Discovery of immune related factors in *Fenneropenaeus chinensis* by annotation of ESTs *

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Abstract A total of 10446 expressed sequence tags (ESTs) are obtained by a large-scale sequencing of a cDNA library from cephalothorax of adult *Fenneropenaeus chinensis*. An EST analysis platform was built up based on local computers and bioinformatic techniques were used to annotate these ESTs in order to promptly find possible functional genes, especially for immune related factors. About 4% of the ESTs show similarity to the coding sequences of such factors, including lectin, serine protease, serpin, lysozyme, etc. These ESTs provide a partial profile of the immune system in *F. chinensis* and useful information for further study on these genes.

Keywords: *Fenneropenaeus chinensis*, bioinformatics, ESTs, immune factors.

An economically important species for aquaculture, *Fenneropenaeus chinensis*, inhabits the east coast of China and the west coast of Korea. A large scale shrimp aquaculture began in the 1970s and the production of *F. chinensis* reached the maximum in 1992. Since 1993, its production has been undergoing great losses due to diseases mainly caused by white spot syndrome virus (WSSV). Prevention and control of diseases are crucial for the *F. chinensis* aquaculture.

Knowledge about shrimp immunity is quite limited. The shrimp immune system is different from that of well studied mammals. Adaptive immunity is assumed to be absent in invertebrates because they lack immunoglobulins and secondary immune response. Therefore vaccination is of little use to prevent shrimp diseases. The sustainability of its aquaculture may depend on selection of disease-resistant shrimps. Thus, identification and isolation of factors related to immune function of shrimp are of great importance.

A number of studies on shrimp immunity have been carried out. The European Union supported a collaborative project "Shrimp Immunity and Diseases Control", researching on *Penaeus monodon* (*P. monodon*), *Litopenaeus vannamei* (*L. vannamei*), *Litopenaeus stylirostris* (*L. stylirostris*), *Marsupenaeus japonicu* (*M. japonicu*), *Penaeus semisulcatus* (*P. semisulcatus*) and *Farfantepenaeus paulensis* (*F. paulensis*). "Immunaqua" is another project supported by the European Commission, with the cooperation of France, Switzerland, Belgium, Brazil, Chile, Thailand and China. Researches have shown that cellular immunity and humoral immunity exist in shrimp[1]. It has been known that some factors, such as lectin, antibacterial peptide and lysozyme, take part in the immune response by regulating immune cascades and cell status. They are called immunomodulators. In cellular immunity, such factors are also needed to recognize pathogens and regulate the reactions. Thus, study on immune mechanisms of shrimp should be focused on these factors.

Getting the coding sequences for these factors is essential to understanding of the defense system in shrimp. Therefore, analysis based on ESTs is a good strategy for understanding the gene expression, getting useful genes and accumulating information for constructing a genetic map.

ESTs are often generated in a large scale with each of about 200 bp ~ 500 bp in length[2]. Proper analytic methods must be employed to effectively and efficiently mine the information contained in the se-

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quences. Bioinformatics is a subject area involving study and use of analysis methods by using informatics techniques such as mathematics, physics and computer technology to store, manage and analyze molecule sequences. In this study bioinformatic techniques are used to find the tentative coding sequences of factors related to immune function in shrimp.

1 Materials and methods

1.1 Source of data

A total of 10446 ESTs were generated by partial sequencing of about 19000 randomly selected clones from a cDNA library[3] constructed from the cephalothorax of female adult *F. chinensis*, through cooperation of our laboratory with Genomics & Bioinformatics Institute, Chinese Academy of Sciences. After removing low quality bases and vector sequences, ESTs longer than 100 bases were picked for further analysis.

These ESTs were assembled by software Phrap (Phil Green)[1]. For the terminal of ESTs, if the similarity of at least 50 continuous bases was more than 95%, the ESTs were theoretically regarded as fragments from the same mRNA or multi-copy of the same gene. They were assembled to a consensus sequence called contig. The ESTs that could not be assembled with others were called singletons.

To compare these ESTs, public accessible sequences in databases were downloaded and integrated into our own computer, including nt database, nr database, Swiss-Prot database and EST database from the National Center for Biotechnology and Information (NCBI), EST data of *Drosophila malenogastor* and *Anopheles gambiae* from the Institute for Genomic Research (TIGR), and InterPro database from European Bioinformatics Institute (EBI).

1.2 Computation platform and software

A PC-cluster with 4 parallel PCs was built and LINUX Red Hat was employed as the operation system. Basic local alignment search tool (BLAST)[4] from NCBI and InterProScan[5] from InterPro website were downloaded and intergrated to construct an analysis platform and a web server of Intranet. ClusterX[6] was used for multiple alignment.

1.3 Data analysis

1.3.1 Sequence alignment by BLAST A strategy for annotating ESTs is to compare them with the sequences of known function, because similarity of sequences suggests a similar function. BLAST is a powerful tool for such a purpose. Candidate ESTs were aligned with known sequences stored in nucleic acid or protein databases. The results generated were evaluated according to the score and E value of matches by BLAST. High score and low E value suggest a high possibility of similar function between sequences. Comparison between ESTs and nucleic acid sequences was done by BLASTN. For ESTs longer than 200 bp, alignment results with E values less than 0.01 were kept for further analysis[7]. BLASTX was used to compare ESTs with protein sequences, and results with E values less than 0.02 were considered significant[8].

1.3.2 Motif search by InterProScan The number of genes discovered from shrimp and its closely related species is quite small in the sequence database. By April 18, 2003, among 16365404 ESTs in dbEST of GenBank, there were only 4519 ESTs from penaeus genus. When we aligned our ESTs with the public accessible sequences in NCBI, only half of them matched. In order to get more ESTs annotated, InterProScan was used to find motifs in our data. Motifs are usually groups of conserved amino acids in peptides, which are related to function and could be used to find similarity among sequences from remote species because they concentrate the search range from the whole sequence to a couple of residues. InterProScan translates inputted sequences in 6 frames and then compares them with the motifs in InterPro database.

2 Results and discussion

2.1 Tentative immune-related factors detected by BLAST

Table 1 lists the factors found from analysis of ESTs that are tentatively related to the immunity. According to their functions in immune response, they are classified into 4 categories: (1) proteins that can induce and regulate immune cascade reaction, (2) proteins that directly destroy pathogen, (3) proteins that coordinate immune response or regulate cell status, and (4) proteins related to immune response,

1) Description of this software is available at http://www.phrap.org/phrap.docs/phrap.html

but the mechanism is not clear.

Table 1. Immunity-related proteins showing similarity to our ESTs by BLASTN and BLASTX

Protein	Number of contigs	Number of singletons	Number of ESTs
Thioredoxin	6	4	108
Serine protease	4	6	46
Lectin, C-type	8	2	43
Heat shock protein 70 kD-APG-2	1		32
Ribosomal protein L10 (QM protein)	2		30
Heat shock protein cognate 3	2		23
Cathepsin B precursor	3	1	22
Translationally controlled tumor protein (TCTP)	1		22
Superoxide dismutase (Mn)	2		14
Serine proteinase inhibitor	2	4	13
Heat shock protein 83 kD	2		9
Transmembrane 4 super family	2		9
Metallothionein	1	4	8
Proteasome 26S subunit	2	3	8
Ribosomal protein S15 (Rig protein)	1	1	6
Ribosomal protein S18 (KE3)	1		6
Heat shock protein 86 kD	1		5
Peroxisomal antioxidant enzyme	1		5
DnaJ	1		2
Heat shock protein STI1	1		2
Prefoldin 6	1		2
Prefoldin 3	1		2
Prohibitin	1		2
11.5 kD antibacterial peptide		1	1
Death associated protein		1	1
Heat shock protein cognate 5		1	1
Heat shock protein SSA1		1	1
Hemocyte transglutaminase		1	1
Lysozyme		1	1
Macrophage asialoglycoprotein-binding protein		1	1
Melanoma-associated antigen 10		1	1
NK-tumor recognition protein		1	1
Total	47	34	428

2.1.1 Proteins that induce and regulate immune cascade reaction There were 59 ESTs possibly encoding serine protease and its inhibitor, which are both potentially involved in two immune cascades, prophenoloxidase cascade (PPO) and clotting cascade, that play important roles in innate immunity.

The 46 ESTs tentatively encoding serine protease may relate to PPO cascade in that these serine proteases are similar to prophenoloxidase activation enzyme (PPA), which resembles trypsin in its carboxyl terminus containing a catalytic serine protease domain. Whereas in the amino terminus, it has a clip domain with six conserved cysteine residues to form three disulfide bonds[9, 10]. A typical serine protease with PPO activating function usually possesses such a domain. Fig. 1 shows the sequence alignment between one of our contigs with prophenoloxidase activating factors from other arthropod species. Source of sequences in Fig. 1 is described in Table 2.

We can see from the sequences that between the clip domain and the protease domain there is a linker region which varies in different species. It is believed that the activation of the zymogen is through a specific protolysis between the linker region and the protease domain, but they are still connected by a disulfide bond after the cleavage.

PPO has been found in 15 invertebrates, including 2 kinds of crustaceans and 13 kinds of insects[11]. Usually observed at the wounded region, it can be activated by components such as glucosan, lipopolysaccharide and peptidoglycan on the cell surface of microbes[12]. Recognition of pathogens activates PPA, which catalyzes prophenoloxidase to phenolocidase(PO). PO participates in the oxidation of phenolic substance into quinones which are converted to melanin. This process is analogous to the activation of alexin in advanced animals. It promotes phagocytosis, release of immune factors and encapsulation of pathogens[13].

PPO needs feedback regulation to prevent the uncontroled extending of the cascade. The serine proteinase inhibitor (serpin) regulates the activation of serine protease and keeps the cascade in normal intensity and range[14].

Together with transgluminase, serine protease and its inhibitor work in a way similar to that in clotting cascade[15, 16]. The soluble precursor of clotting protein would interwind with each other and change into insoluble form to prevent pathogen invasion. The process is somewhat similar to the blood coagulation of mammals.

Table 2. Source of sequences used in multiple alignment

Species	Accession number in GenBank
Tenebrio molitor	CAC12696
Holotrichia diomphalia-I	CAC12665
Holotrichia diomphalia-II	BAC15604
Galleria mellonella	AAN15788
Marsupenaeus japonicus	BAB78483
Litopenaeus vannamei	AAL23948
Penaeus semisulcatus	AAN86085
Penaeus monodon	AAN16375
Anopheles gambiae	AAC47326
Drosophila melanogaster	AAF47445
Musca domestica	PC4062
Heliothis virescens	AAD00078
Cyprinus carpio	BAA95698
Gallus gallus	CAA23711
Bos taurus	AAC37310
Homo sapiens	CAA32175

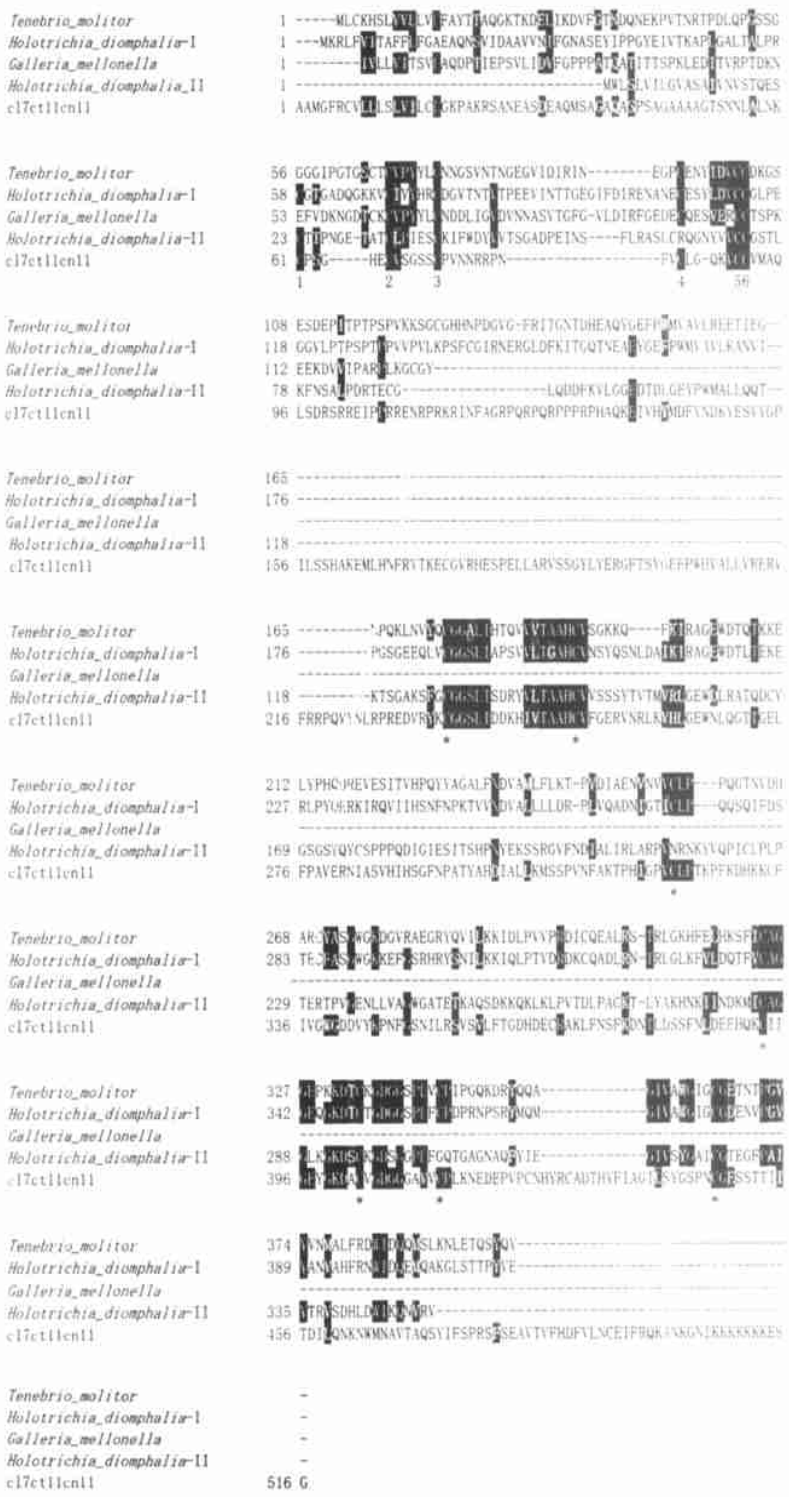

Fig. 1. Multiple sequence alignment of *F. chinensis* contig with PPO activating factors of other arthropod. The arabic numbers below the sequences indicate the conserved cysteine residues that form three disulfide bonds in the clip domain. The cysteine residues that form the disulfide bond within and between the catalytic protease domain are marked by * (source of sequences is described in Table 2).

2.1.2 Antibacterial proteins There was one EST showing similarity to a putative 11.5 kD antibacterial peptide coding sequence from *L. vannamei*[17] (Fig. 2). This peptide was discovered in *Carcinus maenas* and can inhibit the growth of Gram-positive bacteria[18].

Another interesting EST is a lysozyme analogue. The complementary sequence of EST named jh_11950 showed a high similarity to four lysozyme-like proteins from other shrimps (Fig. 3). We can see that from L29 to C80 there is an almost continuous identical region among the sequences, showing a high possibility of being homologous.

> gi| 17223025| gb| AF430071.1| (AF430071) putative antimicrobial peptide mRNA, [*L. vannamei*]
Score= 51.0 bits (105), Expect(2)= 3e-08
Identities= 24/30 (80%), Positives= 24/30 (80%)
Frame= +2/+3

Query: 377 PHAHVSMGPLQPVPTTTSVLASISVASTGV 466
PHAHVS PVPTTTSVLASISVA TGV
Sbjct: 18 PHAHVSTVLPLPVPTTTSVLASISVALTGV 107

Fig. 2. Alignment of our EST (Query) to 11.5 kD antibacterial peptide (Sbjct) in *L. vannamei*.

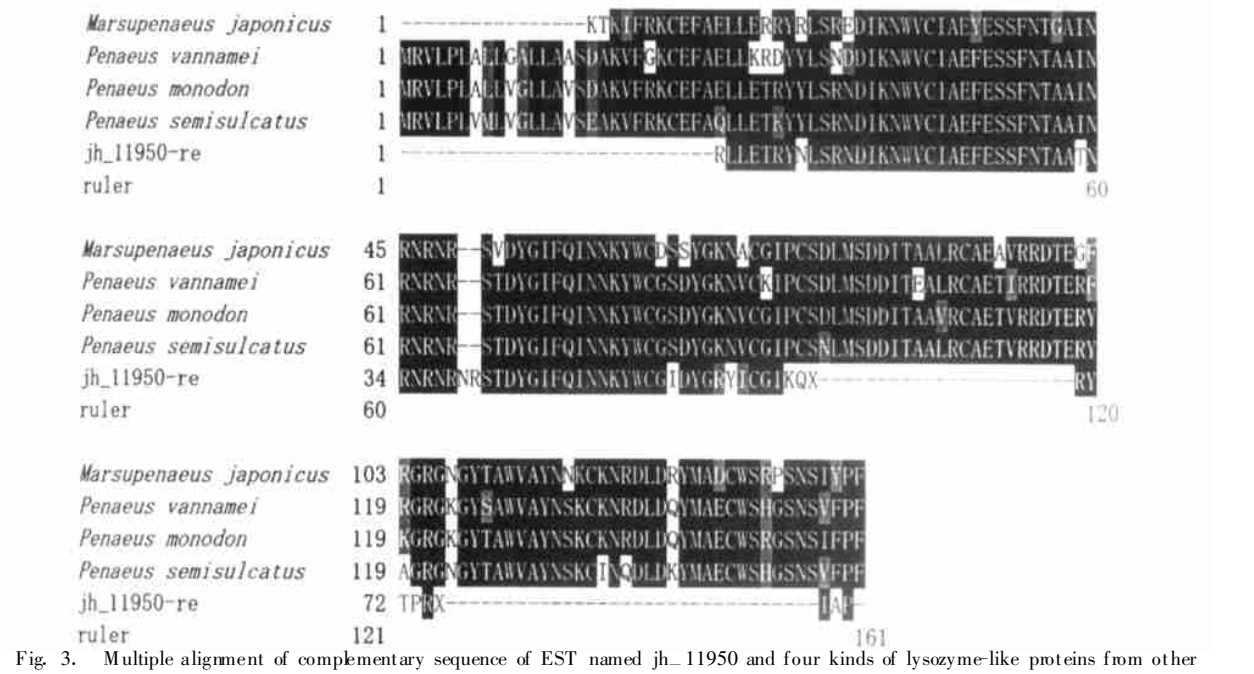

Fig. 3. Multiple alignment of complementary sequence of EST named jh_11950 and four kinds of lysozyme-like proteins from other shrimps (source of sequences is described in Table 2).

Lysozyme can inhibit bacteria by breaking a (1, 4) linkage between two amino sugars, N-acetylmuramic acid and N-acetylglucosamine, of the bacterial peptidoglycan[19,20]. According to Jolles[21], the known lysozyme can be grouped as c (chicken) type, g (goose) type, phage type, bacteria type, plant type and i (invertebrate) type. Through further analysis, these lysozyme-like sequences in Fig. 3 show a higher similarity to c-type lysozyme than other lysozyme types. Fig. 4 shows the multiple alignment result of lysozyme-like sequences of shrimps with c-type lysozymes of other species, including insect, fish, bird, mammal and human. The identical region among these sequences is more dispersed than that in Fig. 3. But there is still a quite conserved region from N47 to C86. In this region E55 and D75 are two amino acids important to the function of lysozyme[22]. They appear in all the sequences in Fig. 4.

2.1.3 Auxiliary factors in immune response This category includes factors related to immunorecognition, molecular chaperone, cell apoptosis and redox.

(1) Immunorecognition by lectin Lectin was discovered in plant at the beginning of the twentieth century. It exists in many kinds of organisms. Different kinds of lectins can bind different types of carbohydrates on bacteria cell wall, cause the activation of serine protease and induce phagocytosis of invading bacteria[23~25]. Although there was only one lectin-like EST found by BLAST search, 43 ESTs containing a C-lectin motif was found by InterProScan search. The role of lectin in immunity is immunorecognition.

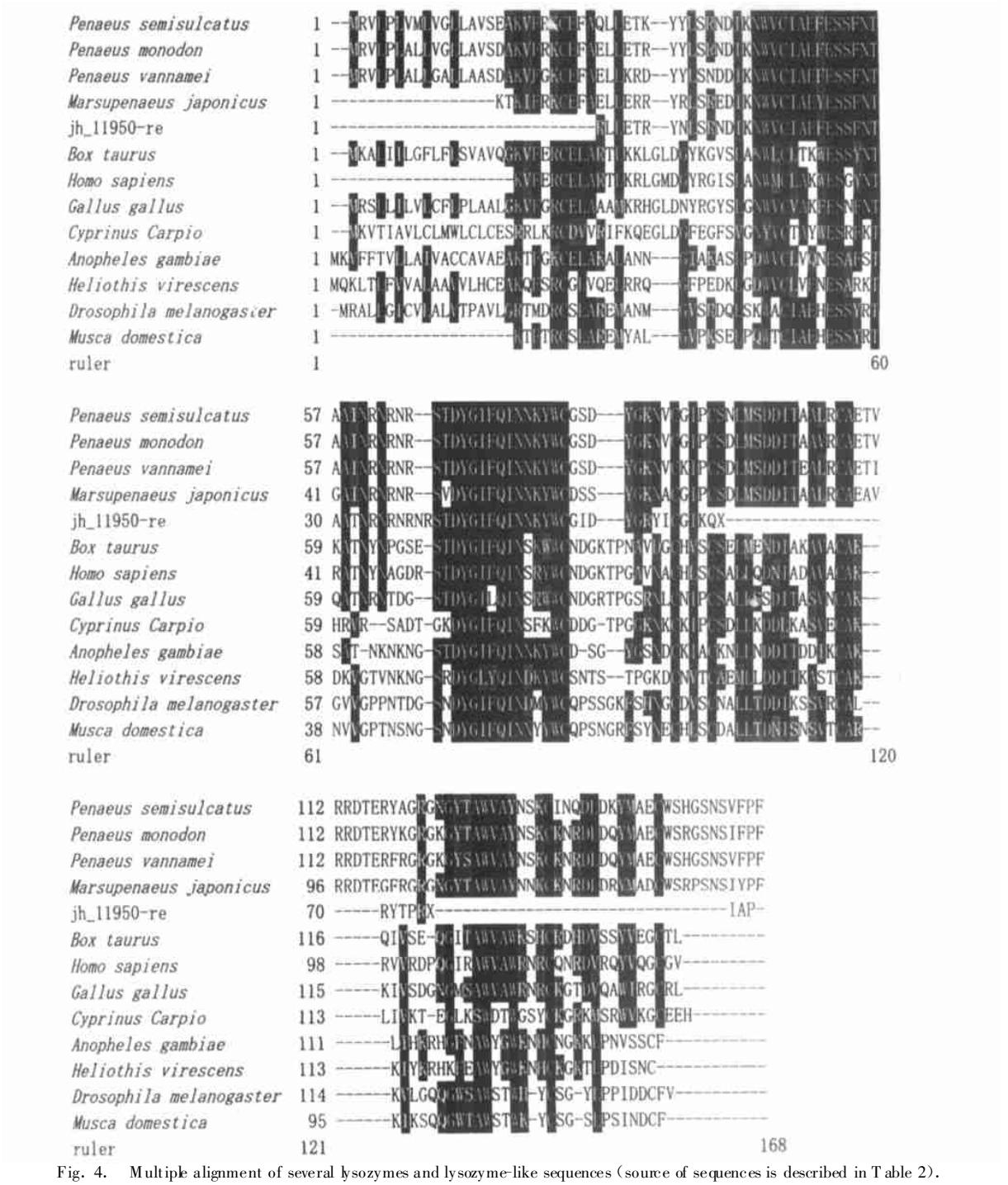

Fig. 4. Multiple alignment of several lysozymes and lysozyme-like sequences (source of sequences is described in Table 2).

(2) Molecular chaperons Table 1 lists 73 ESTs showing similarity to 7 kinds of heat shock proteins (HSP), a kind of molecular chaperon. Threatened by stimulants like virus, bacteria, heat or radiation, some proteins including molecular chaperons will protect cell from damage by keeping the normal structures of proteins and nucleic acids[26]. Besides HSP, chaperonin, prefoldin and DnaJ all function as molecular chaperons[27~29].

(3) Proteins related to redox Table 1 lists 14 ESTs possibly encoding superoxide dismutase (SOD), which is an anti-oxidant enzyme and immunomodulator in shrimp immunity. According to Cmpa-Cordova[30,31], the expression of SOD in haemocytes and muscle increased in the presence of immunostimulants such as β-1, 6 glucan and sulfated polysaccharide. Thioredoxin is another protein with anti-oxidant function. There were 108 ESTs showing

similarity to this protein. Many researches have shown that it modulates immune response through redox control by playing some important roles in regulating the inflammatory process in the primary host defense against infection[32].

(4) Proteins related to apoptosis There were 31 ESTs possibly encoding proteins concerned with cell apoptosis. These proteins are cathepsin, death associated protein, and proteasome 26S subunit. In Rojtinnakorn's research[33], they were found to be up regulated in expression in virus infected shrimps. They may be involved in the elimination of infected cells.

2.1.4 Tumor related genes Table 1 lists 63 ESTs showing similarity to genes encoding tumor related proteins, including transmembrane superfamily 4 (CD37, CD63 and CD9 antigens), prohibitin, translationally controlled tumor protein (TCTP), NK-tumor recognition protein, HSP 90-alpha (HSP 83 tumor specific transplantation 86 kD antigen), KE-3, Rig protein, QM and melanoma-associated antigen 10. Among them, prohibitin and QM can inhibit growth of tumor cells, others are proteins expressed in tumor cells. Besides, TCTP promotes the release of histamine which causes inflammation[34, 35]. Some studies suggest that these genes are concerned with virus infection in shrimp. According to the work of Takashi Aoki et al. expression of TCTP increased in shrimp infected by white spot syndrome virus[33].

It is interesting to see the expression of these tumor related genes in response to virus infection because in mammals many tumors are caused by virus. The shrimp that used to construct the cDNA library was not a pathogen free one, so it was quite likely that there were some latent viruses in its body. Although no symptoms were shown, some of the viruses might have some activities, such as replicating, transcribing or translating themselves, so that was detected by the host, causing expression of tumor/virus related products.

2.2 Motif search results

There are three major goals in doing InterProScan motif search: (1) to annotate the Contig/ESTs that did not show homology through BLAST; (2) to obtain more information, such as the location of functional group, about the already annotated sequences; (3) to obtain more specific information about the role that Contig/ESTs play in biological process.

By motif search, the tentative function of many, although not all, of the unannotated sequences by BLAST were found.

2.2.1 Chitin-binding motif in peritrophin-like protein ESTs showing similarity to peritrophin-like protein comprised about 10% of our data, and were in higher abundance than other proteins. This protein is a kind of peritrophic matrix protein that exists in most arthropods. Peritrophic matrix, with both peritorphin and chitin as components, works like a barrier in the epithelium of some tissues and keeps pathogens from entering[36].

2.2.2 Antifreeze protein (AFP) with C-type lectin-motif We found 87 tentative AFP encoding ESTs in our data and most of them had similarity to type I AFPs, which are Ala-rich, amphiphilic, alpha-helical proteins found in marine teleosts. By binding to ice crystals, AFPs prevent the growth of ice crystals and depress the freezing point. We also found several C-type lectin motifs that were analogous to a domain contained in type II AFP.

2.2.3 Major histocompatibility complex (MHC) It was quite unusual that we found two contigs and one singleton that possibly contained a MHC motif, because it is speculated that such molecules do not exist in invertebrates. The alignment result was composed of only 7 amino acids with a very weak similarity. Of the tumor-related factors we discussed above, some, for example HSP86 (also called tumor specific transplantation antigens), can bind to MHC[37]. Whether MHC exists in crustaceans is still uncertain now and it is highly possible that the homology is false positive or just indicating an intermediate product in evolution. More experiments are needed to test it.

3 Conclusion

The immune system is a complex network consisting of various immunocytes and immune factors. The analysis of the 10446 ESTs provides clues to the discovery of immune-related genes and helps the study of the genes. Further analysis of the ESTs is needed and the next step is to gain the full length genes of interest. Moreover, the analysis of different expression of these ESTs in healthy and infected shrimps is necessary before these genes are to be cloned.

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