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Molecular profile of the unique species of traditional Chinese medicine, Chinese seahorse (*Hippocampus kuda* Bleeker)

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Abstract A cDNA library of male Chinese seahorse (*Hippocampus kuda* Bleeker) was constructed to investigate the molecular profile of seahorse as one of the most famous traditional Chinese medicine materials, and to reveal immunological and physiological mechanisms of seahorse as one of the most primitive vertebrates at molecular level. A total of 3372 expressed sequence tags (ESTs) consisting of 1911 unique genes (345 clusters and 1566 singletons) were examined in the present study. Identification of the genes related to immune system, paternal brooding and physiological regulation provides not only valuable insights into the molecular mechanism of immune system in teleost fish but also plausible explanations for pharmacological activities of Chinese seahorse. Furthermore, the occurrence of high prevalent C-type lectins suggested that a lectin-complement pathway might exert a more dominant function in the innate immune system of teleost than mammal. Carbohydrate recognition domain (CRD) without a collagen-like region in the lectins of seahorse was likely an ancient characteristic of lectins similar to invertebrates.

Key words: Chinese seahorse; Expressed sequence tag; Chinese traditional medicine; C-type lectin; Innate immune; Paternal brooding

1. Introduction

Seahorse, a marine teleost fish, is well known not only for its special medicinal composition but also for its unusual features including male pregnancy. Although seahorses are usually treated as pets, Chinese seahorse (*Hippocampus kuda* Bleeker) is used as one of the most famous and expensive materials of traditional Chinese medicine (TCM). At present, the natural resource of Chinese seahorse has been dramatically reduced as a result of overfishing. Therefore, it is urgent to learn more about this unique creature physiologically and pharmacologically in order to efficiently protect and properly harness this precious species.

Seahorse belongs to *Syngnathidae* of *Syngnathiformes*, in *Steichthyes* of vertebrate phylum. Most animals in *Syngnathidae* can be used as TCM material and were well documented by all versions of China Pharmacopoeia even as early as in the Liang Dynasty (A.D. 502–557). According to the Yin Yang theory described in TCM book, seahorse has the effect on tonifying kidney and activating Yang. The former function is essentially related to the regulation of urinogenital, reproductive, nervous, endocrine and immune systems [1]; the latter is referred to enhance male's sexual function. Recent pharmacological studies suggested that Chinese seahorse not only had hormone-like activities [2,3], boosting hematopoiesis function [4] but also showed activities of anti-tumor [5], anti-aging [6], anti-fatigue [7] and Ca^{2+} channel blocking [5].

Previous studies [5] on the active components of Chinese seahorse were carried out by various methods of medicinal chemistry, which focused on analyses of trace elements, amino acids and soluble components in organic reagents such as fatty acids, phospholipid and steroid. The limitation of these methods is the neglect of water-soluble molecules such as polypeptide, proteins and polysaccharides. Despite there were a few reports on seahorse in terms of heredity, morphology or breeding, the molecular mechanisms of physiology, pharmacology and immunology of seahorse remain unclear. Here, by construction and analysis of a male seahorse cDNA library, we provide a global view of the molecular profile of Chinese seahorse at genes and proteins levels. This is the first report about the gene profile of marine therapeutic organisms documented by authentic TCM book, and provides a novel example of elucidating molecular mechanisms of TCM ingredients.

2. Materials and methods

2.1. Animal material

Adults of *H. kuda* Bleeker were obtained from a local aquaculture farm in Guangdong, China, in November. The whole body of a male fish was collected, except for its internal organs and head since they were not used for the medical ingredients [8].

2.2. cDNA library construction

Total RNA was extracted using Trizol reagent (Gibco-BRL) ac-

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Abbreviations: EST, expressed sequence tag; TCM, traditional Chinese medicine; NKEF, natural killer cell enhancement factor; MBL, mannose binding lectin; CRD, carbohydrate recognition domain; MASP, MBL-associated serine protease; RHL-1, rat hepatic lectin; GSL, GalNAc-specific lectin; MRC, mitochondria-rich cell; TOP, trout ovulatory protein; IGFBP-5, insulin-like growth factor binding proteins-5; PGDS, prostaglandin D synthase; AA, arachidonic acid

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cording to the protocol of the supplier. A cDNA library of male *H. kuda* Bleeker was constructed from the poly(A) RNA using SMART™ cDNA library construction kit (Clontech) following the instruction of manufacturer. The cDNA was ligated into pcDNA3.0 and electroporated into *Escherichia coli* DH5α cells using a gene pulser II electroporation system (Bio-Rad) under the standard condition. The library contained 8×10^5 independent clones based on our estimation, and a total of 10 000 independent cDNA clones were picked randomly and stored at −80°C for further analysis.

2.3. Expressed sequence tag (EST) sequencing

A total of 3372 cDNA clones randomly selected were sequenced from the 5′-end in an automated ABI PRISM 3700 sequencer (Perkin Elmer), using T7 promoter primer and ABI PRISM Big-Dye™ terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems).

2.4. Sequence analysis

Prior to further analysis, the vector sequence was subtracted and the sequences shorter than 100 bases were discarded from the initial EST set. Then, sequences were assembled into many clusters, which were usually referred to those genes containing more than two ESTs. Those ESTs, which only appeared once in the present study, were specially called singletons. The consensus sequence of each cluster was used as the query sequence to search against NCBI BLAST database with FASTA and BLAST X [9]. In the following analysis, annotations of possible protein-coding genes were performed for future study. All sequence alignments were carried out using the Clustal W at www.genome.ad.jp, and the SignalP computer program was used to predict the cleavage site of the signal peptide [10]. The secondary structure of deduced proteins was predicted by using programs at the www.expasy.ch.

3. Results and discussion

3.1. Overall distribution of sequences

In this study, a total of 3372 random cDNA clones were obtained from a male seahorse cDNA library. The average insert length in this library was 0.79 kb, ranging from 0.3 to 2.0 kb, and the occurrences of the readable sequence lengths were shown in Fig. 1. A large fraction of the sequences ranged from 300 bp to 750 bp and the average length of reads was about 484 bp. The initial ESTs contained 1911 consensus sequences, including 345 clusters and 1566 singletons. As

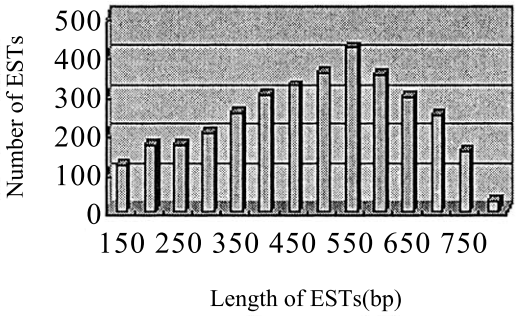

Fig. 1. The distribution of the readable length of ESTs. The readable sequence lengths of the 3372 ESTs ranged from 100 to 800 bp. The average length of reads on which the following analysis was based was 484 bp. Abscissa (50 bp) is the length of sequences, and the *Y*-coordinate is the number of ESTs.

shown in Table 1, there were 23 clusters containing more than nine EST sequences per cluster, accounting for 27.37% of the total clones. In addition, about half of the total clones belonged to singletons, indicating a high complexity of the mRNA population in the adult seahorse. The distribution of cluster sizes was shown in Fig. 2. The cDNA library we used was not a normalized one, thus the number of ESTs corresponding to a particular gene reflected the relative abundance of corresponding transcripts.

The consensus sequence of each cluster was submitted to NCBI to conduct sequence homology searches. According to the BLAST results, 3372 ESTs were divided into three general categories as described in Table 2: (1) 36.45% of total ESTs (1229/3372) representing recognized protein-coding sequences with possibility of chance occurrence less than 10^{-8}; (2) 16.04% (541/3372) being similar to functionally unidentified genes; (3) 47.51% (1602/3372) having no significant similarity to any known sequences including those with match possibility higher than 10^{-8}. The category (3) corresponding to unidentified new genes might be very interesting for further functional analysis.

Table 1
Assembled clusters that correspond to the highest expressed genes

Cluster	Gene identification	Organism	ESTs
H00399	hypothetical 18K protein	*Carassius auratus*	393
H00395	GSL	*Asterina pectinifera*	81
H00005	low-affinity IgE receptor	*Mus musculus*	58
H00386	chondroitin sulfate proteoglycan 3	*Homo sapiens*	40
H00033	cytochrome *c* oxidase subunit III	*Ijimaia dofleini*	39
H00010	procollagen-type I α1 chain	*Danio rerio*	34
H00393	chondroitin sulfate proteoglycan 3	*Rattus norvegicus*	32
H00387	unknown		25
H00389	collagen a2 (I)	*Takifugu rubripes*	23
H00015	skeletal α-actin-type 2	*Coryphaenoides cinereus*	21
H00009	embryonic α-type globin	*Oryzias latipes*	21
H00067	α3-type I collagen	*Oncorhynchus mykiss*	20
H00023	procollagen-type I α1 chain	*D. rerio*	17
H00234	PGDS	*O. mykiss*	14
H00088	unknown		14
H00039	cytochrome *c* oxidase subunit II	*Exocoetus volitans*	13
H00078	unknown		13
H00073	epithelial membrane protein 2	*M. musculus*	12
H00047	S100 calcium binding protein A13	*H. sapiens*	12
H00109	myosin light chain 2	*Pennahia argentata*	11
H00036	troponin C	*Anguilla anguilla*	10
H00139	MHC class II-associated invariant chain	*Gallus gallus*	10
H00007	myosin heavy chain	*Cyprinus carpio*	10

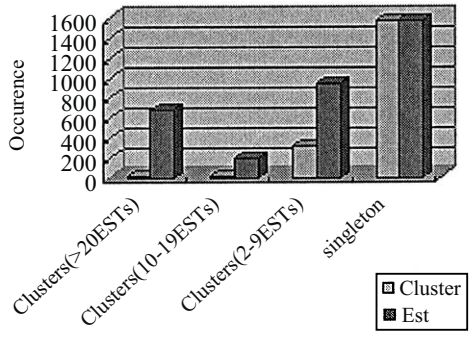

Fig. 2. Prevalence distribution of the cluster size. The initial 3372 ESTs were grouped into 1911 clusters, 11 clusters consisting of more than 20 ESTs each cluster (676 ESTs), 14 clusters including 10–19 ESTs each cluster (193 ESTs), 320 clusters containing two to nine ESTs each cluster (937 ESTs) and 1566 unique sequences.

3.2. ESTs identification and classification

As shown in Table 2, 576 genes with recognized protein-coding sequences were identified, and corresponded to 36.45% (1229/3372) of the total ESTs. Like the previous classification [11], these genes were divided into four major classes as listed in Table 3. Class A contained 80.0% of the recognized protein-coding mRNA transcripts (455 proteins, 975 ESTs), which could be depicted as structural and enzymatic housekeeping proteins associated with the functions of many different cells. Class B, associated with cell–cell communication, contained 65 proteins (183 ESTs). Class C included 25 transcription factors and other gene regulatory proteins (32 ESTs altogether), and class D consisted of 31 specialized terminal differentiation products (39 ESTs). Of these classes, BIII, the genes coding for extracellular matrix proteins and cell adhesion molecules, had the highest redundancy (the total number of ESTs/number of EST clusters) of 5.62, which was consistent with our result of clusters. Of all ESTs in our investigation, only cytochrome *b* gene of *H. kuda* Bleeker was previously reported.

3.3. ESTs similar to immune system-related genes

As listed in Table 4, 60 immune-related proteins (about 331 ESTs) were discovered in the present study, and accounted for approximately 10% of the total ESTs. Chinese seahorse is a teleost fish (*Syngnathiformes* order, *Acanthopterygii* superorder) and its characteristics of immune system remain unclear. Although the immune system of bony fish is considered to share similar organization with mammals, there has been no sufficient evidence regarding the molecular basis of various innate immune responses and adaptive immune responses. The molecular profile of male seahorse cDNA library could provide a global view into the immune system of Chinese seahorse for the first time.

Similar to sea bass (*Perciformes* order, *Acanthopterygii* superorder) [12], seahorse contained immune molecules involved in both the innate and the adaptive immune systems. The former is an evolutionarily ancient form of immunity [13], containing a putative antibacterial peptide, natural killer cell enhancement factor (NKEF), antioxidant protein, detoxifying molecules, hematopoiesis-associated proteins and lectins. The latter appeared early in vertebrate evolution and was consisting of MHC molecules and associated Ii, TCR α chain and T-cell differentiation protein MAL, etc.

Over the past few years, a number of fish cytokine genes have been reported including transforming growth factor-β, interleukin-1β, fibroblast growth factor (FGF), etc. [14]. However, the homologs of B7 homolog 3, B-cell activation protein (regulator of G protein signaling 1, RGS1) and IgD B-cell receptor-associated protein (BAP29) are first discovered in fish [15–18]. These findings will undoubtedly offer valuable

Table 2
The distribution of the genes

	Classes of gene function and annotation	Number of clones	Number of clusters
A	function that many kinds of cells use		
AI	transportation and binding proteins for ions and other small molecules	46	30
AII	RNA processing, polymerizing, splicing, and binding proteins and enzymes	16	13
AIII	cell replication, histones, cyclins and allied kinases, DNA polymerases, topoisomerases, DNA modification	22	14
AIV	cytoskeleton and membrane proteins	389	139
AV	protein synthesis cofactors, tRNA synthetases, ribosomal proteins	160	88
AVI	intermediary synthesis and catabolism enzymes	153	98
AVII	stress response, detoxification and cell defense proteins	107	41
AVIII	protein degradation and processing, proteases	74	25
AIX	apoptosis-related	8	7
	total	975	455
B	cell–cell communication		
BI	signaling receptors, including cytokine and hormone receptors, and signaling ligands	5	5
BII	intracellular signal transduction pathway molecules including kinases and signal intermediates	60	39
BIII	extracellular matrix proteins and cell adhesion	118	21
	total	183	65
C	transcription factors and other gene regulatory proteins		
CI	sequence-specific DNA binding proteins	9	8
CII	non-DNA binding proteins that perform positive or negative roles	21	15
CIII	chromatin proteins other than AIII with regulatory function	2	2
	total	32	25
D	others		
DI	specialized terminal differentiation products	39	31
DII	genes homologous to known sequences of unknown function	541	112
DIII	no significant similarities to known proteins	1602	1223
	total	3372	1911

Table 3
EST sequence similarities, gene description and probability of occurrence by chance

Class	Cluster ID	Accession number	Database entry name	Organisms	Probability
AI. Transportation and binding proteins for ions and other small molecules					
	H01000	AAC28367	S100-like calcium binding protein	*Salvelinus fontinalis*	5.00E-27
	H00270	AAC59937	troponin I slow skeletal muscle isoform	*Coturnix coturnix*	2.00E-51
	H00474	AAF86182	transferrin	*O. tshawytscha*	7.00E-74
	H00145	CAA92147	hemopexin-like protein	*O. mykiss*	6.00E-53
	H00847	CAD12727	high-affinity choline transporter	*Torpedo marmorata*	6.00E-39
	H00846	NP_001437	fatty acid binding protein 7	*H. sapiens*	1.00E-36
	H01023	NP_194568	ADP, ATP carrier-like protein	*Arabidopsis thaliana*	1.00E-69
	H01804	O42175	apolipoprotein A-1	*Sparus aurata*	2.00E-23
	H01605	BAB25124	copper homeostasis protein cutc	*M. musculus*	2.00E-44
AII. RNA processing, polymerizing, splicing, and binding proteins and enzymes					
	H00705	AAC24982	reverse transcriptase	synthetic construct	9.00E-16
	H00208	AAG01404	spliceosomal protein SAP155	*R. norvegicus*	5.00E-52
	H01823	BAB25927	similar to RNA binding protein	*M. musculus*	7.00E-17
	H00655	BAB27510	U2 small nuclear ribonucleoprotein B	*M. musculus*	4.00E-39
	H00432	CAA73349	putative RNA helicase (DEAD box)	*D. rerio*	7.00E-39
	H01723	NP_004809	U5 snRNP 100 kDa protein	*H. sapiens*	9.00E-49
	H00734	NP_112625	polymerase II	*R. norvegicus*	2.00E-40
	H00284	Q63871	DNA-directed RNA polymerases I, II, and III	*M. musculus*	1.00E-18
	H00273	T14855	T14855 reverse transcriptase	*O. latipes*	9.00E-14
AIII. Cell replication: histones, cyclins and allied kinases, DNA polymerases, DNA modification					
	H01025	AAC68476	cyclin D	*Stizostedion vitreum*	1.00E-31
	H00168	NP_002098	H3 histone, family 3A	*H. sapiens*	1.00E-70
	H01872	NP_005316	H1 histone family, member 1	*H. sapiens*	3.00E-22
	H01213	NP_057437	Rec	*H. sapiens*	1.00E-78
	H00214	P06897	histone H2A.1	*Xenopus laevis*	5.00E-47
	H01547	P53782	G1/S-specific cyclin D2	*X. laevis*	2.00E-08
AIV. Cytoskeleton and membrane proteins					
	H00239	AAL37760	ventricular myosin light chain 2	*Canis familiaris*	1.00E-82
	H00134	BAA33452	myosin heavy chain	*Theragra chalcogramm*	2.00E-67
	H00290	BAB28119	actin	*M. musculus*	1.00E-92
	H00741	CAB07851	myosin light chain	*T. rubripes*	1.00E-11
	H01177	1713408A	fibrillin	*H. sapiens*	8.00E-68
	H00219	AAB96361	calcium channel β3 subunit	*T. rubripes*	5.00E-18
	H01814	NP_112188	integral membrane protein 3	*H. sapiens*	1.00E-08
	H01366	AAF31360	ATP synthase subunit B	*Xenopus laevis*	7.00E-53
	H01404	BAA95155	flavocytochrome *b*558	*Tursiops truncatus*	2.00E-39
	H00856	NP_004992	NADH dehydrogenase 1 α subcomplex, 7	*H. sapiens*	2.00E-35
	H00051	NP_006347	ATP synthase	*H. sapiens*	2.00E-52
	H01318	NP_034072	cytochrome *c* oxidase	*M. musculus*	8.00E-36
	H01572	Q9JI55	plectin	*Cricetulus griseus*	2.00E-30
AV. Protein synthesis cofactors, tRNA synthetases, ribosomal proteins					
	H00253	AAD01429	S6 ribosomal protein	*O. mykiss*	1.00E-114
	H00066	AAF61072	40S ribosomal protein S15A	*Paralichthys olivaceus*	5.00E-53
	H00142	AAG33073	ribosomal protein L7	*Rana sylvatica*	8.00E-59
	H00441	AAK11731	ribosomal protein S16	*Heteropneustes fossilis*	1.00E-76
	H00710	AAK63073	60S ribosomal protein L13	*D. rerio*	4.00E-81
	H00283	NP_003742	eukaryotic translation initiation factor 3	*H. sapiens*	5.00E-80
	H01109	NP_143614	50S ribosomal protein L23	*Pyrococcus horikoshii*	8.00E-18
	H01013	AAG50053	eIF4E binding protein 3	*D. rerio*	3.00E-43
	H01925	AAD50290	translation elongation factor 1-α	*Paramecium tetraurelia*	2.00E-23
	H00346	CAA73167	translation initiation factor eIF4A I	*X. laevis*	1.00E-84
	H00658	XP_028985	mitochondrial ribosomal protein S15	*H. sapiens*	3.00E-27
	H00487	Q9GMB8	seryl-tRNA synthetase	*Bos Taurus*	2.00E-76
AVI. Intermediary synthesis and catabolism enzymes					
	H00621	AAC96094	creatine kinase M3-CK	*C. carpio*	3.00E-98
	H01178	AAF14346	uroporphyrinogen decarboxylase	*D. rerio*	4.00E-17
	H01932	AAH12888	similar to *N*-deacetylase/*N*-sulfotransferase	*H. sapiens*	3.00E-10
	H00174	CAA64495	glutathione S-transferase	*Pleuronectes platessa*	4.00E-81
	H00259	NP_001489	glutamate-cysteine ligase	*H. sapiens*	5.00E-56
	H00128	NP_002700	protein phosphatase 1	*H. sapiens*	1.00E-145
	H00299	AAL08021	Ggoose-type lysozyme	*Epinephelus coioides*	1.00E-46
	H00303	NP_006671	malic enzyme 3	*H. sapiens*	1.00E-46
	H00895	NP_075781	pseudouridine synthase 3	*M. musculus*	5.00E-37
	H01590	P11183	glycine cleavage system H protein	*G. gallus*	8.00E-55
	H00282	P54985	peptidyl-prolyl *cis-trans* isomers	*Blattella germanica*	4.00E-76
	H01806	P58108	5*-AMP-activated protein kinase	*B. Taurus*	3.00E-16

Table 3 (*Continued*).

Class	Cluster ID	Accession number	Database entry name	Organisms	Probability
AVII. Stress response, detoxification and cell defense proteins					
	H00216	O13224	heat shock 27 kDa protein	*Poeciliopsis lucida*	1.00E-69
	H00631	T02955	probable cytochrome *P*450 monooxygenase	*Zea mays*	3.00E-11
	H00190	AAF71324	NKEF	*O. mykiss*	1.00E-104
	H00305	AAC27986	Antifreeze protein precursor	*Myoxocephalus*	2.00E-26
	H00681	BAA76887	heat shock protein 70 cognate	*O. latipes*	9.00E-80
	H01089	BAB83525	TBT binding protein	*P. olivaceus*	7.00E-16
	H01631	NP_031478	antioxidant protein 1	*M. musculus*	5.00E-72
	H01195	NP_036226	peroxiredoxin 5	*H. sapiens*	1.00E-44
AVIII. Protein degradation and processing, proteases					
	H00933	AAK93958	ubiquitin-conjugating enzyme	*H. sapiens*	7.00E-57
	H00466	AAK51461	proteasome subunit N3	*O. mykiss*	3.00E-18
	H00094	BAA09853	polyubiquitin	*Cricetulus* sp.	2.00E-53
	H00330	P06813	calcium-dependent protease	*Oryctolagus cuniculus*	7.00E-69
	H00300	XP_004014	similar to prefoldin 1	*H. sapiens*	2.00E-46
	H00241	I50494	serine protease inhibitor	*C. carpio*	1.00E-43
AIX. Apoptosis-related					
	H00650	AAF99551	LUCA-15 protein	*H. sapiens*	1.00E-27
	H00260	AAA85333	DRAL gene product	*H. sapiens*	5.00E-47
	H00721	AAB99847	skeletal muscle-specific calpain	*Sus scrofa*	1.00E-09
	H00127	AAG44955	apoptosis-related protein	*H. sapiens*	4.00E-24
	H00580	NP_035180	programmed cell death 4	*M. musculus*	2.00E-19
	H00872	AAK51490	pyrin	*Otolemur crassicaudatus*	1.00E-26
BI. Signaling receptors, including cytokine and hormone receptors, and signaling ligands					
	H00448	NP_505500	*N*-methyl-D-aspartate receptor-associated protein	*Caenorhabditis elegans*	5.00E-08
	H00480	NP_033301	signal recognition particle receptor	*M. musculus*	1.00E-71
	H01894	CAB71316	5-HT4 receptor	*H. sapiens*	9.00E-37
	H01669	AAH16093	similar to secretagogin	*M. musculus*	7.00E-32
	H00964	AAD00767	progesterone receptor binding protein	*G. gallus*	3.00E-10
	H01839	NP_034648	IGFBP-5	*M. musculus*	2.00E-30
BII. Intracellular signal transduction pathway molecules including kinases and signal intermediates					
	H01738	P02598	calmodulin	*Tetrahymena pyriformis*	
	H01206	XP_031831	serine/threonine kinase 3	*H. sapiens*	1.00E-17
	H01474	NP_033926	calpain 7	*M. musculus*	5.00E-37
	H00574	AAB07998	rhoGap protein	*G. gallus*	6.00E-75
	H00249	AAF34625	regulator of G-protein signaling 2	*M. musculus*	2.00E-42
	H00288	CAB87604	mitogen-activated protein kinase 7	*H. sapiens*	1.00E-12
	H01884	CAC34729	neuronal development-associated protein	*M. musculus*	1.00E-43
	H00717	NP_055141	death-associated protein kinase 2	*H. sapiens*	1.00E-103
	H00596	XP_034172	kinase suppressor of ras	*H. sapiens*	1.00E-75
	H01566	NP_006434	putative c-Myc-responsive	*H. sapiens*	3.00E-25
	H00092	AAD40671	small Rho-like GTPase RhoA	*X. laevis*	3.00E-63
BIII. Extracellular matrix proteins and cell adhesion, e.g. integrins and integrin receptors, and cadherins					
	H01442	AAF61069	galectin	*P. olivaceus*	5.00E-55
	H00386	XP_009327	chondroitin sulfate proteoglycan 3	*H. sapiens*	7.00E-15
	H00827	AAC39659	type XVIII collagen	*H. sapiens*	6.00E-08
	H01272	CAB72265	matrilin-3	*M. musculus*	6.00E-33
	H00307	CAC42527	integrin	*H. sapiens*	7.00E-46
CI. Sequence-specific DNA binding proteins					
	H00755	NP_055610	zinc finger homeobox 1b	*H. sapiens*	3.00E-13
	H01415	NP_109668	zinc finger protein 313	*M. musculus*	4.00E-35
	H00682	AAD13395	transcription factor E4TF1	*T. rubripes*	1.00E-23
CII. Non-DNA binding proteins that perform positive or negative roles					
	H00783	NP_445797	general transcription factor IIa, 2	*R. norvegicus*	3.00E-47
	H01169	O42249	receptor for activated protein kinase C	*Oreochromis niloticus*	1.00E-109
	H01827	AAH09610	activated RNA polymerase II transcription cofactor 4	*H. sapiens*	3.00E-27
	H01267	XP_048384	cofactor required for Sp1 transcriptional activation	*H. sapiens*	4.00E-42
	H00467	CAC09389	a novel protein containing a putative PHD finger domain	*H. sapiens*	3.00E-48
CIII. Chromatin proteins other than AIII with regulatory function					
	H01801	AAC97879	transcription factor WSTF	*H. sapiens*	2.00E-12
	H00465	NP_004999	NHP2 non-histone chromosome protein 2-like 1	*H. sapiens*	5.00E-22
DI. Specialized terminal differentiation products					
	H00920	NP_066969	angiopoietin-like factor	*M. musculus*	4.00E-31
	H01660	CAC13120	low molecular mass polypeptide subunit PSMB9	*T. rubripes*	9.00E-23
	H01422	O42146	metalloproteinase inhibitor 2 precursor	*G. gallus*	2.00E-14
	H01563	AAH12062	similar to DAZ-associated protein 1	*H. sapiens*	3.00E-49
	H01081	NP_003786	sorting nexin 3	*H. sapiens*	7.00E-82
	H01006	NP_075527	augmenter of liver regeneration; ALR	*T. rubripes*	1.00E-10
	H00716	AAK51526	neurabin	*G. gallus*	1.00E-16
	H00733	NP_034515	H2-K region expressed gene 2	*M. musculus*	2.00E-09
	H00463	NP_277050	MacGAP	*H. sapiens*	3.00E-12

Table 3 (*Continued*).

Class	Cluster ID	Accession number	Database entry name	Organisms	Probability
DII. Not enough information to classify					
	H01323	XP_010585	serine/arginine repetitive matrix 1	*H. sapiens*	1.00E-10
	H00569	AAB63598	ovulatory protein 2 precursor	*S. fontinalis*	2.00E-27
	H00493	AAG53983	SMHS2	*R. norvegicus*	5.00E-31
	H01485	AAH10727	RIKEN cDNA 1200014H24 gene	*M. musculus*	4.00E-17
	H00361	AAK40085	brevican-soluble core protein precursor	*X. laevis*	8.00E-08
	H00790	BAA92738	correspond to a region of the predicted gene	*Oryza sativa*	7.00E-28
	H001150	BAB20265	mono ATP binding cassette protein	*H. sapiens*	1.00E-09
	H001690	CAA65729	LR8B	*G. gallus*	3.00E-30
	H00399	JC1348	hypothetical 18K protein – goldfish mitochondrion	*C. auratus*	2.00E-25
	H00074	NP_062308	neuronal protein 15.6	*M. musculus*	5.00E-28
	H01343	NP_077271	hypothetical protein MGC3067	*H. sapiens*	5.00E-13
	H01463	XP_010556	PR domain containing 16	*H. sapiens*	7.00E-13
	H01844	XP_058399	ha0946 protein is Kruppel-related	*H. sapiens*	6.00E-32

evidences to the presence of adaptive immune responses in this fish.

Of the immune molecules related to innate immunity, antioxidant systems are the most important non-specific defense systems, especially in fish [19]. Antioxidant molecules in seahorse library, mainly resulting from blood, included various glutathione S-transferases (12 ESTs), NKEF, antioxidant protein 1, antioxidant enzyme B166, metallothionein A and so on. They may be cooperatively involved in the antioxidation and anti-aging activity of Chinese seahorse as medicine.

Interestingly, more than five anti-tumor genes, including epithelial membrane protein 2, LUCA-15, DRAL, retinoic acid receptor responder 3 and fatty acid binding protein 7, were identified in the current study [20–22], for the first time in fish. Although their roles in pharmacological activities and in seahorse itself are required for the further studies, they will at least provide new insight into studying mechanisms of anticancer activities of seahorse.

In addition, all components of electron transport chain were discovered in this cDNA library, and accounted for ~4.50% of the total ESTs (152/3372). The high prevalence of these molecules may be related to main tissues of seahorse sample, such as muscles and skeletons. Cytochrome *c* oxidase, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase and ferritin subunits, known to contain trace element Fe, were highly prevalent in this cDNA library, which was consistent with high content of Fe in dry Chinese seahorse [23]. These molecules may contribute to the function of anti-fatigue and hematopoiesis by seahorse in conjunction with the usage of other TCMs.

3.4. *ESTs homologous to lectins and their roles in the innate immunity*

Of the immune-related genes, homologs of GalNAc-specific lectin (H00395), chondroitin sulfate proteoglycan 3 (H00386) and low-affinity IgE receptor (H00385), were three most abundant components in the Chinese seahorse, consisting of 81, 72 and 58 ESTs, respectively. However, sequence analyses showed that all of them belonged to C-type lectins. As indicated in Table 4, more than seven different lectins were identified in this cDNA library including C-type mannose binding lectin (MBL, H00011), rhamnose binding lectin OLL (H00065), collectin placenta 1 (H00359) and so on. Of them, MBL has been paid much attention for playing a crucial role in the activation of lectin-complement pathway over the past decade [24].

The lectin-complement-activating pathway, working in innate immune defense, involves carbohydrate recognition receptors, such as MBL and ficolin, and the subsequent activation of MBL-associated serine proteases (MASPs). Mammalian MBL contains a collagen-like domain and carbohydrate recognition domain (CRD). Through its CRD, MBL recognizes and binds mannose and *N*-acetyl-D-glucosamine (GlcNAc) residues in common on microorganisms [24]. Given the discovery of a similar lectin-complement system in ascidian [25], Fujita proposed a model of this activating pathway and its evolution [26].

As for the lectin-activating pathway in fish, there have been many genes encoding MASP found in carp and shark [27]. However, most of MBLs have been identified only in some fresh water fish so far [28,29] instead of in marine fish like seahorse. Sequence analysis (Fig. 3) indicated that four C-type lectins in seahorse, including H00011, H00385, H00395 and H00386, shared six to seven conserved cysteines. Similar to MBL in carp [28] (Fig. 3), all but H00386 contained the QPD motif in the CRD, suggesting specificity for galactose [30]. But the other two conservative residues (positions 3 and 4, Fig. 3) involved in mannose binding were mostly Glu and Asn in seahorse, which was similar to mammals, instead of Gln and Asp in carp (Fig. 3). This result was consistent with the evolutionary status of two species. Like MBLs in sea urchin, starfish and asidian, the N-terminal portion of MBL in seahorse lacked a collagen-like region, which was in contrary to mammalian MBL [24,25] (Fig. 3). The phenomenon might be explained by the immature adaptive immune system of teleost. It is known that the collagen regions are involved in interactions with MASP and assembling MBL–MASP complexes that are able to activate the classical pathway of complement [24]. Since the activation of the classical complement pathway requires antigen–antibody complex, this pathway is not thought to exist in invertebrates, suggesting that it is no use for them to have this collagen region. The classical complement pathway did not arise until appearance of fish indeed, so MBLs in fish may have two types of lectins: with or without the collagen region. Of them, MBL lacking the collagen region might be an ancient form.

H00395, the most prevalent lectin in seahorse, had ~27% amino acid sequence identity with the GSL in starfish, sea urchin and rat hepatic lectin (RHL-1). Its CRD contained 21 highly conserved amino acid residues (Fig. 4), including six cysteines that formed three disulfide bonds in the single polypeptide chain. According to the previous study [31], the

Table 4
Immune-associated genes from the cDNA library of *H. kuda* Bleeker

Cluster ID	Accession number	Gene description	Organisms	Possibility
Immune response molecules				
H00277	AAL11413	major histocompatibility class I receptor	*S. vitreum*	9.00E-24
H00376	NP_571238	β2-microglobulin	*D. rerio*	4.00E-31
H00131	AAA49381	MHC class II α	*Morone saxatilis*	8.00E-53
H00633	AAC64370	MHC class II α chain	*Aulonocara hansbaenschi*	9.00E-42
H00139	AAL91668	MHC class II-associated invariant chain	*G. gallus*	9.00E-27
H01503	AAF97794	T-cell receptor α chain	*T. rubripes*	2.00E-27
H00900	NP_002362	T-cell differentiation protein MAL	*H. sapiens*	2.00E-32
H00483	NP_079516	B7 homolog 3	*H. sapiens*	3.00E-08
H01386	XP_042967	B-cell activation gene	*H. sapiens*	3.00E-20
H01413	XP_027211	similar to IgD B-cell receptor-associated protein (BAP)	*H. sapiens*	4.00E-36
H00020	S34198	CD23	*R. norvegicus*	3.00E-13
H01110	CAC42227	high-affinity IgE receptor gamma subunit	*Ictalurus punctatus*	3.00E-15
H00642	CAC85086	NF-κb inhibitor α (I-KAPPA-B-ALPHA)	*O. mykiss*	5.00E-52
H01278	NP_068569	eosinophil chemotactic cytokine	*H. sapiens*	9.00E-12
H00872	AAK51490	pyrin	*O. crassicaudatus*	2.00E-26
H01368	AAK12121	ovarian immunoreactive antigen	*H. sapiens*	2.00E-28
H00948	90302	FK506 binding protein 9	*H. sapiens*	2.00E-10
H00200	AAK52843	pleurocidin-like prepropolypeptide	*Pseudopleuronectes americanus*	8.00E-14
H00241	I50494	serine protease inhibitor	*C. carpio*	1.00E-43
H01389	BAB25823	retinoic acid repressible protein	*M. musculus*	2.00E-17
Lectins				
H00011	AAM21196	C-type MBL	*O. mykiss*	1.00E-15
H00359	BAC05523	collectin placenta 1	*M. musculus*	2.00E-10
H00386	NP_004377	chondroitin sulfate proteoglycan 3 (neurocan)	*H. sapiens*	1.00E-14
H00395	BAB78598	GSL	*A. pectinifera*	5.00E-16
H00065	BAC11709	rhamnose binding lectin OLL	*Spirinchus lanceolatus*	0.004
H00385	NP_038545	low-affinity IgE receptor (Fc-εRII)	*M. musculus*	5.00E-21
H00157	Q29058	lectin L-36	*S. scrofa*	1.00E-08
H00309	NP_507547	lectin C-type domain short and long forms	*C. elegans*	2.00E-17
Antioxidant molecules, detoxification molecules				
H01631	NP_031478	antioxidant protein 1	*M. musculus*	7.00E-72
H01195	NP_036226	peroxiredoxin 5; antioxidant enzyme B166	*H. sapiens*	2.00E-44
H01306	NP_038836	cysteine-rich protein 3	*M. musculus*	1.00E-73
H00190	AAF71324	NKEF	*O. mykiss*	1.00E-103
H00631	T02955	probable cytochrome *P*450 monooxygenase	*Z. mays*	4.00E-11
H00201	AAL09332	CCTε subunit	*T. pyriformis*	4.00E-53
H00681	Q9W6Y1	heat shock protein 70 cognate	*O. latipes*	1.00E-79
H00216	O13224	heat shock 27 kDa protein	*Poeciliopsis lucida*	2.00E-69
H00474	AAF86182	transferrin	*Oncorhynchus tshawytscha*	1.00E-73
H00420	AAH18534	protective protein for β-galactosidase	*M. musculus*	7.00E-43
H00158	T02995	unspecific monooxygenase	common tobacco	4.00E-30
H00945	1GTU	ligand-free human glutathione S-transferase M1a-1a	*H. sapiens*	9.00E-16
H00506	XP_203966	calcium-regulated heat stable protein CRHSP-24	*M. musculus*	6.00E-15
H00114	NP_079650	methylglyoxalase-I (EC 4.4.1.5)	*M. musculus*	1.00E-78
H00245	P52721	metallothionein A	*Thermarces cerberus*	2.00E-07
H01089	BAB83525	TBT binding protein	*P. olivaceus*	1.00E-15
H00174	CAA64495	glutathione S-transferase	*P. platessa*	6.00E-81
H00084	AAA67270	thioredoxin	*E. coli*	1.00E-59
Anti-tumor proteins				
H00073	XP_148339	epithelial membrane protein 2	*M. musculus*	5.00E-31
H00846	1FDQ	fatty acid binding protein 7, brain; B-FABP	*H. sapiens*	2.00E-36
H00650	AAF99551	LUCA-15 protein	*H. sapiens*	2.00E-27
H01422	O42146	metalloproteinase inhibitor 2 precursor	*G. gallus*	2.00E-14
H00455	XP_043159	retinoic acid receptor responder	*H. sapiens*	1.00E-38
H01606	NP_000295	peripheral myelin protein 22; growth arrest-specific 3	*H. sapiens*	1.00E-25
Hemopoiesis-associated molecules				
H00145	CAA92147	hemopexin-like protein	*O. mykiss*	8.00E-53
H00838	AAL55473	hemin binding protein	*Prevotella intermedia*	2.00E-17
H00139	NP_060938	hematopoietic stem/progenitor cells protein MDSO	*H. sapiens*	2.00E-42
H00377	BAA34951	embryonic β-type globin	*O. latipes*	2.00E-63
H00006	BAA34952	embryonic α-type globin	*O. latipes*	6.00E-57
H01539	BAA34953	ferritin H-subunit	*R. norvegicus*	4.00E-50
H00339	BAA34954	ferritin middle subunit; ferritin M	*Salmo salar*	4.00E-90

structural basis of RHL-1 for selective binding to GalNAc is associated with a histidine at position 256 (RHL-1). Although H00395 had a conserved motif QPD...EDC...NDX_2CX_7C... (where X represent any amino acid) like RHL-1, it lacked the critical histidine at the corresponding position (Fig. 4). How they bind to GalNAc needs a further analysis of its crystal structure. GSL may be involved in the immediate specific cell adhesion and recognition [32], and was also postu-

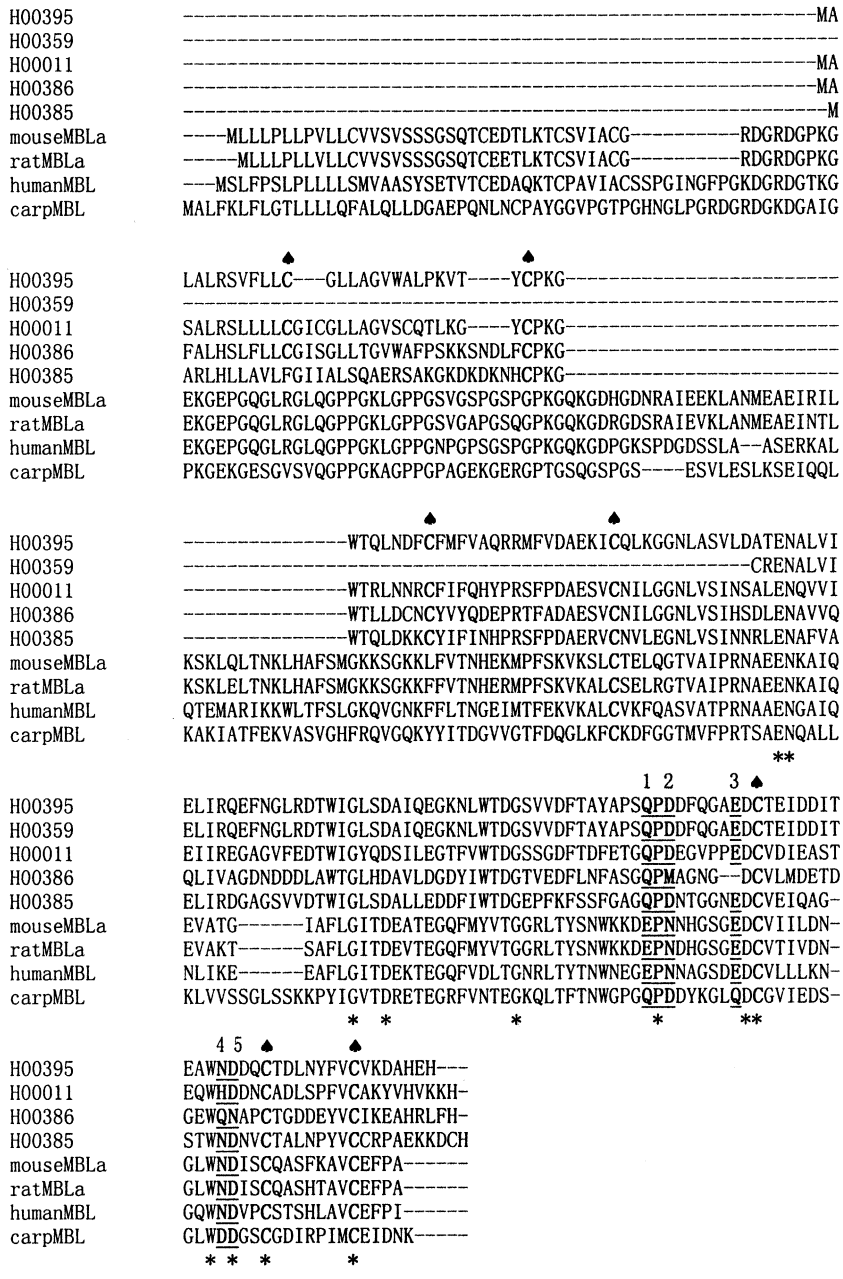

Fig. 3. Alignment of the deduced amino acid sequences of seahorse lectins and MBLs from different species. The alignment was performed using Clustal W 1.81. The amino acids involved in carbohydrate and calcium interaction are underlined with bold and number. The conserved cysteine residues are highlighted with ♠ and bold. Dashes (–) indicate gaps and * denotes identity.

lated to be a primitive and ancient lectin for those homologs traced back to Echinoid and lower vertebrates [33,34].

H00386, a homolog of chondroitin sulfate proteoglycan 3 from human, was actually a new type of lectin, because it only had one fifth of the conserved amino acid residues involved in the binding of galactose and mannose. Therefore, it may be highly selective for some other monosaccharides.

Low-affinity IgE receptor (FcεRII/CD23), a type II membrane-bound glycoprotein highly expressed on activated B-cells [35,36], binds to IgE at a domain homologous to Ca^{2+}-dependent (C-type) animal lectin. However, domain analysis indicated that the homolog of CD23 (H00385) in seahorse lacked the transmembrane region, intracellular and extracellular domain. Therefore, mammalian CD23 may be derived from this lectin.

H00065 is similar to rhamnose binding lectin OLL gene from catfish [37]. Of the two conserved motifs presenting in the OLL of catfish egg, YGR and DPC, only the former appeared in the seahorse lectin. H00065 was assumed to participate in the innate defense during the fertilization and development of eggs, which was correlated with paternal brooding pattern of seahorse [38]. One trait of this lectin was 10 conserved cysteines that might form four interchain and one intrachain disulfide bonds.

3.5. *ESTs specific to male reproduction and paternal brooding pattern*

Six specially expressed genes in male seahorse reproduction system were identified (Table 5). A homolog of seminal plasma glycoprotein 120 was predicted to have sperm immobiliz-

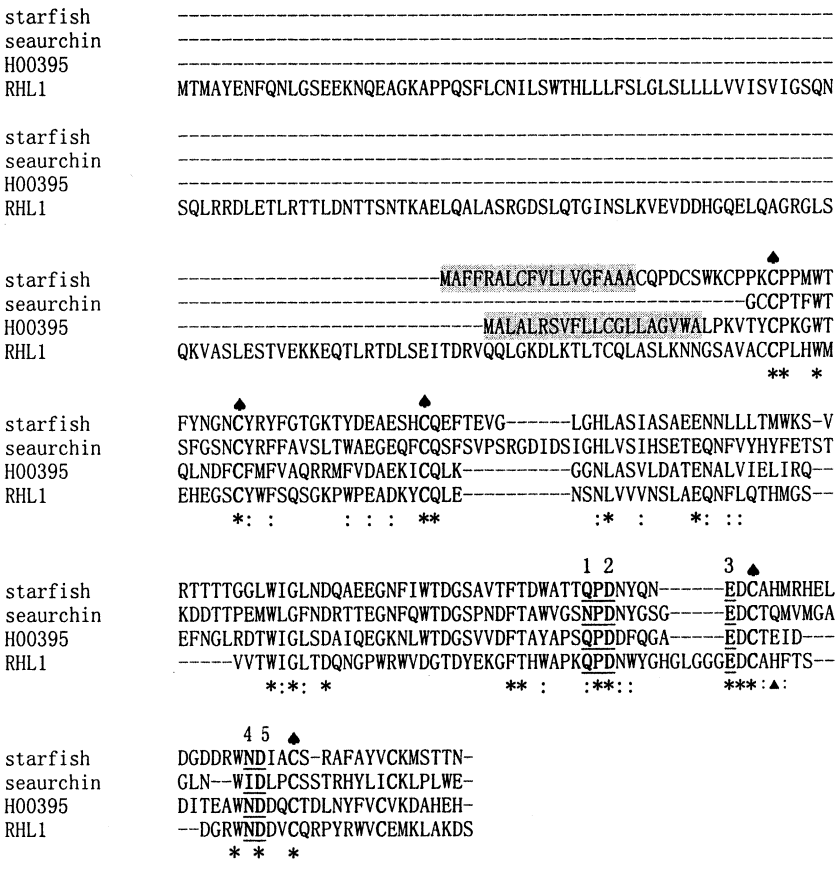

Fig. 4. Amino acid sequence alignment of the seahorse GSL with that of other species. The alignment was performed using Clustal W 1.81. RHL-1 is a major subunit of the hepatic asialoglycoprotein receptor. The amino acids involved in carbohydrate and calcium interaction are underlined with bold and number. The conserved cysteine residues are highlighted with ♠ and bold. The conserved His^{256} residue in RHL-1 is indicated with triangle. * denotes identity and : means positive. Dashes (–) indicate gaps. Signal peptides are shadowed.

ing activity [39]. The appearance of a homolog of testis-specific gene A2 might be associated with male meiotic metaphase chromosome [40]. Surprisingly, a homolog of placental protein 15 was present in the male seahorse cDNA library, whose roles either as nuclear transport factor 2 [41] or others are unclear.

Like the other fish of family *Syngnathidae*, seahorse is characterized by especially pronounced adaptations for paternal care, with a nearly completely enclosed brooding pouch on the belly of male seahorse [42]. This pouch is highly vascularized placenta-like epithelial tissue consisting of mitochondria-rich cells (MRCs) [43]. However, the molecular secret in this pouch had not been well explored yet.

The high prevalence of components of electron transport chain in the male seahorse cDNA library was consistent with the presence of MRCs. The latter, with highly expressed Na^+, K^+-ATPase, maintains the Na^+ concentrations in the brooding pouch near those in the serum rather than seawater during incubation [43].

Interestingly, a homolog of trout ovulatory proteins (TOPs) was identified in the male seahorse library, which was named broodin [44]. Since showing significant amino acid homology to that of TOP (~56%), broodin may protect eggs in the brood pouch from bacterial infection.

The discovery of a series of genes related to brooding suggested the brood pouch might not only provide nourishment, oxygen and osmoregulation, but also protect embryos from infection like the uterus of female mammal [45]. Perhaps there were other antimicrobial peptides present in the male reproductive system of seahorse similar to mammal [46]. If it is true, this type of innate defense pattern in the male mammalian reproduction may have existed earlier in the evolution.

Table 5
Genes related to male reproduction and paternal brooding pattern

Cluster ID	Accession number	Description	Organism	Possibility
H01563	AAH12062	similar to DAZ-associated protein 1	*H. sapiens*	3.00E-49
H01248	NP_079566	testis-specific gene A2 (male meiotic metaphase chromosome-associated acidic protein) (Meichroacidin)	*M. musculus*	4.00E-19
H01651	BAB78539	seminal plasma glycoprotein 120	*O. niloticus*	5.00E-20
H00728	NP_005787	placental protein; nuclear transport factor 2	*H. sapiens*	7.00E-44
H00359	BAC05523	collectin placenta 1	*M. musculus*	2.00E-10
H00569	AAB63598	ovulatory protein 2 precursor	*S. fontinalis*	3.00E-27

Table 6
ESTs related to physiological regulation genes

Cluster ID	Accession number	Gene description	Organism	Possibility
H00448	NP_505500	*N*-methyl-D-aspartate receptor-associated protein	*C. elegans*	5.00E-08
H00581	O42179	somatostatin-like receptor F	*T. rubripes*	6.00E-51
H00123	NP_495277	GABA A receptor-associated protein homolog	*C. elegans*	1.00E-29
H01894	CAB71316	5-HT4 receptor	*H. sapiens*	9.00E-37
H01637	P45883	diazepam binding inhibitor, DBI; ACBP (acyl-CoA binding protein)	*H. sapiens*	7.00E-28
H01669	AAH16093	similar to secretagogin	*M. musculus*	7.00E-32
H00964	AAD00767	progesterone receptor binding protein	*G. gallus*	3.00E-10
H01839	NP_034648	IGFBP-5	*M. musculus*	2.00E-30
H01896	NP_571866	cryptochrome 2a	*D. rerio*	2.00E-23
H00234	BAB88223	lipocalin-type PGDS-like protein	*D. rerio*	4.00E-61

3.6. ESTs relevant to physiological regulation genes

In the current work, 10 genes relevant to regulation of neurotransmission, hormone and circadian rhythm were discovered (Table 6). Of them, homologs of 5-HT receptor and secretagogin were identified for the first time in fish. A homolog of secretagogin may be involved in regulating calcium flux and cell proliferation [47]. The presence of a homolog of insulin-like growth factor binding protein 5 (IGFBP-5) was predicted to associate with male seahorse producing some brooding-related substance [48].

It is noticeable that 14 ESTs of lipocalin-type prostaglandin D synthase (PGDS) were identified. The glutathione-independent PGDS (EC 5.3.99.2) is responsible for the production of PGD2. Functions of PGD2 are interestingly consistent with pharmacological activities of Chinese seahorse such as regulation of nerve, hormone and immune systems [49]. PGD2 and its precursor, arachidonic acid (AA), could induce oxytocin secretion [50] and penile erection [51] by influencing hypophysis-gonad axis. DHA (22:6), the precursor of AA, is a basic material for the sperm generation and related to sperm fertility [52]. Its high content could be served as an evidence for activating Yang of Chinese seahorse documented in TCM [4,53]. Our study may provide a direct lead toward the identification of active ingredients for male's sexual enhancement.

In short, our study has revealed a molecular profile for the unique creature of TCM Chinese seahorse, and established a molecular basis for further interesting analyses.

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References

[1] Song, S.X., Lu, Z.J., Hou, J. and Han, L.Z. (2002) Acta Lab. Anim. Sci. Sin. 10, 101–104.
[2] Zhang, Z.H., Xu, G.J., Xu, L.S. and Wang, Q. (1995) Zhong Yao Cai 18, 197–199.
[3] Zhang, H., Luo, Y. and Luo, S.D. (2001) Mar. Drugs Chin. 2, 39–41.
[4] Zhang, Z.H., Xu, G.J., Xu, L.S. and Wang, Q. (1997) Zhong Yao Cai 20, 142–143.
[5] Zhang, Z.H., Xu, G.J., Xu, L.S. and Wang, Q. (1995) Mar. Drugs Chin. 4, 26–29.
[6] She, M., Wu, L. and Chen, H. (1991) Mod. Appl. Drugs 8, 1–3.
[7] Hu, J.Y., Li, B.F., Li, Z.J. and Lv, P. (2000) Mar. Drugs Chin. 2, 56–58.
[8] Committee of National Pharmacopoeia (2000) Pharmacopoeia of P. R. C., Chemical Industry Press, Beijing.
[9] Benson, D.A., Karsch-Mizrachi, I.I., Ipman, D.J., Ostell, J., Rapp, B.A. and Wheeler, D.L. (2000) Nucleic Acids Res. 28, 15–18.
[10] Nielsen, H., Engelbrecht, J., Brunak, S. and Heijne, G.V. (1997) Protein Eng. 10, 1–6.
[11] Lee, Y.H., Huang, G.M., Cameron, R.A., Graham, G., Davidson, E.H., Hood, L. and Britten, R.J. (1999) Development 126, 3857–3867.
[12] Scapigliati, G. and Romano, N. (2002) Dev. Comp. Immunol. 26, 151–160.
[13] Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. and Ezekowitz, R.A. (1999) Science 284, 1313–1318.
[14] Secombe, C.J., Zou, J., Laing, K., Daniels, G.D. and Cunningham, C. (1999) Aquaculture 172, 93–102.
[15] Chapoval, A.I., Ni, J., Lau, J.S., Wilcox, R.A., Flies, D.B., Liu, D., Dong, H., Sica, G.L., Zhu, G., Tamada, K. and Chen, L. (2001) Nat. Immunol. 2, 269–274.
[16] Moratz, C., Kang, V.H., Druey, K.M., Shi, C., Scheschonka, A., Murphy, P.M., Kozasa, T. and Kehrl, J.H. (2000) J. Immunol. 164, 1829–1838.
[17] Reif, K. and Cyster, J.G. RGS molecule expression in murine B lymphocytes and ability to down-regulate chemotaxis to lymphoid chemokines, (2000) J. Immunol. 164, 4720–4729.
[18] Breckenridge, D.G., Nguyen, M., Kuppig, S., Reth, M. and Shore, G.C. (2002) Proc. Natl. Acad. Sci. USA 99, 4331–4336.
[19] Rudneva, I.I. (1997) Comp. Biochem. Physiol. C118, 255–260.
[20] Wang, C.X., Wadehra, M., Fisk, B.C., Goodglick, L. and Braun, J. (2001) Blood 97, 3890–3895.
[21] Wang, M.S., Liu, Y.E., Ni, J., Aygun, B., Goldberg, I.D. and Shi, Y.E. (2000) Cancer Res. 60, 6482–6487.
[22] Sutherland, L.C., Edwards, S.E., Cable, H.C., Poirier, G.G., Miller, B.A., Cooper, C.S. and Williams, G.T. (2000) Oncogene 20, 3774–3781.
[23] Yue, X.L., Yang, T.W. and Wei, B.H. (1996) Shizhen J. Tradit. Chin. Med. Res. 7, 18–19.
[24] Turner, M.W. (1996) Immunol. Today 17, 532–540.
[25] Sekine, H., Kenjo, A., Azumi, K., Ohi, G., Takahashi, M., Kasukawa, R., Ichikawa, N., Nakata, M., Mizuochi, T., Matsushita, M., Endo, Y. and Fujita, T. (2001) J. Immunol. 167, 4504–4510.
[26] Fujita, T. (2002) Nature 2, 346–353.
[27] Endo, Y., Takahashi, M., Nakao, M., Saiga, H., Sekine, H., Matsushita, M., Nonaka, M. and Fujita, T. (1998) J. Immunol. 161, 4924–4930.
[28] Vitved, L., Holmskov, U., Koch, C., Teisner, B., Hansen, S. and Skjødt, K. (2000) Immunogenetics 51, 955–964.
[29] Mistry, A.C., Honda, S. and Hirose, S. (2001) Biochem. J. 360, 107–115.
[30] Drickamer, K. (1992) Nature 360, 183–186.
[31] Kolatkar, A.R., Leung, A.K., Isecke, R., Brossmer, R., Drickamer, K. and Weis, W.I. (1998) J. Biol. Chem. 273, 19502–19508.
[32] Kijimoto-Ochiai, S. (2002) Cell Mol. Life Sci. 59, 648–664.
[33] Giga, Y., Ikai, A. and Takahashi, K. (1987) J. Biol. Chem. 262, 6197–6203.
[34] Kakiuchi, M., Okino, N., Sueyoshi, N., Ichinose, S., Omori, A.,

Kawabata, S., Yamaguchi, K. and Ito, M. (2002) Glycobiology 12, 85–94.
[35] Sato, T., Konishi, A., Yasuno, S., Arai, J., Kamei, M., Bitoh, M. and Yamaguchi, T. (1997) J. Immunol. Methods 209, 59–66.
[36] Watson, J.L., Jackson, K.A., King, D.P. and Stott, J.L. (2000) Vet. Immunol. Immunopathol. 73, 323–329.
[37] Hosono, M., Ishikawa, K., Mineki, R., Murayama, K., Numata, C., Ogawa, Y., Takayanagi, Y. and Nitta, K. (1999) Biochim. Biophys. Acta 1472, 668–675.
[38] Sasaki, H. and Aketa, K. (1981) Exp. Cell Res. 135, 15–19.
[39] Mochida, K., Matsubara, T., Andoh, T., Ura, K., Adachi, S. and Yamauchi, K. (2002) Mol. Reprod. Dev. 62, 57–68.
[40] Taketo, M.M., Araki, Y., Matsunaga, A., Yokoi, A., Tsuchida, J., Nishina, Y., Nozaki, M., Tanaka, H., Koga, M., Uchida, K., Matsumiya, K., Okuyama, A., Rochelle, J.M., Nishimune, Y., Matsui, M. and Seldin, M.F. (1997) Genomics 46, 138–142.
[41] Grundmann, U., Nerlich, C., Rein, T., Lottspeich, F. and Kupper, H.A. (1988) Nucleic Acids Res. 16, 4721.
[42] Wilson, A.B., Vincent, A., Ahnesjo, I. and Meyer, A. (2001) J. Hered. 92, 159–166.
[43] Watanabe, S., Kaneko, T. and Watanabe, Y. (1999) Cell Tissue Res. 295, 141–149.
[44] Coffman, M.A., Pinter, J.H. and Goetz, F.W. (2001) Biol. Reprod. 62, 928–938.
[45] King, A.E., Fleming, D.C., Critchley, H.O. and Kelly, R.W. (2002) Mol. Hum. Reprod. 8, 341–349.
[46] Li, P., Chan, H.C., He, B., So, S.C., Chung, Y.W., Shang, Q., Zhang, Y.D. and Zhang, Y.L. (2001) Science 291, 1783–1785.
[47] Wagner, L., Oliyarnyk, O., Gartner, W., Nowotny, P., Groeger, M., Kaserer, K., Waldhäusl, W. and Pasternack, M.S. (2000) J. Biol. Chem. 275, 24740–24751.
[48] Arraztoa, J.A., Monget, P., Bondy, C. and Zhou, J. (2002) J. Clin. Endocrinol. Metab. 87, 5220–5228.
[49] Urade, Y. and Hayaishi, O. (1999) Biochim. Biophys. Acta 1436, 605–615.
[50] Kadekaro, M., Terrell, M.L., Bui, V. and Summy-Long, J.Y. (2001) Brain Res. 889, 84–88.
[51] Mizusawa, H., Hedlund, P. and Andersson, K.E. (2002) J. Urol. 167, 757–760.
[52] Gulaya, N.M., Margitich, V.M., Govseeva, N.M., Klimashevsky, V.M., Gorpynchenko, I.I. and Boyko, M.I. (2001) Arch. Androl. 46, 169–175.
[53] Xu, Y.M., Chen, J.W. and Guo, X. (1994) Mar. Drugs Chin. 49, 14.