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Identification and expression analysis of *Dazl* homologue in *Cynops cyanurus*

Yinjiao Zhao[1,2], Ya Du[2], Qinglan Ge[2], Fang Yan[1] and Shu Wei[1]

[1]State Key Laboratory of Conservation and Utilization of Bio-Resources in Yunnan and Center for Life Science, School of Life Sciences, Yunnan University, Kunming, Yunnan, China and [2]Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education and Yunnan Province, School of Chemical Science and Technology, Yunnan University, Kunming, Yunnan, China

Research Article

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Authors for correspondence:
Fang Yan and Shu Wei. State Key Laboratory of Conservation and Utilization of Bio-Resources in Yunnan and Center for Life Science, School of Life Sciences, Yunnan University, Kunming, Yunnan, China.
E-mail: shuwei@ynu.edu.cn; fangyan@ynu.edu.cn

Summary

The *Dazl* (deleted in azoospermia-like) gene encodes an RNA-binding protein containing an RNA recognition motif (RRM) and a DAZ motif. Dazl is essential for gametogenesis in vertebrates. In this study, we report the cloning of *Dazl* cDNA from *Cynops cyanurus*. *Ccdazl* mRNA showed a germline-specific expression pattern as expected. *Ccdazl* expression gradually decreased during oogenesis, suggesting that it may be involved in oocyte development. Phylogenetic analysis revealed that the Ccdazl protein shares conserved motifs/domains with Dazl proteins from other species. Cloning of *Ccdazl* provides a new tool to carry out comparative studies of germ cell development in amphibians.

Introduction

Specification of germ cell lineage, including haploid gametes and their diploid progenitor population called primordial germ cells (PGCs), is fundamentally important for sexual reproduction. Preformation and epigenesis are two independent modes in the process of PGC specification (Extavour and Akam, 2003). In the preformation mode, maternally inherited germ plasm components play a determinant role. Asymmetrical cell division ensures that cells containing germ plasm develop into PGCs, which migrate towards and invade gonads. In contrast, the epigenesis mode relies on signals outside the future PGCs for specification (Swiers *et al*., 2010). Both modes have been found for PGC specification in amphibians. The preformation mode is found in frogs, while the epigenesis mode is found in newts (Smith, 1966; Johnson *et al*., 2001). Why amphibians have evolved two different specification modes is unknown. The detailed mechanisms of PGC specification in amphibians remains to be fully elucidated. For example, how are the conserved germ cell specification components organized? What are the biochemical functions of the key components, such as Dazl?

Germ cell specification is tightly regulated by a variety of factors conserved in many organisms (Lehmann and Ephrussi, 1994; Extavour and Akam, 2003). Of particular importance is the RNA-binding protein Dazl, which is a member of the DAZ (Deleted in Azoospermia) family. The DAZ family contains three members: Daz, Dazl and Boule. Boule is widely present in invertebrates and vertebrates. Daz is currently found only in primates. Dazl is expressed in all vertebrates studied including humans, chickens, mice, *Xenopus*, zebrafish (Yen *et al*., 1996; McNeilly *et al*., 2000; Sekizaki *et al*., 2004; Elis *et al*., 2008; Takeda *et al*., 2009). The RNA recognition motif (RRM) and at least one DAZ domain are evolutionarily conserved in the DAZ family (Cooke *et al*., 1996; Reijo *et al*., 1996). Dazl homologues are specifically expressed in male and female germ cells. It has been proposed that Dazl regulates its target genes by a post-transcriptional mechanism (Reynolds *et al*., 2005, 2007; Shah *et al*., 2010). *Dazl* RNA accumulates in the vegetal cortex of oocytes and the cleavage furrow of embryos in *Xenopus* and zebrafish (Houston *et al*., 1998; Hashimoto *et al*., 2004). Dazl homologues of *Ambystoma mexicanum* and *Cynops pyrrhogaster* have been identified and characterized (Johnson *et al*., 2001; Tamori *et al*., 2004). Dazl has a key role in spermatogonia differentiation and meiosis (Schrans-Stassen *et al*., 2001). Dazl is also required for pluripotency maintenance of embryonic stem cells (ESC) and germ cell differentiation of ESCs *in vitro* (Yu *et al*., 2009). Loss of Dazl functions causes infertility in fish, frogs, mice and humans (Houston and King, 2000; Hashimoto *et al*., 2004; Lin and Page, 2005; Kee *et al*., 2009).

To assist the further study of germ cell development in amphibians, we cloned *Ccdazl* cDNA from *Cynops cyanurus*. Phylogenetic tree analysis indicated that the predicted Ccdazl protein clusters closely with its homologues from other species. Expression analysis of *Ccdazl* in oogenesis and gonads suggested that Ccdazl plays a role in gametogenesis. These results will facilitate germ cell specification studies in this newt species and in amphibians in general.

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Table 1. Primers used in this study

Name	Sequence (5′→3′)	T_m
Cynops-*Dazl*-F1	GAAAGTTGTCGGCGTTCCAG	66°C
Cynops-*Dazl*-R1	TCTCATAATTATCCAAGCCCAGT	
Dazl-RACE-3′F1	GATTACGCCAAGCTTTCCCTGTTCACGAGCCCACTCAG	68–57°C (touchdown)
Dazl-RACE-5′R1	GATTACGCCAAGCTTCCTGGCGTCCACTGTGGGGATAG	
RACE-UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTCTAATACGACTCACTATAGGGC	
Ccdazl-qPCR-F	CATACTGGGGCACTGGACTA	60°C
Ccdazl-qPCR-R	GGGGTTTCTAAAGGATACGG	
Gapdh-qPCR-F	CCACTGTCCATGCTGTGACT	
Gapdh-qPCR-R	CAACCACAGACACATTGGCG	
Ccdazl Fx1-FL	GAAAGTTGTCGGCGTTCC	68–57°C (touchdown)
Ccdazl Rx1-FL	CTTCTCCTGATTGCATTGCT	
Ccdazl Fx2-FL	GGATGCAATTACGAGTTTGG	
Ccdazl Rx2-FL	CTTTTAATCACGGTGCGTTC	

Materials and methods

Animal and embryo collection

Adult males and females of *Cynops cyanurus* were sampled from Chuxiong, Yunnan, China. The embryos in different stages were collected according to previous studies (Wang *et al.*, 1984). All animal experiments in this study were performed under the animal welfare guidelines and were approved by the Institutional Experimental Animal Review Board, Yunnan University.

RNA extraction

Adult tissues of *Cynops cyanurus* (ovary, testis, brain, fat, heart, liver, lung, muscle, skin and spleen) were collected from adult *Cynops cyanurus*. Oocytes from four different stages were collected based on their size and morphology. Total RNA from tissues, oocytes and embryos were extracted using the Animal Total RNA Isolation kit (Foregene, China).

Ccdazl *cDNA cloning*

Here, 1 μg total RNA from ovary was reverse transcribed to generate the *Ccdazl* cDNA using the TransScript Two-step RT-PCR Supermix (TransGen Biotech, China). Primers were designed according to the *Cydazl* homologue sequence (Accession number: AB164065.1; Cynops-*Dazl*-F1/Cynops-*Dazl* -R1; Table 1). A single-band PCR product that met the expected size (1000 bp) was obtained. Then the 5′/3′ rapid amplification of cDNA ends (RACE) primers were designed according to the 1000-bp product. The segments at both ends were obtained using the SMARTer RACE 5′/3′ Kit (TaKaRa Bio Inc., USA; *Dazl*-RACE-3′F1/RACE-UPM, RACE-UPM/*Dazl*-RACE-5′R1; Table 1). Full-length *Ccdazl* cDNA was amplified by primers located at the 5′ and 3′ ends (*Ccdazl* Fx1-FL/*Ccdazl* Rx1-FL, *Ccdazl* Fx2-FL/*Ccdazl* Rx2-FL; Table 1).

Quantitative RT-PCR analysis

The first-strand cDNA was generated from the total RNA of adult tissues and oocytes using the TransScript Two-step RT-PCR Supermix (TransGen Biotech, China). Quantitative PCR (qPCR) was performed using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., USA). The specific qPCR primers for *Ccdazl* were designed according to full-length cDNA sequence (*Ccdazl*-qPCR-F/*Ccdazl*-qPCR-R; Table 1). The internal reference *Gapdh* was designed based on the *Cynops pyrrhogaster Gapdh* sequence (Accession number: AB643658.1; *Gapdh*-qPCR-F/*Gapdh*-qPCR-R; Table 1).

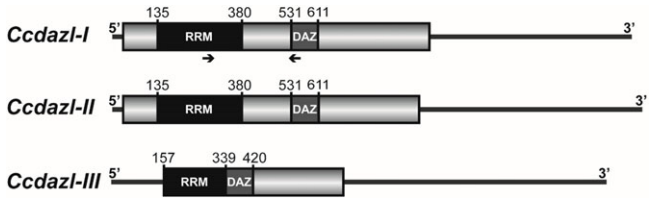

Figure 1. Schematic diagram of *Ccdazl* mRNAs. The open reading frames of three *Ccdazl* mRNAs are indicated by light grey boxes. The black boxes and the darker grey boxes represent the RRM motif and the DAZ repeat, respectively. The arrows indicate the position of the RT-PCR primers.

Amplification of embryo cDNA and qPCR

Full-length cDNA from embryos at different stages were amplified and generated following a protocol for Smart-seq2 (Picelli *et al.*, 2014). qPCR was conducted, as mentioned above (*Ccdazl*-qPCR-F/*Ccdazl*-qPCR-R; Table 1).

Sequence assembly and data analysis

Sequences of PCR segments were assembled using Vector NTI software (ThermoFisher). The similarity analysis for the Dazl sequence referred to previous studies (Stothard, 2000). The multiple amino acid sequence alignments were made using DNAMAN software (LynnonBiosoft). The phylogenetic tree was based on the neighbour-joining (NJ) method and was constructed using MEGA4 software (Saitou and Nei, 1987).

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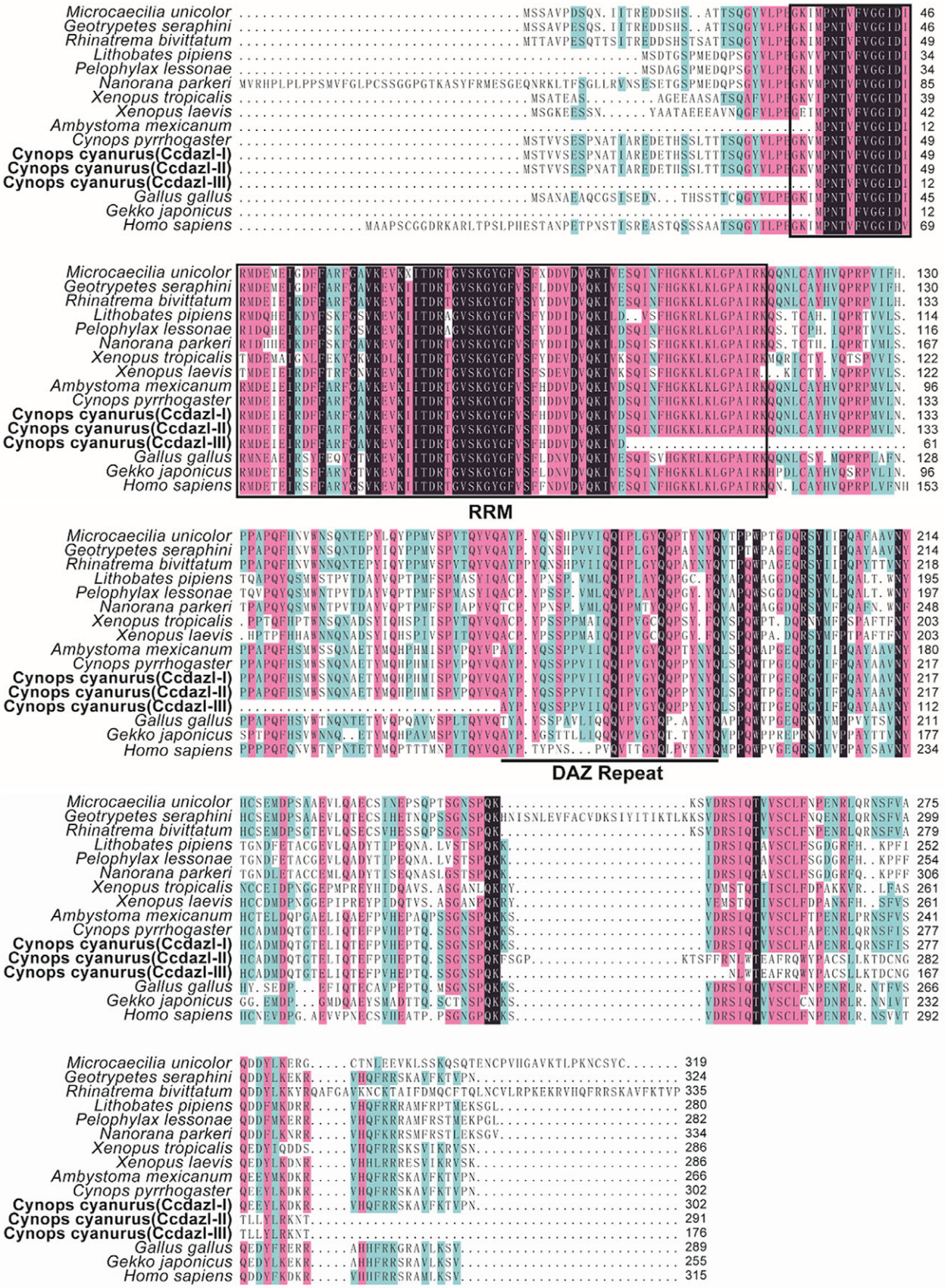

Figure 2. Multiple sequence alignment of Dazl proteins. The alignment was performed using the Dazl sequences from *Cynops cyanurus*, amphibians (*Microcaecilia unicolor*, XP_030058470.1, *Geotrypetes seraphini*, XP_033786595.1, *Rhinatrema bivittatum*, XP_029432885.1, *Lithobates pipiens*, AAV30542.1, *Pelophylax lessonae*, CAM32330.2, *Nanorana parkeri*, XP_018428601.1, *Xenopus tropicalis*, NM_001016823.3, *Xenopus laevis*, NM_001088259.1, *Ambystoma mexicanum*, AY542375.1, *Cynops pyrrhogaster*, AB085215.1), bird (*Gallus gallus*, NP_990039.2), reptile (*Gekko japonicus*, XP_015278700.1), and mammal (*Homo sapiens*, NP_077726.1). The dark blue highlighted sequences indicate that the similarity between homologues is 100%. The purple and light blue highlights represent the similarities of 75–99% and 75–99%, respectively. The RRM motif is shown as black boxes. The DAZ repeat is underlined.

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Results

Cloning of Ccdazl *cDNA from* Cynops cyanurus

To obtain the full-length *Cynops cyanurus dazl* (*Ccdazl*) sequence, we extracted RNA from adult ovary of *Cynops cyanurus* followed by RT-PCR and RACE procedures. Three different *Ccdazl* cDNAs were identified, namely *Ccdazl-I*, *Ccdazl-II* and *Ccdazl-III*. *Ccdazl-I* was 1540 bp in length, including 32 bp of the 5′ untranslated region (5′UTR), 599 bp of the 3′UTR, and 909 bp of the open reading frame (ORF), which was predicted to encode a protein with 302 amino acid residues. Ccdazl-I is identical to a predicted protein (Cydazl) from *Cynops pyrrhogaster* (Tamori *et al.*, 2004), suggesting that the proteins are high conserved in newt species. Separately, the *Ccdazl-II* (1569 bp) was predicted to encodes a protein with 291 amino acid residues. Lastly, *Ccdazl-III* (1466 bp) encoded a protein with 176 amino acid residues. Whether the three Dazl isoforms have different functions remains to be investigated (Fig. 1). Note: Nucleotide sequence data reported are available from the GenBank databases under accession numbers MW803135, MW803136 and MW803137.

Sequence alignment of amino acid sequences of Dazl homologues is shown in Fig. 2. Each isoform of Ccdazls contained one RRM domain and one DAZ motif, suggesting that they all bound to RNA and regulated RNA functions such as translation.

Phylogenetic analysis of Dazl proteins

Pairwise sequence analysis of DAZL protein sequences of different species showed that *Ccdazl-I*, *Ccdazl-II* and *Ccdazl-III* were mostly similar to the sequence from *Cynops pyrrhogaster*, similarity was 100%, 85.0%, and 51.0%, respectively. Identity of Ccdazl-I, Ccdazl-II and Ccdazl-III to the homologues protein of other representative species is shown as follows: *Ambystoma mexicanum* (82.8%, 68.8, 54.1%), *Pelophylax lessonae* (63.7%, 53.6%, 39.0%), *Xenopus laevis* (69.2%, 59.9%, 40.3%), *Microcaecilia unicolor* (78.0%, 67.9%, 42.6%), *Homo sapiens* (70.4, 59.6%, 36.1%), *Gallus gallus* (77.2%,65.3%,40.7%) and *Gekko japonicus* (68.9%, 57.7%, 46.0%) (Fig. 2). The phylogenetic reconstruction results showed that major clades conformed to three taxa of amphibians, including Gymnophiona, Caudata and Anura. *Cynops cyanurus* is clustered with *Cynops pyrrhogaster* (Fig. 3).

Expression pattern of Ccdazl

First, we used quantitative RT-PCR (qRT-PCR) to examine *Ccdazl* RNA expression. *Ccdazl* specific primers were designed according to the consensus sequence of *Ccdazl-I* and *Ccdazl-II*. The qRT-PCR result confirmed that *Ccdazl* mRNA is highly expressed in gonads with little expression in somatic tissues. We noticed that the expression level in the ovary was higher than that in testis (Fig. 4). Next, we measured *Ccdazl* expression from stage I to stage IV oocytes. *Ccdazl* mRNA expression levels decreased gradually during oogenesis (Fig. 5). During embryogenesis, we also observed that expression levels at early stages (stages 6 and 7) were higher than that at later stages (stages 13, 14, and 20) (Fig. 6). In summary, *Ccdazl* is mainly expressed in germlines, suggesting that it functions in germ cell formation and maintenance.

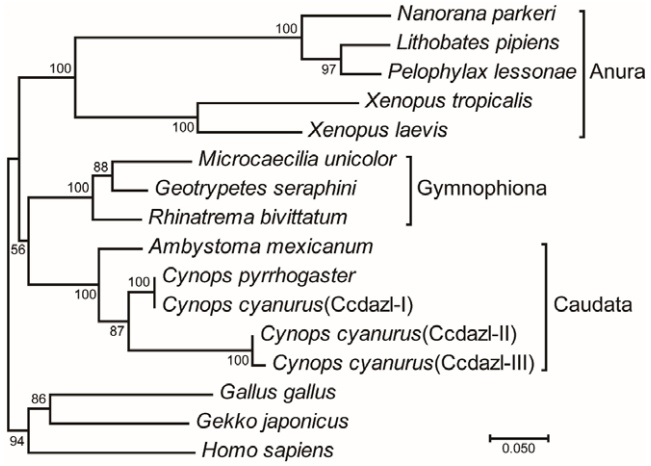

Figure 3. Phylogenetic tree analysis of Dazl proteins. The phylogenetic tree was constructed using the neighbour-joining method. Numbers indicate bootstrap values.

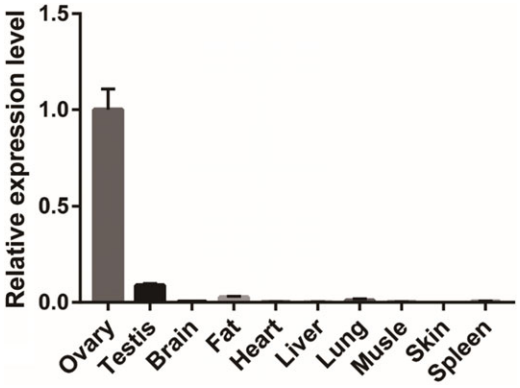

Figure 4. Expression of *Ccdazl* mRNA in adult tissues. *Ccdazl* mRNA expression in adult tissues was detected by quantitative RT-PCR. *Ccdazl* mRNA is abundant in ovary and testis. *Gapdh* was used as the internal reference.

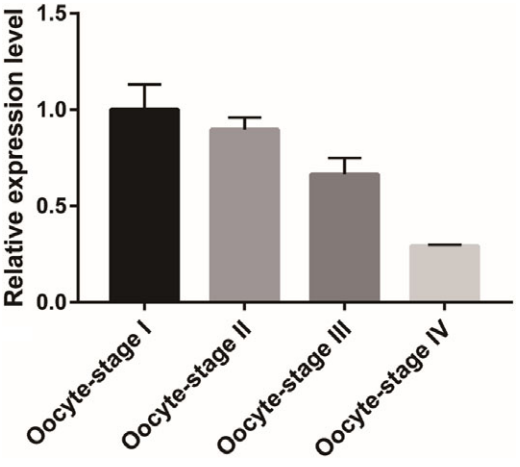

Figure 5. Expression of *Ccdazl* mRNA during oogenesis. *Ccdazl* mRNA is highly expressed in the early stages of oogenesis. Then, the expression level gradually decreased. *Gapdh* was used as the internal reference.

Discussion

The DAZ family proteins have been found in species including *C. elegans*, *Drosophila*, zebrafish, *Xenopus*, mouse, and humans (Eberhart *et al.*, 1996; Maegawa *et al.*, 1999; Karashima *et al.*, 2000). DAZ family proteins play vital roles in meiosis and gametogenesis based on loss of function studies (Eberhart *et al.*, 1996; Ruggiu *et al.*, 1997; Karashima *et al.*, 2000). The RRM domains

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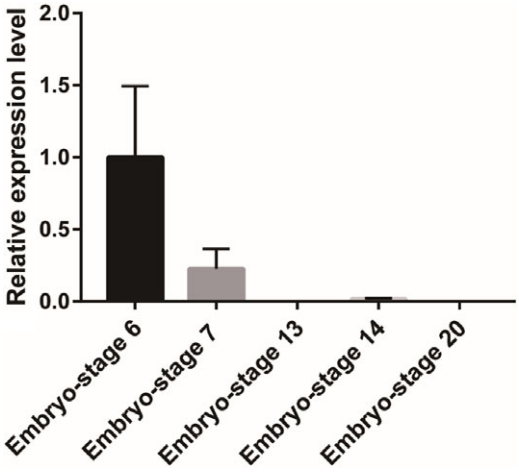

Figure 6. Expression of *Ccdazl* mRNA during embryogenesis. Embryonic expression of *Ccdazl* mRNA during stage 6 is higher than during stage 7, and disappears in the later stage. *Gapdh* was used as the internal reference.

in DAZ, BOULE, and DAZL are critical for their physiological functions, such as fertility, indicating that the RRM domain is essential for biochemical activities of DAZ proteins (Reijo *et al*., 1995). However, biochemical functions of DAZ repeats are unknown (Teng *et al*., 2002).

In this study, we cloned three isoforms of *Ccdazl* cDNAs of *Cynops cyanurus* and examined their expression patterns in adult tissues and early development. As expected, Ccdazls belong to DAZL members but not to BOULE or DAZ. The RRM domain showed marked homology in all DAZL proteins. The sequence of Ccdazl-I was identical to that of Cydazl, and shares an 82.8% identity with the homologue protein of *Ambystoma mexicanum* (Axdazl). Interestingly, the identity of Ccdazl-I to mammals is higher than to frogs. These results are in agreement with the previous studies in newt (Johnson *et al*., 2001; Tamori *et al*., 2004). as mammals and frogs follow the different germ cells specification modes, it is reasonable to speculate that *Cynops cyanurus* uses the epigenesis mode. *Ccdazl* mRNA is abundant in ovary and testis but low or negative in somatic tissues. *Ccdazl* mRNA was highly expressed in ovary with decreasing expression, suggesting that it may play a role during oogenesis. In addition, the disappearance of *Ccdazl* mRNA in late-stage embryos suggested that maternal *Ccdazl* is degraded without zygotic *Ccdazl* transcripts. Cloning of the three isoforms of *Ccdazl* cDNAs will pave the way to study their functions in germ cell formation in this newt species and help comparative studies of germ cell specification in amphibians.

Author contributions. Shu Wei and Fang Yan conceived and designed the study. Yinjiao Zhao performed the experiments. Ya Du and Qinglan Ge analyzed the data. Shu Wei wrote the paper. All authors read and approved the manuscript.

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Competing interests. The authors have declared that they have no competing financial interests.

Ethical approval. All animal experiments in this study were performed under the animal welfare guidelines and were approved by the Institutional Experimental Animal Review Board, Yunnan University.

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