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ORIGINAL ARTICLE

# Discovery of *Nanos1* and *Nanos2/3* as Germ Cell Markers During Scallop Gonadal Development

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## Abstract

*Nanos* are conserved genes involved in germline cell specification and differentiation. However, little is known about the role of different members of *Nanos* family in germ cell development in mollusks. In the present study, we conducted genome-wide identification of *Nanos* family in an economically important scallop *Patinopecten yessoensis*, and detected their expression in adult tissues and during early development. Two *Nanos* genes (*PyNanos1*, *PyNanos2/3*) were identified, both of which have the N-terminal NOT1-interacting motif and C-terminal $(CCHC)_2$ zinc finger domain. Expression profiles showed that *PyNanos1* and *PyNanos2/3* were primarily expressed in the gonads, with *PyNanos1* being localized in the oogonia, oocytes, and spermatogonia, while *PyNanos2/3* being specifically in spermatogonia. The results suggest that *PyNanos* are germ cell specific and may play crucial roles in gametogenesis in the scallop. *PyNanos1* is a maternal gene, which is distributed uniformly at early cleavage, and restricted to 2–3 cell clusters from blastulae to trochophore larvae, suggesting its potential role in the formation of PGCs. Zygotically expressed *PyNanos2/3* displayed a similar signal with *PyNanos1* in the trochophore larvae, suggesting it may also participate in the formation and/or maintenance of PGCs. This study will benefit germplasm exploitation and conservation in bivalves, and facilitate a better understanding of the evolution of *Nanos* family and the role of different *Nanos* in germ cell development in mollusks.

**Keywords** *Nanos* · Gametogenesis · Primordial germ cells · *Patinopecten yessoensis*

## Introduction

As a central component of sexual reproduction in animals, germ cells are responsible for transmitting genetic and epigenetic information across generations (Zeng et al. 2015). Germ cell lineages start with primordial germ cells (PGCs), which differentiate from somatic cells early in embryogenesis, specified either by maternally inherited determinants (preformation) or by inductive signals (epigenesis) (Extavour and Akam 2003; Extavour 2007). PGCs further differentiate into spermatogonia and oogonia, and undergo meiosis to generate sex-specific gametes (i.e., spermatozoa and oocytes) in the adult gonads. In recent years, germ cell markers, such as Nanos, Vasa, and Piwi, are widely used for studying the germ cell fate in different classes of animals (Ewen-Campen et al. 2010).

The *Nanos* genes, encoding CCHC zinc finger proteins that likely function as transcriptional and translational repressors, are especially known for their preserved role in germ cell development in diverse organisms (Reitzel et al. 2016; Wang and Lin 2004). *Nanos* homologs widely exist in vertebrates and invertebrates, with the gene number varying from one to four (De Keuckelaere et al. 2018). For example, the fruit fly and jellyfish have one *Nanos* gene, sea anemone and polyp have two, human and zebrafish have three, and silkworm and fugu have four. Although the *Nanos* gene family is required for germ cell development, previous researches have demonstrated that the paralogs often have

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specific expression patterns and exert distinct functions. For example, mouse *Nanos2* is found in male gonocytes and plays pivotal roles in the sexual differentiation of male germ cells (Saba et al. 2014; Suzuki and Saga 2008). Conditional ablation of postnatal *Nanos2* results in the depletion of spermatogonial stem cells and the progressive defect in spermatogenesis (Sada et al. 2009). *Nanos3* is found in migrating PGCs in mice, and the elimination of this gene results in the complete loss of germ cells in both sexes (Tsuda et al. 2003). Although mouse *Nanos1* is predominantly expressed in the central nervous system, it is in substantial amounts in oocytes (Haraguchi et al. 2003). Therefore, systematic investigation on the *Nanos* gene family facilitates a more comprehensive understanding of the function of *Nanos* genes in different types of germ cells.

Mollusks are the second largest phylum behind arthropods, including cephalopods, gastropods, and bivalves. Many mollusks, such as oysters, scallops, and abalones, are important aquaculture species and still have substantial potential for genetic improvement (Gjedrem and Rye 2018). The germplasm exploitation and conservation of aquatic mollusks required detailed information on the development of germline cells. Till now, a single member of *Nanos* has been reported in two gastropods, *Ilyanassa obsoleta* (Rabinowitz et al. 2008) and *Haliotis asinina* (Kranz et al. 2010), and one bivalve, *Crassostrea gigas* (Xu et al. 2018), which suggest involvement of *Nanos* in PGCs development or oocyte maturation. However, it remains unclear whether different Nanos members exist in mollusks, and what functional differences are between them.

Yesso scallop *Patinopecten yessoensis* is a gonochoristic mollusk with important economic value, mainly distributed along the far eastern Asian coast. According to the statistics from the Food and Agriculture Organization of the United Nations (FAO), the total value of annual global production has exceeded USD 1.5 billion since 2000. The sustainability of aquaculture requires a comprehensive understanding of the molecular mechanisms of germ cell development, which will facilitate the culture and manipulation of germ cells (de Siqueira-Silva et al. 2018; Rivers et al. 2020). Previous studies on the reproduction of *P. yessoensis* generally focus on gametogenesis and sexual differentiation (Li et al. 2018; Osada et al. 2003; Zhang et al. 2020); therefore, our knowledge on the germ cell development is still fragmentary. In present study, we conducted genome-wide identification of *Nanos* gene family and explored their expression patterns in adult tissues and early development. It will contribute to our understanding of the evolution of *Nanos* family and their roles in germ cell development.

## Materials and Methods

### Sample Collection

All scallop samples were collected from the Yantai Marine Seeds Corporation (Shandong Province, China). To obtain embryonic and larval materials, artificial fertilization and larval culture were performed according to the procedure described by Wang and Wang (2008). Zygotes, 2–8 cells, blastulae, gastrulae, and trochophore larvae were collected as described by Wang et al. (2017). The ovaries and testes were dissected from the healthy female and male scallops. Parts of the samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. The other parts were fixed overnight at 4 °C with 4% paraformaldehyde (PFA) followed by washing twice with phosphate-buffered saline (PBS). The fixed samples were then dehydrated with serial methanol (25%, 50%, 75%, and 100%) diluted in 0.01 M PBS, and stored at −20 °C for in situ hybridization (ISH).

### Identification and Phylogenetic Analysis of *Nanos* Genes

Protein sequences of different Nanos from Vertebrata, Chordata, Arthropoda, Lophotrochozoa, and Cnidaria were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) and UniProt (https://www.uniprot.org).

To identify potential *Nanos* genes in Yesso scallop, these Nanos proteins were searched against the *P. yessoensis* transcriptomes and genome (Li et al. 2019; Wang et al. 2017) using tBLASTn, with an e-value of 1e-5. The N-terminal NOT1-interacting motif (NIM) and the C-terminal $(CCHC)_2$ zinc finger domain were predicted using NCBI CDD Tools (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The protein conservation within NIM and the sequence logo were displayed using Jalview (Waterhouse et al. 2009) and WebLogo3 (http://weblogo.threeplusone.com/create.cgi), respectively.

To confirm the identity of the two *PyNanos*, the conserved $(CCHC)_2$ zinc finger domains were retrieved from 39 Nanos proteins, and multiple sequence alignments were performed using ClustalW (Larkin et al. 2007). The phylogenetic tree was constructed by the Neighbor Joining (NJ) method using MEGA 7.0 (Kumar et al. 2016). The genetic distance was calculated by the p-distance method, and the bootstrapping value was set to 1000.

**Table 1** Sequences of primers used for RT-qPCR

| Gene | Primer sequences (5′-3′) | Amplicon length (bp) | Amplification efficiency (%) |
|---|---|---|---|
| *PyNanos1* | F: TGCGGAAATACACCTGTCCC | 145 | 97.63 |
| | R: TTACAGGTGCTGCTACGGAC | | |
| *PyNanos2/3* | F: CATGTGCTCAAGGACACCCA | 141 | 96.54 |
| | R: AGACATTGACCCTAGCCCCT | | |
| *EF1-α* | F: CCATCTGCTCTGACAACTGA | 196 | 102 |
| | R: GGACAATAACCTGAGCCATAA | | |

## RNA Extraction and cDNA Synthesis

Total RNA was isolated from adult gonads and embryos/larvae using the conventional guanidinium isothiocyanate method. DNA contamination was removed by digestion with DNase I (TaKaRa, Shiga, Japan). RNA quality was determined by Nanovue Plus spectrophotometer (GE Healthcare, Piscataway, USA) and agarose gel electrophoresis. First-strand cDNA was synthesized using oligo$(dT)_{18}$ and MMLV reverse transcriptase (TaKaRa, Shiga, Japan). The cDNA products were stored at − 20 °C and used as templates for quantitative PCR (qPCR) and ISH.

## *PyNanos* Gene Expression Analysis

The expression patterns of *Nanos* genes in adult tissues were obtained by analyzing the transcriptome data we generated previously (NCBI Bioproject ID: PRJNA259405) (Li et al. 2016; Wang et al. 2017). For each tissue, there are 3 biological replicates. The raw reads were filtered to obtain high-quality data using a homemade Perl script. The high-quality reads were then mapped to the *P. yessoensis* genome using the STAR software. For each gene, the raw counts were obtained by HTseq, and transformed to transcripts per million (TPM) values using the formula provided by Wagner et al. (2012). The expression level of *PyNanos* genes was quantified using the TPM.

The expression of *Nanos* genes in the embryos/larvae was assayed by qPCR. Gene-specific primers were designed and listed in Table 1. Amplification efficiency of each primer pair was calculated according to the standard curve generated from a two-fold dilution series. The PCR was performed on a Light Cycler 480 Real-time PCR System (Roche Diagnostics, Mannheim, Germany) using the following program: 95 °C for 30 s, and 40 cycles of 95 °C for 10 s and 60 °C for 30 s. There were 3 biological replicates for each stage, and all reactions were conducted in triplicate. Elongation factor 1-α (*EF1-α*) was used as a reference gene based on our previous study (Li et al. 2019). Relative expression levels of the *Nanos* gene were calculated using the $2^{-\Delta\Delta CT}$ method.

To compare the differences in gene expression level among tissues or early developmental stages, one-way ANOVA followed by Turkey test was applied using SPSS. *P* values less than 0.05 were considered statistically significant.

## RNA Probe Synthesis

The probes for ISH were obtained according to the following procedures. The cDNA products of adult gonads were used as templates for *Nanos* gene amplification. Gene-specific primers F and R (Table 2) were designed using Primer Premier v.5.0 to amplify the fragments of *PyNanos* genes. The PCR products were cloned, and confirmed by sequencing. To obtain the templates for in vitro transcription, a second-round PCR was performed using the F-Sp6 and R-T7 primers (Table 2). Finally, the sense and anti-sense RNA

**Table 2** Sequences of primers used for in situ hybridization

| Gene | Primer sequences (5′-3′)* |
|---|---|
| *PyNanos1* | F: AGGCAATAGTTTCGCACCG |
| | R: TGCGCCGTTTCAGGATTA |
| | F-Sp6: ATTTAGGTGACACTATAGAGGCAATAGTTTCGCACCG |
| | R-T7: TAATACGACTCACTATAGGGTGCGCCGTTTCAGGATTA |
| *PyNanos2/3* | F: ATGACGCGGATGGTGAAA |
| | R: CTCGCACAAGATATTTGAAGGA |
| | F-Sp6: ATTTAGGTGACACTATAGATGACGCGGATGGTGAAA |
| | R-T7: TAATACGACTCACTATAGGGCTCGCACAAGATATTTGAAGGA |

*SP6/T7 promoter sequences were underlined

probes were synthesized using DIG RNA labeling mixture (Roche, Mannheim, Germany) and T7/SP6 RNA polymerase (Thermo Fisher Scientific, Waltham, USA) according the manufacturers' instructions.

### In Situ Hybridization

To conduct ISH on gonads, 3 and 5 µm thick paraffin sections were prepared for testes and ovaries, respectively. The sections were then affixed to the polylysine coated glass slides. The following procedures were similar for ISH on gonads and embryos/larvae. Specifically, the samples were rehydrated in a descending series of ethanol solutions and PBST (PBS plus 0.1% Tween-20), followed by digestion with 2 µg/mL proteinase K at 37 °C for 10–20 min. After pre-hybridization in hybridization buffer at 60–65 °C for 3 h, the samples were hybridized with 1 µg/mL RNA probes at 60–65 °C overnight. Then the probes were washed away, and the samples were incubated in blocking buffer (PBST and 0.5% blocking reagent) for 1 h and with 1:2000 ~ 3000 anti-digoxigenin antibody (Roche, Mannheim, Germany) at 4 °C for 16 h. After extensive washing with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-20, pH = 7.5), the samples were incubated with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate solution (Roche, Mannheim, Germany). An extra counterstaining procedure is performed with 1% neutral red solution for ISH on gonads.

## Results

### Identification and Phylogenetic Analysis of *Nanos* Genes

After searching against the Yesso scallop genome, we identified two *Nanos* genes, named *PyNanos1* and *PyNanos2/3*, with the NCBI Accession No. of OWF49530.1 and OWF55054.1. Gene structure analysis showed that both genes were intron-free, with the gene length of 705 and 888 bp, encoding 234 and 295 aa (Fig. 1). The predicted molecular weights of PyNanos1 and PyNanos2/3 were 26.41 and 33.27 kDa, respectively.

Like Nanos from other organisms, PyNanos1 and PyNanos2/3 have a NIM at the N terminus and a typical $(CCHC)_2$ zinc finger domain at the C terminus. The 18-residue NIMs of PyNanos are conserved in some sites, including invariant D7, Y8, L11, and two highly conserved aromatic residues Y2 and F5, which are identical with the Nanos from sea squirt (Fig. 1). The predicted $(CCHC)_2$ zinc finger domains of PyNanos1 and PyNanos2/3 are 53 bp in length, with two conserved CCHC motifs that exist in all the Nanos we examined (Fig. 2).

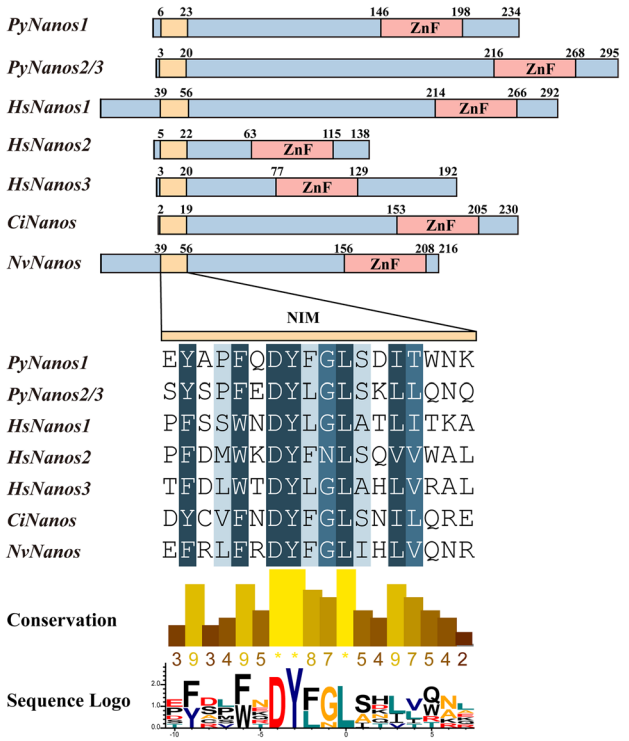


**Fig. 1** The structure of the Nanos proteins from *P. yessoensis* and other three species. All the Nanos proteins have two motifs: N-terminal NOT1-interacting motif (NIM) (yellow) and C-terminal $(CCHC)_2$ zinc finger domain (pink). The numbers indicate the position of the domains and the protein length. The NIMs are aligned, and the conserved residues are highlighted in blue. The species abbreviations are as follows: *Patinopecten yessoensis* (Py), *Homo sapiens* (Hs), *Ciona intestinalis* (Ci), and *Nematostella vectensis* (Nv)

To confirm the identity of the two *PyNanos*, the phylogenetic tree was constructed based on the $(CCHC)_2$ zinc finger domains. As shown in Fig. 2, the *Nanos* genes were clustered into two independent clades (*Nanos1* and *Nanos2/3*). Both *Nanos1* and *Nanos2/3* are present in mollusks, arthropods and vertebrates, whereas cnidarians only have *Nanos1*. It suggests gene duplication occurred independently in the vertebrates and cnidarians, with two copies of *Nanos2/3* (*Nanos2* and *Nanos3*) in the investigated vertebrates and two copies of *Nanos1* in cnidarians. As expected, the two *PyNanos* were dispersed in the *Nanos1* and *Nanos2/3* clades, each clustered with the corresponding *Nanos* of other mollusks.

### Expression of *PyNanos* in Adult Tissues

To screen for germ cell-specific *PyNanos* genes, we analyzed the expression of *PyNanos1* and *PyNanos2/3* in 11 adult tissues, including foot, smooth muscle, striated muscle, eye, mantle, gill, hepatopancreas, kidney, hemocytes, ovary, and testis. Results showed that both genes were

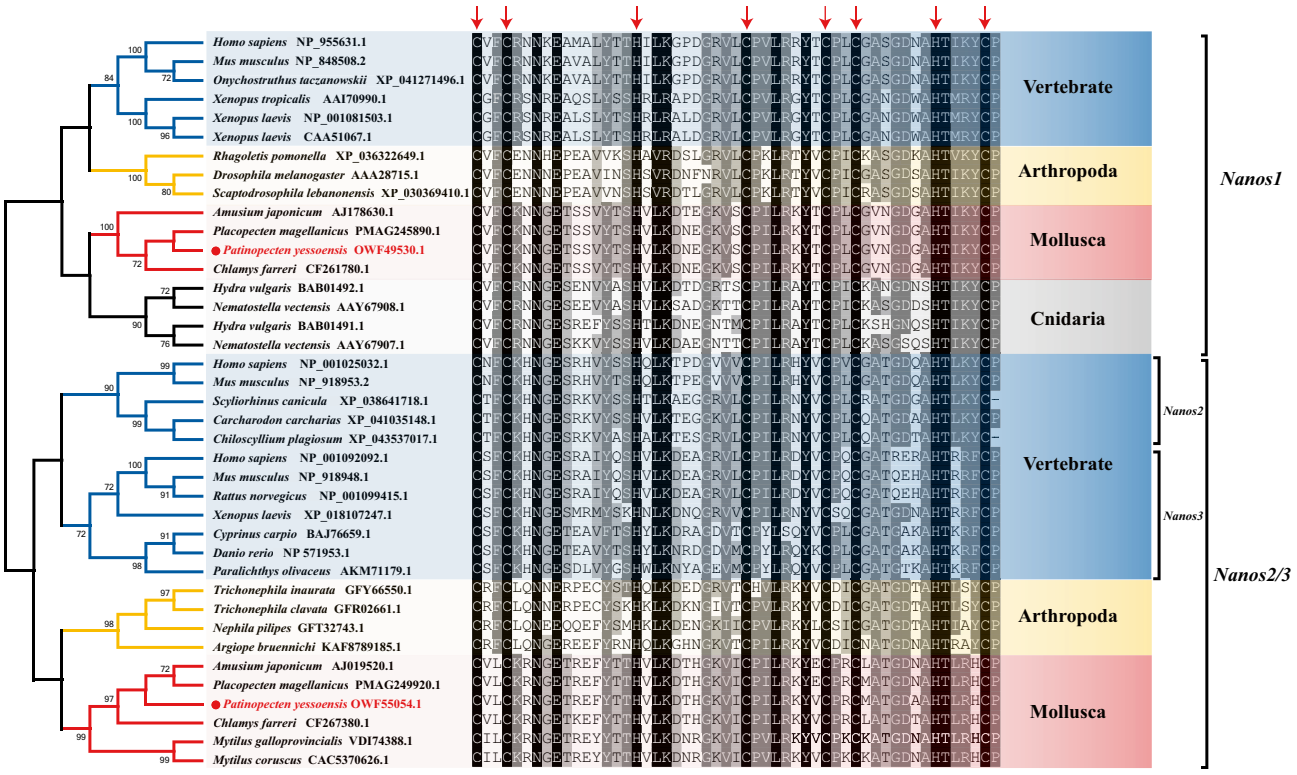


**Fig. 2** Phylogenetic analysis of *Nanos* genes and the multiple alignment of zinc finger domain. The phylogenetic tree was built using the NJ method in MEGA 7.0 software. Nanos proteins of *P. yessoensis* were labeled with red circles. Identical residues in the domain are represented in black and similar residues in gray. The conserved cysteine and histidine residues are indicated with red arrows

specifically expressed in the gonad, and the expression was significantly higher in the ovary than the testis. To be specific, the expression level of *PyNanos1* and *PyNanos2/3* is 30.6- and 0.6-fold higher in the ovary than the testis, respectively (Fig. 3A, B).

Considering that both *PyNanos1* and *PyNanos2/3* were detected in the ovary and testis, we conducted ISH to examine the localization of *PyNanos* in the two tissues. As can be seen, the signal of *PyNanos1* transcripts was strong in the cytoplasm of oogonia and oocytes. In the testis, the signal was primarily detected in the spermatogonia, and no obvious signal was found in the spermatocytes, spermatids, or spermatozoa (Fig. 3C). For *PyNanos2/3*, the anti-sense probe was specifically detected in the spermatogonia, and no obvious signal was detected in the ovary (Fig. 3D). No signal was detected in the ovary or testis using the sense probes. The above results confirmed that both *PyNanos1* and *PyNanos2/3* are germ cell-specific genes.

## Spatiotemporal Expression of *PyNanos* During the Early Development

To investigate the potential role of two *PyNanos* in germ cell development, we analyzed their dynamic expression in five developmental stages including zygotes, 2–8 cells, blastulae, gastrulae, and trochophore larvae. According to the results, *PyNanos1* and *PyNanos2/3* displayed quite different expression patterns during early development. *PyNanos1* showed the highest level in the zygotes, and remained strong in 2–8 cells. The expression level sharply decreased in the subsequent stages (Fig. 4A). In contrast to *PyNanos1*, a smaller expressional variation was found for *PyNanos2/3*. The expression of *PyNanos2/3* reached peaked in gastrulae, significantly higher than the other developmental stages (Fig. 4B).

By using whole-mount ISH, we further determined the localization of *PyNanos1* and *PyNanos2/3* in the five developmental stages (Fig. 4C). Generally, *PyNanos1* can be detected in all five stages, and *PyNanos2/3* was

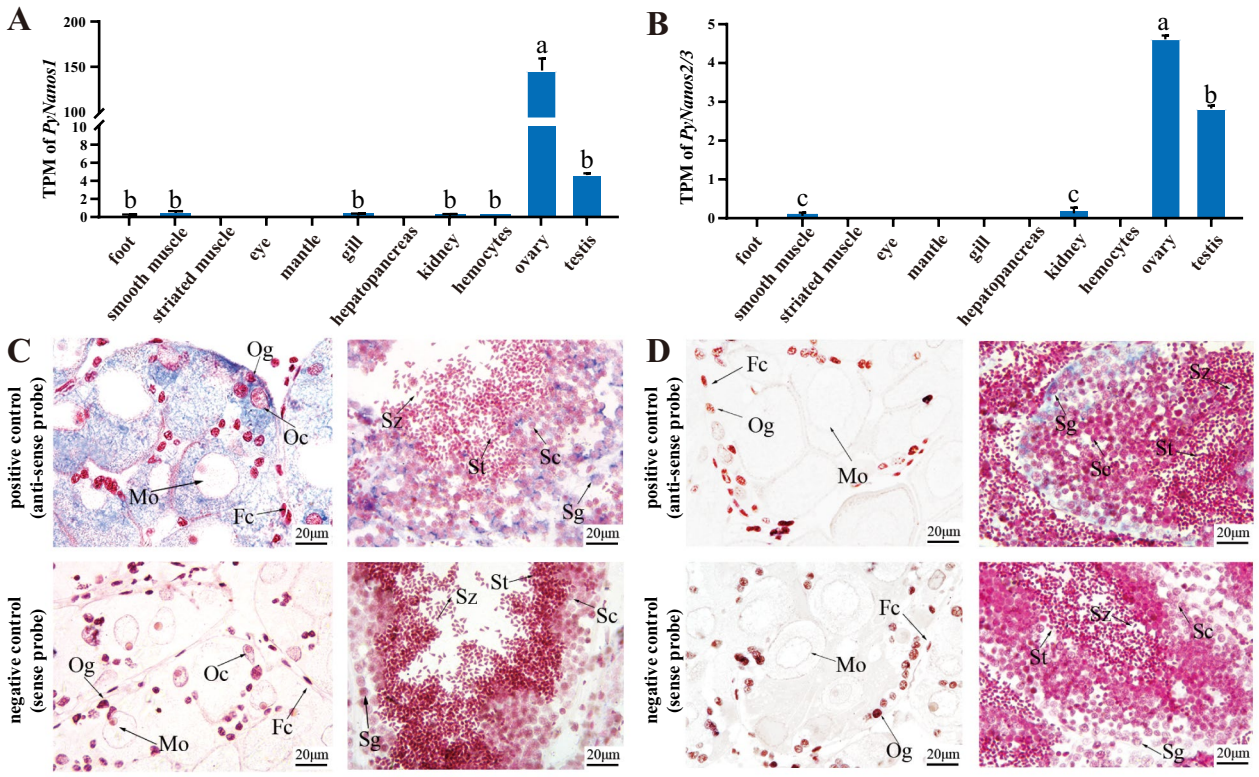


**Fig. 3** Expression profile of the *PyNanos* genes in the adult tissues. **A**, **B** The relative expression of *PyNanos1* and *PyNanos2/3* in eleven tissues. The vertical bars represent the mean ± SEM ($N = 3$). *P*-values were calculated by one-way ANOVA followed by Turkey test. Different letters indicate significant differences ($P < 0.05$). Localization of *PyNanos1* (**C**) and *PyNanos2/3* (**D**) in the ovary and testis by in situ hybridization. Positive signals with an antisense probe are indicated in blue. The red color was given by staining with neutral red as the background. Og, oogonium; Mo, mature oocyte; Sg, spermatogonium; Sc, spermatocyte; St, spermatid; Sz, spermatozoon; Fc, follicle cell

primarily detected in the latter three stages. *PyNanos1* seems uniformly distributed in all cells in the zygotes and 2–8 cells. The signals became segregated in two cell clusters in blastulae, and in three clusters in gastrulae and trochophore larvae. *PyNanos2/3* displayed a diffuse distribution in blastulae and gastrulae, but restricted to two clusters in the trochophore larvae.

## Discussion

In the present study, we found single copy of *Nanos1* and *Nanos2/3* in the genome of *P. yessoensis*. The types and number of *Nanos* members are identical to other mollusks, such as *Placopecten magellanicus*, *Chlamys farreri*, and *Amusium japonicum*. This suggests that no obvious duplication or deletion events occurred during the evolution of *Nanos* genes in Mollusca. Cnidarians also have two *Nanos* genes, but both of them are *Nanos1*, indicating gene duplication may have occurred before the divergence of *Hydra vulgaris* and *Nematostella vectensis*. In contrast to mollusks and cnidarians, the number of *Nanos* members varies a lot in vertebrates and arthropods. For example, most vertebrates have three *Nanos* genes (*Nanos1*, *Nanos2*, and *Nanos3*), indicating gene duplication has occurred for *Nanos2/3*. However, some reptiles and birds have 1–2 *Nanos* (De Keuckelaere et al. 2018), suggesting the occurrence of gene deletion in these organisms. In the arthropods, four *Nanos* orthologues were identified in the silkworm *Bombyx mori*, whereas only one in *Drosophila* (Nakao et al. 2008), suggesting *Nanos* gene has undergone duplications in some arthropods. Together with our previous findings of the *P. yessoensis* genome having outstanding preservation of ancestral karyotype and developmental control (Wang et al. 2017), we speculate that the two *PyNanos* may have preserved more features of the ancestral *Nanos* gene copies than *Nanos* from other phylum.

Nanos are RNA-binding proteins that play vital roles in germ cell development, in which two domains are critically important (De Keuckelaere et al. 2018). One is the evolutionarily conserved C-terminal zinc finger motif $(CCHC)_2$, which is present in all the Nanos proteins we investigated (Fig. 2).

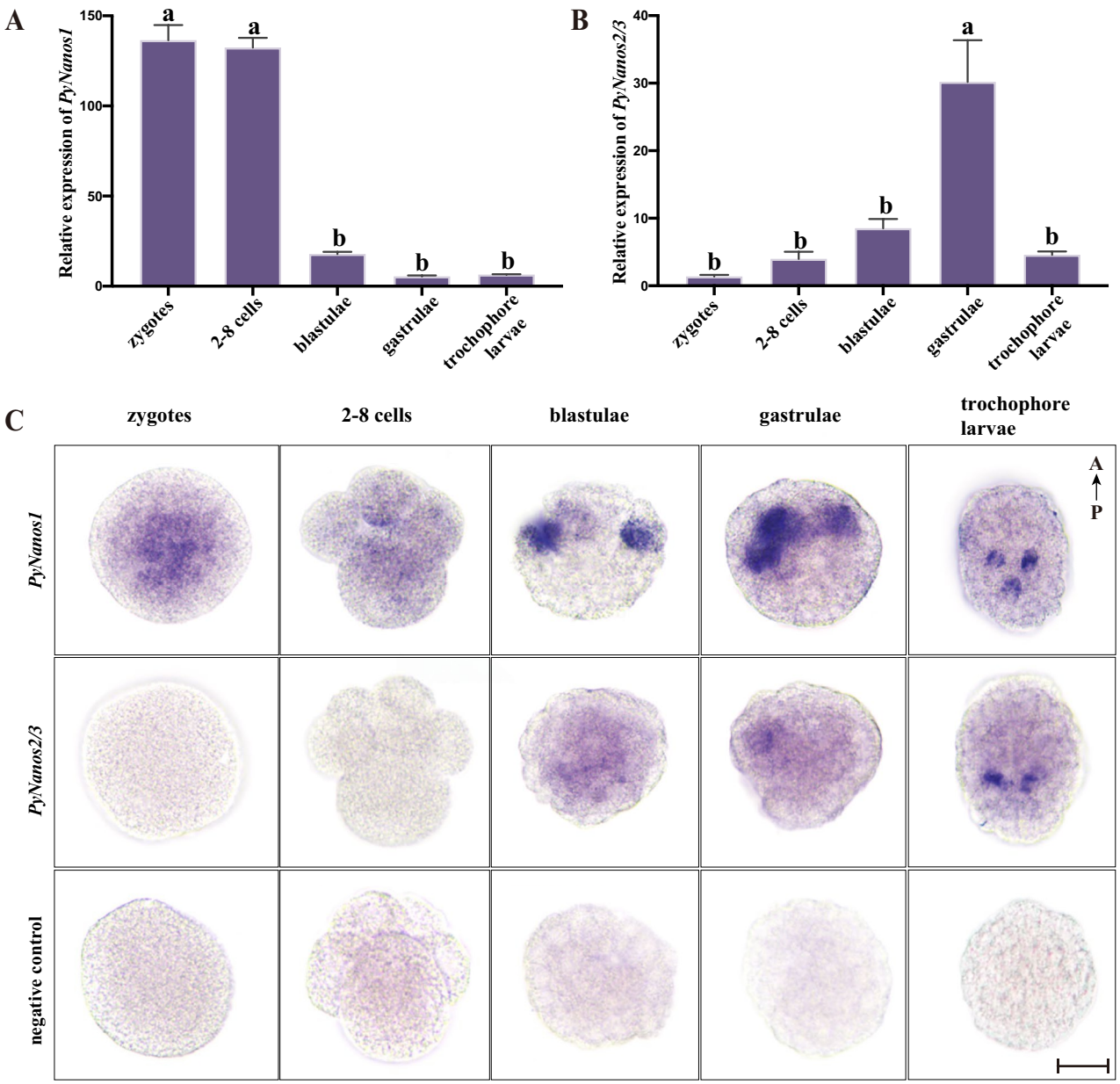


**Fig. 4** Expression of *PyNanos* during early developmental stages. **A**, **B** The relative expression level of *PyNanos1* (**A**) and *PyNanos2/3* (**B**) in zygotes, 2–8 cells, blastulae, gastrulae, and trochophore larvae. The vertical bars represent the mean ± SEM ($N = 3$). Different letters indicate significant differences ($P < 0.05$). **C** Whole-mount ISH detection of *PyNanos1* (upper row) and *PyNanos2/3* (lower row) in the embryos and larvae. The trochophore larvae are in ventral view, with anterior faces up. Scale bars represent 20 μm

This domain mediates binding to RNA and to a conserved Nanos partner Pumilio that confers mRNA target specificity (Bhandari et al. 2014; De Keuckelaere et al. 2018). The other domain is the N-terminal NOT1-interacting motif. Its interaction with the CCR4-NOT deadenylase complex is essential for Nanos-mediated translational repression and mRNA degradation (Bhandari et al. 2014; De Keuckelaere et al. 2018). Both PyNanos1 and PyNanos2/3 have the two domains and the corresponding conserved sites, suggesting they may participate in germ cell development in the way similar to the Nanos from model organisms.

The germ cell-specific expression of *PyNanos* was confirmed by the RNA-seq data of adult tissues and the ISH results of *PyNanos* in the ovary and testis. According to the results, *PyNanos1* is primarily expressed in the oogonia and oocytes. This expression pattern is consistent with *Cg-Nanos-like* from Pacific oyster *C. gigas* (Xu et al. 2018). Although transcriptome analyses revealed that *PyNanos2/3*

was specifically but lowly expressed in the gonads, no positive signal was observed in the ovary, suggesting the expression of *PyNanos2/3* may be too weak to be detected by ISH. Considering the role of Nanos in oocyte development has been demonstrated in adult zebrafish (Draper et al. 2007) and sea urchin (Zhang et al. 2019), we assume the involvement of *Nanos* in the development of female germline cell may be conserved in some bilaterians. In addition to the ovary, both *PyNanos1* and *PyNanos2/3* were detected in the spermatogonia but not in spermatocytes, spermatids, or spermatozoa. This is also consistent with the expression pattern of *Cg-Nanos-like* (Xu et al. 2018). Actually, *Nanos2* and *Nanos3* have been demonstrated to play pivotal roles in the sexual differentiation of male germ cells and maintaining the undifferentiated spermatogonia population in mice, respectively (Lolicato et al. 2008; Saba et al. 2014). The similar expression pattern of *PyNanos1* and *PyNanos2/3* in the scallop testis suggests these two genes may participate in the development of male germline cells as mouse *Nanos2* and *Nanos3*, but the functional difference between them remains to be investigated.

Maternally expressed *Nanos* genes are widely used for the study of germline specification. In the present study, the maternally inherited *PyNanos1* was distributed uniformly at early cleavage, and then accumulated to 2–3 cell clusters from blastulae to trochophore larvae. Similarly in the vetigastropod *Haliotis asinina*, *Nanos* was localized in all micromeres during the first four cleavages and became restricted to 2–3 cell clumps from gastrulae to trochophore larvae (Kranz et al. 2010). In the oyster, *Cg-Nanos-like* was also ubiquitously expressed in early embryos, and the expression was restricted to two cell clumps from gastrulae to umbo-larvae (Xu et al. 2018). According to these studies, the maternally supplied *Nanos* genes are not asymmetrically inherited during early cleavages, indicating PGCs may not be specified exclusively by maternally inherited determinants (preformation) in these organisms. However, the accumulation of *Nanos* expression in specific cells seems to be conserved among mollusks. The specific localization of two cell clusters was also observed for *Vasa* gene in *C. gigas* (Fabioux et al. 2004), corresponding to the two cell clumps observed for *Cg-Nanos-like*. This suggests the cell clusters could be putative PGCs or precursor cells for PGCs. Therefore, we postulate that *PyNanos1* might be involved in the formation of PGCs.

Unlike *PyNanos1*, *PyNanos2/3* is not a maternal gene. It can only be detected in the spermatogonia. Interestingly, its orthologs *Nanos2* and *Nanos3* have been reported to participate in the maintenance of spermatogonia in mice. *Nanos2* is a key stem cell regulator expressed in self-renewing spermatogonial stems cells and is required to maintain the stem cell state during spermatogenesis (Sada et al. 2009). *Nanos3*, which is expressed specifically in undifferentiated spermatogonia after birth, is important for the maintenance of undifferentiated spermatogonia via cell cycle regulation (Lolicato et al. 2008). The expression of *PyNanos2/3* and mouse *Nanos2*, *Nanos3* in spermatogonia suggests this expression pattern may be an original feature shared by these family members. Meanwhile, we found *PyNanos2/3* displayed specific expression in two cell clusters in the trochophore larvae, which may partially overlap with the signal of *PyNanos1*. We therefore postulate that *PyNanos2/3* may be potentially involved in the formation and/or maintenance of PGCs. Actually, similar function has been demonstrated for mouse *Nanos3*, which is found in migrating PGCs, and elimination of *Nanos3* results in the complete loss of germ cells in both sexes (Tsuda et al. 2003). According to these results, it seems the function of *Nanos2/3* members in PGCs and spermatogonia may be conserved between the vertebrates and invertebrates.

## Conclusion

In summary, two *Nanos* genes were identified in *P. yessoensis*. Both of them are germ cell specific and may play crucial roles in gametogenesis in the scallop. As a maternal gene, *PyNanos1* might be involved in the formation of PGCs. *PyNanos2/3* is zygotically expressed and may participate in the specification of PGCs. The comprehensive analysis of *Nanos* family in *P. yessoensis* will facilitate a better understanding of the role of *Nanos* in the development of germline cells in mollusca.

**Author Contribution** Liangjie Liu: investigation, formal analysis, and writing — original draft; Lingling Zhang: conceptualization, supervision, writing — review & editing; Shaoxuan Wu, Yajuan Li, Huilan Wei: investigation. Tian Liu, Lijing Zhang, Ya Shu, Yaxin Yang, Qiang Xing: resources; Shi Wang: project administration.

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## Declarations

**Conflict of Interest** The authors declare no competing interests.

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