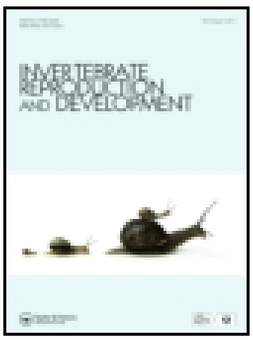

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Genome survey and characterization of reproduction-related genes in the Pacific oyster

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ABSTRACT

Reproductive mechanisms in molluscs have been targets for biological research because of the diverse reproductive strategies exhibited in this phylum. However, little is known about the molecular mechanisms of molluscan reproduction. The whole genome sequence of the Pacific oyster *Crassostrea gigas* together with the transcriptomic data-sets provides a powerful platform for studies on the characterization of the genes involved in bivalve reproduction. Reproduction-related genes were screened in this study through analyzing the Pacific oyster genome. Previously identified molluscan reproduction-related genes were used as queries to a BLASTP search against the *C. gigas* genome to identify gene candidates encoding similar proteins. Transcriptomic data-sets were analyzed to profile gene candidate expression patterns. We obtained 39 gene candidates with high accuracy encoding reproduction-related genes in the *C. gigas* genome, of which 17 are reported in detail, and the rest are identified as new candidate genes involved in reproduction of *C. gigas,* including *nanos*, *piwi*, *dax1*, and *5-hydroxytryptamine* (*5-HT*) receptors. Among 22 gene candidates, seven were gonad-specifically expressed. The set of reproduction-related genes of *C. gigas* identified in this study constitutes a new tool for further research on molecular mechanisms of reproduction in *C. gigas*.

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Crassostrea gigas; oyster reproduction; nanos; piwi; dax1; 5-HT

Introduction

The Mollusca is a hugely successful and spectacularly diverse phylum of animals that exhibits diverse modes of sexual reproduction (ect- and entaquatic, internal, copulatory organs, spermatophores) (Haszprunar & Wanninger 2012). In molluscs, there are dioecious and hermaphroditic species, as well as species capable of sex change (Coe 1943), and even parthenogenesis does occur occasionally (Haszprunar & Wanninger 2012). Mollusc reproduction has held the interest of biologists over the centuries. Many studies have been performed to elucidate the mechanism of molluscan reproduction from various research areas, including physiology, evolution, behaviour, ecology, endocrinology, and molecular biology (Wilbur & Yonge 1966; Joosse 1972; Reid 1990; Fabioux et al. 2004).

Although progress has been made in clarifying the mechanisms underlying molluscan reproduction, knowledge of the molecular mechanisms of molluscan reproduction is still limited. Several genes involved in sex determining pathways, gonad development, and germ cell development have been identified in a few molluscs (Matsumoto et al. 2008; Rabinowitz et al. 2008; Feng et al. 2010). Recently, emerging next-generation sequencing techniques and bioinformatics tools have facilitated research on the molecular mechanisms of molluscan reproduction. Numerous transcriptomic studies on gametogenesis, sex determination, and gonad development in molluscs have been conducted and screened hundreds of reproduction-related gene candidates (Boutet et al. 2008; Dheilly et al. 2012; Zhang et al. 2014). The long-waited availability of molluscan genomes provides a unique opportunity to mine the reproduction-related genes with high accuracy. Matsumoto et al. (2013) obtained more than 40 gene models encoding orthologs of reproduction-related genes in other molluscs by analyzing the pearl oyster draft genome.

The Pacific oyster *Crassostrea gigas* is the most widely cultivated marine bivalve mollusc, having been introduced from Asia to all continents but Antarctica (Mann 1979; Hedgecock et al. 2015). Global annual production is conservatively estimated as 555,914 metric tons in 2013

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(Hedgecock et al. 2015). Gonad appearance and condition are important factors in marketability of oysters, as the gonad is visually the most obvious organ of a shucked oyster and largely influences the overall flavor and texture of the meat (Hand & Nell 1999). The marketability of *C. gigas* drops in summer and autumn when oysters are either gravid or spent (Nell 2002). Thus, research has been performed on the controlled reproduction in *C. gigas* (Allen & Downing 1991; Guo et al. 1996; Nell 2002). Furthermore, *C. gigas* has a very complex mode of sexual reproduction. In *C. gigas*, most individuals are dioecious and functional hermaphroditism is also present at a low frequency. Sex change appears to be common in this species. *C. gigas* has an extremely high fecundity, and females can spawn tens to hundreds of millions of eggs into the water where fertilization occurs (Massapina et al. 1999). Because of its commercial importance and complex mode of sexual reproduction, *C. gigas* is becoming an interesting model for highly fecund marine metazoans. Understanding the mechanisms regulating reproduction of *C. gigas* is, thus, a major area of interest. However, current understanding of the molecular mechanisms of oyster reproduction remains limited. Less than ten genes were confirmed to be related to reproduction in *C. gigas* (Matsumoto et al. 2003; Fabioux et al. 2004; Moy & Vacquier 2008; Naimi et al. 2009a, 2009b).

The availability of the *C. gigas* genome (Zhang et al. 2012) and hundreds of transcriptomic data not only offers a good opportunity for a genome-wide survey of reproduction-related genes, but also provides a novel platform for in-depth research on the molecular mechanism of oyster reproduction. In this study, we analyzed the *C. gigas* genome to screen reproduction-related gene candidates that had high homologies to the previously described genes involved in reproduction in other molluscs. The expression patterns of the *C. gigas* reproduction-related genes were analyzed based on the transcriptomic data. The group of genes related to reproduction with high accuracy described in this study provides a new tool for further research on the molecular mechanisms of reproduction in *C. gigas*.

Materials and methods

Gene identification from the C. gigas *genome*

Gene identification was performed through BLASTP of *C. gigas* genome at the NCBI website (https://blast.ncbi.nlm.nih.gov/). Thirty-nine reproduction-related complementary DNAs (cDNAs) sequences reported in molluscs were BLASTP searched against the *C. gigas* genome (optional of organism was limited to taxid: 29,159 in non-redundant database), and the obtained gene candidates (predicted transcripts) were reciprocally BLASTP searched against the NCBI non-redundant database to confirm the best-hit sequence.

The obtained ortholog genes were annotated as follows: when a gene candidate showed a significant and reciprocal BLASTP hit in response to the query sequence, the gene candidate was named 'Cg-xxx-like' (e.g. 'Cg-*nanos*-like'). If the gene candidate was previously characterized detailed in *C. gigas*, it was then named 'Cg-xxx' (e.g. 'Cg-*vasa*'). When more than two gene candidates with strong structural conservation were identified for the query sequence, a letter was added as a suffix to distinguish them (e.g. 'Cg-*nanos*-like A, B, C').

Transcriptome sequences analysis

Illumina RNA-Seq data were also downloaded from the GigaScience database (ftp://climb.genomics.cn/pub/10.5524/100001_101000/100030/RNA-Seq/), including nine RNA-Seq data-sets (paired-end reads, GEO accession number GSM768396-GSM768404) mainly from nine tissues, and 35 different development time-point data-sets (single-end reads, GEO accession number GSM768406-GSM768414, GSM768416-GSM768428, GSM768430-GSM768433, GSM768435-GSM768443). The software RSEM (Li & Dewey 2011) was used for expression analysis of genes to obtain FPKM (fragments per kilobase of transcript per million fragments sequenced) values (Trapnell et al. 2010). We used the tissue-specific index to evaluate gonad-specific expression of reproduction-related gene candidates. The index was calculated as described by Yanai et al. (2005). The expression value of each mRNA was quantified as FPKM. The genes were considered as tissue-specific genes when the tissue-specific index was above 0.90. The results of tissue-specific index among 22 gene candidates are shown in Supplementary Table S1. FPKM in 34 different larval development time-points (from eggs to spats) were exclusively calculated to analyze the expression profile of germline development gene candidates including *nanos* and *piwi* (Table S2).

Alignment and molecular phylogeny

Amino acid sequences were aligned using DNAMAN 8 (http://www.lynnon.com/) for finding the most conserved domain motifs. Phylogenetic trees were constructed using full length amino acid sequences with MEGA5.0 software utilizing the neighbor-joining method (Saitou & Nei 1987). The bootstrap values were replicated 1000 times to obtain the confidence value for the analysis. The GenBank accession numbers of amino acid sequences used in the multiple sequence alignments and phylogenetic analysis are available in Supplementary Table S3. The signal sequence and cleavage sites were predicted through the SignalP3.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

Results and discussion

In the *C. gigas* genome, 39 gene candidates were identified encoding orthologs of reproduction-related genes reported in other molluscs. A complete list of the 39 annotated genes is shown in Table 1. Among these gene candidates, 17 have been previously characterized in *C. gigas* and described in detail (Table 1), including one germline determination gene (*vasa*), five genes related to sex determination (*dml*, *foxl2*, *dsx*, *soxE*, *β-catenin*), nine genes related to gonad development (*vtg*, *estrogen receptor*, four types of *GnRH* receptor, *gonadal-TGFβ*, *wnt4, insulin receptor-related receptors*), one gene for egg-laying hormones (*ELH*) and one fertilization-related gene (*bindin*). In this study, we focused on the other 22 new annotated gene candidates, of which seven were gonad-specifically expressed (Table 1; Supplementary Table S1).

Reproductive processes were divided into the following categories: germline development, gonad development, oocyte maturation and spawning, and others. The 22 reproduction-related genes of *C. gigas* are analyzed on the basis of these categories.

Germline development

Nanos

The *nanos* gene, which encodes an RNA binding zinc finger protein, was first identified as a maternal-effect gene in *Drosophila* (Wang & Lehmann 1991). In the absence of maternal *nanos*, pole cells fail to migrate into the gonads and do not become functional germ cells (Kobayashi et al. 1996). To date, *nanos* orthologs have been cloned in both vertebrates and invertebrates, such as *Danio rerio* (Köprunner et al. 2001), *Mus musculus* (Tsuda et al. 2003), *Caenorhabditis elegans* (Subramaniam & Seydoux 1999) and *Helobdella robusta* (Pilon & Weisblat 1997). In these organisms, maternally derived *nanos* plays a critical role in early development, more specifically, in maintenance and development of primordial germ cells (PGCs) (Subramaniam & Seydoux 1999; Tsuda et al. 2003).

In molluscs, *nanos* orthologs have been observed in embryos of *Ilyanassa obsoleta* (Rabinowitz et al. 2008), *Haliotis asinina* (Kranz et al. 2010) and *Pinctada fucata* (Matsumoto et al. 2013). In *I. obsoleta*, morpholino knockdown of *nanos* resulted in invalid regulation of the blast

Table 1. List of reproduction-related genes identified in the *Crassostrea gigas* genome.

Gene annotation	Gene ID	BLAST best hit to NCBI database Accession number (species name)	Gonad-specific	References for the BLAST best hit
Cg-*nanos*-like	LOC105348851	ETE70556 (*Ophiophagus hannah*)	Ovary	Vonk et al. (2013)
Cg-*piwi1*-like	LOC105339049	AGI95996 (*Branchiostoma floridae*)	No	Zhang et al. (2013)
Cg-*piwi2*-like	LOC105337341	NP_001296620 (*Hydra vulgaris*)	No	Juliano et al. (2014)
Cg-*dax1*-like	LOC105338842	AFU35437 (*Chlamys farreri*)	No	Li et al. (2014)
Cg-*phb1*-like	LOC105322860	AEI91930 (*Octopus tankahkeei*)	No	Mao et al. (2012a)
Cg-*phb2*-like	LOC105328645	KF601690 (*Chlamys farreri*)	No	Han et al. (2015)
Cg-*IGFBP7*-like	LOC105339347	AEE01360 (*Haliotis diversicolor*)	No	Li et al. (2012)
Cg-*5-HT*$_1$-like A	LOC105348009	BAE72141 (*Mizuhopecten yessoensis*)	No	Tanabe et al. (2010)
Cg-*5-HT*$_1$-like B	LOC105344141	AIW04132 (*Pinctada fucata*)	No	Wang & He (2014)
Cg-*5-HT*$_2$-like	LOC105330687	NP_001232921 (*Aplysia californica*)	No	Nagakura et al. (2010)
Cg-*5-HT*$_4$-like	LOC105330597	NP_001240691 (*Aplysia californica*)	No	
Cg-*5-HT*$_7$-like	LOC105345175	AAQ84306 (*Planorbella trivolvis*)	No	Mapara et al. (2008)
Cg-*TSSK1*-like A	LOC105333853	EZA58692 (*Cerapachys biroi*)	Testis	Oxley et al. (2014)
Cg-*TSSK1*-like B	LOC105328495	AEM36057 (*Mytilus edulis*)	Testis	Ciocan et al. (2011)
Cg-*TSSK1*-like C	LOC105328496	AMY26492 (*Atrina pectinata*)	Testis	Li et al. (2016)
Cg-*TSSK4*-like	LOC105321145	NP_081949 (*Mus musculus*)	Testis	Jha et al. (2013)
Cg-*TSSK5*-like	CGI_10024523	ELR54884 (*Bos mutus*)	Testis	Qiu et al. (2012)
Cg-*SCA*-like	LOC105339661	ES469345 (*Argopecten purpuratus*)	Testis	Boutet et al. (2008)
Cg-*Calcineurin*-like B	CGI_10013607	ACI96107 (*Pinctada fucata*)	No	Li et al. (2009)
Cg-*CDA*-like A	LOC105335466	ABW90692 (*Haliotis diversicolor supertexta*)	No	Wu et al. (2009)
Cg-*CDA*-like B	LOC105334462		No	
Cg-*ADAR*-like A	LOC105326427	NP_001103 (*Homo sapiens*)	No	Li et al. (2015)
Cg-*Vasa*	LOC105335166	AY423380 (*Crassostrea gigas*)	No	Fabioux et al. (2004)
Cg-*DMl*	LOC105338304	EU046234 (*Crassostrea gigas*)	No	Naimi et al. (2009b)
Cg-*Dsx*	CGI_10019568	KJ489413 (*Crassostrea gigas*)	Testis	Zhang et al. (2014)
Cg-*Vtg*	CGI_10021817	BAC22716 (*Crassostrea gigas*)	Ovary	Matsumoto et al. (2003)
Cg-*ER*	CGI_10024100	BAF45381 (*Crassostrea gigas*)	No	Matsumoto et al. (2007)
Cg-*SoxE*	LOC105340517	JX040450 (*Crassostrea gigas*)	No	Matsumoto et al. (x2014)
Cg-*β-catenin*	LOC105320742	AFL93714 (*Crassostrea gigas*)	No	
Cg-*FoxL2*	LOC105319597	FJ68956 (*Crassostrea gigas*)	No	Naimi et al. (2009a)
Cg-*GnRH*-R	LOC105330405	AJ890150 (*Crassostrea gigas*)	No	Rodet et al. (2008)
Cg-*GnRH*-RII-S	LOC105330405	CAP17414 (*Crassostrea gigas*)	No	
Cg-*GnRH*-RII-L	LOC105330405	CAP17413 (*Crassostrea gigas*)	No	
Cg-*GnRH*-R-TF	LOC105330405	CAP17415 (*Crassostrea gigas*)	No	
Cg-*IR*	LOC105348544	CAD59674 (*Crassostrea gigas*)	No	Gricourt et al. (2006)
Cg-*bindin*	CGI_10005529	ACH72077 (*Crassostrea gigas*)	No	Moy & Vacquier (2008)
Cg-*gonadal-TGFβ*	CGI_10004608	ABU50369 (*Crassostrea gigas*)	No	Fleury et al. (2008)
Cg-*Wnt4*	LOC105346960	EKC23277 (*Crassostrea gigas*)	No	Zhang et al. (2012)
Cg-*ELH*	LOC105347951	EKC39528.1 (*Crassostrea gigas*)	No	Stewart et al. (2014)

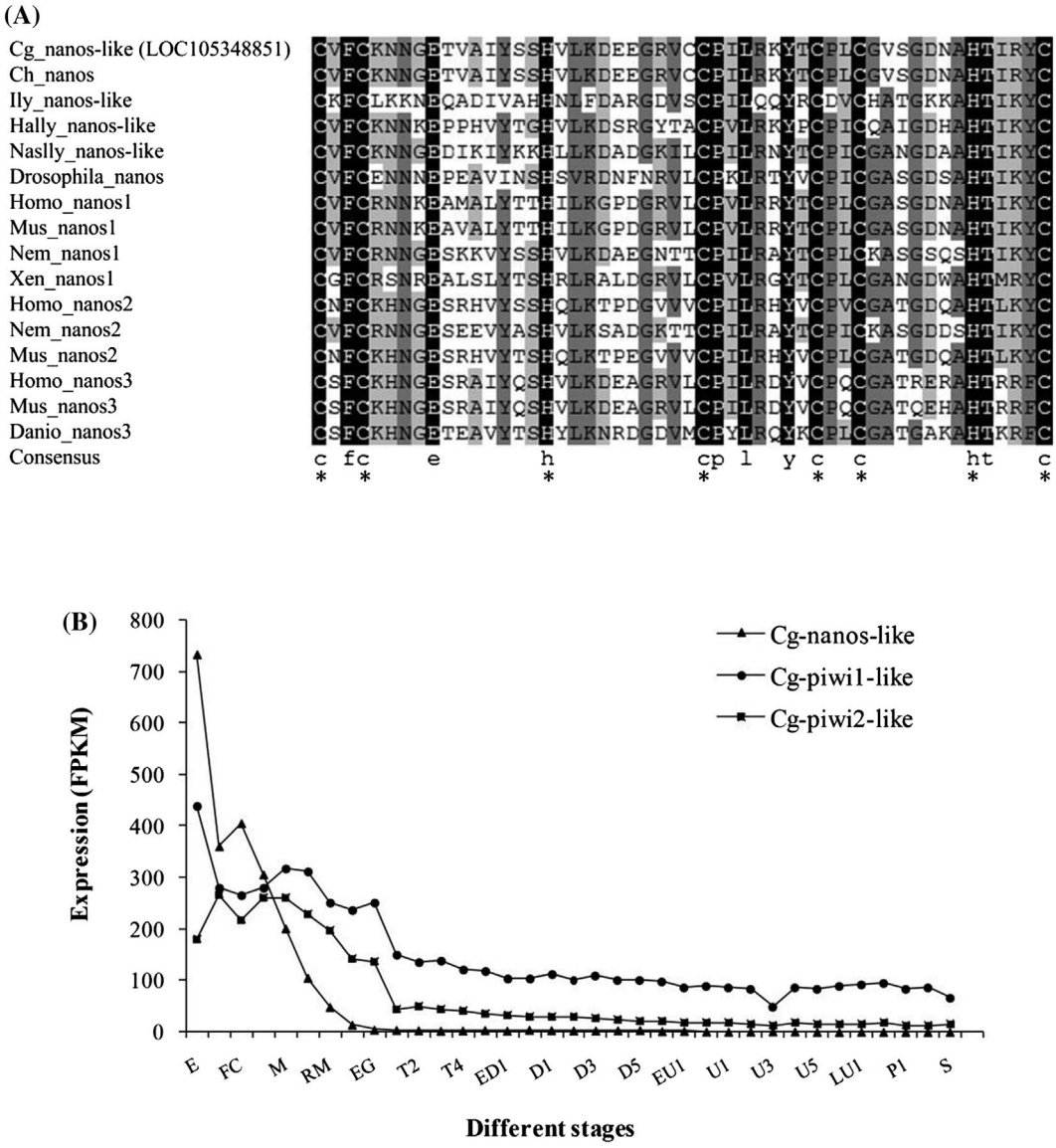

Figure 1. Cg-*nanos*-like identified in *C. gigas* genome and its expression profile. (A) Amino acid sequence alignment of the Cg-*nanos*-like predicted RNA-binding domain with that of other known vertebrate and invertebrate *nanos* orthologs. Two highly conserved CCHC zinc finger motifs are indicated by asterisks. (B) Expression pattern of Cg-*nanos*-like, Cg-*piwi1*-like and Cg-*piwi2*-like during larval development. The meanings of abbreviations of development stage are shown in Table S2.

cells derived from the 4d cell (Rabinowitz et al. 2008). However, the functions of molluscan *nanos* orthologs in the development of PGCs remain unknown.

In the *C. gigas* genome, we found a gene candidate that encoded a *nanos* ortholog containing a *nanos* RNA-binding domain. Amino acid sequence alignment of the predicted *C. gigas nanos* RNA-binding domain with other orthologs indicated high conservation of two characteristic CCHC zinc finger motifs (Figure 1(A)). BLASTP in NCBI showed that the deduced amino acid sequence of the gene candidate (LOC105348851) shared highest 72% identity with that of *Ophiophagus hannah* (e-value = e^{-23}). This gene candidate was annotated as 'Cg-*nanos*-like' (Table 1). Cg-*nanos*-like was specifically expressed in the mature female gonad (Table S1), suggesting the putative important role of Cg-*nanos*-like in *C. gigas* female reproduction. Maternal-effect Cg-*nanos*-like was an egg specific gene candidate during *C. gigas* larval development, as the transcripts of Cg-*nanos*-like appeared soon after fertilization and subsequently decreased rapidly (Figure 1(B)). These results support a possible role of Cg-*nanos*-like in female germline stem cell differentiation.

Piwi

The *piwi* represents the first class of evolutionarily conserved genes required for stem cell self-renewal and maintenance (Cox et al. 1998). In the model organisms *C. elegans*, *D. rerio* and *M. musculus*, mutation in *piwi* led

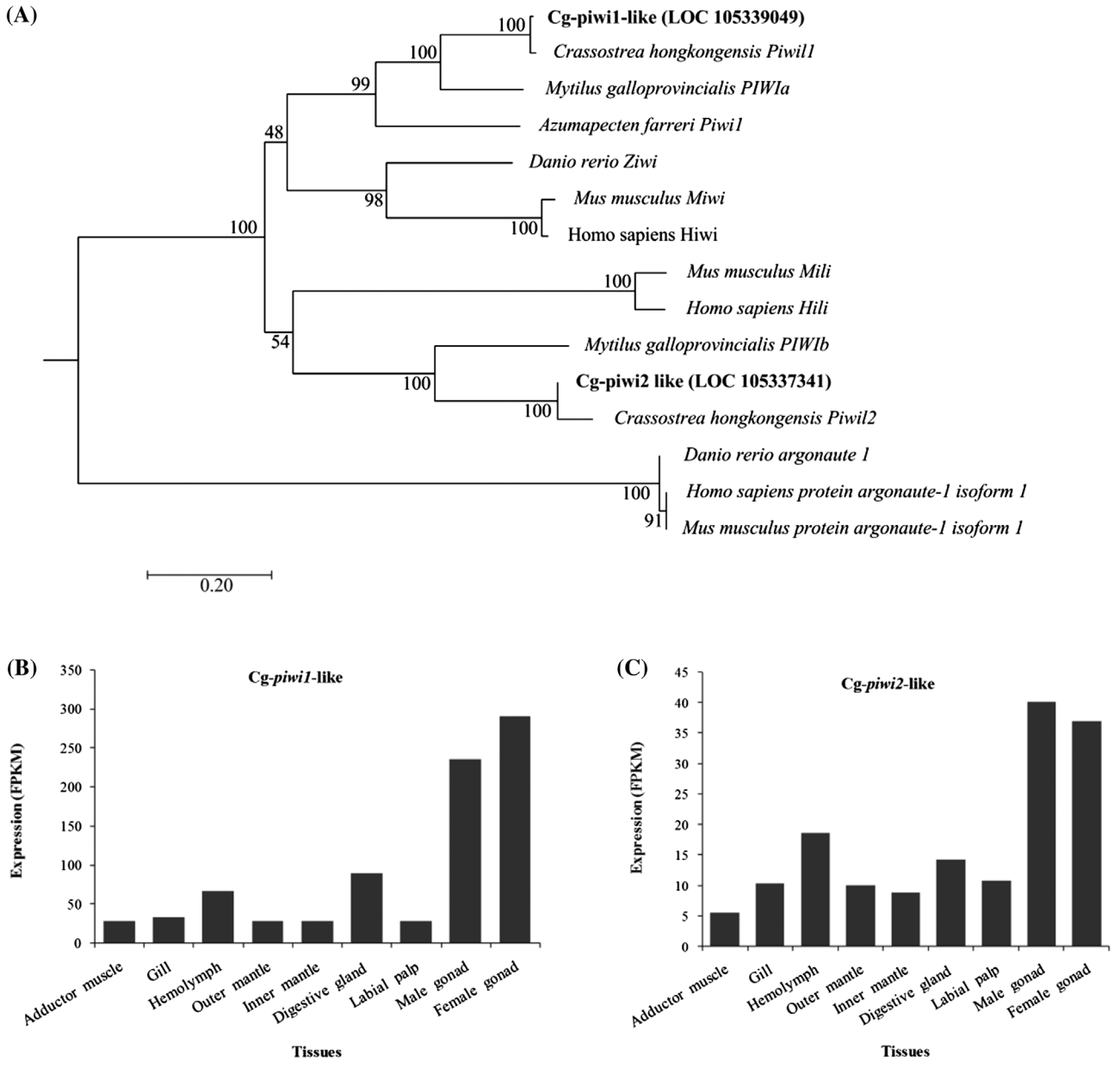

Figure 2. Cg-*piwi1*-like and Cg-*piwi2*-like identified in *C. gigas* genome and their expression profiles. (A) Molecular phylogenetic tree of Piwi/Ago family proteins. Numbers in the tree are bootstrap values. (B) Expression profiles of Cg-*piwi1*-like in *C. gigas* adult tissues. (C) Expression profiles of Cg-*piwi2*-like in *C. gigas* adult tissues.

to a block in development of stem cells (Klattenhoff & Theurkauf 2008). In most animals reported so far, Piwi proteins are specifically expressed in the germline (Cox et al. 2000; Tong et al. 2015). These observations revealed that *piwi* was involved in the reproductive process.

The Argonaute family can be subdivided into two distinct subfamilies, Piwi and Argonaute (Ago). Piwi proteins are characterized by a PAZ-domain in the middle and a highly conserved PIWI-domain at the C-terminal region. In molluscs, a *piwi* ortholog was firstly characterized in *Crassostrea hongkongensis* (Tong et al. 2015). In *C. hongkongensis*, transcripts of *piwi* were exclusively expressed in developing germ cells including oogonia and early vitellogenic oocytes, which was detected by mRNA *in situ* analysis (Tong et al. 2015). However, the precise role of *piwi* genes in molluscan germline development remains unclear, and awaits further investigation.

In the *C. gigas* genome, two gene candidates encoding orthologs of the PAZ domain and PIWI-domain transcription factors were identified. Phylogenetic analysis revealed that two gene candidates fell within the Piwi subfamily rather than the Ago subfamily (Figure 2(A)). Within the *piwi* subfamily, we found that the gene candidate LOC105339049 was most closely related to the PIWI-like 1 protein of *C. hongkongensis*, so we named this gene 'Cg-*piwi1*-like'. The other gene candidate LOC105337341

clustered with *piwi* like 2 sequences from other organisms, which was thus annotated 'Cg-*piwi2*-like'. The results of the BLASTP search of each gene candidate are shown in Table 1. The strongest similarities were as follows: Cg-*piwi1*-like to *Branchiostoma floridae* PIWI 1 protein (e-value = 0.0, identities 68%), Cg-*piwi2*-like to *Hydra vulgaris* PIWI 2 (e-value = 0.0, identities 59%). Cg-*piwi1*-like was expressed at a low level in various tissues with no significant difference between male and female gonads (Figure 2(B)), while Cg-*piwi2*-like was rarely expressed in all tissues (Figure 2(C)). During different developmental stages of *C. gigas* larvae, Cg-*piwi1*-like and Cg-*piwi2*-like expression were the highest at the initiation of fertilization and decreased to achieve minimum level after the early gastrula stage (Figure 1(B)).

Gonad development

Dax1

Dax1 is a member of the nuclear hormone-receptor (NR) superfamily. Receptors in this superfamily possess two conserved domains, ligand-binding domain (LBD) in the C-terminal and DNA-binding domain (DBD) in the N-terminal. *Dax1* is involved in female sex determination in mammals (Swain et al. 1996) and birds (Smith et al. 2000). However, recent studies in the mouse have shown that it may have a critical role in testicular development (Richard et al. 1998; Park et al. 2005). Such a function for *dax1* has also been demonstrated in lower vertebrates such as amphibians (Sugita et al. 2001) and teleosts (Wang et al. 2002). Compared with the extensive studies of *dax1* in vertebrates, information of *dax1* orthologs is poorly understood in invertebrates. To date, in molluscs, a *dax1* ortholog has been identified in the scallop (*Cf-dax1*) (Li et al. 2014). In this study, the mRNA level of *Cf-dax1* showed a sex dimorphic feature in favor of testis, which is similar to many vertebrates (Li et al. 2014). The functions of molluscan *dax* genes in gametogenesis remain to be determined.

Based on analysis of the *C. gigas* genome, a gene candidate (LOC105338842) encoding a *dax1* ortholog was identified. *Cg-dax1*-like contained two putative domains of the NR superfamily, a C-terminal LBD and an N-terminal DBD. However, unlike those of vertebrates, it lacked an LXXLL-like motif in the putative DNA binding region. A BLASTP search of LOC105338842 showed the highest similarity to *C. farreri dax1* (e-value = $1e^{-138}$, identities 54%) (Table 1). The result of phylogenetic analysis (Figure 3(A)) demonstrated that the oyster *dax1* appeared to be most closely related to scallop *dax1*. It is evident that *dax-1*s of molluscs are in a group different from that of vertebrates. Thus, LOC105338842 was annotated 'Cg-*dax1*-like'. Tissue distribution analysis revealed that Cg-*dax1*-like was expressed widely in adult tissues at a high level, with no differences between female and male gonads in the mature stage (Figure 3(B)).

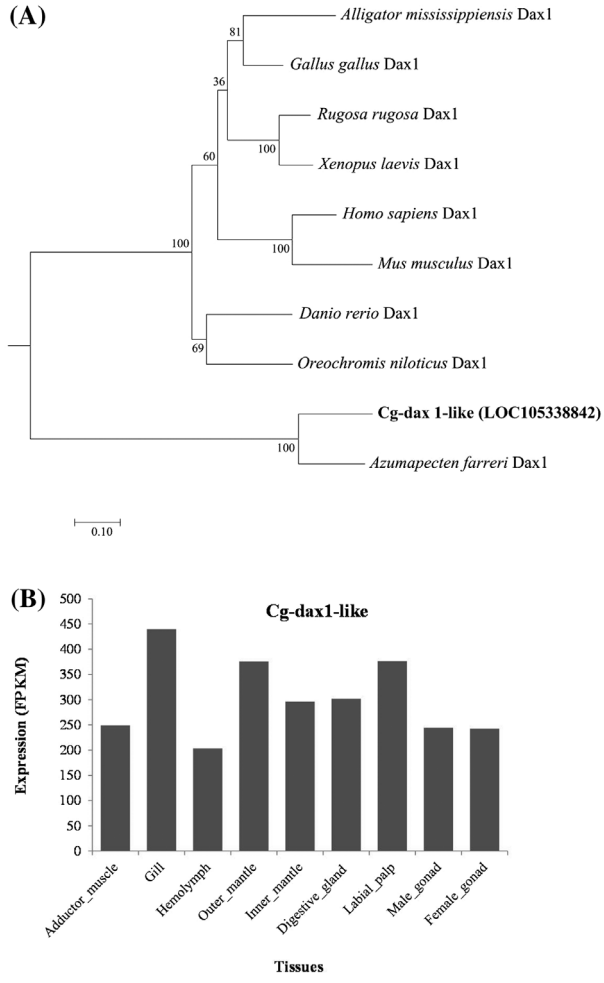

Figure 3. Cg-*dax1*-like identified in *C. gigas* genome and its expression profile. (A) Phylogenetic tree of ligand-binding domains from selected *dax1* gene in *C. gigas* and reference species. Numbers in the tree are bootstrap values. (B) Expression profile of Cg-*dax1*-like in *C. gigas* adult tissues.

Prohibitin-1/-2

Prohibitin-1(*phb1*) and its homologous *phb2*, members of PHB family, were characterized by three domains, including the well-conserved SPFH domain (also known as the PHB domain), transmembrane domain, and coiled-coil domain. *Prohibitins* have been isolated from various vertebrates and invertebrates, and several studies have shown evidence of their essential role of gametogenesis in invertebrates (Mao et al. 2012a, 2012b). In the model organism *C. elegans*, RNAi-mediated knockdown of *phb1* led to gametogenesis-defective sterile adults (Sanz et al. 2003). Transcripts of *phb1* are abundant in spermatids of the octopus *Octopus tankahkeei* and *Eriocheir sinensis* (Chinese mitten crab), suggesting a role in spermiogenesis (Mao et al. 2012a, 2012b). In the scallop *Cypripedium farreri*

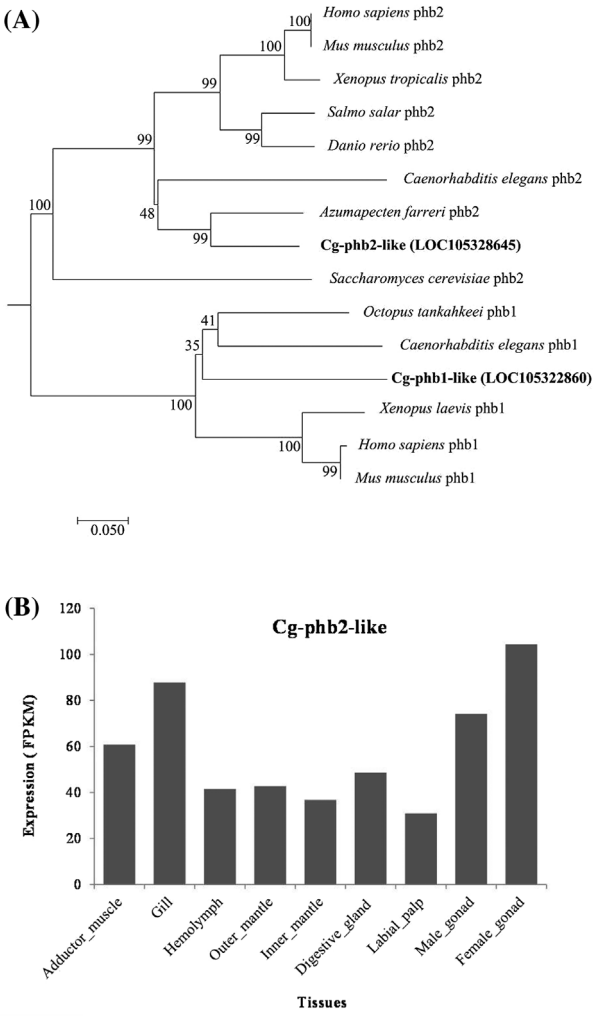

Figure 4. Cg-*phb1*-like and Cg-*phb2*-like identified in *C. gigas* genome and their expression profiles. (A) Phylogenetic relationship of PHB. Bootstrap values were indicated at each branch node. (B) Expression profile of Cg-*phb2*-like in *C. gigas* adult tissues.

(Han et al. 2015), *phb2* may have a similar role as those in the octopus and the Chinese mitten crab.

In the *C. gigas* genome, we identified two gene candidates that encode the PHB domain. The constructed phylogenetic tree presented two distinct branches of *phb1* and *phb2*, and LOC105322860 was clustered to the *phb1* subfamily, while LOC105328645 was clustered to the *phb2* subfamily (Figure 4(A)). LOC105322860 and LOC105328645 were thus annotated 'Cg-*phb1*-like' and 'Cg-*phb2*-like', respectively. Cg-*phb2*-like was widely expressed in various tissues and it was more abundant in the female gonad than male gonad (Figure 4(B)).

Insulin-like growth factor binding protein 7 (IGFBP7)

Members of the IGF-binding protein (IGFBP) superfamily are evolutionary conserved N-terminal cysteine-rich extracellular proteins that are involved in the regulation of IGF and insulin signaling both in vertebrates and invertebrates (Duan & Xu 2005; Li et al. 2012). The IGFBP family consists of two groups, high affinity for IGF I and II binding protein (*IGFBP1-6*) and low affinity for these ligands-related proteins (*IGFBP-rP1-IGFBP-rP10*). These low-affinity proteins showed structural similarity to an N-terminal conserved IB domain of other IGFBPs. *IGFBP7*, also known as IGFBP-related protein 1 (IGFBP-rP1), is the only member of the identified IGFBP superfamily that bind strongly to insulin (Corkins et al. 1995). In molluscs, the cDNA structure of an *IGFBP7*-like has been reported in the abalone (Li et al. 2012).

In the *C. gigas* genome, we identified a gene candidate (LOC105339347) encoding a protein with high similarities with IGFBPs. In the BLASTP, LOC105339347 showed the highest similarity to *H. diversicolor IGFBP7* (e-value = $1e^{-55}$, identities 43%) (Table 1). The phylogenetic tree constructed based on the overall amino acid sequences of other IGFBP proteins revealed that LOC105339347 was aligned to a clade of *IGFBP7*-like protein (Figure 5(A)). Therefore, LOC105339347 was annotated 'Cg-*IGFBP7*-like'. A signal peptide was predicted in Cg-*IGFBP7*-like with the cleavage site between Gly_{19} and Gln_{20}. The mRNA transcripts of Cg-*IGFBP7*-like could be detected in all examined tissues, and were most abundance in *C. gigas* adductor muscle (Figure 5(B)).

Oocyte maturation and spawning

5-HT receptor

5-HT (serotonin) acts through receptors to mediate various functions in both vertebrates and invertebrates. Hormonal control of oocyte maturation has been widely studied in molluscs. In gastropods, the neurohormone 5-HT has been linked to various behavioral and physiological processes such as sensation (Rathouz & Kirk 1988), feeding (Kawai et al. 2011), locomotion (Satterlie & Norekian 1995) and circadian rhythms (Levenson et al. 1999). In bivalves, administration of 5-HT has been shown to induce oocyte maturation and spawning (Hamida et al. 2004; Tanabe et al. 2006).

In bivalves, the *in vitro* treatment of 5-HT to isolated fragments of an ovary in the mature stage stimulates the release of the oocytes, and induces germinal vesicle break down (GVBD) in the released oocytes (Matsutani & Nomura 1987). In addition, oocyte maturation is generally characterized by GVBD of the oocyte (Eppig 1982). Oocyte maturation of bivalves are mediated by 5-HT via 5-HT receptors on the oocyte surface (Krantic et al. 1993; Gobet et al. 1994).

In mammals, 5-HT receptors have been classified into seven types according to their pharmacological characterizations except for type 3 (*5-HT$_3$*), which are ligand-gated

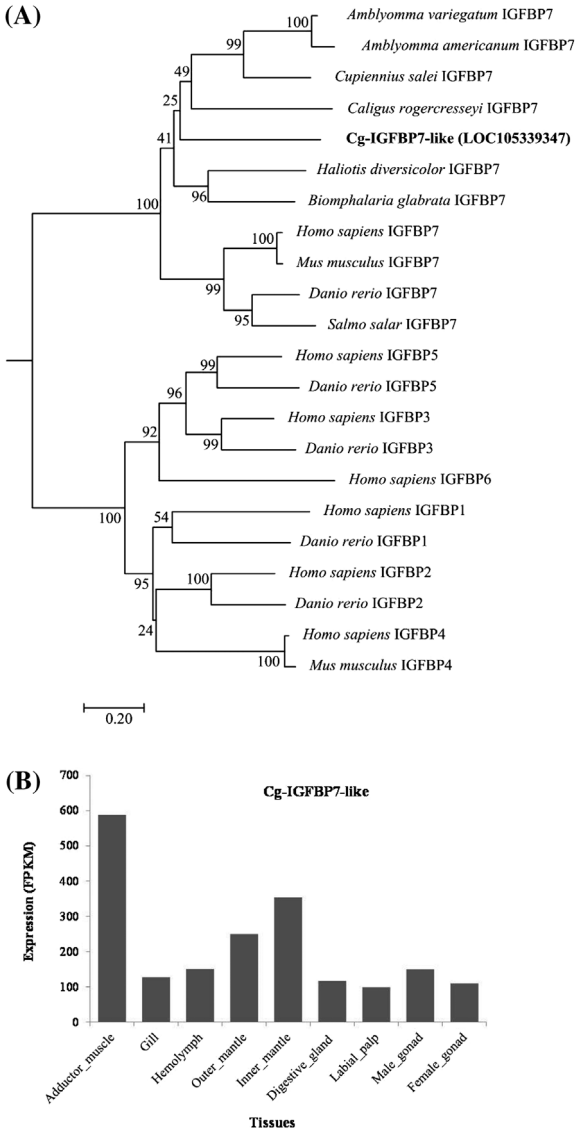

Figure 5. Cg-*IGFBP7*-like identified in *C. gigas* genome and its expression profile. (A) Molecular phylogenetic tree of the IGF-binding protein superfamily proteins. Bootstrap values are indicated at each branch node. (B) Expression profile of Cg-*IGFBP7*-like in *C. gigas* adult tissues.

ion channels, the other six types belong to the superfamily of G-protein-coupled receptors (GPCRs) (Gerhardt & van Heerikhuizen 1997). Until now, several molluscan 5-HT receptor genes have been cloned from the central nervous system and the sperm of gastropods: two are *5-HT*$_{Lym}$ and *5-HT2*$_{Lym}$ from the pond snail *L. stagnalis* (Sugamori et al. 1993; Gerhardt et al. 1996) and the other four from *A. californica* (Li et al. 1995; Angers et al. 1998). In the Japanese scallop, *Patinopecten yessoensis*, the 5-HT receptor on the surface of the oocyte membrane has been pharmacologically characterized as a mixed profile of *5-HT*$_1$/*5-HT*$_2$ subtypes that was distinct from any mammalian 5-HT receptors. Obviously, invertebrate 5-HT receptors need a more definitive way to be identified and classified because of significant differences in pharmacological properties between vertebrate and invertebrate receptors (Tierney 2001).

In the *C. gigas* genome, we identified five gene candidates that encode 5-HT receptor orthologs. All these genes are members of GPCRs superfamily. In the BLASTP search, the gene candidates with the highest similarity were as follows: LOC105344141 to *P. fucata* 5-HT$_1$ receptor (e-value = 4e^{-179}, identities 71%), LOC105348009 to *M. yessoensis* 5-HT receptor (e-value = 4e^{-176}, identities 64%), LOC105330687 to *A. californica* 5-HT$_2$ receptor (e-value = 8e^{-31}, identities 71%), LOC105330597 to *A. californica* 5-HT$_4$ receptor (e-value = 6e^{-123}, identities 62%) and LOC105345175 to *Planorbella trivolvis* type 7 serotonin receptor (e-value = 6e^{-40}, identities 71%). In phylogenetic analysis with 5-HT receptors of various species, the five gene candidates clustered appropriately to different types of 5-HT receptors, as shown in Figure 6. LOC105348009 and LOC105344141 were classified into 5-HT$_1$ receptor family indicating that they belong to different subtypes of 5-HT$_1$ receptors. The two gene candidates were annotated 'Cg-*5-HT*$_1$-like A' and 'Cg-*5-HT*$_1$-likeB', respectively. The other three gene candidates were annotated as follows: Cg-*5-HT*$_2$-like (LOC105330687), Cg-*5-HT*$_4$-like (LOC105330597), Cg-*5-HT*$_7$-like (LOC105345175).

Others

To investigate the reproductive mechanisms of bivalves at a molecular level, several reproduction-specific genes were isolated from the Chilean scallop *Argopecten purpuratus* and the mussel *Mytilus edulis* (Boutet et al. 2008; Ciocan et al. 2011). Of these genes, testis-specific serine/threonine-protein kinase (*TSSK*), spermatogenesis and centriole associated 1 (*SCA*), calcineurin (*CN*), cytidine deaminase (*CDA*), RNA-specific adenosine deaminase (*ADAR*) were used as a query to BLASTP search against the *C. gigas* genome.

TSSK

Several studies from various animal phyla have shown that TSSK proteins are almost exclusively expressed in the testis, suggesting an indispensable role in spermatogenesis and/or function of spermatozoa. In mammals, five members of the testis specific serine/threonine kinase family have been identified that show differences in their localization and timing of expression. Target deletion of *Tssk1/Tssk2* and *SSTK* genes in mice resulted in male sterility (Spiridonov et al. 2005; Xu et al. 2008). In molluscs, *TSSK* is exclusively expressed in the Chilean scallop *A. purpuratus* and the mussel *M. edulis* testis, suggesting its role in molluscan spermatogenesis and/or sperm function. However, the mussel homolog is upregulated in immature/early-developing

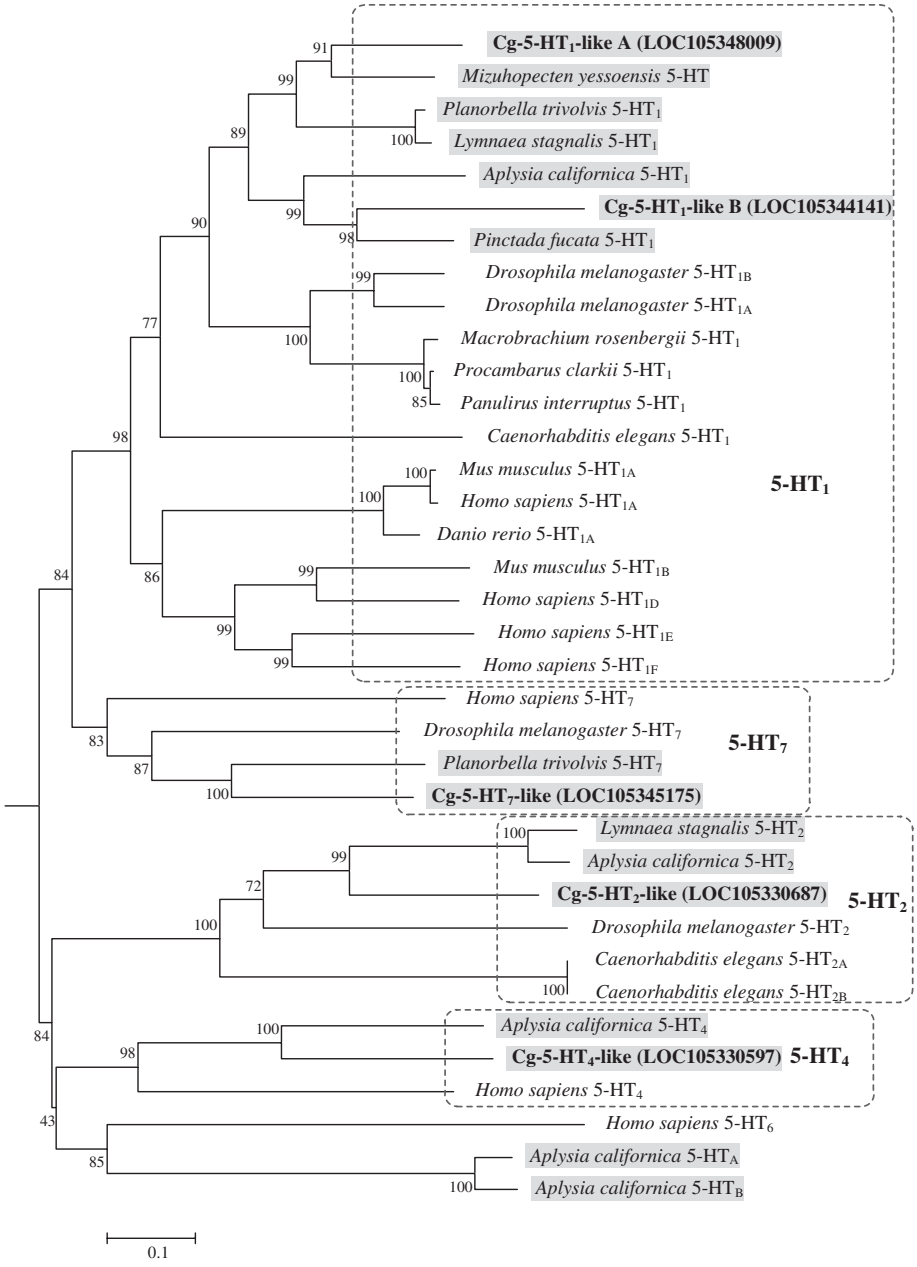

Figure 6. Neighbor-joining phylogenetic tree of 5-HT receptors proteins. Bootstrap values are indicated at each branch node. Groups of 5-HT receptors are shown as different receptor types and are distinguished by a gray dotted line. Molluscan 5-HT receptors are shaded in gray.

gonads, yet the scallop homolog is downregulated at this stage of gametogenesis (Boutet et al. 2008; Ciocan et al. 2011). In the *C. gigas* genome, we found five gene candidates with sequences similar to the *TSSK* family proteins in mammals (Table 1).

SCA

Spermatogenesis and centriole associated 1 protein, also called speriolin, is another testis-specific protein. Boutet et al. (2008) observed that *SCA* mRNA was highly specific-expressed in the scallop immature testis and at a low level in other tissues, which is consistent with the research on mRNA expression pattern of mouse testis (Goto & Eddy 2004). Speriolin was identified as a novel spermatogenic cell-specific protein that co-localizes with pericentrin in the centrosome in mouse spermatocytes and continues to be present in spermatid centrosomes in the absence of pericentrin. Our search in the *C. gigas* gene models identified one gene candidate similar to the scallop *SCA*, which was also exclusively expressed in the testis.

CN

CN is a calcium/calmodulin-activated serine/threonine-specific phosphatase. CN was initially isolated in

mammalian brain as a heterodimer consisting of a catalytic subunit (CNA) and a regulatory subunit (CNB). In molluscs, research on the scallop *P. yessoensis* demonstrated that *CNA* and *CNB* were both expressed in the testis and their expression was correlated to the maturation cycle of testis, suggesting that *CN* is involved in spermatogenesis (Uryu et al. 2000). Other research showed that *CNA* was also expressed in the ovary of the scallop *A. purpuratus* (Boutet et al. 2008). In the *C. gigas* genome, a gene candidate was identified with a sequence similar to *Calcineurin B* (*CNB*), while no gene candidates similar to *CNA* were found.

ADAR and CDA

In a previous study conducted on the scallop *A. purpuratus*, *ADAR* was present at a higher mRNA level in the immature gonad than in other tissues, and that *CDA* mRNA expression was higher in the ovary than in other tissues (Boutet et al. 2008). In other molluscs, two genes encoding *ADAR* have been characterized from the squid *Loligo opalescens* (Palavicini et al. 2009) and one *CDA* gene has been identified in the abalone *H. diversicolor supertexta* (Wu et al. 2009). In the *C. gigas* genome, one gene candidate for *ADAR* and two gene candidates for *CDA* were identified (Table 1).

Conclusion

In the present study, 39 reproduction-related gene candidates of the Pacific oyster *C. gigas* were identified. Identification of these genes constitutes a new tool for research on the bivalve reproductive process and enables us to provide a better understanding of bivalve reproduction at a molecular level. More specific and precise individual investigation is needed to elucidate their role in future studies, using functional studies such as RNA interference.

Disclosure statement

No potential conflict of interest was reported by the authors.

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