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Research paper

Molecular characterization and expression of *Piwil1* and *Piwil2* during gonadal development and treatment with HCG and LHRH-A_2 in *Odontobutis potamophila*

Cheng Zhao[a,b], Wenxu Zhu[a,b], Shaowu Yin[a,b,*], Quanquan Cao[a,b], Hongye Zhang[a,b], Xin Wen[a,b], Guosong Zhang[a,b], Wanli Xie[a,b], Shuqiao Chen[c]

[a] College of Life Sciences, Key Laboratory of Biodiversity and Biotechnology of Jiangsu Province, Nanjing Normal University, Nanjing, Jiangsu 210023, China
[b] Co-Innovation Center for Marine Bio-Industry Technology of Jiangsu Province, Lianyungang, Jiangsu 222005, China
[c] Nanjing Institute of Fisheries Science, Nanjing, Jiangsu 210036, China

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ABSTRACT

Piwi proteins play an important regulatory role in germ cell division during gametogenesis and gonad development. In order to understand the function of *Piwi* genes in the reproductive process of the dark sleeper, we identified and characterized *Piwil1* and *Piwil2* from gonad tissue. The tissue distribution demonstrated that *Piwils* were highly expressed in the gonad of the dark sleeper. During gonad development, higher expression was observed in stage I of both the testes and ovaries than in subsequent stages at mRNA and protein levels. The results of immunohistochemistry demonstrated that Piwils were predominantly distributed in the spermatogonia, spermatocytes, and early oocytes. When treated with the HPG axis hormone (HCG and LHRH-A2), the expression of *Piwils* was significantly decreased in the testes and ovaries at mRNA and protein levels. All of these results indicated that *Piwils* play a vital role in gonad development and gametogenesis. Our findings provide valuable evidence to further clarify the underlying modulation mechanism of *Piwils* in teleosts.

1. Introduction

The Argonaute family of proteins functions as a primary element of the RNA-induced silencing complex that delivers small RNAs to specific mRNA targets. The Argonaute family is comprised of two subclasses: Ago and Piwi (Carmell et al., 2002; Hock and Meister, 2008; Zhou et al., 2010). Ago proteins are prevalent among all cell types and preferentially bind with siRNA and miRNA. Piwi proteins, meanwhile, are involved in a particular Piwi-interacting RNA (piRNAs) pathway and are less understood. *Piwi* genes are characterized by highly homologous PAZ and PIWI domains (Filipowicz, 2005). The PAZ domain is found at the center of the nucleotide sequence while the PIWI domain is located at the C-terminus (Tolia and Joshua-Tor, 2007). The PAZ domain consists of 100–200 amino acids at the N-terminus and preferably binds with the 3′ end of short RNAs. The PIWI domain has approximately 300 amino acids at the C-terminus and is structurally similar to the RNAse H domain (Cerutti et al., 2000).

Piwi genes were first found in *Drosopila* as an essential factor in maintaining germline stem cells in the reproduction process, and Piwi protein mutants are defective in gametogenesis for *Drosopila* (Klattenhoff et al., 2009). The *Piwi* subfamily contains three homologs (*Miwi1*, *Miwi2*, and *Mili*) in mouse (*Mus musculus*), all of which showed male-specific expression (Kuramochi-Miyagawa et al., 2001). Mouse *Piwi* genes have been shown to be central to male fertility, and when *Miwi* are eliminated, sterility can occur due to arrested spermatogenesis and apoptosis of the germ cells in the male mouse (Deng and Lin, 2002). Moreover, mutations in *Mili* and *Miwi2* can result in the loss of male germ cells at three and six months, respectively (Carmell et al., 2007; Unhavaithaya et al., 2009). There are four homologous of the *Piwi* subfamily in humans—*HIWI*, *HIWI2*, *HIWI3*, and *HILI*, which are expressed in spermatocytes and round spermatids (Qiao et al., 2002). As for teleosts, *Ziwi* (*Piwi*-like 1, *Piwil1*) and *Zili* (*Piwi*-like 2, *Piwil2*) were firstly elucidated in zebrafish (*Danio rerio*) (Tan et al., 2002). Studies have shown *Ziwi* and *Zili* to be exclusively expressed in the germline of zebrafish and further indicate that germ cell apoptosis can result from *Ziwi* mutation and that *Zili* is required for germ cell differentiation and

Abbreviations: HPG, hypothalamic–pituitary-gonadal; GnRH, Gonadotropin-releasing hormone; GTH, gonadotropins; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHRH, luteinizing hormone-releasing hormone; HCG, human chorionic gonadotropin; ORF, open reading frame; PGC, primordial germ cell; GSI, gonadosomatic index; Mw, molecular weight

* Corresponding author at: College of Life Sciences, Key Laboratory of Biodiversity and Biotechnology of Jiangsu Province, Nanjing Normal University, Nanjing, Jiangsu 210023, China.
E-mail address: 08272@njnu.edu.cn (S. Yin).

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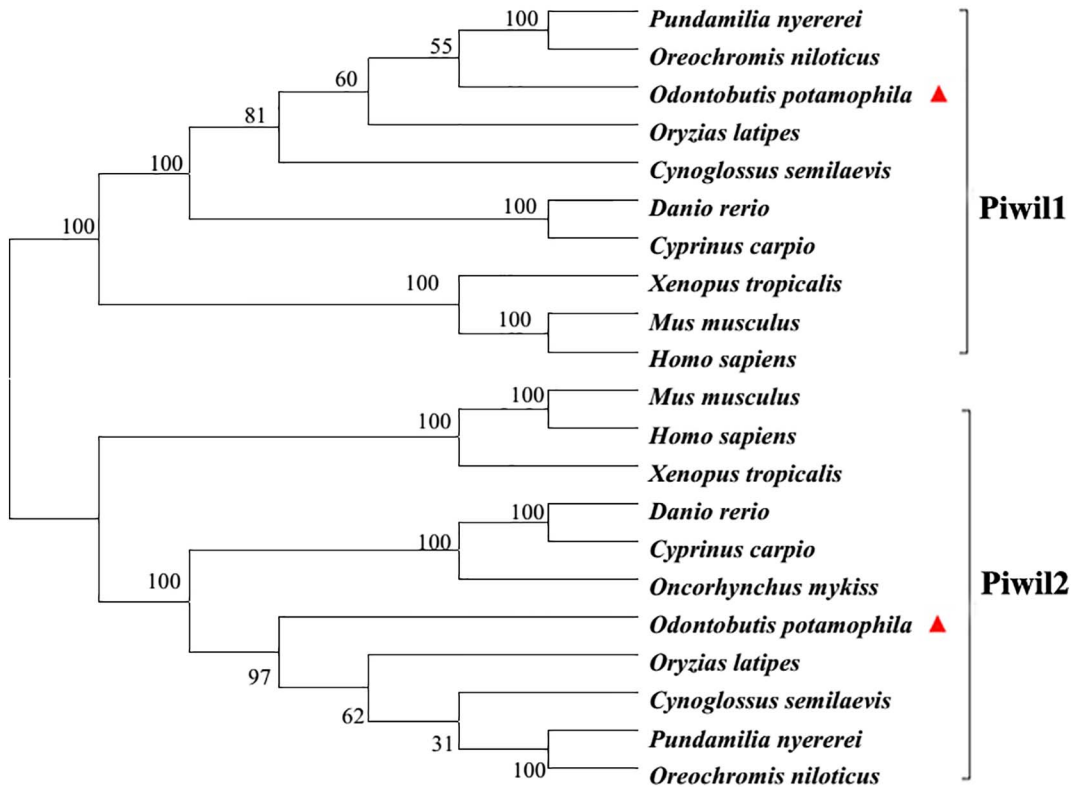

Fig. 1. Phylogenetic analysis for Piwil1 and Piwil2 protein from the dark sleeper and other vertebrates. The bootstrap values are based on 1000 resampling replicates. The GenBank accession numbers for amino acids sequences of Piwil1 used are as follows: *Cynoglossus semilaevis* (XP_008335747.1); *Cyprinus carpio* (KTF80228.1); *Danio rerio* (NP_899181.1); *Homo sapiens* (NP_060538.2); *Mus musculus* (NP_067286.1); *Oreochromis niloticus* (XP_013127041.1); *Oryzias latipes* (NP_001153908.1); *Pundamilia nyererei* (XP_005729611.1); and *Xenopus tropicalis* (XP_002940227.2); *Odontobutis potamophila* (MF_346061.1). The GenBank accession numbers for amino acids sequences of Piwil2 used are as follows: *Cynoglossus semilaevis* (AHZ97877.1); *Cyprinus carpio* (AEN55535.1); *Danio rerio* (NP_001073668.2); *Homo sapiens* (NP_060538.2); *Mus musculus* (NP_067283.1); *Oncorhynchus mykiss* (NP_001117714.1); *Oreochromis niloticus* (XP_003445710.1); *Oryzias latipes* (NP_001153909.1); *Pundamilia nyererei* (XP_005733624.1); and *Xenopus tropicalis* (NP_001106470.1); *Odontobutis potamophila* (MF_346060.1).

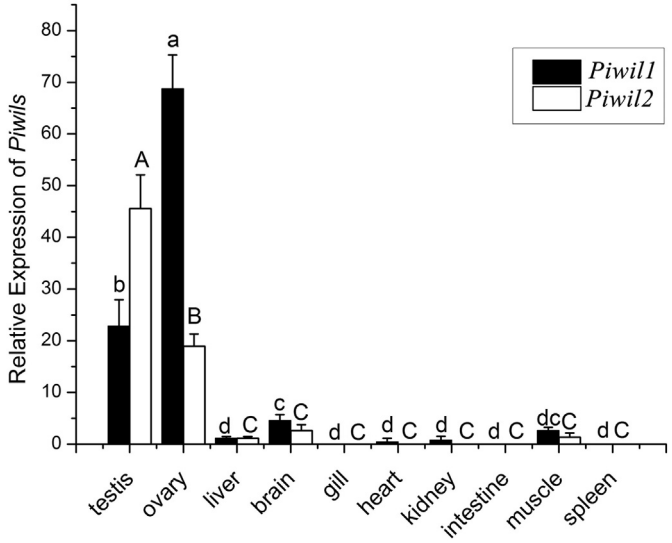

Fig. 2. Tissue distribution analysis of *Piwil1* and *Piwil2* transcripts of the dark sleeper using qRT-PCR. The relative expression of *Piwils* at the mRNA level in each tissue was calculated by the $2^{-\Delta\Delta Ct}$ method. β-actin and GAPDH served as an internal reference gene. Error bars represent a standard deviation of three replicates. Different lowercase or capital letter superscripts indicate significant difference ($P < .05$) between different tissues.

meiosis (Houwing et al., 2007; Houwing et al., 2008). *Piwi* homologous genes have also been reported in several teleosts, including *Cyprinus carpio*, *Oreochromis niloticus*, and *Cynoglossus semilaevis*, all of which are involved in the reproductive process (Zhou et al., 2012; Xiao et al., 2013; Zhang et al., 2014).

In teleosts, the activities of gonads are primarily regulated by the hypothalamic–pituitary-gonadal (HPG) axis (Weil and Crim, 1985). Gonadotropin-releasing hormone (GnRH) is synthesized and released by the hypothalamus cells, which act on the pituitary gland to stimulate the synthesis of gonadotropins (GTH), including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LHRH-A2 (luteinizing hormone-releasing hormone, an analog of GnRH) and HCG (an analog of LH) are important endogenous hormone analogs of the reproductive axis that enable maturation of the gonads. As such, they are widely used to induce fish spawning in aquaculture (Miura et al., 1999; Mylonas et al., 2010). In previous studies, exogenous sex hormones such as fadrozole, finasteride, estrogen, and luteinizing hormone were reported to affect the expression of Piwi-piRNA pathway genes in mice, *Xenopus*, as well as in certain fish species (Zhang et al., 2010; Pan et al., 2012; Zhou et al., 2012; Xiao et al., 2013). Thus, *Piwi* may play an important role in the process of HPG axis hormonal regulation.

The dark sleeper (*Odontobutis potamophila*) is an important commercial fish species widely distributed in the river systems of southeastern China (Hou et al., 2014). In recent years, the dark sleeper has become a very promising aquaculture species in the country due to its delicious taste and high nutritional value. Because male individuals grow substantially larger and at a quicker rate than females (Mei and Gui, 2015), males have greater economic value than females. Despite all this, investigation into the molecular mechanisms of its development is far from sufficient. Research that clarifies the expression patterns of *Piwils* can provide a foundation for further study of the dark sleeper due to their important role in the regulation of reproduction.

In this study, we cloned and identified *Piwil1* and *Piwil2* in the dark