, 6 x 75 kDa hexameric unit oligomeric quaternary structure has been refined for hemocyanin from crab *Eriphia verrucosa* (Dolashki *et al.,* 2015). As understood from the sequence-level features and domain analysis of Mr_HC_1, the hemocyanin_N is all alpha domains in all chains with beta-fold recognized in the hemocyanin_M and hemocyanin_C domains. Further, the independent amino acid residue properties were examined using Ramachandran plot accessing the SWISS-MODEL server (Fig. 5B). Most of the residues were distributed in the core and allowed regions of Ramachandran plot such as in between 90° to 180° ψ and -90° to -180° φ and 0° to -90° ψ and -90° to -180° φ. In assessing the MolProbity results, a score of 1.78 was obtained with residue clash score of 0.60 and Ramachandran favored residues of 91.42%. Fig. 5C shows residues clashes between B109Met-B144His, F398Phe-F402His, B313Arg-F316Asp, and D398Phe-D402His, restricted to B, D and F chains of this homo-hexameric protein.

Further, the assessment showed that 2.28% of residues were Ramachandran outliers, 5.71% Rotamer outliers, and 113 residues showed C-Beta deviations. 405 out of 43422 were considered as bad angles while no bad bonds were noticed. The local quality estimate and normalized QMEAN4 scores for the predicted model is statistically shown in Fig. 5D and Fig. 5E, respectively. QMEAN is a scoring function that identifies the best model from an ensemble of alternative models on which our protein structure prediction relies (Benkert *et al.,* 2008).

In conclusion, the *in silico* characterization suggests that Mr_HC_1 belongs to γ-subunit type hemocyanins. High sequence identity with Mn_HC_1 identifies the putative role of Mr_HC_1 in antibacterial defense regulated by copper accumulation. This study is a preliminary attempt to understand the biological function and regulation of hemocyanin and to advance the research on multifunctionality and polymorphism in hemocyanins.

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Add-on Information

Authors' contribution: B.B. Patnaik, J. Mohanty: Conceptualization and supervision of research plans, writing of the manuscript; **S. Baliarsingh, J.M. Chung, Y.S. Lee:** Transcriptome annotation, data curation and in silico analysis; **S. Sahoo, I. Nayak:** Data validation and software support.

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