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Cloning, expression and methylation analysis of piwil2 in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

piwi is an important regulator gene in germ cell division during spermatogenesis. *piwi* homologous genes are involved in gametogenesis and germline specification, and knocking down these genes could affect germ cell meiotic progression. To understand the function of *piwi*-related genes in spermatogenesis, we cloned a Piwi-subfamily member (*piwil2* gene) from the gonad of *Cynoglossus semilaevis*. The full-length of *piwil2* cDNA was 3314 bp, including a 3162 bp open reading frame (ORF), a 60 bp 5′-UTR, along with a 92 bp 3′-UTR, and encoded a predicted protein of 1053 amino acid residues. Phylogenetic analysis showed that the PIWIL2 putative protein belonged to the Argonaute protein family, and Piwi-subfamily, with typical PAZ and Piwi domains. Ultrathin sections of different gonadal stages, and real-time quantitative PCR showed that the relative expression of the *piwil2* gene couldn't be detected until day 95 (95 days) larvae, when germ cell divided rapidly in *C. semilaevis*. The *piwil2* transcripts were more abundant in gonads of males and neo-males than in females, and weakly expressed in other tissues and organs. Compared with the relative expression of *piwil2* in gonads, the CpG methylation levels were significantly higher in females. Chromosomal fluorescence in situ hybridization (FISH) showed that the *piwil2* gene was located on the Z sex chromosome of *C. semilaevis*. These results suggest that *piwil2* plays an important role in spermatogenesis of *C. semilaevis*.

1. Introduction

Spermatogonium is the only adult germ cell in testis that could differentiate into spermatozoa. In invertebrates, spermatogenesis is a process regulated by internal and external factors. But in vertebrates, this process is mainly influenced by internal factors, for example, some protein families. A crucial member of these protein families is the Argonaute protein family, which becomes functional by combining with piRNA and forming RNA-induced silencing complexes (RISC). The Argonaute protein family is considered to play an important role in stem cell self-renewal, RNA interference (RNAi) and translational regulation, and is the best defined protein component of the RNA interference (RNAi) machinery (Faehnle and Joshua-Tor, 2007; Liu et al., 2007; Li et al., 2010). The Argonaute protein family is composed of three members: the Ago-subfamily, the Piwi-subfamily, and the *Caenorhabditis elegans*-subfamily (Kawaji and Hayashizaki, 2008). The Piwi subfamily, which is found in animals only and is specifically expressed in germ cells and embryos, has two principal domains: the PAZ domain in the middle of the nucleotide sequence, and the Piwi domain at the C-terminus (Parker and Barford, 2006; Peters and Meister, 2007; Tolia and Joshua-Tor, 2007). The PAZ domain is an RNA-binding motif that binds to the 3′ end of short RNAs, while the Piwi domain is structurally similar to the RNaseH catalytic domain (Simon et al., 2011).

Lin and Spradling (1997) found the *piwi* gene in *Drosophila* firstly and confirmed that *piwi* gene may encode nucleoplasmin. Moreover, by participating in post-transcriptional modification of nuclear mRNA, the *piwi* gene may play an important role in maintaining germ cell activity in *Drosophila* (Lin and Spradling, 1997). Up to now, several homologous genes of the *Drosophila* Piwi-family have been found in a variety of animals, such as *Homo sapiens*, *Mus musculus*, *Caenorhabditis elegans* and *Bombyx mori* (Kuramochi-Miyagawa et al., 2001; Lac et al., 2001; Qiao et al., 2002; Tatsuke et al., 2014). In addition, *piwi* homologous genes has been reported in several teleost, including *Cyprinus carpio*, *Carassius carassius*, *Oreochromis niloticus* and *Danio rerio* (Tan et al., 2002; Houwing et al., 2008; Zhou et al., 2012, 2014; Xiao et al., 2013). PIWI protein can regulate the expression of the functional gene

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Table 1
Primers used in this study.

Primers	Sequences (5′to 3′)	Purpose used
CseF382F	ATTCACTGACCCCTGAGAGC	Genetic sex identification
CseF382R	TGGCACCATCATTGTAAAACTA	Genetic sex identification
Piwil292-979 F	GGAGGAGCCAGAGGAGTATGTC	Partial fragment cloning
Piwil292-979 R	CAGGGATGTGATTTGAAGCGA	Partial fragment cloning
Piwil273-745 F	GGGAGAAGTCGTGGCGTTTGA	Partial fragment cloning
Piwil273-745 R	TGCGGTTGTTGTAGCGGGTG	Partial fragment cloning
Piwil707-1431 F	TGGTCGGCAGCGTCGTCA	Partial fragment cloning
Piwil707-1431 R	GATGTAGCAGTCAGTGCGGTCGT	Partial fragment cloning
Piwil1370-1863 F	GCGTCCGACTGGAGAAAC	Partial fragment cloning
Piwil1370-1863 R	GGAGTACAGCATGGTGTTGATT	Partial fragment cloning
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′ or 3′ RACE, Touchdown PCR
UPM-short	CTAATACGACTCACTATAGGG	5′ or 3′ RACE, Touchdown PCR
NUP	AAGCAGTGGTATCAACGCAGAGT	5′ or 3′ RACE Nested PCR
P-GSP5′	GCGAGTCAGCAACGGCGGCA	5′ RACE, Touchdown PCR
P-NGSP5′	CAAAAACACTGCCTCTGCCCA	5′ RACE Nested PCR
P-GSP3′	CGTCTTCTACCCCCGCCGCTGT	3′ RACE, Touchdown PCR
P-NGSP3′	CGACCGCACTGACTGCTACATC	3′ RACE Nested PCR
Piwil1036-1206 F	CGTCACCTTCGCTCCAAAT	RT-qPCR of *piwil2*
Piwil1036-1206 R	TCTTCGTCGTCCGTTCGC	RT-qPCR of *piwil2*
Actin-F	GAGTAGCCACGCTCTGTC	RT-qPCR of *β-actin*
Actin-R	GCTGTGCTGTCCCTGTA	RT-qPCR of *β-actin*
Piwil-FISH-F	AGTCGTGGCGTTTGATGGTTC	Chromosomal localization
Piwil-FISH-R	GCATTCTCGGGGTTGTAGTGATT	Chromosomal localization
Left M primer	AGTTTTAGATTTGTGGAAGAAGAAC	Bisulfite-sequencing PCR
Right M primer	AAAAAACGATTTATACCATACCGAA	Bisulfite-sequencing PCR
Left U primer	GTTTTAGATTTGTGGAAGAAGAATG	Bisulfite-sequencing PCR
Right U primer	AAAAAACAATTTATACCATCCAAA	Bisulfite-sequencing PCR

at a post-transcription level by associating specifically with piRNAs and generating appropriate physiological effects, including the induction of histone and DNA methylation, deletion of DNA sequences, mRNA breakdown, and inhibition of translation (Kuramochi-Miyagawa et al., 2008; Lu and Clark, 2010; Siomi et al., 2010). In males, the overexpression of *piwi* homologous genes can promote the proliferation of germ cell, while gene mutation or deletion may result in a progressive loss of germ cell and affect the formation of sperm, which will result in reproductive defects (Houwing et al., 2007).

The half-smooth tongue sole (*C. semilaevis*) is an important economic marine fish species in China, and is highly valued because of its delicious taste. So far, some studies were performed on the identification and cloning of sex-related molecular markers and genes in half-smooth tongue sole (Chen et al., 2007; Deng et al., 2008, 2009). Especially recently the whole-genome sequencing and assembly of the half-smooth tongue sole have been done (Chen et al., 2014), and a first generation BAC-based physical map has been constructed (Zhang et al., 2014). However, study on spermatogenesis-related genes is not available in this fish. In this study, we cloned full length cDNA of *piwil2* from *C. semilaevis* and assessed its relative expression in different tissues and organs of sexually mature fish and early larval stages. To analyze the expression differences among different sexes, promoter methylation detection and fluorescence in situ hybridization (FISH) were conducted. The results will be useful in the future construction of a prokaryotic expression vector that will contain an enhancer promoter of *piwil2*.

2. Materials and methods

2.1. Specimen and tissue collection

Sexually mature *C. semilaevis* over one-year old were obtained from a commercial hatchery (Haiyang 863 High-tech Experimental Base, Haiyang, China). Eleven tissues and organs (spleen, liver, heart, intestine, brain, skin, kidney, gonad, muscle, gill and blood) were collected from three male and three female, snap frozen in liquid nitrogen, and stored at −80 °C until use. In the same way, testis of three sexually mature neo-males and three triploid-males were collected. In addition, gonads of larvae 20 days, 35 days, 50 days, 65 days, 80 days, 95 days, 120 days and 150 days after hatching were collected and stored at −80 °C (Shao et al., 2013). Half of the gonad from each sexually mature *C. semilaevis* was collected and stored in liquid nitrogen. Simultaneously, another half of the gonad were collected and fixed in 2.5% glutaraldehyde solution for histology. To determine the genetic sex, the fin samples of each individual were collected and stored in 100% ethanol for DNA extraction.

2.2. Phenotypic sex and genetic sex identification

Gonads were fixed in 2.5% glutaraldehyde solution for more than 24 h, then, washed with 75% ethanol. After dehydration through ascending concentrations of ethanol, xylol clearing and paraffin wax embedding, tissue sections of 5–6 μm thick were cut. The sections were stained in hematoxylin and eosin (H&E), observed and photographed under a light microscope (Nikon ECLIPSE 80i fitted with a Nikon Digital Sight Ds-Fi2 camera) (Deng et al., 2009). The histology pictures are shown in Fig. 4.

Genomic DNA from fin samples of each individual was extracted using a phenol-chloroform protocol (see Chen et al., 2007). The quality and concentration of DNA were measured with a NanoVue™ Plus Spectrophotometer (GE Healthcare, USA), then stored at −20 °C for future use. A pair of female-specific PCR primers (CseF382F and CseF382R, in Table 1) was used for PCR amplification (Chen et al., 2007, 2012). PCR was carried out as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C (30 s), 66 °C (30 s), and 72 °C (30 s), then 72 °C 10 min for a final extension. A 291 bp female specific band was amplified from genetic female individual, while there was no band in genetic male individual. In addition, the *C. semilaevis* that was phenotypic male and genetic female was considered to be neo-male (Hu and Chen, 2013).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality and concentration of total RNA were assessed by agarose gel electrophoresis and measured with NanoVue™ Plus Spectrophotometer (GE Healthcare, Piscataway, NJ, USA), then stored at −80 °C until use. Reverse transcription and cDNA synthesis was carried out using a PrimeScript RT

reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions.

2.4. Primer design and confirmation of partial piwil2 cDNA

Three pairs of primers, Piwil273-745 (F/R), Piwil707-1431 (F/R), and Piwil1370-1863 (F/R) (in Table 1) were designed to verify the correctness of the cDNA sequence according to the result of whole-genome sequencing (Chen et al., 2014). PCR was performed with an initial denaturation step of 95 °C for 5 min and 35 cycles of amplification followed by a 7 min extension at 72 °C. Each cycle included denaturation at 95 °C for 30 s, annealing at 64.7 °C (56 °C for piwil1370-1863 (F/R)) for 30 s, and extension at 72 °C for 35 s. Five clones were sequenced to obtain the partial cDNA sequences of *piwil2*.

2.5. Rapid amplification of cDNA ends

Both 5′- and 3′-RACE were performed to obtain the 5′ and 3′ cDNA ends of *piwil2* using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Dalian, China) according to the manufacturer's instructions (Chen et al., 2014). Two pairs of gene-specific primers, P-GSP5′ and P-NGSP5′, P-GSP3′ and P-NGSP3′ (in Table 1) were designed according to the partial *piwil2* cDNA sequence above. Two rounds of PCR were conducted for 5′-RACE amplification. The P-GSP5′ and universal primers Mix (UPM), including UPM-long and UPM-short, were used for Touchdown PCR, while the P-NGSP5′ and Nested Universal Primer (NUP) were used for Nested PCR. 3′-RACE amplification was in accordance with the 5′-RACE amplification above. The Touchdown PCR reaction was carried out as follows: 95 °C for 4 min; 95 °C for 30 s, reducing 0.5 °C per cycle from 70 °C to 65.5 °C for 30 s, 72 °C for 2 min, for 9 cycles; 95 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, for 30 cycles; and 72 °C 10 min for elongation. The conditions of Nested PCR were: 95 °C for 4 min; 40 cycles of denaturation at 94 °C for 30 s, annealing temperature for 30 s, extension at 72 °C for 2 min; and final extension at 72 °C for 7 min.

The PCR products were separated by 1% agarose gel electrophoresis using a DL 2000 marker (Takara). The expected bands were separated from the gel and purified with a Zymo Gel DNA Recovery Kit (Zymo Research, USA). The purified fragments were ligated into PMD18-T (Takara), and propagated in *Escherichia coli* Top 10 (Tiangen, Beijing, China). Six positive clones were selected out and sequenced by BGI tech.

2.6. Bioinformatics analysis and phylogenetic tree

The coding sequence (CDS) was predicted and translated into putative amino acid sequences using DNASTAR 5.0 (Chen et al., 2010). Multiple alignments of the amino acid sequence with other known vertebrates were performed by Vector NTI 10. The unique domain structure was analyzed with an NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/structure). The protein molecular weight and isoelectric point (PI) were deduced using the website (http://www.expasy.org/proteomics). The signal peptide was predicted by Signalp3.0 (http://www.cbs.dtu.dk/services/SignalP/).

The nucleotide and amino acid sequence of *piwil2* in other vertebrates were retrieved from GenBank. The phylogenetic tree was constructed with the Neighbor-Joining (NJ) method using MEGA 4.0 software (Saitou and Nei, 1987). Reliability of the NJ tree was assessed by the bootstrap method with 10,000 replications.

2.7. Real-time quantitative PCR (RT-qPCR)

At least three samples of different tissues and organs, and developmental stages of gonads were processed by RT-qPCR, which was conducted on a 7500 Real time PCR system (Applied Biosystems, USA). The reaction was carried out at a final volume of 20 μl, containing 0.3 μl cDNA template, 10 μl SYBR Premix Ex Taq™ (Takara), 0.4 μl ROX Reference Dye II (Takara), 0.4 μl of Piwil1036-1206 F and Piwil1036-1206 R (Actin-F and Actin-R as reference gene) and 8.5 μl ddH_2O. PCR amplification was performed in triplicate wells, using the following conditions: 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. Dissociation curve analysis was performed after each assay to determine target specificity. The primers Piwil1036-1206 F and Piwil1036-1206 R were used to amplify the *piwil2* fragment. In addition, the primers Actin-F and Actin-R were used for *β-actin* expression, which was used as the internal control for *piwil2* gene expression analysis (Deng and Chen, 2008; Xu et al., 2009; Hu and Chen, 2013). The (delta) (delta) Cq method was used to quantify the mRNA expression levels. Finally, a PASW Statistics 18.0 software was used to perform discrepancy analysis among different samples.

2.8. Prediction of methylation sites and methylation specific PCR

Gonads of each one-year old female, male and neo-male were used to extract genomic DNA, by using a TIANamp Marine Animals DNA Kit (Tiangen) according to the manufacturer's instructions. Then DNA of the gonads of each gender was mixed and the quality and concentration were measured with NanoVue™ Plus Spectrophotometer (GE Healthcare, Piscataway, NJ, USA). Prediction of methylation loci and design of BS-PCR primers were conducted using the website (http://www.urogene.org/methprimer/index1.html). The DNA mixture was modified by an EZ DNA Methylation-Gold Kit™ (Zymo Research). A primer pair (Left M primer, Right M primer) was used for PCR amplification before the DNA template was disposed by bisulfite sodium, while another primer pair (Left U primer, Right U primer) was used for Methylation Specific PCR (MSP). After cloning into a PMD18-T vector (Takara), and propagation in *E. coli* Top 10 (Tiangen), eight positive clones were sequenced by BGI. The site-specific methylation measurements at selected CpG and core promoter sites were done by BiQ-Analyzer software.

2.9. Chromosome preparation and Fluorescence In Situ Hybridization

FISH was used to determine the chromosomal localization of the *piwil2* gene in *C. semilaevis*. The chromosomes of male and female were derived from the head and kidney tissues of sexually mature *C. semilaevis*, and prepared according to an established procedure as described by Xie et al. (2012).

According to *piwil2* whole-genome sequencing (Chen et al., 2014), a pair of primers Piwil-FISH-F and Piwil-FISH-R were designed and used to screen out *piwil2* gene from bacterial artificial chromosome (BAC) library of *C. semilaevis* (Shao et al., 2009). The positive clones were inoculated and propagated, and then the plasmids were extracted using a Mini Plasmid Kit (Biomed, Beijing) which was used to prepare FISH probes using a DIG-Nick Translation Mix (ROCHE, Germany). The FISH probes were hybridized with the prepared chromosome slide, and incubated with goat anti-DIG antibody (Molecular, USA) and stained with FITC-labeled donkey anti-sheep IgG antibodies (Molecular).

After air drying in the dark, slides were counterstained with the fluorescence quenching agent with PI (Vector, USA) and covered with a cover slip. Slides were observed under a fluorescence microscope (Nikon ECLIPSE 80i) equipped with 60× oil immersion lens (Nikon Plan Fluor 100X 1.30 Oil) and photographed by a NICK camera (Nikon Digital Sight Ds-Fi2).

3. Results

3.1. Isolation and characterization of piwil2 cDNA

With the primers P-GSP5′, P-NGSP5′, P-GSP3′, P-NGSP3′, the adaptor primers UPM and NUP, a 3314 bp *piwil2* cDNA was obtained after RT-PCR and RACE (GenBank accession number KF937805) (Fig. 1 a, b). The complete cDNA sequence contains a 60 bp 5′-UTR, a 3162 bp open reading frame (ORF), along with a 92 bp 3′-UTR. The ORF encodes

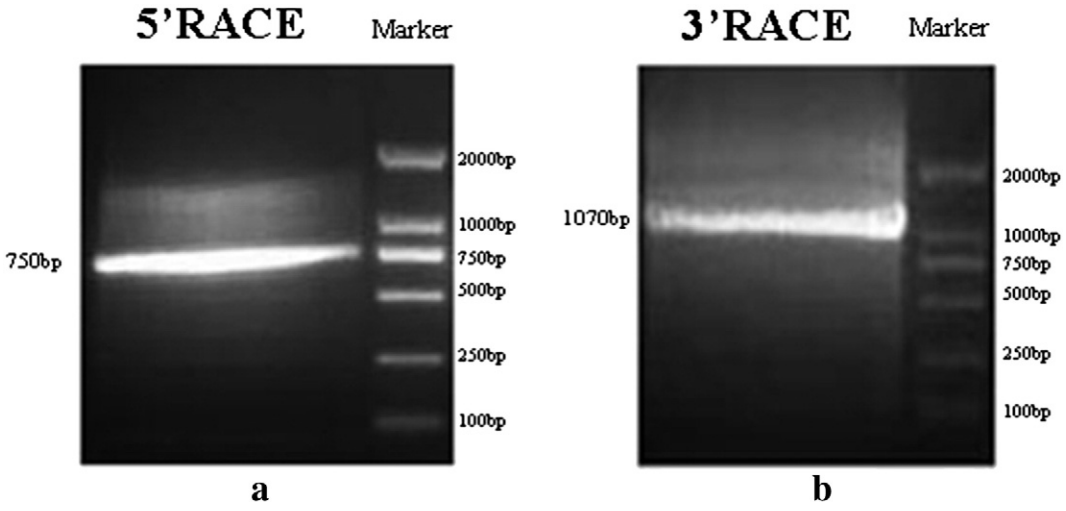

Fig. 1. Electrophoresis of *piwil2* RACE products, 5′-RACE (a) and 3′-RACE (b). Arrows indicated target bands.

a putative protein with 1053 amino acid residues, with a predicted molecular weight of 117 kDa and an isoelectric point (pI) of 8.81.

Based on bioinformatics analysis, two principal domains of the Piwi-subfamily were found in this sequence without a signal peptide site (Fig. 2). One was the RNA-binding PAZ domain from 1528 bp to 1809 bp in the middle of the sequence, and the other was the RNase-like Piwi domain starting from 2295 bp to 3177 bp in the end (Cerutti et al., 2000). In addition, a nucleic acid binding interface site was identified in the PAZ domain region, and at the same time, a 5′RNA guide strand anchoring site was found in the Piwi-like domain.

3.2. Homology and phylogenetic analysis

The amino acid sequence alignment of PIWIL2 in *C. semilaevis* and other vertebrates demonstrated that the PIWIL2 protein of *C. semilaevis* shared many protein features with those of other fishes and mammals. Based on Blastp at the NCBI website, the PIWIL2 protein of *C. semilaevis* displayed the closest homology to that of *O. niloticus* (XP_003445710), with a 68% identity. In addition, it was 53% identical to the PIWIL2 proteins of *H. sapiens* (NP_060538) and *M. musculus* (NP_067283).

A phylogenetic tree for the PIWIL2 protein was constructed based on the deduced amino acid sequences to study the relationship between *C. semilaevis* and other known members of the Piwi-subfamily by the Neighbor-Joining method. The accession numbers in GenBank data libraries are recorded in Table 2. As shown in Fig. 3, the tree branched as two distinct clusters, within the fish and non-fish factors. *C. semilaevis*, along with *Takifugu rubripes*, *O. niloticus*, *Pundamilia nyererei*, *Maylandia zebra* and *Oryzias latipes* were grouped into a fish cluster, while *Danio rerio*, *Oncorhynchus mykiss*, and *Cyprinus carpio* were grouped into another cluster in the phylogenetic tree.

3.3. Gonad histological observations of one-year old C. semilaevis

The gonad ultrathin sections of sexually mature *C. semilaevis* are shown in Fig. 4. In the one-year old ovary, the ovarian cavity was filled with stage III primary oocytes. The oocytes were round and big with a large oil droplet in the middle of the cell. In the testis of normal diploid male, most of the seminiferous lobules were full of spermatozoa, along with a large amount of mature sperm in the sperm duct and seminal vesicle. Almost no spermatids or spermatozoa occurred in seminiferous lobules of the testes of triploid-male.

3.4. Tissues and organs distribution and expression of piwil2 in the different developmental stages of gonads

Using RT-qPCR, the mRNA expression of the *piwil2* gene in different tissues and organs of sexually mature male and female were analyzed. In male, *piwil2* transcripts were highly abundant in testis, weakly expressed in the brain, and negligibly expressed in spleen, liver, heart, intestine, skin, kidney, muscle, gill and blood (Fig. 5). For female, high levels of *piwil2* transcripts were detected in the ovary, with low levels in the liver and brain, and almost no expression in the spleen, heart, intestine, skin, kidney, muscle, gill and blood ($p < 0.05$) (Fig. 5).

To examine the differences in expression levels of *piwil2* in gonads of sexually mature fish, we detected four kinds of gonads from different individuals including male, female, neo-male and triploid-male by RT-qPCR. The RT-qPCR results showed that the relative expression of *piwil2* to *β-actin* in the ovary and testis was significantly different (Fig. 6). The relative expression in the ovary was weaker than that in the testis of the male, neo-male and triploid-male, and there were no significant differences ($p > 0.05$) between male, neo-male and triploid-male.

The relative expression of the *piwil2* gene to *β-actin* at different developmental stages of *C. semilaevis* gonads was also analyzed. From 20 days after hatching to 80 days, an extremely low level of expression was observed in larvae. From 95 days after hatching, the *piwil2* expression quantity increased sharply. Along with development, the expression level was higher at 120 days than 95 days, and an even higher level of transcription was found at 150 day. Moreover, there were obvious gaps between the testis and ovary during the different developmental stages of gonads, especially from 80 days after hatching ($p < 0.05$) (Fig. 7).

3.5. Differences in the degree of DNA methylation

In this study, we analyzed the degree of methylation of CpG and core promoter sites located in the 5′ flanking region of the *piwil2* DNA sequence utilizing the bisulfite sodium sequencing method (bisulfite-sequencing PCR, BSP). As shown in Fig. 8, in female, male and neo-male, fourteen potential methylation loci were studied in the CpG and core promoter region of *piwil2*. With a distribution of 84% (94/112) CpG methylation, the methylation level of female was higher than the 33% (37/112) in male and 63% (71/112) in neo-male. In the female sample, seven loci had 100% CpG methylation, while none of the loci had 100% non-methylation (Fig. 8 A). However, a relatively lower level of

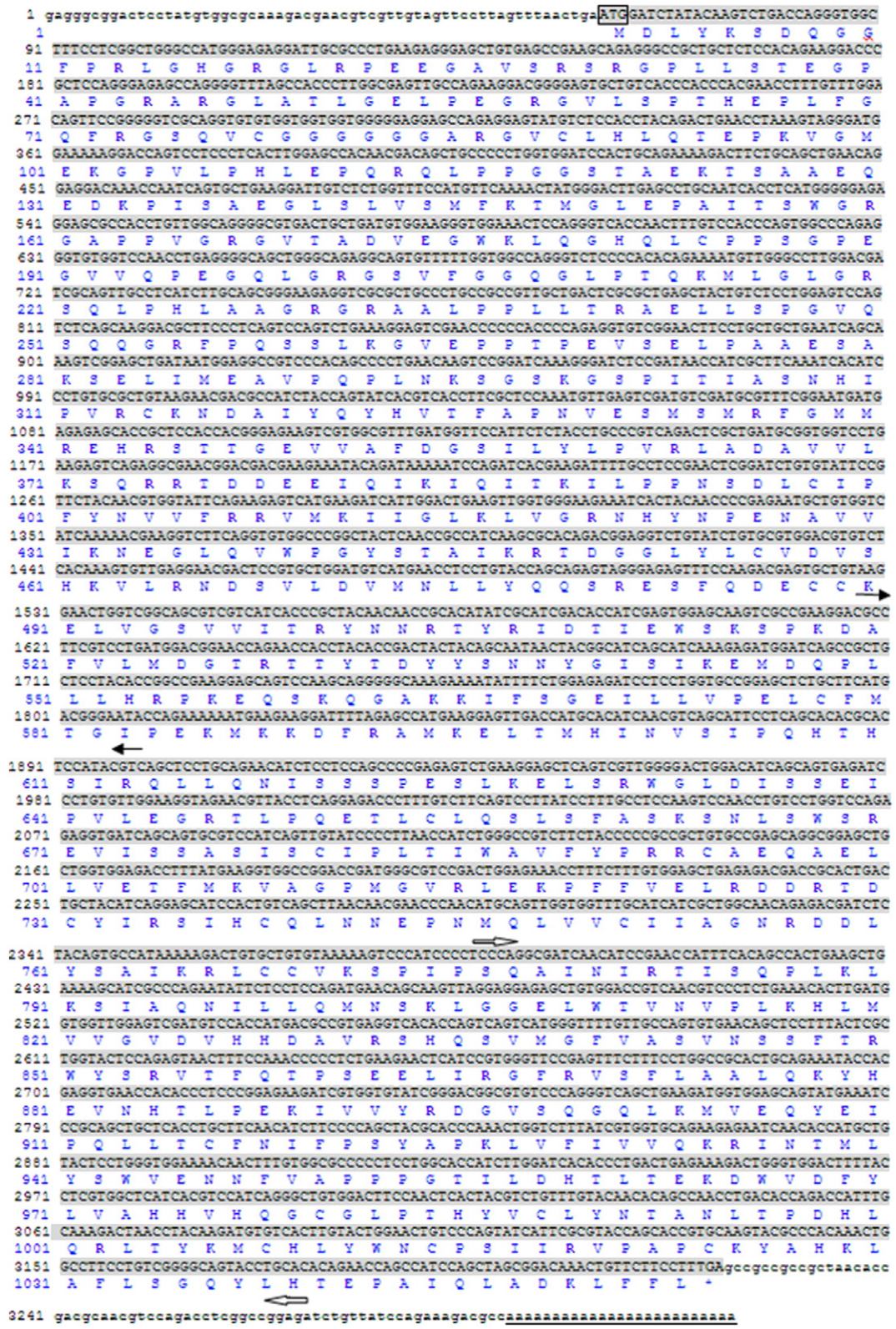

Fig. 2. Full length of nucleotide sequence (above) and deduced amino acid sequence (below) of *piwil2* in *C. semilaevis*. Nucleotides were numbered from the first base at 5′end and numbered to the left of each line. 5′-UTR and 3′-UTR were marked in lowercase, respectively. Black arrows indicated PAZ domain (middle of sequence) and white arrows indicated Piwi domain (C-terminal side). The start codon ATG was boxed. The stop codon was indicated by an asterisk and the poly (A) signal was underlined.

methylation was observed in testis of male and neo-male since male gametes were not methylated at some differentially methylated regions. For male samples, only three loci had 100% CpG methylation, and ten loci had fewer than 12.5% CpG methylation (Fig. 8 B). For neo-male samples, in which only three loci were 100% CpG methylated, the degree of methylation was in between (Fig. 8 C).

Table 2
The sequences used for multiple alignments and the construction of phylogenetic tree were retrieved from GenBank data libraries. Their accession numbers are as follows.

Species name	GenBank accession number
C. semilaevis	KF937805
Pundamilia nyererei	XP_005733624
Oryzias latipes	NP_001153909
Oreochromis niloticus	XP_003445710
Oncorhynchus mykiss	NP_001117714
Mus musculus	NP_067283
Maylandia zebra	XP_004558604
Cyprinus carpio	AEN55535
Takifugu rubripes	XP_003974746
Danio rerio	NP_001073668
Gallus gallus	AEL88238
Xenopus (Silurana) tropicalis	NP_001106470
Homo sapiens	NP_060538

3.6. Chromosomal localization of piwil2

In this study, the target gene, *piwil2*, was positioned in the well Hind III 005 J03 of the *C. semilaevis* BAC library (Shao et al., 2009), and signals were acquired from the chromosome slides of male and female with the aid of a microscope (Fig. 9). FISH analysis for *piwil2* indicated that two pairs of signals were captured on one pair of male chromosome terminals, while only one pair of signals could be found on one chromosome terminal of female. We can therefore confirm that the *piwil2* gene is located on the Z sex chromosome of *C. semilaevis*.

4. Discussion

In this study, a *piwil2* gene of the Piwi-subfamily from *C. semilaevis* was reported for the first time, along with its expression analysis, methylation detection, and chromosomal localization. The ORF of *piwil2* cDNA was 3162 bp, encoding a putative protein consisting of 1053 amino acid residues. The deduced protein analysis showed that the PIWIL2 protein shared a high identity with that of *O. niloticus* and *M. zebra*. Moreover, with two highly conserved structures, the PAZ domain and Piwi domain, the second half of sequences was more conservative than the other region.

In recent years, there have been many studies that have demonstrated that *piwi* homologous genes are expressed in germ cell by regulating transposons and other targets to maintain genome integrity (Leu and Draper, 2010). In *Drosophila*, the *piwi* mutant demonstrated that *piwi* was essential for gametogenesis, and it was a key regulator of germ cell in female (Suzuki et al., 2012). In *O. latipes*, the *piwil2* homologous gene was required not only for determining the number of germ cell, but also for controlling germ cell migration (Li et al., 2012). In human and mouse, the PIWIL2 protein also played an important role in maintenance and differentiation of adult germ cell during gametogenesis (Cox et al., 1998; Kuramochi-Miyagawa et al., 2001).

In sexually mature *C. semilaevis*, we demonstrated that the *piwil2* gene was expressed abundantly in the testes of male and ovaries of female, but there was negligible expression in other tissues or organs ($p < 0.05$). The expression pattern in *C. semilaevis* was consistent with the *piwi* homologues gene in adult zebrafish (Tan et al., 2002), but markedly different from that of homologues genes in mammals. For example, in human and mouse, the *piwi* homologues genes were specifically expressed in testis, rather than in ovary or other tissues (Deng and Lin, 2002; Qiao et al., 2002; Beyret and Lin, 2011). Although expressed exclusively in gonads, the differences of relative expression between testis and ovary were striking. Compared to the female, the *piwil2* expression levels of the male and neo-male were significantly different, which suggests that the expression in male and neo-male was three or four times higher than the expression in female ($p < 0.05$). Surprisingly, the *piwil2* expression of triploid-male was inconsistent with the expression of female, but showed a similar expression with male and neo-male. Although triploid-male could not produce functional sperm, their testes were well developed, and spermatogonia were seen in the seminiferous lobules. These findings indicated that the *piwil2* gene was essential for the development of germ cell, and was

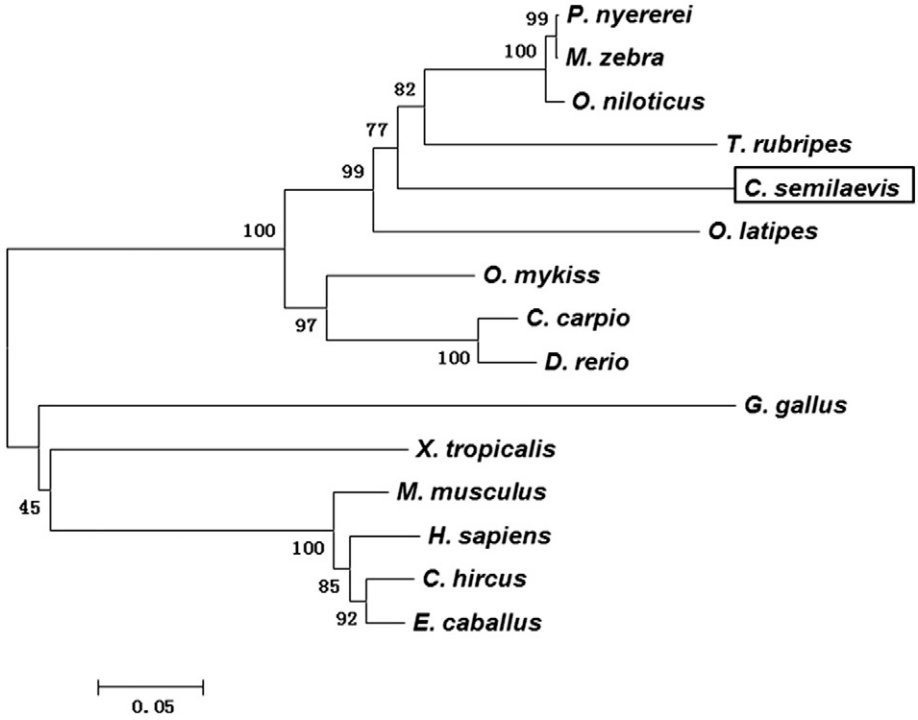

Fig. 3. Phylogenetic analysis of PIWIL2 protein in *C. semilaevis* and other vertebrates. The bootstrap values based on 10,000 resampling replicates. The branch length scale in terms of genetic distance was indicated above the tree. The *piwil2* protein in *C. semilaevis* was boxed.

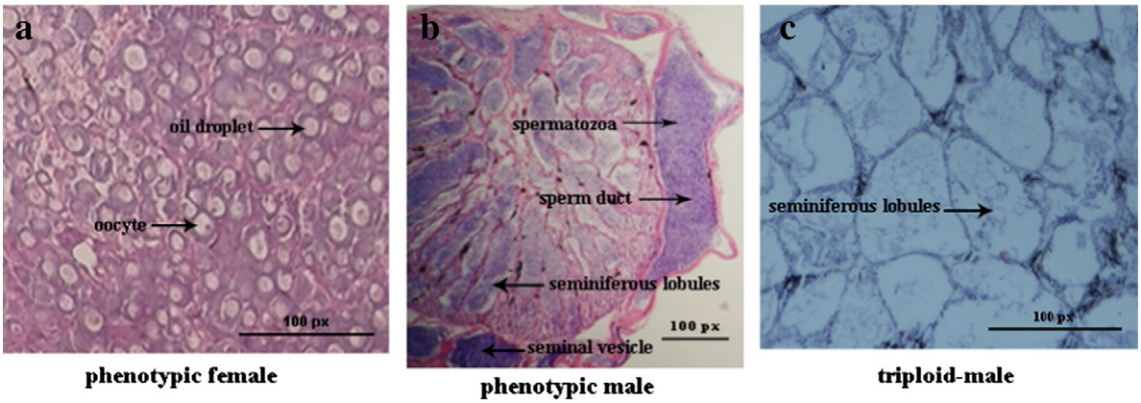

Fig. 4. The gonad ultrathin sections of sexually mature *C. semilaevis*, phenotypic female (a), phenotypic male (b) and testis of triploid-male (c). Neo-male was in accordance with phenotypic male. Fig. 4 b were observed under light microscope equipped with ×40 lens, while Fig. 4 a c was observed under light microscope equipped with ×100 immersion lens, and photographed by NICK camera.

especially important for the development of spermatogonium in the testis during spermatogenesis. However, it was not the abnormal expression of *piwil2* that lead to spermatogenesis disorder of triploid-male in our study, which implied that the *piwil2* gene might begin to function during the early stages of spermatogenesis. The expression of *piwil2* at early developmental stages of *C. semilaevis* gonads revealed that the *piwil2* gene didn't come into effect until day 95 after hatching. In the comparison of female and male, a marked increase appeared in the latter sex from day 95 after hatching, the time when sex differentiation has already finished and the germ cell has started to divide rapidly. But in the gonads of sexually mature *C. semilaevis*, the expression of *piwil2* gene was lower than that in 150 day larvae. In common carp and crucian carp, Zhou et al. found that the expression of *Piwil2* decreased during final mature stage of gonads in ovary (Zhou et al., 2012, 2014). Pan et al. found that estrogen may show a negatively effect on *piwi* expression in final maturation of ovary in mice (Pan et al., 2012). In *C. semilaevis*, the expression level of *Piwil2* also decreased in mature testis and ovary than in testis and ovary at 150 days. While it still needs to be check out that whether the increase of steroids could lead to a decrease of *piwil2* expression in *C. semilaevis*. Combined with our experimental results shown above, we draw the conclusion that the product of the *piwil2* gene was an important component of germ cell proliferation and differentiation at the early stages of spermatogenesis. Although not a candidate gene for sex determination in *C. semilaevis*, the function of *piwil2* in spermatogenesis needs further study.

When occurring in the DNA promoter region, DNA methylation is a key heritable epigenetic modification (Wong et al., 2012; Zhou, 2012). Promoter DNA methylation plays an essential role in the regulation of gene expression and causes phenotypic alteration without altering the DNA sequence (Kirino, 2011; Daugela et al., 2012; Wong et al., 2012). To explore the reason why the mRNA expression of *piwil2* varied among gonads in different sexes of sexually mature *C. semilaevis*, the degree of promoter DNA methylation was detected in male, female, and neo-male respectively. Our results showed clear differences among the three groups, that the degree of methylation in female was higher than that in male and neo-male at most CpG loci, which provided a reasonable explanation to the question above. In other words, these results indicate that the high level of promoter methylation in female may lead to low expression of *piwil2*, while in male and neo-male, the *piwil2* gene is expressed actively as a result of a low degree of promoter DNA methylation.

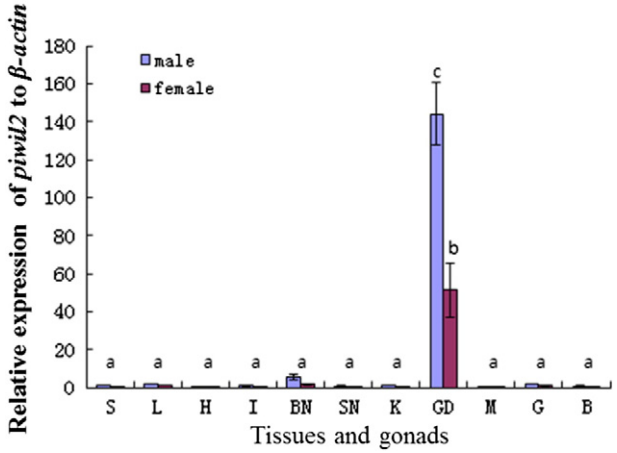

Fig. 5. RT-qPCR analysis of *piwil2* expression in various tissues and organs of sexually mature male and female. S: spleen, L: liver, H: heart, I: intestine, BN: brain, SN: skin, K: kidney, GD: gonad, M: muscle, G: gill, B: blood. The *β-actin* gene was used to calibrate the cDNA template for all of the samples. All data were expressed as mean ± S.E. from three separate individuals (n = 3). Bars with different letters differed at $p < 0.05$, and calculated by one-way ANOVA. *piwil2* mRNA was highly expressed in testis and ovary.

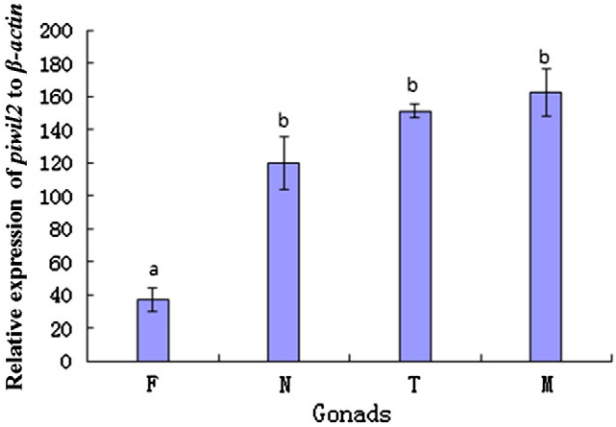

Fig. 6. The relative expression of *piwil2* in gonads among female, neo-male, triploid-male and male. F: female, N: neo-male, T: triploids-male, M: male. The expression amount was normalized to the *β-actin* transcript level. The data were analyzed by Duncan comparison tests using PASW statistics 18.0. All data are expressed as mean ± S.E. from three separate individuals (n = 3). Bars with different letters differed at $p < 0.05$, and calculated by one-way ANOVA. The *piwil2* expression was higher in testis than in ovary.

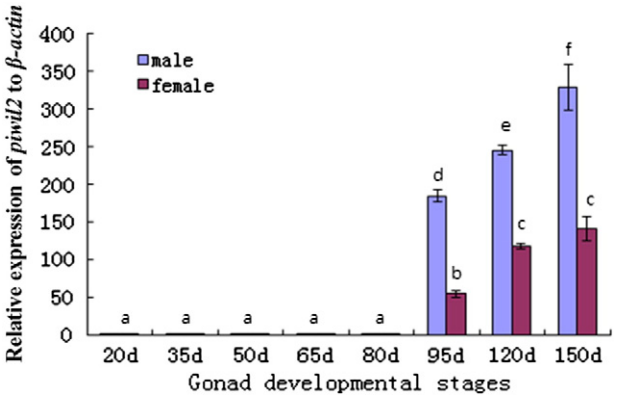

Fig. 7. The expression profile of *piwil2* in gonads at different stages of development. The *β-actin* gene was used to calibrate the cDNA template for all the samples. 20 d: 20 days after hatch, 35 d: 35 days after hatch, 50 d: 50 days after hatch, 65 d: 65 days after hatch, 80 d: 80 days after hatch, 95 d: 95 days after hatch, 120 d: 120 days after hatch, 150 d: 150 days after hatch. The data were analyzed by Duncan comparison tests using PASW statistics 18.0. All data were expressed as mean ± S.E. from three separate individuals ($n = 3$). Bars with different letters differed with statistical significance at $p < 0.05$, and calculated by one-way ANOVA.

The molecular mechanisms and subcellular localization of the Piwi-subfamily protein in spermatogenesis has been widely researched (Cox et al., 2000; Bak et al., 2011; Kim et al., 2012). By contrast, chromosomal location of the *piwi*-related gene is poorly studied. To locate the *piwil2* gene on *C. semilaevis* chromosomes, FISH was used to analyze the expression pattern of the *piwil2* gene in sexually mature female and male. In vertebrates, there are two kinds of genetic sex-determining system, the XX/XY-type and the ZZ/ZW-type (Yoshimoto and Ito, 2011). The sex-determining system of *C. semilaevis* is a typical ZZ/ZW-type. The homogametic sex is genetic male (ZZ) and the heterogametic sex is genetic female (ZW), as in birds (Smith et al., 2009). From the results mentioned above, it can be seen that there were two pairs of signals captured in male, while only one pair in female. By combining the karyotype analysis of the ZZ/ZW-type with the results of FISH, we conclude that the *piwil2* is a Z-linked gene and located on the Z sex chromosome. Since genes located in sex chromosomes are often expressed in some physiological processes involved in reproduction, *piwil2* might play an important role in spermatogenesis of phenotypic male *C. semilaevis*. This is why the *piwil2* transcript was abundant in testis rather than in other tissues or organs.

In summary, we cloned a *piwi* homologous gene *piwil2* from *C. semilaevis*. The expression of *piwil2* in various tissues and organs, mature gonads, as well as developmental stages of larval gonads was

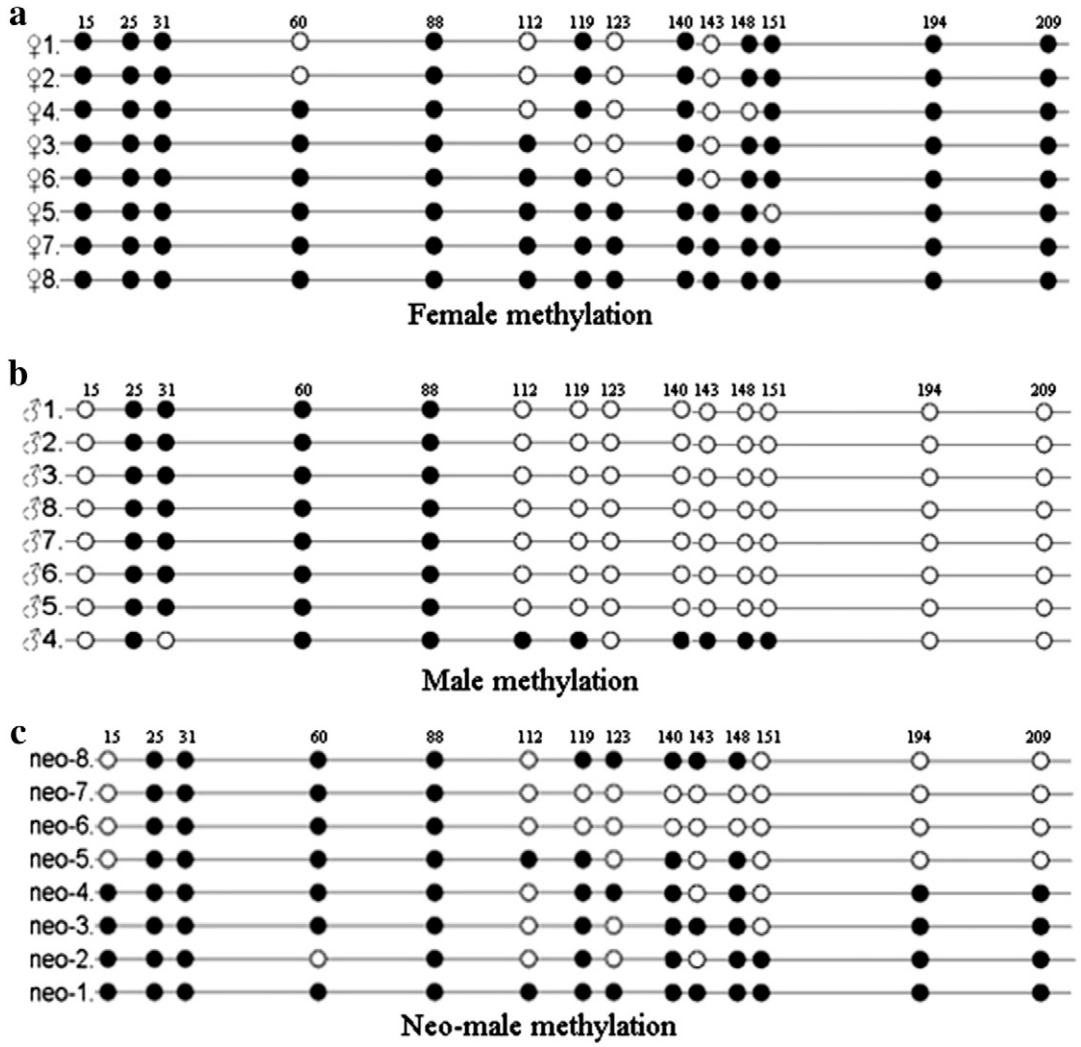

Fig. 8. The methylation profiles of *piwil2* in gonads of one-year old *C. semilaevis* female (A), male (B) and neo-male (C). The data were analyzed by BiQ-Analyzer. Numbers 15–209 correspond to potential CpG methylation sites. Filled (black) circles correspond to methylated Cs, and unfilled (white) circles correspond to unmethylated Cs. The methylation levels of *piwil2* were higher in female than in male and neo-male.

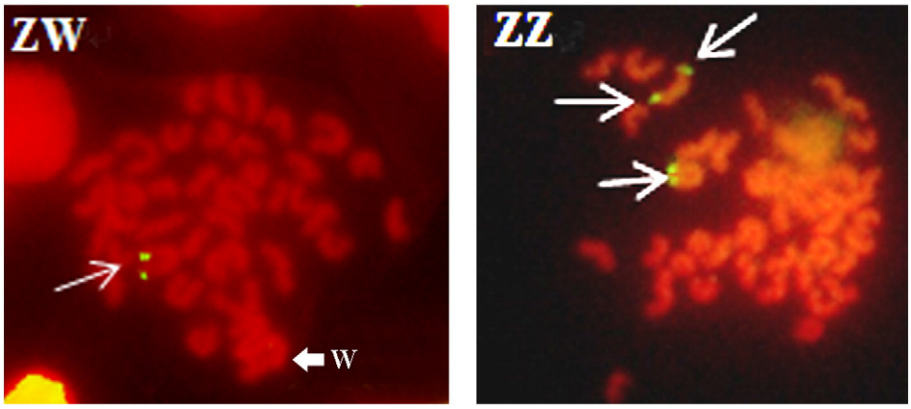

Fig. 9. Chromosomal localization of the *piwil2* gene in *C. semilaevis* (ZW, ZZ). White arrows indicated the hybridization signals, and bold arrows indicated W chromosome. Pairs of signals were located in the terminals of chromosome arms. Two pairs of signals were captured in male, while one pair in female.

detected by RT-qPCR. The degree of CpG methylation in the promoter region was tested using the bisulfite sodium sequencing method, and the results indicated that the highest methylation level was detected in female, and lowest in male. After FISH, conducted by the DIG-dUTP method, we identified the chromosome where the *piwil2* gene was located. All these results imply that *piwil2* has a vitally important function in gametogenesis of phenotypic male. However, further studies are needed to elaborate the precise role and mechanism of the *piwil2* gene in *C. semilaevis* during spermatogenesis.

Acknowledgments

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