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RESEARCH ARTICLE

Comparative genomic analysis of crustacean hyperglycemic hormone (CHH) neuropeptide genes across diverse crustacean species [version 1; referees: 2 approved with reservations]

Wai Hoong Chang , Alvina G. Lai

Nuffield Department of Medicine, University of Oxford, Oxford, Oxfordshire, OX3 7FZ, UK

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Abstract

Background: Recent studies on bioactive peptides have shed light on the importance of these compounds in regulating a multitude of physiological, behavioral and biological processes in animals. Specifically, the neuropeptides of the crustacean hyperglycemic hormone (CHH) superfamily is known to control a number of important functions ranging from energy metabolism, molting, osmoregulation to reproduction.

Methods: Given the importance of this peptide family, we employed a conservative approach utilizing extant transcriptome datasets from 112 crustacean species, which not only include important food crop species from the order Decapoda, but also from other lower order crustaceans (Branchiopoda and Copepoda), to identify putative CHH-like sequences.

Results and conclusions: Here we describe 413 genes that represent a collection of CHH-like peptides in Crustacea, providing an important staging point that will now facilitate the next stages of neuroendocrine research across the wider community.

Keywords

Crustaceans, Neuropeptides, Crustacean Hyperglycemic Hormone (CHH), Transcriptomics, Comparative genomics

Open Peer Review

Referee Status:

	Invited Referees	
	1	2
version 1 published 23 Jan 2018	 report	 report
1 Abigail Elizur , University of the Sunshine Coast, Australia Tuan V. Nguyen , University of the Sunshine Coast, Australia		
2 Chi-Ying Lee , National Changhua University of Education, Taiwan Jean-Yves Toullec , Station Biologique de Roscoff, France		

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Corresponding author: Alvina G. Lai (alvina.lai@ndm.ox.ac.uk)

Author roles: **Chang WH:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Lai AG:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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Introduction

Crustaceans and insects from the phylum Arthropoda have longstanding histories in peptide biology research, principally in areas related to the roles of peptide hormones in physiology and neuroendocrine signaling. Early discoveries have demonstrated that compounds in the crustacean nervous system were responsible for chromatophore control^{1–3}. Four decades later, it was revealed that a compound known as the red pigment concentrating hormone functions as the first crustacean/invertebrate neuropeptide⁴. Since then, multiple studies have shed light on the highly pleiotropic functions of crustacean neuropeptides implicated in the regulation of a myriad of physiological processes such as light adaptation, molt inhibition, carbohydrate metabolism, reproduction and ion transport^{5–9}.

The crustacean hyperglycemic hormone (CHH) represents a neuropeptide superfamily that is unique to arthropods^{6,10–12}. This superfamily is made up of peptides containing ~70 amino acids originally isolated from the X-organ-sinus-gland system of the decapod *Carcinus maenas*¹³. Given their high degree of structural similarities, and the conservation of six cysteine residues, the molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) were considered as part of this family collectively known as CHH/MIH/GIH. To date, at least 150 CHH peptides have been isolated and characterized, mainly in decapods through comparative studies on endocrinology^{5–7,14–21}. Although there are reports on CHH peptides in other crustacean taxa such as *Armadillidium vulgare* (Isopoda)^{22,23}, *Daphnia pulex* (Cladocera)²⁴ and *Daphnia magna*¹⁵, investigations beyond decapods have remained scant and the sequences of CHH/MIH/GIH genes in other crustacean taxa have remained elusive.

Here, we took advantage of the growing number of high-throughput crustacean datasets on public repositories to perform transcriptome mining of the CHH/MIH/GIH superfamily. To this end, we looked at crustacean species from three Classes (Figure 1) and annotated CHH/MIH/GIH genes. This high confidence set of genes identified using our *in silico* framework provides an important basis for understanding neuropeptide biology underpinning physiological adaptations across diverse crustacean species.

Methods

Transcriptome datasets and query sets

We retrieved complete transcriptome datasets for 112 crustacean species available at the time of manuscript preparation from the European Nucleotide Archive. Five non-crustacean arthropod proteomes were retrieved from Uniprot. A complete list of accessions used in this study is provided in Supplementary Table 1. We retrieved a list of query sequences used in subsequent homology searches from Uniprot and GenBank.

Identification of CHH/MIH/GIH peptides

To identify CHH/MIH/GIH gene orthologs, we used multiple Basic Local Alignment Search Tool (BLAST)-based approaches such as BLASTp and tBLASTn with varying Blocks Substitution matrices based on a previously published workflow²⁵. The BLAST results were filtered by e-value of $< 10^{-6}$, best reciprocal BLAST hits against the GenBank non-redundant (nr) database and redundant contigs having at least 95% identity were collapsed using CD-HIT. We then utilized HMMER (version 3.1) employing hidden Markov models (HMM) profiles²⁶ to scan for the presence of CHH Pfam domains²⁷ on the best reciprocal nr

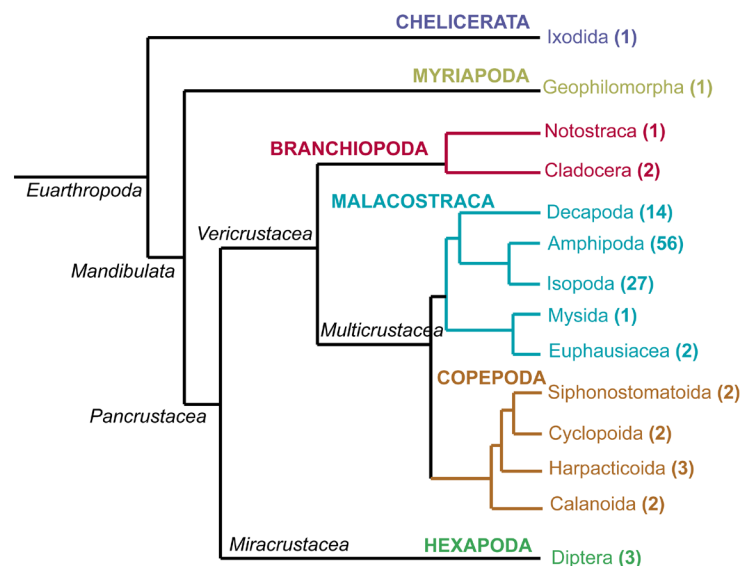


Figure 1. Phylogenetic relationship of Crustacea. The number of species within each taxon is denoted in parentheses.

BLAST hits to compile a final non-redundant set of crustacean CHH/MIH/GIH orthologs. Pfam annotations, associated e-values and fasta sequences are provided in [Dataset 1²⁸](#) and [Dataset 2²⁹](#).

Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignments of CHH protein sequences were performed using [MAFFT](#) (version 7)³⁰. Phylogenetic tree was built from the MAFFT alignment using RAXML WAG + G model to generate best-scoring maximum likelihood trees³¹. [Geneious](#) (version 7) was used to generate multiple sequence alignment images as well as graphical representations of the Newick tree³².

Results and discussion

We have annotated CHH/MIH/GIH genes from 112 crustacean transcriptome datasets representing three Classes: Malacostraca (Amphipoda: 56 species, Decapoda: 14 species, Isopoda: 27 species, Euphausiacea: 2 species and Mysida: 1 species), Branchiopoda (3 species), and Copepoda (9 species) ([Supplementary Table 1](#)). We also looked at 5 non-crustacean species from Arthropoda: Insecta (3 species), Arachnida (1 species) and Chilopoda (1 species) ([Supplementary Table 1](#)). Using sequence and motif similarity based approaches, we have conservatively identified a total of 413 genes from these transcriptomes ([Figure 2](#); [Dataset 1²⁸](#) and [Dataset 2²⁹](#)).

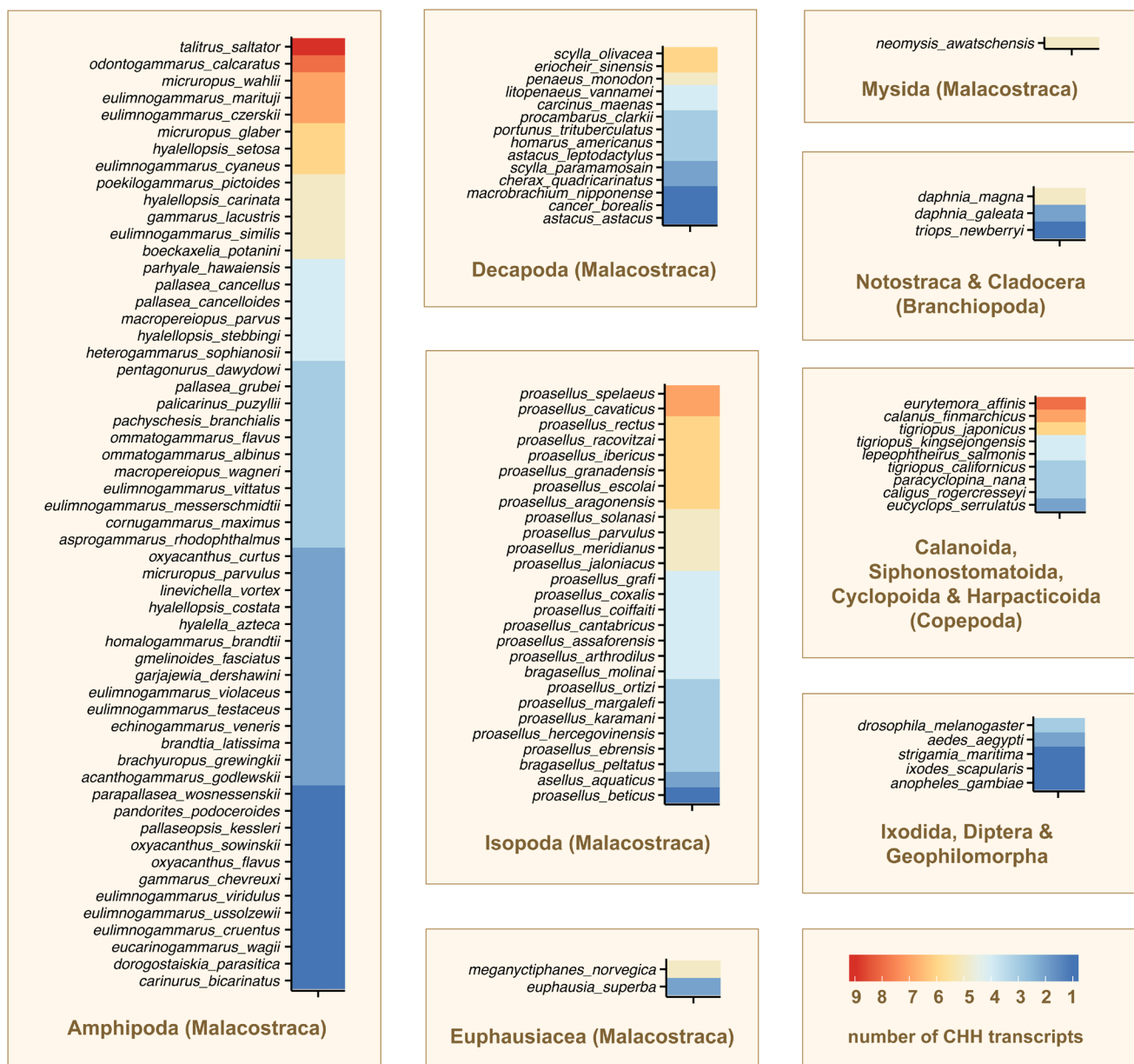


Figure 2. CHH/MIH/GIH genes in Crustacea. Heat maps denote the number of CHH/MIH/GIH genes identified from each crustacean species. CHH/MIH/GIH genes from five non-crustacean species within Arthropoda are also shown.

Multiple sequence alignment analyses on representative CHH/MIH/GIH sequences revealed the presence of a conserved set of six cysteine residues (Figure 3), likely contributing to the formation of disulfide bonds³³. Comparison of insect sequences from *Drosophila melanogaster*, *Anopheles gambiae* and *Aedes aegypti* demonstrated sequence identities of at least 46% (Supplementary Table 2). Within crustacean taxa, a range of sequence identities were observed: Branchiopoda (~25% to 93%), Copepoda (~12% to 30%) and Malacostraca (~10% to 98%) (Supplementary Table 2). This is reflected in the phylogeny where CHH/MIH/GIH sequences from related individuals form distinct clusters (Figure 4). It was previously reported that multiple gene duplications of CHH family peptides occurred in the decapod lineage leading to a high degree of genetic polymorphism¹⁵, hence providing an explanation for our current observation. Two separate clusters of CHH genes exhibiting antagonistic patterns of expression were identified in the decapod *Metapenaeus ensis*, posited to represent an ancient gene duplication event³⁴. Although it is not possible to pinpoint the genomic loci of CHH sequences identified from this study, it is likely that paralogous copies offer mechanisms for evolving new functions through functional divergence. CHH-like genes arising from duplication of the ancestral copy are subjected to reduced selective pressure and therefore

may lose their hyperglycemic activity to adopt more specialized roles¹⁵. Further biochemical studies will be required to unravel the functions of the novel genes identified from this study.

Dataset 1. Fasta file for CHH/MIH/GIH sequences in crustaceans and other arthropods

<http://dx.doi.org/10.5256/f1000research.13732.d191194>

Dataset 2. List of Pfam annotated CHH/MIH/GIH genes and associated e-values in crustaceans and other arthropods

<http://dx.doi.org/10.5256/f1000research.13732.d191195>

Conclusions

We have generated a high confidence list of CHH/MIH/GIH sequences from distantly related crustaceans. As a fundamental step in a broader endeavor this data is now available to the wider community to allow detail functional analyses pertinent to the next stages of neuropeptide research. Given the paucity of CHH sequences beyond decapod crustaceans, our analysis forms a promising basis for studies ranging from biochemistry to the evolution of this elusive superfamily.

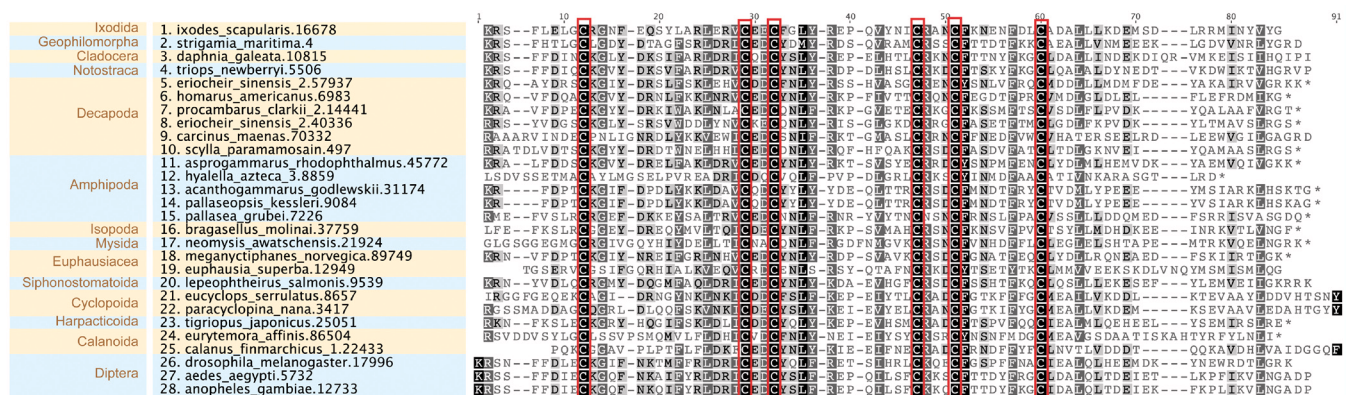


Figure 3. Multiple sequence alignment of representative CHH/MIH/GIH proteins from each taxon. Six conserved cysteine residues are annotated within red boxes.

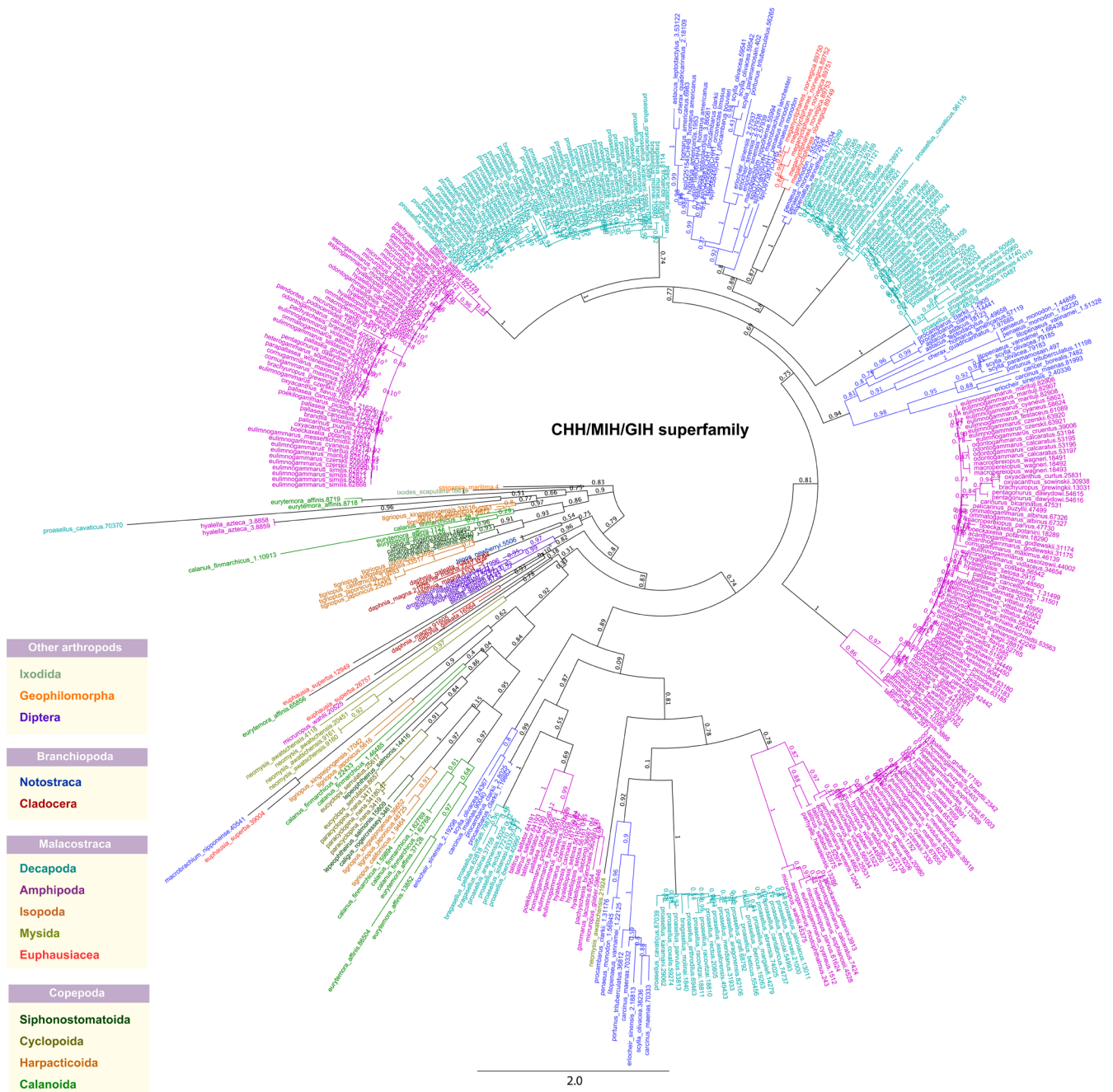


Figure 4. Crustacean CHH/MIH/GIH phylogeny. The tree was constructed using the maximum-likelihood method from an amino acid multiple sequence alignment. The node labels of each taxon are marked with distinctive colors denoted in the figure inset. Bootstrap support values ($n=1000$) are denoted as branch labels.

Data availability

Data supporting the conclusions of this study are provided as [Supplementary Material](#) and [Dataset 1](#) and [Dataset 2](#).

Dataset 1: Fasta file for CHH/MIH/GIH sequences in crustaceans and other arthropods. [10.5256/f1000research.13732.d191194](https://doi.org/10.5256/f1000research.13732.d191194)²⁸

Dataset 2: List of Pfam annotated CHH/MIH/GIH genes and associated e-values in crustaceans and other arthropods. [10.5256/f1000research.13732.d191195](https://doi.org/10.5256/f1000research.13732.d191195)²⁹

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary material

Supplementary Table 1: List of accession numbers for species used in this study.

[Click here to access the data.](#)

Supplementary Table 2: Protein distance matrix (% identity) constructed from a multiple sequence alignment of CHH/MIH/GIH sequences.

[Click here to access the data.](#)

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Open Peer Review

Current Referee Status:



Version 1

Referee Report 20 April 2018

doi:10.5256/f1000research.14919.r30523



Chi-Ying Lee¹, Jean-Yves Toullec²

¹ Department of Biology, National Changhua University of Education, Changhua, Taiwan

² Sorbonne Universités, UPMC Université Paris 06, UMR 7144 CNRS, Equipe ABICE, Station Biologique de Roscoff, Roscoff, France

The authors collected and analyzed transcriptome datasets for 112 crustacean species (representing three Classes - Malacostraca, Branchiopoda, and Copepoda), as well as those for 5 non-crustacean (arthropod) species (Insecta, Arachnida and Chilopoda) (Supplementary Table 1; Fig. 1), retrieved from public repository.

Collectively, 413 genes are annotated based on sequence and motif similarity as encoding peptides belonging to the so-called CHH/MIH/GIH family. The number of CHH/MIH/GIH genes identified from each crustacean species is shown as heat maps (Fig. 2). Protein sequence, annotation information, etc. are listed (Dataset 1 and 2). Between-species % identity is reported (Supplementary Table 2). Multiple sequence alignment on representative CHH/MIH/GIH sequences is presented in Fig. 3 to show the 6 conserved cysteine residues (Fig. 3). A phylogenetic tree is built based on the multiple sequence alignment data (Fig. 4).

The main drawback of this work is it only results in a 413-sequence inventory of CHH/MIH/GIH family peptides, but with little information (nucleotide length, amino acid sequence).

The authors did not describe how the tree presented in Fig. 1 was built with what sorts of data. If they took a constructed tree from other source, this should be cited. There are obviously other current interpretations of a phylogenetic relationship of Crustacea, in addition to the one shown in the manuscript.

For annotation information (Dataset 2), it should be more discerning in that peptide should be assigned as CHH, MIH/GIH, MOIH (mandibular organ-inhibiting hormone) or ITP (ion transport peptide), instead of only giving an inclusive description - Crustacean CHH/MIH/GIH neurohormone family.

Results derived from further analysis of the sequence data (Figs, 2, 3, and 4) are not adequately discussed or lead to conclusions that have been previously established.

For example, the only conclusion made with Fig. 3 is the 6 highly cysteine residues, a signature of the family already extensively described. Figure 4 is left without discussion. Moreover, it is not mentioned which type of peptide (CHH, MIH/HIV, or ITP) the different clusters highlighted in the tree belong to.

Part of the discussion (left column, p. 4) starting with "It was previously reported that multiple gene

duplications of CHH family peptides.....” is not particularly relevant to the data being discussed and is again making a point already extensively described and discussed. It should also be mentioned that genomic data are more adequate than transcriptomic data when discussing gene duplication and gene copy number (Fig. 2).

Figure 2 is also left without discussion. In addition, data presented in this figure might be suffering from the criticism that the number of CHH/MIH/GIH genes assigned to each species is likely biased as the number will be absolutely influenced by the type of tissue used for sequencing (eyestalk ganglia, other tissue, or whole animal) and sequencing depth. Accordingly, the relevant information should be given in Supplementary Table 1 and comparison should only be made among species with the same sequenced tissue type (e.g., eyestalk ganglia) and comparable sequencing depth.

Overall, while the methods employed are largely (but not entirely) appropriate for the intended analysis, the goal of the study is not clearly set and this work only produces a sequence inventory without novel finding or solid discussion. Additional analyses expected to yield novel finding should be added to the manuscript. For example, with a vast amount of sequence information that could be extracted from a set of 413 peptides from animals encompassing 3 Classes, would it not be possible to uncover some function-defining sequence characteristics or motif? This piece of information would be useful for functional analysis, which the authors thought should be an important aspect of the next stage of research.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Endocrinology

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Referee Report 26 February 2018

doi:10.5256/f1000research.14919.r31165

**Abigail Elizur , Tuan V. Nguyen**

Genecology Research Center, Faculty of Science, Health, Engineering and Education, University of the Sunshine Coast, Sippy Downs, Qld, Australia

The current study is an interesting "*in silico*" approach that mine for CHH/MIH/GIH neuropeptide in a wide range of crustacean. Introduction is updated and concise. However, there are some minor points that will need to be addressed to provide clarity information for readers. Materials and methods is sound and appropriate, although I raise some particular suggestions to further expand the quality of the MS. Results and discussions are overall good. One major point that needs to be fixed is the phylogenetics tree, whereas the color code system does not correlate between legend and figure.

In addition, I have specific comments, (the fact that it does not appear as numbered pages and lines makes it a bit awkward, but see below):

Page 2: ...known as CHH/MIH/GIH- There are also Mandibular Inhibiting hormone (MOIH) and also ITP (Ion Transport Protein) to be part of the super family. See Ohira (2016)¹.

...from three Classes - Please elaborate on this, are these branchiopoda, malacostraca and copepoda?

(Figure 1) – How was Figure 1 generated ? Is this a phylogenetics based tree built on mitochondrial genome or it is just an illustration?

Genebank nr database... What about TSA (Transcriptome Shotgun Assembly) database? As that will increase the chance that the algorithm will discover more CHH/MIH/GIH? Since you mention in the introduction "Here, we took advantage of the growing number of high-throughput crustacean datasets on public repositories to perform transcriptome mining of the CHH/MIH/GIH superfamily." Adopting the TSA database will be better suited for the MS.

...and redundant contigs... Why 95%?, let's assume that CHH prepropeptide can be roughly more than 120aa. For instance, two distinct CHH in *Homarus americanus* CHH-A (P19806) and CHH-B (Q25154) have 127/134 aa that are similar (~95%). Will they be collapsed with CD-HIT?

Page 4

Within crustacean taxa... Similarity was pretty high in Branchiopoda and Malacostraca (93% & 98%), but significantly lower in Copepoda. Can you propose an explanation for that?

It was previously reported that... In light of this, Veenstra (2016)² has some very intensive mining of CHH in multiple decapod species. Why not provide a section that compares between the number of CHH/MIH/GIH predicted from your dataset with the above study?

Figure 4 - Color coding system is misleading, for instance Decapoda was assigned as teal in legends, but actually blue in the phylogenetics tree.

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2. Veenstra JA: Similarities between decapod and insect neuropeptidomes. *PeerJ*. 2016; 4: e2043 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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