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Expressed sequence tags from eyestalk of kuruma prawn, *Marsupenaeus japonicus*

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Abstract

We analyzed the expressed sequence tags (ESTs) obtained from a cDNA library of the eyestalk of the kuruma prawn, *Marsupenaeus japonicus*, to examine gene expression profile with special focus on female reproduction. The assembly of 1988 ESTs created 136 contigs from 738 ESTs; however 1250 ESTs remained singletons. Significant similarities (blast score ≥ 50 bits) to the DNA sequences in the databank were found for only 16.7% of the 1386 sequences (136 contigs plus 1250 singletons), suggesting that the eyestalk library contains many unknown genes. Ribosomal RNA and mitochondrial respiration enzymes with significant similarities were found abundantly in the ESTs, whereas genes related to maturation or endocrine systems were scarce. Three ESTs were assumed to encode novel eyestalk hormones with marked similarities to pigment-dispersing hormone, molt-inhibiting hormone and crustacean hyperglycemic hormone. Sequences encoding a product highly homologous to farnesoic acid *O*-methyltransferase, an enzyme that produces methyl farnesoate, were also found.

Keywords: CHH; Crustacean; Hormone; MIH; PDH; Reproduction; EST; Eyestalk; Farnesoic acid *O*-methyltransferase

1. Introduction

The crustacean eyestalk has the unique ability of regulating a wide range of physiological functions through neuropeptides produced in the X-organ, a cluster of nerve cell bodies in the eyestalk. A substantial number of eyestalk neuropeptides and/or cDNAs encoding the neuropeptides have been discovered in various crustacean species including shrimps, lobsters, crabs and isopods. To date, crustacean hyperglycemic hormone (CHH), gonad-inhibiting hormone (GIH), molt-inhibiting hormone (MIH), mandibular organ-inhibiting hormone (MOIH), pigment-dispersing hormone (PDH) and red pigment-concentrating hormone (RPCH) have been found in the eyestalk (De Kleijn and Van Herp, 1995; Chan et al., 2003). These data also indicated that CHH, GIH, MIH and MOIH share a structural similarity, constituting the CHH peptide family. In contrast, the structural similarity makes it difficult to elucidate the function of each hormone. Multiple variants that can be categorized into the same hormone class, based on the similarities of gene or protein sequences, have been found in many species studied (Chan et al., 2003). Moreover, a single hormone can exert multiple physiological functions (Yang et al., 1995; Khayat et al., 1998). Thus, the function of individual hormones in the eyestalk has not been fully understood. The mechanisms of production, secretion and action of eyestalk hormones also remain to be clarified.

The mechanism of the sexual maturation of the kuruma prawn (*Marsupenaeus japonicus*), has been of special interest to shrimp farmers and fisheries scientists, especially that maturation of this species in captivity is difficult. Therefore, fertilized eggs for the production of juveniles for shrimp culture and seed enhancement are usually obtained from fully mature females caught in the wild. Presently, among eight CHH family peptides reported in the kuruma prawn (Yang et al., 1995, 1996, 1997; Ohira et al., 2005), based on molecular similarity, two are similar to MIH and the rest to CHH; yet no GIH has been identified in this or in any other shrimp species. Nevertheless, GIH has been reported in three species of lobsters (Soyez et al., 1987; Edomi et al., 2002; Marco et al., 2002), in one of crayfish (Aguilar et al., 1992) and in one of

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Table 1
Summary of contig creation and blastn search

Number of EST creating a contig	Number of contig or singleton	Blast score		
		<50	50–100	≥100
Singleton	1250	1077	76	97
2	92	63	11	18
3	22	8	4	10
4	5	4	1	
5–9	12	3	1	8
10–49	4			4
50	1			1
Total	1386	1155	93	138

isopods (Greve et al., 1999). Consequently, the role of the eyestalk hormone in the maturation of shrimps is almost unknown. In the present study, as a first step understanding the molecular mechanism of maturation, expressed sequence tag (EST) analysis was carried out on the eyestalk of the kuruma prawn. The results of EST analysis are summarized and the newly found genes assumed to relate to the hormonal system are further discussed.

2. Materials and methods

2.1. Sampling

Immature female kuruma prawn, *M. japonicus* (24.9–36.2 g) were obtained from a commercial farm in Okinawa prefecture, Japan. Ablation of the eyestalk of the shrimp stimulated gene expression of vitellogenin in the ovary (data not shown), suggesting that the eyestalk secretes a factor that down-regulates sexual maturation. The nerve fibers containing the X-organ were excised from the eyestalks of 10 shrimp and pooled. Most parts of the compound eye were eliminated from the samples; however, an appreciable quantity of retinular cells remained, because complete elimination of the compound eye would cause loss of nerve cell bodies present around the boundary between the medulla externa and the compound eye. The average mass of the sample per eyestalk was 0.57 mg.

2.2. Construction of complementary DNA (cDNA) library

Total RNA was extracted from the eyestalk, and poly (A)+ RNA was purified by passing it twice through an oligo (dT)-cellulose column. Synthesis of cDNA and unidirectional cloning into the pSPORT1 vector were conducted using a kit, superscript plasmid system with gateway technology for cDNA synthesis and cloning (Invitrogen). The vector was transformed into *Escherichia coli*-competent cells, and one-pass sequencing from the 5′-end was applied to 2304 independent colonies by the Takara dragon genomics center (Mie, Japan). The average length of nucleotide sequences with Phred quality value >15 for the 2304 readings was 487 base pairs (bp). After removal of vector-based sequences, 1988 readings (86.3%) contained 100 bp or longer sequences, and the ESTs were analyzed further. Nucleotide sequences of the ESTs were registered in the DNA data bank of Japan (DDBJ).

2.3. Contig formation and homology search

The assembly of ESTs to create contigs was done on the genetyx-mac ATSQ program (ver 4.2.1). The database of DNA sequences in DDBJ was searched using the blastn program (Altschul et al., 1997). ESTs and contigs were ranked into 3 groups based on the blast score: high homology (≥100 bits), low homology (50≤~<100) and unidentified (<50). ESTs and contigs that showed homologies only to either an untranslated region of compared sequences or non-characterized sequences were classified as unidentified, even when the blast score was more than 50 bits.

2.4. Phylogenetic analysis

Mature peptide sequences of CHH family peptides were aligned by the clustal V method on the MegAlign software, ver. 5.51 (DNASTAR inc.). For the clustal V method, a PAM100 residue weight table was used with the gap penalty set to 10 and the gap length penalty set to 10. A phylogenetic tree was then created by the software. Aligned sequences obtained from DDBJ were CHHs for *M. japonicus* (AB007507, AB035724, D87864, AB007508, AB007509), *Penaeus monodon* (AY346378, AY346381, AY346382), *Metapenaeus ensis* (AF109775, AF247160), *Macrobrachium rosenbergii* (AF219382), *Nephrops norvegicus* (AY285782), *Homarus americanus* (S76846), *Procambarus clarkii* (AF474408) and *Carcinus maenas* (AF286081); MIHs for *M. japonicus* (AB004652, AB162448), *P. monodon* (AY496454, AY496455), *M. ensis* (AF076276, AF294648), *Cancer magister* (AF031493) and *C. pagurus* (AJ245380); MOIHs for *Libinia emarginata* (AF144660) and *C. pagurus* (AJ245378, AJ245379); GIHs for *H. americanus* (X81821) and *N. norvegicus* (AF163771).

3. Results

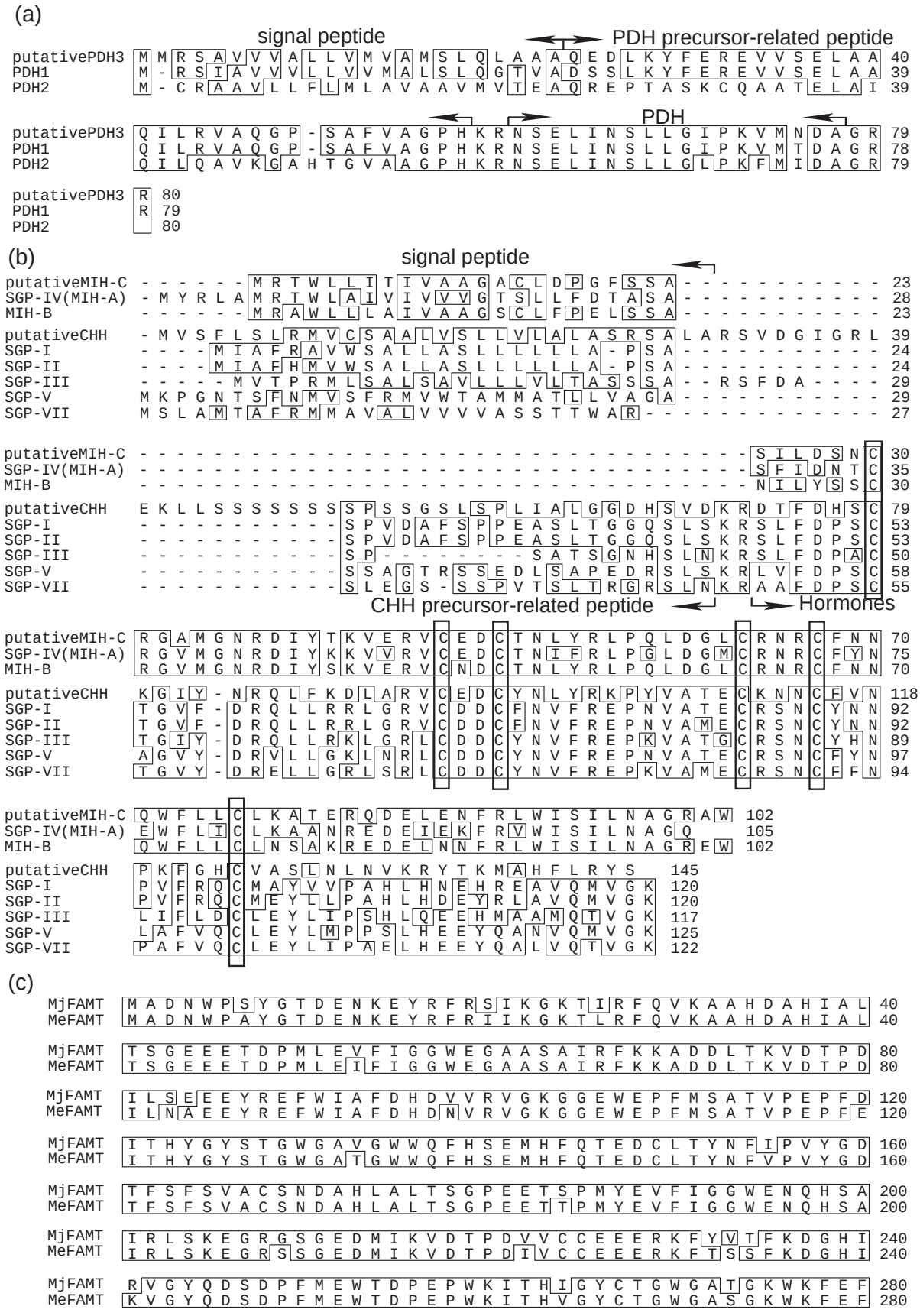
The assembly of 1988 ESTs obtained from the eyestalk cDNA library of the kuruma prawn created 136 contigs from 738 ESTs (37.1%) and 1250 singleton ESTs (62.9%) (Table 1). The DNA database in DDBJ was then searched for the counterparts of the 1386 sequences (136 contigs and 1250 singleton ESTs).

Table 2
Types of gene products obtained by blastn search using ESTs

Types of product	Number of EST	Percentage
Ribosomal RNA	368	18.5
Enzyme	148	7.4
Structural protein, cytoskeleton	46	2.3
Visual pigment and related protein	31	1.6
Ribosomal protein	30	1.5
Intercellular modification, signal transduction	27	1.4
DNA replication, transcription, translation	20	1.0
Carrier protein	15	0.8
Neurotransmitters and related protein	6	0.3
Hormone, growth factor	5	0.3
Membrane protein	5	0.3
Others	29	1.5
Total	730	36.7

Table 3
List of genes identified in the DNA database with blast score more than 300 bits

Product	Blast score (bits)	Species	Accession number
Enzyme			
Cytochrome *c* oxidase subunit I, mitochondrial gene	1661	*Melicertus canaliculatus* (penaeid shrimp)	AY264893
Cytochrome *c* oxidase subunit II, mitochondrial gene	406	*Penaeus monodon* (black tiger shrimp)	AF217843
Cytochrome *c* oxidase subunit III, mitochondrial gene	369	*Farfantepenaeus notialis* (penaeid shrimp)	X84350
Cytochrome *b*, mitochondrial gene	461	*Penaeus monodon* (black tiger shrimp)	AF125382
Phosphoenolpyruvate carboxykinase	767	*Litopenaeus vannamei* (Pacific white shrimp)	AJ250829
Arginine kinase	1128	*Penaeus monodon* (black tiger shrimp)	AF479772
Farnesoic acid *O*-methyltransferase	481	*Metapenaeus ensis* (sand shrimp)	AF333042
Na+/K+ ATPase, alpha subunit	686	*Callinectes sapidus* (blue crab)	AF327439
Phosphopyruvate hydratase (enolase)	710	*Penaeus monodon* (black tiger shrimp)	AF100985
Sarco/endoplasmic reticulum Ca2+−ATPase	454	*Procambarus clarkii* (red swamp crayfish)	AF025848
C-type lysozyme	381	*Marsupenaeus japonicus* (kuruma prawn)	AB080238
Fumarate hydratase class II	674	*Bordetella bronchiseptica* (bacterium)	BX640449
14-3-3 zeta-like type II	661	*Penaeus monodon* (black tiger shrimp)	AF441716
ATP synthase lipid-binding protein	1174	*Marsupenaeus japonicus* (kuruma prawn)	AB079892
Osmolarity-sensing histidine-kinase	504	*Pseudomonas fluorescens* (bacterium)	AY542493
Methylmalonate-semialdehyde dehydrogenase	601	*Bordetella bronchiseptica* (bacterium)	BX640439
D-isomer specific 2-hydroxyacid dehydrogenase	515	*Pseudomonas putida* (bacterium)	AE016780
Ribosomal RNA			
16S ribosomal RNA, mitochondrial gene	997	*Melicertus canaliculatus* (penaeid shrimp)	AY264907
28S ribosomal RNA, mitochondrial gene	805	*Litopenaeus vannamei* (Pacific white shrimp)	AF124597
Ribosomal protein			
Ribosomal protein S18	325	*Cherax destructor* (Australian freshwater crayfish)	AY014897
Ribosomal protein L27a	432	*Drosophila yakuba* (fruit fly)	AY232080
Structural protein and cytoskeleton			
Actin	1302	*Marsupenaeus japonicus* (kuruma prawn)	AB055975
Actin 2	1021	*Penaeus monodon* (black tiger shrimp)	AF100987
Tropomyosin	963	*Metapenaeus ensis* (sand shrimp)	U08008
Structural protein and cytoskeleton			
Alpha tubulin	1052	*Drosophila melanogaster* (fruit fly)	M14643
Beta-I tubulin	463	*Homarus americanus* (American lobster)	U41811
Intercellular modification and signal transduction			
Heat shock protein 70	967	*Penaeus monodon* (black tiger shrimp)	AF474375
Heat shock protein 90	466	*Chiromantes haematocheir* (crab)	AY528900
Nitrogen regulation protein	547	*Pseudomonas syringae* (bacterium)	AE016857
Ras	930	*Marsupenaeus japonicus* (kuruma prawn)	AF338471
Adaptor-related protein complex 2. Beta 1 subunit	258	*Rattus norvegicus* (Norway rat)	NM_080583
G protein beta 1 subunit	484	*Homarus americanus* (American lobster)	AF044735
Hormone			
Pigment dispersing hormone precursor	585	*Litopenaeus vannamei* (Pacific white shrimp)	Y11723
DNA replication, transcription, translation			
LexA repressor	373	*Pseudomonas chlororaphis* (bacterium)	AF502251
Eukaryotic initiation factor 4A	1164	*Marsupenaeus japonicus* (kuruma prawn)	AB074896
Elongation factor-1 alpha	606	*Libinia emarginata* (portly spider crab)	U90050
Elongation factor-2	381	*Libinia emarginata* (portly spider crab)	AY305506
Carrier protein			
ATP-binding ABC transporter protein	573	*Bordetella bronchiseptica* (bacterium)	BX640444
Calmodulin	369	*Phytophthora infestans* (fungus)	M83535
Penicillin-binding protein 1A	1009	*Escherichia coli* (bacterium)	X02164
Neurotransmitters and related protein			
Synaptosome-associated protein of 25 kDa	391	*Procambarus clarkii* (red swamp crayfish)	AB063359
Membrane protein			
Putative membrane protein	373	*Bordetella bronchiseptica* (bacterium)	BX640440
Others			
tRNA-Val, mitochondrial gene	353	*Pseudomonas syringae* (bacterium)	AE016870
Alpha2-macroglobulin homolog	1065	*Marsupenaeus japonicus* (kuruma prawn)	AB108542
Histone H3.3B	414	*Drosophila melanogaster* (fruit fly)	NM_176719
GGDEF domain protein	367	*Pseudomonas putida* (bacterium)	AE016777


(a)
signal peptide
PDH precursor-related peptide
PDH
putativePDH3
PDH1
PDH2
(b)
signal peptide
putativeMIH-C
SGP-IV(MIH-A)
MIH-B
putativeCHH
SGP-I
SGP-II
SGP-III
SGP-V
SGP-VII
CHH precursor-related peptide
Hormones
(c)
MjFAMT
MeFAMT

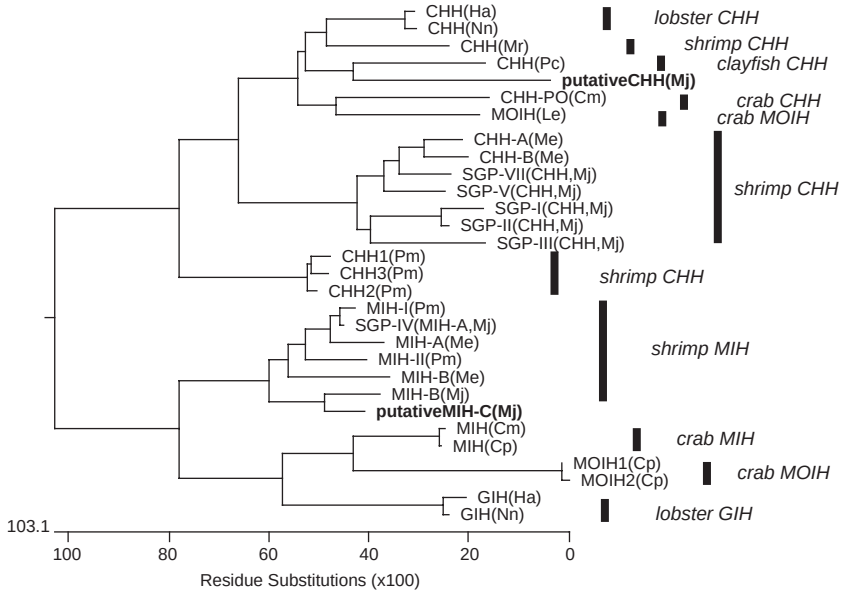


Fig. 2. Phylogenic tree based on the analysis of mature peptide sequences of a CHH family peptide. CHH, crustacean hyperglycemic hormone; GIH, gonad-inhibiting hormone; MIH, molt-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; SGP, sinus gland peptide; Mj, *Marsupenaeus japonicus*; Pm, *Penaeus monodon*; Me, *Metapenaeus ensis*; Mr, *Macrobrachium rosenbergii*; Nn *Nephrops norvegicus*; Ha, *Homarus americanus*; Pc, *Procambarus clarkii*; Cm *Carcinus maenas*; Cm, *Cancer magister*; Cp, *Cancer pagurus*; Le, *Libinia emarginata*.

Only 138 sequences (10.0%) were ranked in the high homology group (blast score ≥100), and 93 sequences (6.7%) in the low group (score: 50–100; Table 1); both groups included 36.7% of the total number of ESTs. The remaining 1155 sequences (83.3%) covering 63.3% of ESTs, were classified as unidentified (score <50, Table 1). The EST and contig sequences in high and low homology groups were categorized by the types of gene products (Table 2). The most abundant category was ribosomal RNA (rRNA), and the next most abundant category was enzymes, including mostly mitochondrial respiration enzymes (82 of 148 ESTs). Moreover, various types of products were found in the counterparts of ESTs, although the proportion of each category was less than a small percentage. The products with a score of more than 300 bits are listed in Table 3; most of them were derived from crustaceans, while some were bacterial gene products.

Among the 1386 sequences, only three (all singleton ESTs) demonstrated close similarities to nucleotide sequences of the CHH peptide family: one to PDH (blast score 585 bits), and two to MIH (111 and 178). Since these ESTs contained partial sequences for these genes, cDNA sequences for whole open reading frames (ORF) were subsequently determined. The three cDNA sequences, as well as the deduced amino acid sequences, were distinct from the known kuruma prawn PDHs and MIHs. The deduced amino acid sequence of the PDH-like sequence demonstrated 94.4% identity to kuruma prawn PDH1 in the mature peptide region and 83.3% to the PDH2 mature peptide, and was thereby designated as putative PDH3 (Fig. 1a). The signal peptide and the PDH precursor-related peptide of the putative PDH3 clearly showed higher homologies to those of PDH1 than to those of PDH2. The deduced amino acid sequence of one MIH-like sequence (originating from the EST with a blast score of 178) showed high identities to MIHs: 83.5% to the mature hormone region of kuruma prawn MIH-B and 72.7% to that of MIH-A (Fig. 1b). All cysteine in the mature peptide region was conserved. This was thus thought to be the third type of kuruma prawn MIH, namely MIH-C. In contrast, the other MIH-like sequence (originating from the EST with a blast score of 111) seemed similar to CHHs rather than MIHs (Fig. 1b). The sequence (putative CHH) contained the CHH precursor-related peptide sequence found in CHHs, but not in MIHs, and showed closer similarity to CHHs than to MIHs; percent identities to mature peptides of CHHs (SGP-I, II, III, V and VII) were 51.9–55.8%, whereas those to MIHs were 39.7–42.9%. The putative CHH sequence appeared unique, however, because similarity among the known kuruma prawn CHHs (percent identities: 65.8–90.5%) were obviously stronger than that of putative CHH to the known CHHs. Therefore, the putative CHH was further compared with all members of a CHH family from various crustacean species by creating phylogenetic tree (Fig. 2). Each hormone class was well grouped except for MOIH of *L. emarginata*. The putative CHH was assembled within a cluster of CHHs, but it showed a closer phylogenetic relation to crab and crayfish CHHs than to shrimp CHHs.

Fig. 1. Deduced amino acids of putative eyestalk hormones and farnesoic acid *O*-methyltransferase (FAMT), and alignment with their homologs. (a) A pigment-dispersing hormone (PDH), (b) CHH-family peptides including crustacean hyperglycemic hormone (CHH), sinus gland peptide (SGP), and molt-inhibiting hormone (MIH) and (c) FAMT. In (a) and (b), all the sequences are derived from the kuruma prawn. The conserved cysteine residues that are essential for secondary structures of CHH and MIH are indicated by rectangles. Mj, *Marsupenaeus japonicus*; Me, *Metapenaeus ensis.*

Sequences of one singleton EST and one contig consisting of two ESTs showed marked similarities to farnesoic acid *O*-methyltransferase (FAMT), an enzyme in the final step of production of methyl farnesoate (MF), a crustacean homolog of insect juvenile hormone. As the sequences of the contig and the EST overlapped each other only a short length, the program did not create any contig consisting of the three ESTs. Examination of the subsequent nucleotide sequences of these ESTs confirmed that those ESTs encode the same product. The deduced amino acid sequence of putative FAMT was highly homologous (93.2% identity) to that of *M. ensis* (Fig. 1c).

4. Discussion

By EST analysis we examined the genes expressed in the eyestalk, a mainstay of crustacean physiology including maturation. Lehnert et al. (1999) previously reported ESTs of the eyestalk, the cephalothorax and the pleopod of the black tiger shrimp (*P. monodon*); eyestalk ESTs of 55 nuclear genes were analyzed in the report. We obtained 1988 ESTs from the cDNA library of the eyestalk of the kuruma prawn, and although the ESTs contained rRNA (18.5% of total EST) and mitochondrial products like respiration enzymes (4.1%), our study was conducted on a larger scale than aforementioned one. Nevertheless the present study is also not an exhaustive enough analysis of genes expressed in the eyestalk, especially that a high percentage of singleton EST (63.9%) after the creation of contigs implies that numerous unexamined sequences still remain in the cDNA library. Despite its small size, the eyestalk seems to express a large variety of genes.

Three ESTs that showed sequences with high homologies to those of known eyestalk hormones are, by comparing their deduced amino acid sequences, thought to encode novel PDH, MIH and CHH. The putative PDH and MIH peptides showed high identities especially to mature peptide regions of kuruma prawn PDH1 (94.4%) and MIH-B (83.5%), respectively. The six cysteine residues in the mature MIH peptide, which are essential for the correct three-dimensional shape (Katayama et al., 2004), were conserved. These results suggested that putative PDH and MIH are functional. On the other hand, putative CHH showed a rather weaker similarity, and the phylogenic analysis indicated that the putative CHH had a stronger similarity to crab and crayfish CHHs than to known kuruma prawn CHHs. Thus, functional analyses of gene products are prerequisite for confirming its biological role. To date, sequences for peptides and/or cDNA of two PDHs and eight CHH family hormones of the kuruma prawn have been reported (Yang et al., 1995, 1996, 1997; Ohira et al., 2005). All of the putative hormones found in the present study were different from the known hormones in their sequences of both nucleotides and amino acids. Nevertheless, this does not mean that the shrimp used in this study did not express those known genes, because cDNAs of the known hormones from the cDNA library were amplifiable by the PCR method using specific primers for each hormone (data not shown). This indicates that numerous sequences, possibly encoding unknown hormones, remain unexamined.

ESTs encoding FAMT, an MF-producing enzyme, were found in the eyestalk library. Although its biological function is not well understood, MF is, from its chemical structure, speculated to be a crustacean equivalent of insect juvenile hormone and is, therefore, a possible candidate to be a factor related to crustacean growth and maturation (Laufer et al., 1987a). The synthesis site of MF is the mandibular organ in crustacean species (Laufer et al., 1987b; Tobe et al., 1989; Sagi et al., 1991; Ding and Tobe, 1991), and the synthesis is negatively controlled by the eyestalk (Tsukimura and Borst, 1992). Recently, the wide distribution of FAMT including that from the eyestalk has been demonstrated, strongly suggesting ubiquitous, rather than a single source of, MF production (Silva Gunawardene et al., 2002). Our study supports the production of MF from the eyestalk.

Roughly speaking, the sequences ranked in a high homology group can encode homologs of comparable gene, whereas those ranked in a low homology group have only partial similarities to comparable genes. Scores less than 50 rarely mean marked similarity, and are, thereby, classed as unidentified. Despite the large scale of genomic analyses in humans, mice, African clawed frog, pufferfish, fruit fly, and such, it is surprising that more than 60% of EST sequences obtained from the kuruma prawn eyestalk disclosed no counterparts. This indicates that crustaceans possess a large number of unique genes that remain to be clarified. The fact that an EST obtained by one-pass sequencing does not always include a coding region is a methodological problem and could be another reason for ESTs classified as unidentified.

Since ESTs are obtained after numerous processes, their variety and proportion to each EST component in the library does not correctly reflect the original conditions of the tissues or organs used. However, since no specific genes were concentrated or subtracted intentionally when we constructed the EST library, the conditions of gene expression in the eyestalk may be roughly speculated from the variety and proportion of genes encoded by ESTs. Over 18% of total ESTs encoded rRNA. Because of the completion of the study on the nucleotide sequence of rRNA in penaeid species (Wilson et al., 2000), ESTs ranked as unidentified no longer include rRNA sequences. Accordingly, cDNAs for rRNA could occupy about 18.5% in the library used, and it is known that rRNA occupies about 95% of total RNA. Despite the purification of mRNA by oligo (dT) column, the contamination of rRNA seems unavoidable probably due to A-rich sequences in rRNA. Considerable contamination of cDNA originating in rRNA has been described (Lehnert et al., 1999). Moreover, rRNA sequences were also often found in sequences deposited as ESTs in the DNA data bank. Rhodopsin, an eye pigment protein, and arrestin, a deactivator of rhodopsin mediated signaling in the retina, were often found, being derived from the contaminated compound eye in the tissue sample. Genes related to cellular metabolism, like ribosomal proteins, heat shock proteins, respiratory enzymes, were also found in abundance. Compared with such genes, eyestalk hormones or hormone-related genes were scarce. The application of methods for concentrating tissue-specific genes might be effective in uncovering rare genes.

To summarize, we obtained 1988 ESTs from the cDNA library of the eyestalk of the kuruma prawn. A variety of homologous genes were found by searching DNA database; however counterparts for a large number of ESTs still could not be identified. Although only a small number of EST was speculated to encode the products related to the endocrine system, three noble putative hormones were found among them. To further understand the regulation of maturation through eyestalk factors, the profiling of the gene expression of those ESTs is our next study.

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