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4 **Discovery of germline-related genes in Cephalochordate amphioxus: A**
5 **genome wide survey using genome annotation and transcriptome data**
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Abstract

The generation of germline cells is a critical process in the reproduction of multicellular organisms. Studies in animal models have identified a common repertoire of genes that play essential roles in primordial germ cell (PGC) formation. However, comparative studies also indicate that the timing and regulation of this core genetic program vary considerably in different animals, raising the intriguing questions regarding the evolution of PGC developmental mechanisms in metazoans. Cephalochordates (commonly called amphioxus or lancelets) represent one of the invertebrate chordate groups and can provide important information about the evolution of developmental mechanisms in the chordate lineage. In this study, we used genome and transcriptome data to identify germline-related genes in two distantly related Cephalochordate species, *Branchiostoma floridae* and *Asymmetron lucayanum*. *Branchiostoma* and *Asymmetron* diverged more than 120 MYA, and the most conspicuous difference between them is their gonadal morphology. We used important germline developmental genes in several model animals to search the amphioxus genome and transcriptome dataset for conserved homologs. We also annotated the assembled transcriptome data using Gene Ontology (GO) terms to facilitate the discovery of putative genes associated with germ cell development and reproductive functions in amphioxus. We further confirmed the expression of 14 genes in developing oocytes or mature eggs using whole mount *in situ* hybridization, suggesting their potential functions in amphioxus germ cell development. The results of this global survey provide a useful resource for testing potential functions of candidate germline-related genes in cephalochordates and for investigating differences in gonad developmental mechanisms between *Branchiostoma* and *Asymmetron* species.

Keywords: Amphioxus, primordial germ cell, reproduction, development, evolution, GO annotation.

1. INTRODUCTION

The germline cells of metazoan animals are segregated from somatic cell lineages during development either by the inheritance of specific maternal gene products during early cleavages (preformation) or by induction mechanisms that occur during embryogenesis (epigenesis) (Extavour and Akam, 2003; Johnson et al., 2011). Despite the great contrast between these two types of mechanism, once primordial germ cells (PGCs) are specified they usually show distinct morphological features and express a suite of conserved genes, most commonly including *Vasa*, *Nanos*, *Piwi/Ago*, *Tudor*, *Boule/DAZ*, and *bruno* (Juliano and Wessel, 2010; Voronina et al., 2011). Interestingly, many of these genes, including *Vasa*, *Nanos*, and *Piwi* are also expressed in multipotent somatic stem cells, especially in those cells that have great ability to differentiate, proliferate, self-renew and regenerate. These observations have led to the hypothesis that PGCs and somatic stem cells may have evolved from a common pluripotent progenitor cell population that employs a conserved gene regulatory network for its development and maintenance (Juliano et al., 2010; Solana, 2013). Indeed, certain cell types that can produce both gametes and somatic cells, such as the I-cells in *Hydra* and the archeocytes in sponges (David, 2012; Funayama, 2010), have been identified in early branching metazoan animals, and these conserved genes are expressed in these pluripotent cells. In planarians, neoblasts represent totipotent stem cells that can lead to the regeneration of the entire body (including the germline); these cells also require the expression of a set of similar genes for their formation and normal function (Rink, 2013). Thus, the evolution of diverse germline developmental mechanisms in metazoans may reflect modifications of gene repertoire that interact with this core genetic network as well as changes in the timing or regulatory control to operate this core genetic network in different animals.

The phylogenetic position of Cephalochordata (commonly called amphioxus or lancelets) makes this animal group a useful reference point for studying the evolution of the genome and developmental mechanisms. Currently there is broad consensus that cephalochordates represent the earliest branching group within chordates, and tunicates are the sister group to vertebrates (Bourlat et al., 2006; Delsuc et al., 2006; Delsuc et al., 2008). Compared to tunicates, however cephalochordates retained more ancestral

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4 chordate characteristics and experienced less genome reorganization or gene loss after
5 they diverged from other chordate lineages (Holland et al., 2008; Holland et al., 2004;
6 Louis et al., 2012; Paps et al., 2012). Moreover, the cephalochordate genome did not
7 undergo the two-round (2R) whole genome duplication events that occurred during early
8 vertebrate evolution (Putnam et al., 2008). Therefore, cephalochordates provide a suitable
9 model for comparing the evolution of genome and of gene function between invertebrate
10 chordates and vertebrates and even between chordates and other metazoan animals
11 (Bertrand and Escriva, 2011).
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19 To study the evolution of germline developmental mechanisms in chordates, our
20 group previously searched the genome of the model amphioxus *Branchiostoma floridae*
21 (Holland et al., 2008; Putnam et al., 2008) and identified several conserved germline
22 markers including *vasa*, *nanos*, *piwi*, and *tudor-domain-containing* genes (Wu et al.,
23 2011; Zhang et al., 2013). We showed that transcripts of these genes are maternally
24 deposited in the matured oocytes; after fertilization, these transcripts are asymmetrically
25 localized in the early developing embryos and are subsequently inherited by the specific
26 blastomere that is destined to differentiate into the germline cells in amphioxus (Wu et al.,
27 2011; Zhang et al., 2013). Our results from studies of various *Branchiostoma* species
28 further suggested that *Branchiostoma* amphioxus use maternal gene products to specify
29 their germline cells during early embryogenesis (i.e. a preformation mechanism), in
30 contrast to the previous idea that cephalochordates use an inductive mechanism
31 (epigenesis) to form their germ cells (Extavour and Akam, 2003; Extavour, 2007; Frick
32 and Ruppert, 1997). These new findings raised a question regarding the generality of
33 using maternal gene products to specify germline cells in cephalochordates. Expanding
34 our knowledge of the gene complements and mechanisms that control germline
35 specification to cover more distantly related cephalochordate species would facilitate our
36 understanding of how mechanisms for germline specification have evolved in chordates.
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52 In addition to *Branchiostoma* species, which have paired gonads, there are two
53 other groups of cephalochordates: *Epigonichthys* and *Asymmetron*, which have gonads
54 only on the right side (Holland, 2011; Nishikawa, 2004; Ruppert, 1997). Little is known
55 about the biology of *Asymmetron* and *Epigonichthys* amphioxus; only recently,
56 researchers successfully obtained laboratory spawning from an *Asymmetron* amphioxus
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(*Asymmetron lucayanum*) and described its early development (Holland and Holland, 2010). On the basis of mitochondrial DNA, *Epigonichthys* was placed as the sister group of *Branchiostoma*, and *Asymmetron* was branched outside the *Branchiostoma* + *Epigonichthys* clade (Kon et al., 2007; Nohara et al., 2005). According to the molecular clock based on mitochondrial DNA and on nuclear gene sequence data, divergence between *Asymmetron* and *Branchiostoma* is estimated to have occurred 120-160 million years ago (MYA), suggesting wide divergence between these two groups (Nohara et al., 2005; Yue et al., 2014).

In this study, we conducted a comprehensive survey of conserved germline-related genes in two amphioxus species, *Branchiostoma floridae* and *Asymmetron lucayanum*, that represent the two diverged cephalochordate groups. We also searched for potential germline cell developmental genes within the available genome or transcriptome resources in these two species using annotated GO terms. Finally, we tested the validity of our search approach by performing *in situ* hybridization on *B. floridae* ovarian tissue and mature eggs to examine the expression patterns of a partial list of these identified genes. The identification of these germline-related genes in cephalochordates will contribute to the understanding of the evolution of mechanisms underlying germline specification in chordates and could provide a basis for investigating differences in gonad developmental processes in *Branchiostoma* and *Asymmetron* amphioxus.

2. MATERIALS AND METHODS

2.1 Sequence sources

B. floridae proteome sequences were downloaded from <http://genome.jgi-psf.org/Brafl1/Brafl1.home.html> following procedures described in Yue et al. 2014. The two sets of *A. lucayanum*'s likely protein-coding transcripts (asymAD and asym20h) were predicted by TransDecoder (<http://transdecoder.sourceforge.net>) based on the transcriptome assemblies of two pooled *A. lucayanum* libraries (asymAD for adults and asym20h for larvae) (NCBI BioProject accession SRP035506). The corresponding protein sequences were used as two sets of *A. lucayanum* proteomes. Details of these two transcriptome assemblies were described in Yue et al. 2014. The proteomes of four well-studied model organisms, (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio* and *Mus musculus*), as well as those of six other species (*Ciona intestinalis*, *Petromyzon marinus*, *Latimeria chalumnae*, *Xenopus tropicalis*, *Gallus gallus* and *Homo sapiens*) were downloaded from Ensembl (release 78); the proteomes of *Trichoplax adhaerens*, *Amphimedon queenslandica*, *Nematostella vectensis*, *Lottia gigantea*, *Tribolium castaneum* and *Strongylocentrotus purpuratus* were downloaded from EnsemblMetazoa (release 24). The proteome of *Callorhinchus milii* was downloaded from <http://esharkgenome.imcb.a-star.edu.sg/>.

2.2 Identifying conserved germline-related genes in cephalochordates

We surveyed the existing literature to create a list of known genes that are crucial to the germline cell development process of four well-studied model organisms, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio* and *Mus musculus* (Table S1). We defined these genes as the candidate genes for germline cell development. We identified the representative protein sequence of each gene based on the annotation information provided by Ensembl (www.ensembl.org), FlyBase (<http://flybase.org>), WormBase (<http://www.wormbase.org>), ZFIN (www.zfin.org) and MGI (<http://informatics.jax.org/>). A Pfam (v27.0) search (<http://pfam.xfam.org/>) was

performed for each candidate gene to identify its characteristic protein domain structure. For each characteristic domain, we used the hmmsearch program from the hmmer package (v3.1b1) (Eddy. 2011) to search the *B. floridae* and *A. lucayanum* proteomes to call out the *B. floridae* gene models and *A. lucayanum* transcripts that contain such specific domains based on the hidden Markov model (HMM) pre-built by Pfam (<http://pfam.xfam.org/>). This provided an inclusive preliminary list for each candidate gene with its potential homologs in the two cephalochordate species.

We then used Muscle (v3.8) (Edgar, 2004) to align the characteristic domain regions and employed FastTree (v2.1.7) (Price et al., 2009) to build the phylogenetic tree. The orthology relationships were inferred based on the topology of the phylogenetic tree. The full domain structure of each complete gene was further checked and compared with the original candidate genes. We also performed BLASTP searches for our candidate genes against the *B. floridae* and *A. lucayanum* proteomes (e-value cutoff = 1E-6) to cross-validate our orthology inferences based on the phylogenetic tree. Two candidate genes, *bam* and *pgc*, lacked any detectable characteristic domains; we therefore directly used the BLASTP result to determine whether there are orthologous genes in cephalochordates. Finally, for all inferred cephalochordate orthologous counterparts of our candidate genes, we performed a BLASTP search against the NCBI nr database to confirm that the top hits were indeed the orthologs of our original candidate genes as a final confirmation of our orthology inference.

To better characterize the genomic structure of *piwill* genes in cephalochordates, we retrieved the genome assembly, proteome sequences and gene annotation information of *B. becheri* from http://mosas.sysu.edu.cn/genome/download_data.php (v18h27.r3 version) (Huang et al., 2014). The *piwill* genes in *B. becheri* were identified in the same way as their *B. floridae* counterparts. The gene arrangement of *piwill* gene cluster in *B. floridae* and *B. belcheri* were plotted using GenomeTools' AnnotationSketch module (Steinbiss et al., 2009). The annotation of neighboring genes around the *piwill* gene cluster in *B. floridae* and *B. becheri* were performed by BLASTP searches against Genbank nr database. The acronym names for these neighboring genes were assigned based on the information from the HUGO Gene Nomenclature Committee (HGNC) (<http://www.genenames.org>).

2.3 Identifying potential germline-related genes in cephalochordates by GO annotation

In Yue et al. 2014, we conducted BLAST2GO annotations for both the *B.floridae* and *A. lucayanum* proteomes. In that analysis, we performed a BLASTP search against the SwissProt and NCBI nr databases and used the standard BLAST2GO pipeline (v2.8) (Conesa et al., 2005). In the current study, we screened the *B. floridae* and *A. lucayanum* proteomes based on our previous BLAST2GO proteome annotations and called out those *B. floridae* gene models and *A. lucayanum* transcripts that are associated with the GO term "germ cell development" (GO:0007281). These cephalochordate gene models/transcripts constituted a second set of germline cell development-related genes in cephalochordates. We also performed Fisher's exact test (Fisher, 1922) with false discover rate (FDR) correction (Benjamini and Hochberg, 1995) to identify GO terms that are overrepresented by these genes against the genomic background.

2.4 Identifying non-redundant *B. floridae* - *A. lucayanum* orthologous gene pairs for our identified germline cell development-related genes in cephalochordates.

We employed Proteinortho (v5.11) (Lechner et al., 2011) to identify the orthologous gene/transcript groups within the *B. floridae* proteome and the two *A. lucayanum* proteomes (asymAD and asym20h). Proteinortho made its inference based on reciprocal best hits in all against all BLASTP searches. Within each identified cephalochordate orthologous gene group, if there were members from both the asymAD and asym20h libraries, we selected the longer sequence as the non-redundant representative sequence for *A. lucayanum*. To further illuminate the orthology relationship, we incorporated all the other species sampled in this study to ensure our orthology inference based on the topology of their joint phylogenetic tree.

2.5 Retrieving available ESTs/cDNA clones information for our identified *B. floridae* germ cell development genes

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4 For our identified *B. floridae* germline cell development-related gene models, we
5 located the available ESTs/cDNA information in both the *B. floridae* cDNA database and
6 the NCBI EST database (Wang et al., 2012; Yu et al., 2008). Using the protein sequence
7 of the corresponding *B. floridae* gene models, we performed a TBLASTN search using
8 an e-value cutoff of 1E-6. When searching against the NCBI EST database, we limited
9 our search organism to *B. floridae* (NCBI taxid: 7739) only.
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17 2.6 *In situ* hybridization and image acquisition

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21 To verify the expression of identified *B. floridae* germline-related genes, cDNA
22 clones identified from the *B. floridae* cDNA database were picked to obtain their plasmid
23 DNA and subsequently used as templates to synthesize digoxigenin (DIG)-labeled anti-
24 sense RNA probes for *in situ* hybridization. Template preparation, probe synthesis, and *in*
25 *situ* hybridization were performed as previously described (Lu et al., 2012; Wu et al.,
26 2011). Images were obtained using a Zeiss Axio Imager A2 microscope with a Zeiss
27 AxioCam MRc CCD camera. Adobe Photoshop CS4 was used to minimally adjust the
28 brightness of images and Adobe Illustrator CS4 was used to construct the data figure.
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3. RESULTS AND DISCUSSION

3.1 Identification of conserved germline-related genes in *B. floridae* and *A. lucayanum* by the candidate gene approach

To identify conserved germline-related genes in amphioxus, we compiled a list of candidate genes that have known functions in germline formation and maintenance in four well-studied model organisms (*Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio* and *Mus musculus*) for our survey (Supplementary Table S1). For each candidate gene compiled in Table S1, we searched for orthologs in the two amphioxus species, *B. floridae* and *A. lucayanum*. The major domain that each candidate gene encodes was characterized to facilitate our search. We successfully identified orthologs of *nanos*, *vasa/glh*, *arrest/bruno/celf2*, *bgn/ythdc2*, *boule/dazl*, *csul/pmr5*, *gcl glt/qki*, *mael*, *mago nashi*, *mex3*, *orb/cpeb*, *ovo*, *prdm1/blimp1*, *prdm14*, *pumilio/puf/fbf*, *smB*, *smD3*, *smE/snr6* and *tsunagi/rmb8a* in both amphioxus species (Table 1). The orthologous *B. floridae* gene models that we identified for *nanos* and *vasa/glh* is consistent with previous findings (Wu et al., 2011). Despite an exhaustive search approach, we were unable to find amphioxus orthologs for *bam*, *buc*, *cxcl12a/sdf1a*, *cxcr4b*, *dnd1*, *dppa3/stella*, *meg*, *mex*, *nanog*, *oma*, *oskar*, *pgc*, *pie*, *pos1*, *pou5f1/oct4*, and *smG/snr7*, suggesting that amphioxus most likely do not possess orthologs of these genes. Some of these genes, such as *the oskar* in insects (Ahuja and Extavour, 2014; Ewen-Campen et al., 2012), and the *buc*, *nanog* and *pou5f1/oct4* in vertebrates (Bontems et al., 2009; Takatori et al., 2008), are likely lineage specific genes and are thus not present in the amphioxus genome. Another possibility is that some of these genes might have diverged too widely to be recognized by standard BLAST and Pfam searches and that more sophisticated search strategies (e.g., (Roch et al., 2014) will be required to retrieve their homologs. Alternatively, amphioxus may have simply lost those genes during evolution. Further analysis will be needed to confirm whether these genes are absent in the amphioxus lineage.

The *piwi*, *dmrt* and *tdrd* genes in our candidate gene list come from Piwi-encoding, DM-encoding and TUDOR-encoding multigene families, respectively

(Supplementary Table S1). As a result, many homologs of those genes can be found in both amphioxus species (Table 2-4). For example, in *B. floridae*, we found 7 Piwi-domain encoding, 8 DM-domain encoding and 16 TUDOR-domain encoding gene models. To further determine the phylogenetic relationship among different homologous gene members, we incorporated 16 other species and constructed phylogenetic trees for each gene family. Figures S1-S3 show the full 18-way phylogenetic trees, and Figures S4-S6 display the downsampled trees. Using this method, some gene members can be confidently assigned to certain orthologous clades annotated by known genes within the clade. For example, *B. floridae* gene models 126411, 126413 and 91824 should be *piwi-like 1* whereas the gene models 276092 should be *piwi-like 2*; the gene model 89999 did not fall into either clade (*piwi-like 1* or *piwi-like 2*) so we defined it as just *piwi-like* (Table 2 and Figures S1, S4). As reported previously (Zhang et al., 2013), we found that the 3 *piwi-like 1* gene models (126411, 126413, 91824) are co-localized in a ~104.3 kb region on the same scaffold (Bf_V2_18 on v2.0 assembly or scaffold214 on v1.0 assembly; Figure 1A). We examined this region more carefully by extracting genomic regions including both this 104.3 kb segment and its flanking 20 kb regions on both sides for re-annotation to rule out mis-annotation in the original reference genome annotation. We carried out the re-annotation using Softberry's FGENESH program (<http://www.softberry.com/berry.phtml?topic=fgenes>) (Salamov and Solovyev, 2000) with *B. floridae* specific gene discovery parameters provided by Softberry. FGENESH reported 4 Piwi-domain-encoding gene models, for which the gene models of 126411 and 91824 are the same as the *B. floridae* reference annotation, whereas the gene model 126413 was divided into 2 individual Piwi-encoding gene models, suggesting that this 104.3kb genome segment includes at least 3 and possibly 4 Piwi-gene models. It is known that the process of collapsing two allelic haploid genome copies into one representative copy (e.g., generating the haploid v2.0 *B. floridae* reference assembly from the diploid v1.0 assembly) is prone to introduce artifacts in the cases in which two allelic gene copies are placed sequentially on the merged scaffold, resulting in a scenario that is indistinguishable from a tandem gene cluster. However, because this 104.3 kb *piwi-like* gene cluster is already present on the same scaffold in the v1.0 assembly, the result is unlikely to be due to the haplotype-merging process. Therefore, it is our opinion that this

104.3 kb genome segment more likely represents a true piwi-like gene cluster, a finding that indicates the presence of at least 3 *Piwi-like 1* gene models in the *B. floridae* genome. To further confirm the existence of this *piwi-like* gene cluster, we examined the *piwill* gene arrangement in the recently available genome assembly from another amphioxus species, *B. becheri* (Huang et al., 2014). Interestingly, we found not only one set, but two sets of this syntenic *piwill* gene clusters in *B. becheri* (Figure 1B). These two *B. becheri* *piwill* gene clusters are located on the same genomic scaffold (scaffold:178) and the two clusters (one contains three *piwill* gene models and the other contains two) are roughly 300 kb apart. These two *B. becheri* *piwill* gene clusters both have similar syntenic structure of the neighboring genes compared to that in *B. floridae*, although one of them is in opposite direction. This suggests that a further segment duplication might have occurred in *B. becheri*.

For the DM-domain- and TUDOR-domain-containing protein families, we identified amphioxus orthologs for *dmrt1/a2*, *dmrt2*, *dmrt3* in the DM-domain-encoding gene family (Table 3 and Figures S2, S5) and *snd1*, *stk31*, *smn*, *tdrd5*, *tdrd6*, *tdrd9* and *tdrdkh* in the TUDOR-domain-encoding gene family (Table 4 and Figures S3, S6). These phylogenetic classifications were further confirmed by our BLAST search against the NCBI nr database. We also identified other putative DM-domain-encoding genes and TUDOR-domain-encoding genes whose orthologous affiliations cannot be confidently assigned (Table 3 and Table 4; Supplementary Figures S2, and S5). The best hits for these genes in the NCBI nr database are listed in Table 3 and Table 4 as a basis for future detailed characterization.

3.2 Identifying potential molecular players in the development of amphioxus germ cells by GO annotation

In addition to our candidate gene approach, we also identified a set of amphioxus gene models/transcripts that may be involved in germline cell development by searching gene models/transcripts that are associated with the GO term “germline-cell development” (GO:0007281) in our previous BLAST2GO annotation. In total, we found 63 such *B. floridae* gene models and 69 such *A. lucayanum* transcripts from 88 cephalochordate

orthologous groups. Of these 88 cephalochordate groups, 9 were also identified by the candidate gene approach (Table 5 and Supplementary Table S2). These cephalochordate orthologous groups are tabulated in Table 5, Supplementary Tables S2 and S3. In Table 5, the GO term associations have support from both the *B. floridae* gene models and the *A. lucayanum* transcripts, whereas in Table S2 the GO term association is only supported by the *B. floridae* gene models and in Table S3 the GO term association is supported only by the *A. lucayanum* transcripts.

We performed Fisher's exact test to determine whether certain molecular function GO terms are also enriched in *B. floridae* gene models and *A. lucayanum* transcripts associated with the GO term germline-cell development (GO:0007281). We identified 52 molecular function GO terms that are consistently enriched compared with genomic background across our 3 cephalochordate datasets. The top 10 enriched molecular function GO terms are listed in Table 6 as representatives; the entire list can be found in Supplementary Table S8. Many of the GO terms enriched in this search are related to DNA/RNA-binding functions or to RNA helicase activities, suggesting that many of those genes encode proteins that interact with RNAs. This is consistent with the notion that the products of many germ-cell-specific genes encode evolutionarily conserved proteins that are involved in post-transcriptional gene regulation, especially in regulating the localization, stability, and translational level of mRNAs or interacting with non-coding small RNAs to exert their regulatory functions (Ewen-Campen et al., 2010; Hale et al., 2014; Voronina et al., 2011).

3.3 Verification of new molecular markers for the amphioxus germ cell development

For the *B. floridae* germline-related genes that we identified by the candidate gene approach and by the GO term association approach, we further searched for respective cDNA clone/EST hits in the *B. floridae* cDNA database (Supplementary Tables S4 and S5) and NCBI EST database (Supplementary Tables S6 and S7). It is worth noting that for each query the e-values of the best hits from the two databases are not directly comparable due to difference in the database size. This cDNA/EST information will be very helpful in future functional studies of these genes.

To determine whether these identified genes are expressed in germline cells, we conducted an initial screening in which representative cDNA clones of 47 genes in Supplementary Tables S5 and S6 were selected and used to synthesize riboprobes. Using whole-mount *in situ* hybridization of *B. floridae* ovaries and mature eggs, we were able to detect clear expression patterns of 14 genes (Figure 2). The signals were usually more apparent in small oocytes, while the signals in intermediate sized oocytes were mostly diffused and difficult to focus under microscope, likely due to the accumulation of the yolk materials during oogenesis. Thus, in Figure 2 we mostly present images focusing on the smaller oocytes to show the positive *in situ* hybridization signals in the ovaries. Transcripts of *vasa* and *piwil1* were detected in both developing oocytes and mature eggs (Fig. 2, B-C, B'-C'); more importantly, these transcripts were asymmetrically localized near the vegetal pole of the unfertilized eggs (Fig. 2, B'-C'). This pattern is consistent with our previous demonstration that *vasa* and *piwil1* transcripts are components of the vegetal germ plasm in amphioxus eggs (Wu et al., 2011; Zhang et al., 2013). Transcripts of *piwil2* could be observed in ovarian tissue and in developing oocytes (Fig. 2D); however, *piwil2* transcripts exhibited a uniform distribution pattern in the mature egg (Fig. 2D'). Transcripts of *pumilio*, *mago nashi*, *mex-3*, and one *tdrd* gene (cDNA cluster 08482) could be detected in developing oocytes (Fig. 2, E-H), but the transcripts of these genes were asymmetrically localized toward the animal pole of the mature egg (Fig. 2, E'-H'). Transcripts of two other *tdrd* genes (cDNA clusters 15630 and 06837) and of *maelstrom* were also detected in the ovaries (Fig. 2, I-K), and the transcripts of these genes were localized near the cortex of the animal pole in mature eggs (Fig. 2, I'-K'). Weak transcript signals of another *tdrd* gene (cDNA cluster 04486) could be detected uniformly distributed in developing oocytes (Fig. 2L), but the signals were localized near the vegetal cortex of the mature egg (Fig. 2L'), similar to the distribution pattern of germ plasm. Weak signals of *smE/snr-6* were also detected uniformly in developing oocytes (Fig. 2M); however, in mature eggs, the signals of *smE/snr-6* were localized toward the animal pole (Fig. 2M'). Transcript signals of *bgn/ythdc2* and another *tdrd* gene (cDNA cluster 16581) were detected in developing oocytes (Fig. 2, N-O), but no clear evidence of subcellular localization of these transcripts could be observed in the mature egg (Fig. 2, N'-O').

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4 In sum, in addition to the three previously described genes *vasa*, *piwil1* and *piwil2*,
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6 our initial screening demonstrated the expression patterns of 11 novel genes in female
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8 germ cells. From our previous studies, we know that the labeling of *Vasa* and *Nanos* gene
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10 products near the vegetal cortex appear to be co-localized with sheets of electron-dense
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12 particles with ER and mitochondria (Wu et al., 2011). The maternal transcripts of the
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14 newly identified *tdrd* (04486) gene were observed distributing in the same pattern near
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16 the vegetal cortex (Figure 2L'). As to the transcripts localized near the animal cortex, we
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18 suspect they are likely associated with the remnant of the germinal vesicle that is
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20 originally in the animal pole of the developing oocyte (Holland and Holland, 1992;
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22 Holland and Holland, 1991). Because only ovaries and mature eggs were used in this
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24 study, further analysis using male gonadal tissue and embryonic samples will be needed
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26 to determine whether the other 33 genes identified in this survey are expressed in testis or
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28 during the developmental stages.

29 30 **4. CONCLUSION**

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33 In the current study, a comprehensive survey of germline-related genes was
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35 conducted using the genome and transcriptome datasets of two amphioxus species. Our
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37 computational search resulted not only in the identification of homologs of evolutionary
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39 conserved germline-related genes, but also in the discovery of potential candidate genes
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41 for germline cell development and function in cephalochordates. In addition, our *in situ*
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43 hybridization demonstrated specific expression patterns of 11 novel genes in *B. floridae*
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45 ovaries and mature eggs, suggesting that these genes likely have some functions in
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47 female germ cells. We anticipate that further studies using male germ cells and
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49 embryonic samples will provide additional evidence that will enable us to determine the
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51 potential functions of the identified genes. The germline-related genes reported here will
52
53 facilitate future expression analysis and functional studies of the mechanisms of germline
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55 development in cephalochordates and should also provide important insights into the
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57 evolution of germline specification mechanisms in chordates.

58 59 **ACKNOWLEDGEMENTS**

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FIGURES

A

B. floridae *piwil1* cluster on scaffold:Bf_V2_18

B

B. belcheri *piwil1* cluster on scaffold:178

Figure 1. Genomic neighborhoods of *piwil1* gene cluster in *B. floridae* (A), and the two duplicated *piwil1* gene clusters in *B. belcheri* (B). The *piwil1* genes are indicated in black color. Annotation of the neighboring genes was inferred based on BLASTP searches against Genbank nr database. *3-ksr*: 3-keto-steroid reductase; *gtpbp*: GTP-binding protein; *nlr*: NOD-like receptor; *prodh1*: proline dehydrogenase 1; *prodh2*: proline dehydrogenase 2; *rogdi*: rogdi homolog; *rpsd4*: RNA pseudouridylate synthase domain-containing protein 4; *tcn1*: transcobalamin-1; *tlr*: toll-like receptor.

Figure 2. The expression patterns of 14 germline-related genes in *B. floridae* ovaries and mature eggs detected by whole mount *in situ* hybridization. A-O, Developing oocytes dissected from female gonads; A'-O', Mature eggs (unfertilized) with the animal pole oriented to the top and vegetal pole to the bottom. (A, A') Sense-riboprobe synthesized from *vasa* was used as a negative control. (B-O, B'-O') Transcripts of 14 identified genes can be observed in ovaries (white arrows) and/or in mature eggs (black arrows). The white arrow in A' indicates the position of a yolk-free area (YFA), which contains the second meiotic spindle and can be used to recognize the animal pole of the egg. The cDNA cluster ID for anti-sense riboprobe synthesis is indicated below the name of each. The scale bar in A is 100 μ m, and can be applied to all images.

TABLES

Table 1. Amphioxus homologs of conserved genes involved in germline cell development.

Gene name	Major domain encoded	<i>B. f.</i> gene models	<i>A. l.</i> transcripts (non-redundant)
<i>nanos</i>	zf-nanos	85485*	asymAD_comp37714_c0_seq1_m.14292
<i>vasa/glh</i>	DEAD, Helicase_C	214468*	asymAD_comp42457_c0_seq2_m.23103
<i>arrest/bruno/celf2</i>	RRM_1	114402	asymAD_comp46076_c0_seq8_m.32483
<i>bgn/ythdc2</i>	HA2, OB_NTP_bind	123593	asym20h_comp78808_c0_seq2_m.38179
<i>boule/dazl</i>	RRM_1	89711	asym20h_comp73083_c0_seq1_m.25638
		89712	asym20h_comp77866_c0_seq2_m.35437
<i>csul/pmrt5</i>	PRMT5	114443	asymAD_comp46212_c0_seq1_m.32865
<i>gcl</i>	BTB	209210	asymAD_comp45519_c0_seq3_m.30820
<i>gld/qki</i>	KH_1	236368	asym20h_comp78683_c0_seq2_m.37777
<i>mael</i>	Maelstrom	121310	asymAD_comp43714_c0_seq1_m.26082
<i>mago nashi</i>	Mago_nashi	114879	asymAD_comp46168_c2_seq1_m.32739
<i>mex3</i>	KH_1	126022	asymAD_comp15843_c0_seq1_m.716
<i>orb/cpeb</i>	RRM_1	85610	asymAD_comp38638_c0_seq6_m.15671
<i>ovo</i>	zf-C2H2	226363	asymAD_comp42374_c0_seq1_m.22917
<i>prdm1/blimp1</i>	zf-C2H2	120365	asym20h_comp78929_c0_seq2_m.38578
<i>prdm14</i>	zf-C2H2	240852	asym20h_comp79040_c0_seq3_m.38916
<i>pumilio/puf/fbf</i>	PUF	286861	asym20h_comp79141_c0_seq2_m.39316
<i>smB</i>	LSM	94837	asymAD_comp42845_c0_seq2_m.23966
<i>smD3</i>	LSM	114009	asymAD_comp38720_c0_seq3_m.15856
<i>smE/snr6</i>	LSM	268096	asym20h_comp72130_c1_seq26_m.23962
<i>tsunagi/rmb8a</i>	RRM_1	251578	asym20h_comp66831_c0_seq3_m.15573

Footnotes:

1. Gene models labeled with asterisk (*) were also identified by Wu et al. (2011)
2. We did not find orthologous genes in cephalochordates for *bam*, *buc*, *cxcl12a/sdf1a*, *cxc4b*, *dnd1*, *dppa3/stella*, *meg*, *mex*, *nanog*, *oma*, *oskar*, *pgc*, *pie*, *pos1*, *pou5f1/oct4*, *smG/snr7*

Table 2. Piwi-encoding gene models/transcripts in *B. floridae* and *A. lucayanum*

Gene name	Major domain	<i>B. f.</i> gene models	<i>A. l.</i> transcripts (non-redundant)	Top hit description	Top hit ID	Identity	E-value	Bit score	Note
piwi	Piwi	126411*		PREDICTED: piwi-like protein 1 [Latimeria chalumnae]	ref XP_005987243.1	54%	0	943	
		126413*	asymAD_comp47783_c0_seq1_m.3 8647	Piwi-like 1 protein [Branchiostoma floridae]	gb AGI95996.1	80%	0	2262	<i>piwi-like 1</i>
		91824*	asym20h_comp66860_c0_seq1_m.1 5617	Piwi-like 1 protein [Branchiostoma floridae]	gb AGI95996.1	87%	6.00E-74	248	
		276092*	asymAD_comp46117_c0_seq1_m.3 2603	Piwi-like 2 protein [Branchiostoma floridae]	gb AGI95997.1	94%	0	1395	<i>piwi-like 2</i>
		89999*		Piwi-like 2 protein [Branchiostoma floridae]	gb AGI95997.1	32%	5.00E-126	408	<i>piwi-like</i>
argonaut	Piwi	77832*	asymAD_comp42174_c0_seq1_m.2 2473	translation initiation factor 2C, putative [Ixodes scapularis]	ref XP_002413188.1	76%	0	1431	
		81672	asymAD_comp45264_c0_seq1_m.3 0080	translation initiation factor 2C, putative [Ixodes scapularis]	ref XP_002413188.1	81%	0	1422	

Footnote:

1. For each cephalochordate orthologous group, the top hit information was based on the corresponding *B. floridae* gene model (when *B. floridae* gene model is available) or *A. lucayanum* transcripts (when *B. floridae* gene model is not available)
2. Gene models labeled with asterisk (*) were also identified by Zhang et al. (2013)

Table 3. DM-encoding gene models/transcripts in *B. floridae* and *A. lucayanum*.

Gene name	Major domain encoded	<i>B. f.</i> gene models	<i>A. l.</i> transcripts (non-redundant)	Top hit description	Top hit ID	Identity	E-value	Bit score	Note
dmrt	DM	233458		doublesex- and mab-3-related transcription factor A2 [Takifugu rubripes]	ref NP_001033039.1	98%	7.00E-33	126	<i>dmrt1/a2-like</i>
		126562		PREDICTED: doublesex and mab-3 related transcription factor 3-like [Saccoglossus kowalevskii]	ref XP_002732510.1	38%	6.00E-56	205	<i>dmrt2-like</i>
		92186		PREDICTED: doublesex and mab-3 related transcription factor 3-like [Saccoglossus kowalevskii]	ref XP_002732510.1	41%	2.00E-54	202	<i>dmrt3-like</i>
		246825		double sex and Mab-3 related transcription factor 2 [Pinctada martensi]	gb ADD97887.1	67%	4.00E-22	96.3	
		233494	asym20h_c mp62618_c 0_seq2_m.1 0981	double sex and mab-3 related transcription factor [Saccoglossus kowalevskii]	ref NP_001164688.1	89%	3.00E-35	133	
		212199		doublesex- and mab-3-related transcription factor A2 [Takifugu rubripes]	ref NP_001033039.1	45%	1.00E-06	53.5	
		65698		ATPase [Actinosynnema mirum]	ref WP_015805386.1	63%	7.00E-09	186	
		69907		Doublesex- and mab-3-related transcription factor 2 [Chelonia mydas]	gb EMP30458.1	35%	2.00E-09	65.5	

Footnote:

For each cephalochordate orthologous group, the top hit information was based on the corresponding *B. floridae* gene model (when *B. floridae* gene model is available) or *A. lucayanum* transcripts (when *B. floridae* gene model is not available)

Table 4. TUDOR-encoding gene models/transcripts in *B. floridae* and *A. lucayanum*.

Gene name	Major domain encoded	<i>B. f.</i> gene models	<i>A. l.</i> transcripts (non-redundant)	Top hit description	Top hit ID	Identity	E-value	Bit score	Note
tdrd	TUDOR	117294	asymAD_comp45765_c0_seq1_m.31529	PREDICTED: staphylococcal nuclease domain-containing protein 1-like [Saccoglossus kowalevskii]	ref XP_002732557.2	57%	2.00 E-94	307	<i>snd1-like</i>
			asymAD_comp47611_c0_seq2_m.37793	serine/threonine-protein kinase 31 [Rattus norvegicus]	ref NP_001178900.1	26%	5.00 E-83	306	<i>stk31-like</i>
		121736	asym20h_comp73447_c0_seq5_m.26323	PREDICTED: survival motor neuron protein [Cavia porcellus]	ref XP_003461660.1	35%	2.00 E-19	95.5	<i>smn-like</i>
			asym20h_comp76266_c0_seq1_m.31846	Tudor domain-containing protein 5 [Crassostrea gigas]	gb EKC34343.1	30%	3.00 E-90	306	<i>tdrd5-like</i>
		125037	asym20h_comp79605_c0_seq1_m.41219	Tudor domain-containing protein 1 [Crassostrea gigas]	gb EKC34332.1	25%	4.00 E-116	5250	<i>tdrd6-like</i>
			asymAD_comp45668_c0_seq1_m.31259	PREDICTED: putative ATP-dependent RNA helicase TDRD9-like [Saccoglossus kowalevskii]	ref XP_006817051.1	49%	0	1370	<i>tdrd9-like</i>
		67212	asym20h_comp74389_c1_seq4_m.28149	PREDICTED: tudor and KH domain-containing protein isoform X5 [Gallus gallus]	ref XP_423400.4	32%	1.00 E-72	266	<i>tdrdkh-like</i>
			asym20h_comp73791_c0_seq4_m.26914	tudor domain-containing 7 protein [Branchiostoma floridae]	gb AGI95999.1	96%	0	2134	
		72207	asym20h_comp63166_c0_seq6_m.11486	Tudor and KH domain-containing protein [Stegodyphus mimosarum]	gb KFM62854.1	33%	1.00 E-32	330	
			asym20h_comp3999_c0_seq1_m.3482	PREDICTED: putative ATP-dependent RNA helicase TDRD12-like [Saccoglossus kowalevskii]	ref XP_006814119.1	45%	8.00 E-18	89.4	
		88929		PREDICTED: tudor domain-containing protein 1-like [Saccoglossus kowalevskii]	ref XP_006824064.1	31%	7.00 E-93	549	
			asym20h_comp55522_c1_seq1_m.7228	Tudor domain-containing protein 12 [Crassostrea gigas]	gb EKC42887.1	31%	3.00 E-	717	

					152		
116968	asymAD_comp47682_c0_seq1_m. 38082	PREDICTED: tudor domain- containing protein 7 [Orycteropus afer afer]	ref XP_00794 5180.1	29%	1.00 E-57	273	
74965	asymAD_comp46963_c0_seq1_m. 35225	PREDICTED: tudor domain- containing protein 1 [Latimeria chalumnae]	ref XP_00600 2877.1	28%	3.00 E-61	506	
90728	asym20h_comp68196_c0_seq2_m. 17625	PREDICTED: putative leucine- rich repeat-containing protein DDB_G0290503-like [Saccoglossus kowalevskii]	ref XP_00681 1719.1	27%	1.00 E-12	83.6	
176882	asymAD_comp47341_c0_seq7_m. 36652	A kinase anchor protein 1, mitochondrial [Crassostrea gigas]	gb EKC1806 6.1	53%	4.00 E-55	196	
100917		Schlafen-like protein 1 [Zootermopsis nevadensis]	gb KDR0968 3.1	33%	8.00 E-09	67.8	
	asym20h_comp73843_c0_seq1_m. 27012	PREDICTED: tudor domain- containing protein 7-like [Saccoglossus kowalevskii]	ref XP_00682 1058.1	27%	3.00 E-50	194	
	asym20h_comp68194_c0_seq2_m. 17619	PREDICTED: RING finger protein 17 isoform X7 [Mus musculus]	ref XP_00651 9175.1	30%	3.00 E-46	296	
	asymAD_comp44373_c1_seq1_m. 27575	Tudor domain-containing protein 6 [Tauraco erythrolophus]	gb KFV0967 6.1	25%	2.00 E-81	1635	
	asym20h_comp23968_c0_seq1_m. 987	Tudor domain-containing protein 1 [Pelecanus crispus]	gb KFQ5907 3.1	48%	1.00 E-09	100	
	asym20h_comp55522_c0_seq1_m. 7225	Tudor domain-containing protein 12 [Crassostrea gigas]	gb EKC4288 7.1	37%	6.00 E-48	223	
	asymAD_comp47812_c0_seq1_m. 38821	RING finger protein 17 [Crassostrea gigas]	gb EKC1907 1.1	34%	0	778	
	asymAD_comp45215_c0_seq2_m. 29922	PREDICTED: putative leucine- rich repeat-containing protein DDB_G0290503-like [Saccoglossus kowalevskii]	ref XP_00681 1719.1	39%	3.00 E-10	76.3	
	asym20h_comp30294_c0_seq1_m. 1746	tudor domain-containing protein, putative [Ixodes scapularis]	ref XP_00240 8421.1	31%	7.00 E-08	57.8	
	asym20h_comp59727_c0_seq1_m. 8979	RING finger protein 17 [Zootermopsis nevadensis]	gb KDR1474 9.1	31%	8.00 E-17	241	

Footnote:

1. For each cephalochordate orthologous group, the top hit information was based on the corresponding *B. floridae* gene model (when *B. floridae* gene model is available) or *A. lucayanum* transcripts (when *B. f.* gene model is not available).
2. Gene models labeled with asterisk (*) were also identified by Zhang et al. (2013).

Table 5. Cephalochordate-orthologous groups in which both *B. floridae* gene models and *A. lucayanum* transcripts are associated with the GO term germline-cell development (GO:0007281).

<i>B. f.</i> gene models	<i>A. l.</i> transcripts (non-redundant)	Top hits in NCBI_nr database by the corresponding <i>B. f.</i> gene model					
		Top hit description	Top hit ID	Identity	E-value	Bit score	Note
85485	asymAD_comp37714_c0_seq1_m.14 292	nanos-like protein [Branchiostoma floridae]	gb ADM26639.1	100%	0	587	<i>nanos</i>
89712	asym20h_comp77866_c0_seq2_m.3 5437	protein boule-like [Otolemur garnettii]	ref XP_003803032.1	45%	3E-28	122	<i>boule/</i> <i>dazl</i> <i>prdm</i> <i>1/bli</i>
120365	asym20h_comp78929_c0_seq2_m.3 8578	blimp1 [Branchiostoma floridae]	gb ACH72078.1	96%	0	1434	<i>mp1</i>
214468	asymAD_comp42457_c0_seq2_m.23 103	vasa-like protein [Branchiostoma floridae]	gb AGI96004.1	100%	0	764	<i>vasa</i>
268096	asym20h_comp72130_c1_seq26_m. 23962	Small nuclear ribonucleoprotein E [Dicentrarchus labrax]	emb CBN80607.1	88%	8.00E-52	168	<i>smE/s</i> <i>nr-6</i>
240852	asym20h_comp79040_c0_seq3_m.3 8916	PREDICTED: PR domain zinc finger protein 14-like [Saccoglossus kowalevskii]	ref XP_002739286.2	86%	0	617	<i>prdm</i> <i>14</i>
89711	asym20h_comp73083_c0_seq1_m.2 5638	PREDICTED: protein boule-like [Saccoglossus kowalevskii]	ref XP_006818817.1	37%	1.00E-33	140	<i>boule/</i> <i>dazl</i>
115963	asymAD_comp42235_c0_seq1_m.22 637	PREDICTED: U2 small nuclear ribonucleoprotein A'-like [Lepisosteus oculatus]	ref XP_006628826.1	73%	7.00E-123	362	
230456	asym20h_comp59976_c0_seq1_m.9 111	PREDICTED: serine/threonine-protein kinase Chk2-like [Aplysia californica]	ref XP_005112692.1	65%	5.00E-166	485	

200665	asymAD_comp40204_c0_seq1_m.18503	PREDICTED: probable ATP-dependent RNA helicase DHX40 isoform X2 [Pelodiscus sinensis]	ref XP_006138715.1	61%	0	913
125404	asymAD_comp37090_c0_seq1_m.13397	PREDICTED: bone morphogenetic protein 5 isoform X2 [Poecilia reticulata]	ref XP_008428025.1	55%	1.00E-101	313
265042	asymAD_comp39341_c0_seq1_m.16898	small nuclear ribonucleoprotein D2-like protein [Azumapecten farreri]	gb AAM94277.1	96%	1.00E-74	228
122387	asymAD_comp44568_c2_seq1_m.28089	Ras-related protein Rab-18 [Salmo salar]	ref NP_001133260.1	78%	8.00E-119	348
100379	asymAD_comp47635_c0_seq10_m.37880	Putative Profilin [Rhizopus microsporus]	emb CEG81615.1	41%	6.00E-29	113
80164	asymAD_comp45660_c1_seq8_m.31240	PREDICTED: bcl-2 homologous antagonist/killer-like [Strongylocentrotus purpuratus]	ref XP_793197.1	51%	7.00E-46	160
280395	asymAD_comp46055_c1_seq1_m.32423	PREDICTED: DNA mismatch repair protein Msh2 [Saccoglossus kowalevskii]	ref XP_002740239.1	71%	0	1360
59675	asymAD_comp46357_c0_seq1_m.33314	PREDICTED: putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15-like isoform X2 [Aplysia californica]	ref XP_005092201.1	86%	0	1263
117669	asym20h_comp72765_c0_seq5_m.25104	PREDICTED: pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16-like [Xiphophorus maculatus]	ref XP_005810482.1	71%	0	1785
68495	asymAD_comp45366_c0_seq1_m.30361	RecName: Full=Targeting protein for Xklp2 homolog; Short=TPX2 homolog; AltName: Full=Microtubule-associated protein TPX2 homolog [Patiria pectinifera]	sp E2RYF8.1 TPX2_PATPE	46%	5.00E-147	467
264344	asymAD_comp36658_c0_seq1_m.12856	small nuclear ribonucleoprotein Sm D1 [Xenopus (Silurana) tropicalis]	ref NP_001004780.1	90%	3.00E-56	182
71433	asym20h_comp76525_c0_seq1_m.32413	PREDICTED: H/ACA ribonucleoprotein complex subunit 4 [Callorhinchus milii]	ref XP_007889523.1	77%	0	685
113775	asymAD_comp43709_c3_seq1_m.26078	G10-like protein [Branchiostoma belcheri]	gb AAK84395.1 AF397147_1	100%	6.00E-100	295
205455	asymAD_comp47154_c1_seq2_m.35945	PREDICTED: superkiller viralicidic activity 2-like 2-like [Saccoglossus	ref XP_006825596.1	80%	0	1620

kowalevskii]

66988	asymAD_comp45593_c0_seq2_m.31047	ubiquitin-conjugating enzyme E2 [Penaeus monodon]	gb ACD13594.1	88%	5.00E-103	307
		PREDICTED: glutamate receptor-interacting protein 1 isoform X1 [Callorhinchus milii]	ref XP_007903149.1	53%	0	1089
143690	asymAD_comp38746_c1_seq3_m.15889	PREDICTED: U5 small nuclear ribonucleoprotein 200 kDa helicase [Orycteropus afer afer]	ref XP_007957626.1	80%	0	3665
124682	asymAD_comp47769_c0_seq2_m.38566	PREDICTED: activin receptor type-1 [Caprimulgus carolinensis]	ref XP_010162237.1	68%	0	624
221588	asymAD_comp43940_c0_seq2_m.26599	PREDICTED: serine/threonine-protein kinase mTOR [Callorhinchus milii]	ref XP_007895970.1	75%	0	2144
86149	asymAD_comp47778_c0_seq1_m.38624	PREDICTED: U6 snRNA-associated Sm-like protein LSm4 [Diaphorina citri]	ref XP_008476479.1	75%	7.00E-56	181
281075	asymAD_comp45149_c1_seq7_m.29737	retinoic acid receptor beta, isoform 4 [Gallus gallus]	emb CAA40615.1	61%	3.00E-161	473
116859	asymAD_comp45723_c0_seq1_m.31417	nucleostemin-like protein [Saccoglossus kowalevskii]	ref NP_001161618.1	52%	0	634
63599		PREDICTED: mediator of RNA polymerase II transcription subunit 6 [Callorhinchus milii]	ref XP_007891615.1	66%	3.00E-108	324
202253	asymAD_comp35738_c0_seq1_m.11614	PREDICTED: inhibitor of growth protein 1 [Chrysochloris asiatica]	ref XP_006851578.1	52%	3.00E-85	268
130314		PREDICTED: putative ATP-dependent RNA helicase DHX33 [Strongylocentrotus purpuratus]	ref XP_792543.2	63%	1.00E-139	422
124728	asymAD_comp42509_c0_seq1_m.23212	PREDICTED: gamma-secretase subunit PEN-2-like [Strongylocentrotus purpuratus]	ref XP_791533.1	63%	2.00E-43	147
260605	asymAD_comp41171_c0_seq1_m.20351	PREDICTED: SNW domain-containing protein 1-like [Saccoglossus kowalevskii]	ref XP_002741369.2	72%	0	766
277402	asymAD_comp46344_c0_seq2_m.33274	Bcl-2-like protein 1 [Crassostrea gigas]	gb EKC30554.1	40%	9.00E-28	112
247509	asymAD_comp46023_c0_seq5_m.32337	PREDICTED: helicase SKI2W [Anolis carolinensis]	ref XP_003218045.1	48%	2.00E-160	604
40636	asymAD_comp40636_c1_seq1_m.19357					

		PREDICTED: putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15-like [Oryzias latipes]	ref XP_004079718.1	43%	0	594
90438	asymAD_comp46262_c0_seq1_m.33042	ubiquitin-conjugating enzyme e2 d4-like [Lichtheimia corymbifera JMRC:FSU:9682]	emb CDH55607.1	32%	4.00E-17	82.8
78802	asymAD_comp42095_c1_seq1_m.22295	PREDICTED: CUGBP Elav-like family member 4 [Callorhinchus milii]	ref XP_007895323.1	68%	8.00E-70	269
89423	asym20h_comp76564_c0_seq10_m.32490					

Footnote:

The highlighted gene models were also identified by our candidate gene approach.

Table 6. Enriched molecular function GO terms for *B. floridae* gene models associated with the GO term germline-cell development (GO:0007281).

GO ID	GO description	FDR*
GO:1901363	heterocyclic compound binding	3.69E-18
GO:0097159	organic cyclic compound binding	1.11E-17
GO:0003724	RNA helicase activity	6.36E-14
GO:0003676	nucleic acid binding	9.95E-14
GO:0004386	helicase activity	1.97E-12
GO:0000166	nucleotide binding	2.89E-12
GO:1901265	nucleoside phosphate binding	2.90E-12
GO:0008026	ATP-dependent helicase activity	1.23E-11
GO:0070035	purine NTP-dependent helicase activity	1.23E-11
GO:0035639	purine ribonucleoside triphosphate binding	1.44E-11

Footnote:

FDR(*): false discovery rate.