



Article Cloning and Expression Profiling of the Gene *vasa* during First Annual Gonadal Development of Cobia (*Rachycentron canadum*)

Qian Ma^{1,2}, Jiehua Kuang¹, Gang Chen^{1,2,*}, Jiandong Zhang^{1,2}, Jiansheng Huang^{1,2}, Feifan Mao¹ and Qiling Zhou¹

- ¹ College of Fisheries, Guangdong Ocean University, Zhanjiang 524025, China; mfm_0624@163.com (Q.M.); kuangjh3@haid.com.cn (J.K.); yzxzjd@126.com (J.Z.); huangjs@gdou.edu.cn (J.H.); ff954366@163.com (F.M.); 18738627206@163.com (Q.Z.)
- ² Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang), Zhanjiang 524025, China
- * Correspondence: cheng@gdou.edu.cn

Abstract: The vasa gene is essential for germ cell development and gametogenesis both in vertebrates and in invertebrates. In the present study, vasa (Rcvasa) cDNA was cloned from cobia (Rachycentron canadum) using the RACE amplification method. We found that the full-length cDNA sequence of Rcvasa comprises 2571 bp, containing a 5'-UTR of 145 bp, a 3'-UTR of 341 bp, and an open reading frame (ORF) of 2085 bp, encoding a protein of 694 aa. The deduced amino acid sequence contains 8 conserved motifs of the DEAD-box protein family, 7 RGG repeats, and 10 RG repeats in the Nterminal region. Comparisons of the deduced amino acid sequence with those of other teleosts revealed the highest percentage identity (86.0%) with Seriola quinqueradiata. By using semiquantitative RT-PCR, Rcvasa appeared to be specifically expressed in the testis and ovary, among 13 tissues analyzed. In addition, annual changes in Rcvasa expression levels were examined in the gonads by quantitative real-time PCR (qRT-PCR). The expression of Rcvasa in the testis first increased significantly at 120 dph (stage II-III), then stabilized as the testis developed from 185 dph (stage III) to 360 dph (stage V). During the development of the ovary (stage I to II), the expression of Rcvasa first increased and reached the highest level at 210 dph (stage II), then decreased. Furthermore, the results of chromogenic in situ hybridization (CISH) revealed that Rcvasa mRNA was mainly expressed in germ cells and barely detected in somatic cells. In the testis, Rcvasa mRNA signal was concentrated in the periphery of spermatogonia, primary spermatocytes, and secondary spermatocytes and was significantly weaker in spermatids and spermatozoa. In the ovary, Revasa mRNA signal was uniformly distributed in the perinuclear cytoplasm and was intense in early primary oocytes (stage I and II). These findings could provide a reference for understanding the regulatory mechanisms of vasa expression during the development of germ cells in cobia.

Keywords: Rachycentridae; *Ddx4*; molecular characterization; gametogenesis; qRT-PCR; mRNA distribution

1. Introduction

Germ cell development is the basis of vertebrate reproduction and plays a vital role in transmitting species-specific genomic information between generations. In teleosts, germ cells originate from primordial germ cells (PGCs) which were segregated from somatic cells in the early stage of embryogenesis; then, PGCs migrate into the primary gonad and become gonadal germ stem cells (oogonia in the ovary and spermatogonia in the testis) [1]. During gonad maturation, oogonia and spermatogonia undergo meiosis and then transform into ovum and sperm, respectively. The process from formation of PGCs to maturity is regulated by many factors, including genes, hormones, and environment [2]. To date, the mechanism through which germ cells produce either ovum or sperm has been



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). unclear. Recently, germ cell markers including *vasa* have been widely used in gonadal development studies [3–7], as they could be effectively used to trace the development of germ cells. These studies would facilitate the identification of the mechanisms underlying the specification and development of germ cells.

The vasa gene, also called *Ddx4* (DEAD box polypeptide 4), is an ATP-dependent RNA helicase belonging to the DEAD (Asp–Glu–Ala–Asp)-box protein family [8,9]. It also plays an indirect role in the metabolism of RNA [10] and in the regulation of the expression of transcription factors [11]. This gene was initially identified as a maternal-effect gene in *Drosophila*, and mutations in it could hamper the development of germ cells [12]. In the past few decades, several germ cell-specific molecular markers have been discovered and used for identifying the germline in fish [13]. Among these genes, *vasa* is one of the most documented germ cell markers in teleosts [14–19]. Extensive progress has been made in understanding the function of *vasa* in PGC determination; however, little information is available about the function of *vasa* in gonadal tissues. As reported, the *vasa* gene is also expressed in mature gonads, and significant interspecies differences in the expression patterns of *vasa* during gametogenesis were found [20].

Cobia (*Rachycentron canadum*; Rachycentridae, Perciformes) is a migratory pelagic fish widely distributed across tropical and subtropical waters, except in the eastern Pacific [21]. Interest in this species for cage and other intensive aquaculture systems has raised because of its rapid growth, strong disease resistance, and excellent meat quality [22]. To date, the production of cobia in southern China (amounting up to an average of about 40,000 tons per year, according to the China Fishery Statistical Yearbook) has kept increasing and is ranked high among all the fish species maricultured in China. The recent growth of cobia culture in southeast Asia [23] has raised needs for breeding technology development. In order to facilitate the large-scale breeding of cobia and improve the source of germplasm, further research on its gonadal development and gametogenesis is necessary.

In this study, the full-length *vasa* cDNA of cobia (*Rcvasa*) was cloned and identified. We further investigated *Rcvasa* expression patterns by qRT-PCR and detected the localization of *Rcvasa* mRNA by CISH during the first annual gonadal development. These results can help understand the role of *vasa* in spermatogenesis and oogenesis and will ultimately provide a basis for clarifying the mechanisms underlying the migration and differentiation of PGCs in cobia.

2. Materials and Methods

2.1. Biological Samples

From June 2019 to April 2020, cobia individuals at 90 dph (days post hatching), 120 dph, 150 dph, 185 dph, 210 dph, and 360 dph (more than three female and three male fish in each period) were obtained from an indoor aquaculture farm in the town of Mata (Maoming, Guangdong, China). A total of 18 male (weight 230.0–4225.0 g, length 29.2–64.5 cm) and 23 female (weight 215.0–5050.0 g, length 29.8–68.5 cm) cobia were collected. All the fish were reared in metal circular tanks with a diameter of 9 m in 2.5 m-deep water. During the experimental period, water temperature, salinity, and pH were 25.0–30.0 °C, 27.0–30.5, and 7.6–8.0, respectively.

The two-lobed gonads of the same fish were stored separately: one lobe, used for chromogenic in situ hybridization, was fixed in 4% paraformaldehyde (PFA) for 24 h, then stored in 70% ethanol with diethyl pyrocarbonate (DEPC) until histological processing; the other was first rinsed with 0.1% DEPC-treated water, then fixed in RNAlater overnight at 4 °C and stored at -80 °C. As tissue analysis of 150 dph juvenile cobia, 13 tissues (ovary, testis, heart, brain, muscle, liver, gill, intestine, stomach, spleen, kidney, skin, and eye) were extracted and fixed immediately in RNAlater overnight at 4 °C, then stored at -80 °C until used.

2.2. Total RNA Extraction, Cloning, and Phylogenetic Analysis of the vasa Gene

Total RNA from the gonads at different stages during development (90–360 dph) and various tissues of 150 dph juvenile cobia were isolated with the Trizol Reagent (Invitrogen). The concentration and quality of total RNA was determined by a SimpliNano microvolume UV–Vis Spectrophotometer (Biochrom), and its integrity was measured by electrophoresis on a 1.5% agarose gel. First-strand cDNA and 5'/3' RACE-ready cDNA were synthesized using the EasyScript One-step cDNA Synthesis Kit (TransGen) and SMARTer[®] RACE 5'/3' Kit (Clontech), respectively, according to the manufacturer's instructions.

The CDS sequence of the vasa gene was extracted from the genome-wide database of cobia, which was obtained previously by our research team (it has not been uploaded to the NCBI database). For amplification of vasa cDNA fragments, two pairs of specific primers (vasaF/R, Table 1) were designed from the 5'- and 3'- ends of the CDS sequence. The PCR cycling conditions were as follows: predenaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 3 min; a final extension was performed at 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel, purified using the Gel Extraction Kit (TransGen, Beijing, China), cloned into the pMD-18T vector (Takara), and propagated in *E. coli* DH-5 α (Takara, Dalian, China). The positive clones were sequenced by the sequencing service of Sangon Biotech Co., Ltd. (Shanghai, China). To obtain the upstream and downstream sequences, two forward primers (Rcvasa3'-F1/F2) and two reverse primers (Rcvasa5'-R1/R2) were designed according to fragments of Rcvasa (Table 1). Subsequently, nested PCR was performed using the SMARTer[®] RACE 5'/3' Kit (Clontech, Mountain View, CA, USA). The products from the first PCR (1 µL each) were used as templates in the following PCR reactions. The PCR conditions for 5'/3' RACE were as follows: predenaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s (Rcvasa3'-F1/F2, Rcvasa5'-R1) or at 63 °C for 30 s (Rcvasa5'-R2), and extension at 72 °C for 1 min 30 s; final extension at 72 °C for 10 min.

Primer	Sequence (5'–3')	Usage	
Rcvasa-F	ATGAAAAGAGAACCAGGCGCGA	CDS coquence dening	
Rcvasa-R	CTACTCCCATTCTTCATCATCAGCTG	CD3 sequence cioning	
Rcvasa3'-F1	TGACCTCCCCAACAACATAGACG		
Rcvasa3'-F2	TGGGAGGGCGGTGTCTTTC	3' RACE	
Rcvasa5'-R1	GCCAGCAGAAATGATGGGGGAT		
Rcvasa5'-R2	CTCCCTGATCTCCACCTTGTCTG	5 RACE	
Long-UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE universal primer	
Short-UPM	CTAATACGACTCACTATAGGGC	KACE universai primer	
Rcvasa-F1	GGTTGGCAGGAGCAGTAACT	PCR amplification	
Rcvasa-R1	TGTGGTTGTGATCTCCGGTG	I CK antpinication	
β-actin-F	AGGGAAATTGTGCGTGAC	internal control gene	
β-actin-R	AGGCAGCTCGTAGCTCTT	Internal control gene	
Rcvasa-Pro	CAGGACGTTACACCCCGGCATATTTCTC	probe of CISH	

Table 1. Primer sequences used in this study.

Homology comparison and identity analysis of the deduced amino acid sequence of the obtained cDNA were carried out using the NCBI BLAST server. Alignment of the deduced amino acid sequences was performed using the clustalX1.83, and the output of the alignment results through GenDoc. Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method with MEGA 5.0. Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches.

2.3. Semiquantitative RT-PCR

Total RNA extraction and cDNA synthesis were performed using 13 tissues (ovary, testis, heart, brain, muscle, liver, gill, intestine, stomach, spleen, kidney, skin, and eye). The tissue distribution pattern of *Rcvasa* was examined by semiquantitative RT-PCR. *Rcvasa*

transcripts were amplified by using a pair of specific primers (Rcvasa-F1/R1, Table 1) spanning a 210 bp cDNA fragment. As an internal control, the β -actin cDNA was amplified using the primers β -actin-F/R (Table 1). The PCR cycling conditions were as follows: predenaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 20 s; final extension at 72 °C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and the gel was visualized on a Tanon 4100 GEL imaging system.

2.4. Quantitative Real-Time PCR (qRT-PCR)

The expression patterns of *Rcvasa* transcripts during gonadal development (90–360 dph) were determined by qRT-PCR. The total RNA of a gonad (2 µg) was reverse-transcribed into cDNA using the EasyScript One-step cDNA Synthesis Kit (TransGen). The sequences of the specific primers for *Rcvasa* (Rcvasa-F1/R1) and the internal control β -actin (β -actin-F/R) are listed in Table 1. The qRT-PCR assays were performed with a SYBR[®] Premix Ex TaqTM kit (Takara) on an ABI 7500 Real-Time PCR Detection System (ThermoFisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's protocol. The *vasa* expression level was normalized against β -actin expression level to generate a Δ Ct value, and the relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method described previously [24]. For each data point, triplicate reactions were carried, out and the experiment was repeated three times.

To identify statistically significant differences in *vasa* relative expression by qRT-PCR, one-way ANOVA was employed, followed by a Duncan's multiple range test, using SSPS 19.0 software (IBM). In each case, differences were accepted as statistically significant at p < 0.05.

2.5. Chromogenic In Situ Hybridization (CISH)

In situ hybridization by chemical staining with BCIP/NBT substrates on histological sections of gonads from cobia at 120 dph, 210 dph, and 360 dph was performed in accordance with some previously reported procedures [3,17]. The classification of gonad differentiation was based on histological observations of cobia gonadal development [25], and the staging criteria were in accordance with Liu (1993) [26]. A *Rcvasa* oligonucleotide probe (Table 1) labeled with digoxigenin (DIG) was synthesized by Servicebio Biological Technology Co., Ltd. (Wuhan, Hubei, China). Briefly, the testes and ovaries were dissected, fixed in 4% PFA, and then embedded into paraffin, and 6 μ m sections were cut. After deparaffinization, hydration, and digestion with proteinase K (20 μ g/mL), the samples were hybridized with the probe at 37 °C overnight. The signals were detected by phosphatase-conjugated anti-DIG-AP and NBT/BICP as the chromogenic substrates.

3. Results

3.1. Cloning of Rcvasa cDNA and Phylogenetic Analysis

The full-length cDNA sequence of cobia *vasa* (*Rcvasa*) was cloned by RACE amplification (GenBank accession No. MW436698). The *Rcvasa* cDNA appeared to consist of 2571 bp, comprising an ORF (open reading frame) of 2085 bp, a 5'-UTR (untranslated region) of 145 bp, and a 3'-UTR of 341 bp, and to encode a 694-amino acid protein.

The predicted RcVasa protein contains 8 conserved motifs of the DEAD-box protein family, including motif I (AQTGSGKT), motif Ia (PTRELI), motif Ib (GG), motif II (TPGRL), motif III (DEAD), motif IV (SAT), motif V (RGLD), and motif VI (HRIGRTGR) (Figure 1), and the N-terminal region contains 10 arginine–glycine (RG) repeats and 7 arginine–glycine-glycine (RGG) repeats. The RcVasa protein also has two highly conserved domains, the DEXDc domain (275–486 aa) and the HELICc domain (522–603 aa). Multiple sequence alignment revealed that the deduced amino acid sequence of Rcvasa has 62.6–86.0% identity with those of Rcvasa proteins in other teleosts. The highest identity was found between cobia Rcvasa amino acid sequence and that of the corresponding protein in *Seriola quin-queradiata* (86.0%), followed by *Thunnus thynnus* (84.4%), *Euthynnus affinis* (82.6%), and

1	MKREPGANKEQVLYPPWPLRPPVKCKALAEVSLNLKKSHAAGRYIKMDVWEEQETPTSSVALTSHAPSECSQGDFR <mark>N</mark> SDGG <mark>EFGRGRGGRG</mark>	91
1	TSHTLSEGTKGDSW <mark>N</mark> TNGGEFGRGRGGRGMDEW <mark>EEB</mark> GT-TSTVALTSHTLSEGTKGDSW <mark>N</mark> TNGGEFGRGRGGRG	44
1	TSHTSSEGTQGDFW <mark>N</mark> TNGGEFGRGRGGRGMDEWEEBCN-TSTITLTSHTSSEGTQGDFW <mark>N</mark> TNGGEFGRGRGGRG	44
1	T\$HAS\$Q G \$Q GD F1M}\$DG-EFGRGRGGRG	44
1	TSHNPTEGNQGGSW <mark>y</mark> <mark>IDDWDE</mark> GEA-TSTTTLTSHNPTEGNQGGSW <mark>y</mark> <mark>T</mark> SSG <mark>EYGRGRGGR</mark> G	44
1	FGGAGNDKSN <mark>SEG</mark> TE <mark>G</mark> SSWKMTGDS <mark>F-RGRGGR</mark> GG	51
1	MEENWOTE LETEKPTY VPNFSTLETEN TDNY SAYSND IN AQVYDSERS FGNRGOY RSERS PSNFNRGSR	71
1	RSEEQAWMANS <mark>G</mark> RPNSPSLRFS <mark>S</mark> RPSSPLS <mark>G</mark> FPG <mark>R</mark> P	55
1	PVFEKDKYSS <mark>G</mark> AN <mark>GDTFN</mark> RTSASS- <mark>E</mark> MED G PS <mark>GR</mark> DMGDE DWEAE ILKPHVS <mark>S</mark> YVPVFEKDKYSS <mark>G</mark> AN <mark>GDTFN</mark> RTSASS- <mark>E</mark> MED G PS <mark>GR</mark> D	53
1	PifekDrySQen <mark>QDNFN</mark> RTPASSSEMDD <mark>G</mark> PSR <mark>R</mark> D	52
92	<mark>RCRRCGF</mark> TNSPS <mark>SDCDEDRNNANSWNNTOGERCG</mark> FRC <mark>RCGC</mark> GC <mark>RCRCFV-TD</mark> RSDFSGC	148
45	RG-RGGFK\$SYS\$GCDENDENSWNNA0GERCGFRGRGRGRGFGRTDQSEFNGD	96
45	RG_RCGFKSSTSSCODCNDEDKWNAAGERCGFRCRCGQCGGRCFCRVDQSEFNDD	99
45	RGRRGGFS8SFS8GCDEHGXGCDSWXX11GERDGFRGRGRORGRGFG-GXDCLEFGG	101
45	RC-CCCFKSADENEXCDDCGNNNAAGERCGLRCRCGRCRCRCFCRQFCRQ	97
52		105
72	IE KOKOKOFOLINAKUNI SSERDIV FODDEKTOVIKOF POROG	148
56	NSPFFGSQANOSLAAROLAKSLPVQHULIQISSSKESVIRPNEDQPVIRPQEXSSSKPQEXS	126
54		107
55		152
140		210
97		167
100	NORENOVEG	171
102		178
98		159
106	DOWKGESSG	212
149		237
127	POVODOGERVP-GIPOSKCENSERNS	199
168	SESDODOGTORGGGLEDSRKPAASDSGNGDTYQSRS, SGRGQVKGLNEEVVTGSGKNSWKSETEGGESSI SQCPKVTYTPPP	250
133	NDLDPDECMORTGGLFQSRRPVLSGTGNGDTSQSRSqSqSGSERGQVKGLNEEVITGSGKNSWKSEAFGGESSUTGCPKVTYIPPPP	217
	MotifI	
220	PEDEDSIFAHYESGINFNKYDDILVDVSGINPPOAINSFDEAALCESLRKNVSKSGVVKPTPV9KHGIPIISAGRDLMAGAQIGSGKTAAFLLPILØQLMIDGVAASQFSELQEPE	335
220 168	PEDEDS IFAHYESG INFNKYDDTLVD VSGTNP PQA INSFD EAALCESLRKNVSKSGVVKPTPVQKHG IPIIS AGRDLMAQAQTGSGKTAAFLLPILQQLMTDGVAASQFSELQEPE PEDEDT IFSHYESG INFDKYDDIMVD VSGTNP PQA VMTFD EAALCESLRKNVSKSGVVKPTPVQKHG IPIIS AGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASRFSELQEPE	335 283
220 168 172	PEDEDSIFAHYESGINFNKYDDTLVDVSGTNPPQAINSFDEAALCESLRKNVSKSGVVKPTPVQKHGIPIISAGRDLMAQAQTGSGKTAAFLLPILQQLMTDGVAASQFSELQEPE PEDEDTIFSHYESGINFDKYDDTMVDVSGTNPPQAVMTFDEAALCESLRKNVSKSGVVKPTPVQKHGIPIISAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASRFSELQEPE PEDEDSIFSHYETGINFDKYDDTMVDVSGTNPPQAVMTFDEAALCESLRKNVSKSGVVKPTPVQKHGIPIISAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASRFSELQEPE	335 283 287
220 168 172 179	PEDEDS IF AHYESG INFNKYDD I LYD VSGTNP POA INS FDEAALCESLRKNY SKSGYV KPTPVQKHG IP IIS AGRDLMAGAQTGSGKTAAFLLPILQQLMID GVAASQFSELQEPE PEDEDT IF SHYESG INFDKYDD INYD VSGTNP POA VMTFDEAALCESLRKNY SKSGYV KPTPVQKHG IP IIS AGRDLMAGAQTGSGKTAAFLLPILQQLMAD GVAASQFSELQEPE PEDEDS IF SHYETG INFDKYDD INYD VSGTNP POA VMTFDEAALCESLRKNY SKSGYV KPTPVQKHG IP IIS AGRDLMAGAQTGSGKTAAFLLPILQQLMAD GVAASQFSELQEPE PEDEDS IF SHYETG INFDKYDD INYD VSGTNP POA IMTFAEAALCESLRKNY SKSGYV KPTPVQKHG IP IIS AGRDLMAGAQTGSGKTAAFLLPILQQLMAD GVAASQFSELQEPE	335 283 287 294
220 168 172 179 160	PEDEDS IF AHYESG INFNKYDDI LVD VSGTNPPQA INSFDEAALCESLRKNVSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDT IFSHYESG INFDKYDDINND VSGTNPPQAVMTFDEAALCESLRKNVSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDED SIFSHYETG INFDKYDDINND VSGTNPPQAVMTFDEAALCESLRKNVSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDED SIFSHYETG INFDKYDDIINND VSGTNPPQAVMTFDEAALCESLRKNVSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDED SIFSHYETG INFDKYDDIIND VSGTNPPQAIMTFAEAALCESLRKNVSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIFAHYETG INFDKYDDIIND VSGTNPPQAIMTFAEAALCESLRKAVTKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE	335 283 287 294 275
220 168 172 179 160 213	PEDEDS IF AHVESCINFNKYDDI LVD VSGTNPPQA INSFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDI IFSHYESGINFDKYDDI MVD VSGTNPPQA VMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDI SIFSHYETGINFDKYDDI MVD VSGTNPPQA VMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDI SIFSHYESGINFNKYDDI IVD VSGTNPPQA IMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDI SIFSHYESGINFNKYDDI IVD VSGTNPQA IMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIFSHYESGINFNKYDDI IVD VSGTNPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIFSHYATGINFDKVDDI IVD VSGTNPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIFSHYATGINFDKVDDI IVD VSGTNPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIFSHYATGINFDKVDDI IVD VSGTNPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE	335 283 287 294 275 328
220 168 172 179 160 213 238	PEDEDS IF AHVESC INFNKYDDI LVD VSGTNPPQA INSFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDT IFS HVESG INFDKVDDI MVD VSGTNPPQA VMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IFS HVETG INFDKVDDI MVD VSGTNPPQA VMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IFS HVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IF AHVESG INFNKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES IF AHVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKNVSKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASSFSELQEPE PEDES IF AHVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASSFSELQEPE PEDES IF AHVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMTDGVAASSFSELQEPE PEDES IF AHVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQQLMTDGVAASSFSELQEPE PEDES IF AHVETG INFDKVDDI MVD VSGTNPPQA IMTFDEAALCE SLRKAVTKS GVV KPTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQU MTDGVAASSFSELQEPE PEDES IF AHVETG INFDKVDDI MVG VGKDVPPA ILLFEEAGLCD SLRKAVTASGVV KMTPVQKHG IP II SÄGRDLMAQAGTGSGKTAAFLLPILQU MTDGVAASSFSELQEPE PDGEDN FRQYÖSG INFDKVDE ILVD VTGKDVPPA ILLFEEANLCE TLRRVARAGVKLTPVQKHS IP I IMAGRDLMAQAGTGSGKTAAFLLPILSYMNEGITASQVLDLQEPE	 335 283 287 294 275 328 353
220 168 172 179 160 213 238 200	PEDEDS IF AHVESC INFNKYDDILVD VSGTNPPQA INSFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDT IF SHYESG INFDKYDDIMVD VSGTNPPQA VMI FDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IF SHYETG INFDKYDDIMVD VSGTNPPQA VMI FDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IF AHYESG INFDKYDDIMVD VSGTNPPQA VMI FDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IF AHYESG INFDKYDDIMVD VSGTNPPQA IMI FDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES IF AHYKTG INFDKYDDIMVD VSGTNPPQA IMI FDEAALCE SLRKAVTKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIF SHYATG INFDKYDDIMVD VSGTNPPQA IMI FDEAALCE SLRKAVTKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIF SHYATG INFDKYDDIMVD VSGTNPPQA IMI FDEAALCE SLRKAVTKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES SIF SHYATG INFDKYDDIMVD VSGTNPPA ILLFEEAGLCD SLRKAVTKS GYV KPTPVQKHG IP II SAGRDLMAQAQTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PDGEDNIFRQYGSG INFDKYDE ILVD VTGKDVPPA ILLFEEANLCE TIR RNV ARAGVVKLTPVQKHS IP I MAGRDLMAQAQTGSGKTAAFLLPILQV MANGGVTASAFQVLQEPE PEDEQ SIF ACYQSG INFDKYDE ILVD VTGKDVPPA ILLFEEANLCE TIR RNV ARAGVVKLTPVQKHS IP I MAGRDLMAQAQTGSGKTAAFLLPILGVTANKDGVTASAFQVLQEPE PEDEQ SIF ACYQSG INFDKYDE INFORMAGD FAGTAFAGTIRKNTSKT GYSKLTPVQKHS IP I MAGRDLMAQAQTGSGKTAAFLLPILGVTANKDGVTASAFQVQOEPQ	 335 283 287 294 275 328 353 315
220 168 172 179 160 213 238 200 251	PEDEDS IF A HYESG INFNKYDD I LVD VSGTNPPQA IMFFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDS IF SHYESG INFDKYDD IMVD VSGTNPPQA VMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDEDS IF SHYETG INFDKYDD IMVD VSGTNPPQA VMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDEDS IF AHYESG INFDKYDD IMVD VSGTNPPQA IMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDEDS IF AHYESG INFDKYDD IMVD VSGTNPPQA IMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDES SIF AHYKTG INFDKYDD IMVD VSGTNPPQA IMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDES SIF SHVATG INFDKYDD IMVD VSGTNPPQA IMIFDEAALCE SLRKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLMA DGVAASGFSELQEPE PEDES SIF SHVATG INFDKYDD IMVD VSGNPPKA IMIFEEAGLCD SLSKWYSKS GYV KPTPVQKHG IP II SAGRDLMACAQTGSGKTAAFLLPILQQLM DGVAASSFSELQEPE PDGEDN IF RQYGSG INFDKYDE ILVD VTGKDVPPA ILLFEEASILCE TLRRWA RAGVYKLTPVQKHS IP IMAGRDLMACAQTGSGKTAAFLLPILQVMANGGYTAASAFVSE IQEPE PEDEQS IF AFYYG SG INFDKYDE ILVD VTGKDVPPA ILLFEEASILCE TLRRWA RAGVYKLTPVQKHS IP IMAGRDLMACAQTGSGKTAAFLLPILGYMNDG IT ASGYT ASAFQXQ DEPE PEDEQS IF AFYG SG INFDKYDE ILVD VTGKDVPPA ILLFEEANLCE TLRRWA RAGVYKLTPVQKHS IP IMAGRDLMACAQTGSGKTAAFLLPILGYMNDG IT ASGYT ASAFQXQEPQ PEDEQS IF AFYG SG INFDKYDE ILVD VGGHDAPPA ILLFEEANLCE TLRRWA RAGVYKLTPVQKHS IP IV QAGRDLMACAQTGSGKTAAFLLPILGYMNDG IT ASGYT ASAFQXQEPQ PEDEDS SIFAHYG INFDKYDT ILVE VSGHDAPPA ILLFEEANLCE TLRRWA RAGVYKLTPVQKHS IP IV QAGRDLMACAQTGSGKTAAFLLPILAHMRDGIT ASGYT GASFKELQEPE	 335 283 287 294 275 328 353 315 366
220 168 172 179 160 213 238 200 251 218	PEDEDS IF ANY ESCINFNKYDDILVD VSGTNPPQA IMSFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMT BGVAASQFS ELQEPE PEDED TIFS HY ESCINFDKYDDINVD VSGTNPPQA VMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFS HY ETGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFS HY ETGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFAHYESGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDES SIFS HY ETGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINAGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDES SIFS HY TGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINGAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDES SIFS HY TGINFDKYDDINVD VSGTNPPQA IMTFDEAALCE SLRKNV SKS GYV KPTPVQKHG IP IIS AGRDINGAQTGSGKTAAFLLPILQRM DGVAASSFS ELQEPE PEDED SIFAGYØGS INFDKYDE TILVD VTGKDVPPA ILLFEEANLCE TILRNV ARA GYV KLTPVQKHS IP IIMAGRDINAGAQTGSGKTAAFLLPILQRM DGVASKFS ELQEPE PEDED SIFAGYØGS INFDKYDE CAVE MSGLDPPA ILLFEEANTAG TILKNIS TIG VSKLTPVQKHS IP VIQ AGRDINGAQTGSGKTAAFLLPILDING MAGNGGT ASAFCKOEPE PEDED SIFAGYØGS INFDKYDE GAVE MSGLDPPA ILLFEEANTAG TILKNIS TIG VSKLTPVQKHS IP VIQ AGRDINGAQTGSGKTAAFLLPILDFINNFGGT ASAFCKOEPE PEDED SIFAGYØGS INFDKYDE GAVE MSGLDPPAPILLFEEANTAG TILKNISTIG VSKLTPVQKHS IP VIQ AGRDINGAQTGSGKTAAFLLPILDFING SITASQYT DLOEPE PEDED SIFAGYØGS INFDKYDE GAVE MSGLDPPAPILLFEEANTAG TILKNISTIG VSKLTPVQKHS IP VIQ AGRDINGAQTGSGKTAAFLLPILAFLMDAGT IN SAFCKELQEPE PEDED SIFAGYØGS INFDKYDE GAVE MSGLDPAPA ILLFEEANTAG TILNN TAKAGYTKLTPVQKYS IP VLAGTGINGAQTGSGKTAAFLLPILAFLDFING TASAFCKELQEPE PEDED SIFAGYØGS INFDKYDE GAVEN SGCDAPPA ILLFEEANTER CONTINN TAKAGYTKLTPVQKYS IP VLAGTGINGAQTGSGKTAAFLLPILAFLEPID TASRFKELQEPE	 335 283 287 294 275 328 353 315 366 333
220 168 172 179 160 213 238 200 251 218	PEDEDS IF AHYESG INFNKYDD I LVD VSGTNP POA IMSFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMT BGVAASQFS ELQEPE PEDEDT IF SHYESG INFDKYDD INVD VSGTNP POA VMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASQFS ELQEPE PEDEDS IF SHYETG INFDKYDD INVD VSGTNP POA VMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASQFS ELQEPE PEDEDS IF AHYESG INFNKYDD ILVD VSGTNP POA IMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASGFS ELQEPE PEDEDS IF AHYESG INFNKYDD ILVD VSGTNP POA IMTFD EAALCESLRKAV KS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASGFS ELQEPE PEDES SIF SHY AG INFDKYDD ILVD VSGTNP POA IMTFD EAALCESLRKAV KS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASGFS ELQEPE PEDES SIF SHY AG INFDKYDD ILVD VSGTNP POA IMTFD EAALCESLRKAV KS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQQLMA DGVAASGFS ELQEPE PEDES SIF SHY AG INFDKYDD ILVD VSGTNP POA IMTFD EAALCESLRKAV KS GYV KPTPVQKHG IP II S AGRDLMAGAQTGSGKTAAFLLPILQSU MAGAYT SGKTAAFLLPILQE INFOX PEDES SIF SHY AG INFDKYDD ILVD VSGSNP PRA IMTFE EAALCE THRNN AR AGVV KLTPVQKHS IP II NS AGRDLMAGAQTGSGKTAAFLLPILQSU MAASGFSE IQEPE PEDES SIF SHY AG INFDKYDE ILVD YGKDVPPA ILLFFE EAALCE THRNN AR AGVV KLTPVQKHS IP VIQAGAQTGSGKTAAFLLPILQSUNFGGT ASGAFLOPEPE PEDEDS IF ACYGS GINFDKYDE CAVE MSGLDPPAPILLFE EAANLCE THRNN AR AGVV KLTPVQKHS IP VIQAGAQTGSGKTAAFLLPILD FUNKING O'T ASAFGKOEPEP PEDEDS IF ACYGS GINFDKYDE GAVE MSGLDPPAPILLFE EAANLCE THRNN AR AGVV KLTPVQKYS IP VIL AGRDLMAGAQTGSGKTAAFLLPILAHMARDGIT ASAFFKELQEPE PEDEDS IF ACYGS GINFDKYDE GAVE MSGLDPPAPILLT FE EAANLCE THRNN AR AGVY KLTPVQKYS IP VIL AGRDLMAGAQTGSGKTAAFLLPILAHMARDGIT ASAFFKELQEPE PEDEDS IF ACYGS GINFDKYDE GAVE MSGLDPAPA ILLFFE EAANLCE THNN IAK AGYT KLTPVQKYS IP VIL AGRDLMAGAQTGSGKTAAFLLPILAHMARDGIT ASAFFKELQEPE PEDEDS IF AFYG TG INFDKYD TILVE VSGHD APPA ILLFFE EAANLCE THNN IAK AGYT KLTPVQKYS IP VIL AGRDLMAGAQTGSGKTAAFLLPILAHMARDGIT ASAFFKELQEPE PEDEDS IF AFYG TG INFDKYD TILVE VSG	335 283 287 294 275 328 353 315 366 333
220 168 172 179 160 213 238 200 251 218	PEDEDS IF AHYESC INFNKYDD I LVD VSGTNP POA IMSFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASQFS ELQEPE PEDED TIF SHYESC INFDKYDD INND VSGTNP POA VMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASQFS ELQEPE PEDED SIF SHYETC INFDKYDD INND VSGTNP POA VMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDED SIF SHYETC INFDKYDD INND VSGTNP POA VMTFD EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDEDS IF AHYESC INFDKYDD INND VSGTNP POA IMTFA EAALCESLRKNV SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDEDS IF AHYESC INFDKYDD INND VSGTNP POA IMTFA EAALCESLRKAV TKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDEDS IF SHY AG INF FOXD IN D VSGTNP POA IMTFE EAGLCD SLK WY SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDES SIF SHY AG INF FOXD IN D VSGTNP POA IMTFE EAGLCD SLK WY SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDES SIF SHY AG INF FOXD IN D VSGTNP POA IMTFE EAGLCD SLK WY SKS GYV KPTPVQKHG IP II S AGRDLMAAQTGSGKTAAFLLPILQUMADGVAASGFS ELQEPE PEDES SIF SHY AG INFORVIDE ILVD YGGKDVPPA ILL FEEAGLCD SLK WY SKS GYV KLTPVQKHS IP II NAGRDLMAAQTGSGKTAAFLLPILAR FUT DCVAASKFS ELQEPE PEDEDS IF AHYG GINFDKYDE CLYD EXGLDPPAPILAFEEANLCE TIR RN ARAGYK KLTPVQKHS IP II QAGRDLMAAQTGSGKTAAFLLPILAHLMIKDCIT ASGFKELQEPE PEDEDS IF AHYG GINFDKYDE ILVD YGGNDPAPILAFEEANLCE TIR RN ARAGYK KLTPVQKYS IP IVL AGRDLMAAQTGSGKTAAFLLPILAHLMIKDCIT ASGFKELQEPE PEDEDS IF AHYG GINFDKYD TILVE VSGHD	335 283 287 294 275 328 353 315 366 333 IV
220 168 172 179 160 213 238 200 251 218 336 284	INTELL PEDEDS IF AHY ESC INFINIVUD I UV USGTNP POATING FD EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASQFS ELQEPE PEDED TIFSHYESG INFINIVUD USGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFSHYETG INFINIVUD USGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFSHYETG INFINIVUD UV USGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFSHYETG INFINIVUD IND VSGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFSHYATG INFINIVUD IND VSGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMADGVAASGFS ELQEPE PEDED SIFSHYATG INFINITION VSGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMT GGVAASGFS ELQEPE PEDED SIFSHYATG INFINITION VSGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP II SAGRDLMAAQTGSGKTAAFLLPILQQLMT GGVAASGFS ELQEPE PEDED SIFSHYATG INFINITION VSGTNP POATMIFED EAALCE SLRKNV SKS GVV KPTPVQKHG IP IS SGRDLMAAQTGSGKTAAFLLPILQQLMT GGVAASGFS ELQEPE PEDED SIFSHYATG INFOKYDD INFO VSGSNP RATUTFEEAALCE SLRKNV SKS GVV KPTPVQKHG IP IS SGRDLMAAQTGSGKTAAFLLPILQQLMT GGVAASGFS ELQEPE PEDEOS SIFARY GO INFOKYDE INTO TING VSGSNP RATUTFEEAALCE SLRKNV SKS GVV KTPPVQKHS IP II SGRDLMAAQTGSGKTAAFLLPILARTHNKGGT TASGFKELQEPE PEDEOS SIFARY G	335 283 287 294 275 328 353 315 366 333 IV 451 399
220 168 172 179 160 213 238 200 251 218 336 284	PEDEDS IF AHYESG INFNKYDDI LVD VSGTNPPQA IMSFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQUMD GYAASQFSELQEPE PEDEDTIFSHYESG INFDKYDDI MVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQUMD GYAASQFSELQEPE PEDEDTIFSHYESG INFDKYDDI MVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQUMD GYAASQFSELQEPE PEDEDSIFAHYESG INFDKYDDI MVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQUMD GYAASGFSELQEPE PEDEDSIFAHYESG INFDKYDDI MVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQUMD GYAASGFSELQEPE PEDESIFAHYESG INFDKYDDI MVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS SGRUMAQAGTGSGKTAAFLLPILQUMD GYAASSFSELQEPE PEDESIFAHYESG INFDKYDDI MVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS SGRUMAQAGTGSGKTAAFLLPILQUMD GYAASSFSELQEPE PEDESIFAHYESG INFDKYDDI MVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS SGRUMAQAGTGSGKTAAFLLPILQUMD GYAASSFSELQEPE PEDESIFAHYESG INFDKYDEILUD VTGKDVPPAILLFEEAALCET IRRWYKAR GYV KITPVQKHS IP IIN GRDLMAQAGTGSGKTAAFLLPILGYMNK GYTASSFSELQEPE PEDEOSIFACYGSG INFDKYDEILUD VTGKDVPAILLFEEAALCET IRRWYKAR GYV KITPVQKHS IP IIN GRDLMAQAGTGSGKTAAFLLPILGYMNK GYTASAFOKQUEPE PEDEOSIFACYGSG INFDKYDEILUD VTGKDVPAILLFEEAALCET IRRWYKAR GYV KITPVQKHS IP IV QAGDLMSQAGTGSGKTAAFLLPILGYMNK GYTASAFOKQUEPE PEDEOSIFACYGSG INFDKYDTTILVEVSGHDAPPAILAFEEAALCET IRRWYKAR GYK KITPVQKYS IP IV LGGDLMAQAGTGSGKTAAFLLPILAHMARGGT ASFRKELQEPE PEDEOSIFACYGSG INFDKYDTTILVE VSGHDAPPAILTFEEAALCET IRRWYKAR GYT KITPVQKYS IP IVL AGRDLMAQAGTGSGKTAAFLLPILAHMARGGT ASFRKELQEPE PEDEDSIFAHYGTG INFDKYDTTILVE VSGHDAPPAILTFEEAALCET INN TAKAGYT KITPVQKYS IP IIL AGRDLMAQAGTGSGKTAAFLLPILAHMARGGT ASFRKELQEPE MOOTIF IA MOOTIF ID MOOTIF INFORMANG GYT GRGWNGU KYSTUPVQKYS IP IIL AGRDLMAQAGTGSGKTAAFLLPILAHMARGTASFRKELQEPE PEDEDSIFAHYGTG INFDKYDTTILVE VSGHDAPPAILTFEEANLCOTINN TAKAGYT KITPVQKYS IP IIL AGRDLMAQAGTGSGKTAAFLLPILAHMARGTASFRKELQEPE PEDEDSIFAHYGTG INFDKYDTIVYGYST HOUTIFVGGYST HOUTINN TAKAGYT KITPVQKYS IP IIL GRDLMACA	335 283 287 294 275 328 353 315 366 333 IV 451 399 403
220 168 172 179 160 213 238 200 251 218 336 284 288 295	PEDEDS IF AHYESG INFNKYDDILVD VSGTNPPQA IMSFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGGSGKTAAFLLPILQQLMI DGVAASQFSELQEPE PEDEDI IFSHYESG INFDKYDDINVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IFSHYETG INFDKYDDINVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IFSHYETG INFDKYDDINVD VSGTNPPQAVMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDEDS IFSHYETG INFDKYDDINVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES IFSHYETG INFDKYDDINVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS SGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES IFSHYATG INFDKYDDINVD VSGTNPPQAIMTFDEAALCESLRKWYSKS GYV KPTPVQKHG IP IIS AGRDLMAQAGTGSGKTAAFLLPILQQLMADGVAASGFSELQEPE PEDES IFSHYATG INFDKYDEILVD VTGKDVPPAILTFEEANLCE IIRKWARAGVY KLTPVQKHS IP IIN GODLMAQAGTGSGKTAAFLLPILSYMINEGITASGYL GLGEPE PEDEDS IFSHYATG INFDKYDEILVD VTGKDVPPAILTFEEANLCE TIRKWARAGVY KLTPVQKHS IP IVQ GODLMSQAGTGSGKTAAFLLPILSYMINEGITASGYL GLGEPE PEDEDS IFAHYGTG INFDKYDEILVD VTGKDVPPAILTFEEANLCE TIRKWARAGVY KLTPVQKHS IP IVQ GODLMSQAGTGSGKTAAFLLPILSYMINEGITASGYL GLGEPE PEDEDS IFAHYGTG INFDKYDEILVD VTGKDVPAILTFEEANLCE TIRKWARAGVY KLTPVQKHS IP IVQ GAGDLMSQAGTGSGKTAAFLLPILSYMINEGITASGYL GLGEPE PEDEDS IFAHYGTG INFDKYDTILVE VSGHDAPPAILTFEEANLCE TIRKWARAGYTKLTPVQKYS IP IVQ GAGDLMSQAGTGSGKTAAFLLPILAHMINGGTASRFKELQEPE PEDEDS IFAHYGTG INFDKYDTILVE VSGHDAPPAILTFEEANLCO TLNNTIAKAGYTKLTPVQKYS IP IIL GCDLMAQAGTGSGKTAAFLLPILAHMINGGTASRFKELQEPE PEDEDS IFAHYGTG INFDKYDTILVE VSGHDAPPAILTFEEANLCO TLNNTIAKAGYTKLTPVQKYS IP IIL GCDLMAQAGTGSGKTAAFLLPILAHMINHGITASRFKELQEPE PEDEDS IFAHYGTG INFDKYDTILVEVSGHDAPATITFEEANLCO TLNNTIAKAGYTKLTPVQKYS IP IIL GCDLMAQAGTGSGKTAAFLLPILAHMINHGITASRFKELQEPE PEDEDS IFAHYGTG INFDKYDTIVVSGVSTCHQIREICRGCVVCCTFGRILDVIG GRGKVALVKLRYIVIDEADRULDMGFEDMRIKVSSFGVPSKENRGTLMFSAT AIVAPTREILINQI VLEARKFAFGTCVRPVVVSGVSTCHQIREICRGCVVCCTFGRILDVIG GRG	335 283 287 294 275 328 353 315 366 333 IV 451 399 403 410
220 168 172 179 160 213 238 200 251 218 336 284 288 295 276	PEDEDS IF ALVESS INFORVED ILVD VSGTNP POALMS FD EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS OF SELOEPP PEDED TIF SHYESG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS OF SELOEPP PEDED SIF SHYETG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS GRS ELOEPP PEDED SIF SHYETG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS GRS ELOEPP PEDED SIF AVYTG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS GRS ELOEPP PEDES SIF SHYETG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS GRS ELOEPP PEDES SIF SHYETG INFORVED INVED VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS SFS ELOEPP PEDES SIF SHYETG INFORVED INVERTIONE VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS SFS ELOEPP PEDES SIF SHYETG INFORVED INVERTIONE VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS SFS ELOEPP PEDES SIF SHYETG INFORVED INVERTIONE VSGTNP POALMIFED EAALCE SLRKNV SKS GYV KPTPVOKHG IP ILS AGREDUNA AQTGSGKTAAFLLPILOOLMED VAAS SFS ELOEPP PEDED SIF ACYOS GINF BKYDE ILV DUT SKRDV PAALLEFE EANFAOTLEKINIS SKT GYS KLTPVOKHS IP VID AGREDUNG AQTGSGKTAAFLLPILOV MAS KEN ENGLOPPE PEDED SIF ACYOS GINF BKYDE CAVE MSGLD PAPA ILL FE EANFAOTLEKINIS SKT GYS KLTPVOKHS IP VID AGREDUNG AQTGSGKTAAFLLPILOV MAN BOGT AS AFRKELOEPPE PEDED SIF ACYOS GINF BKYDE GINF BKYDE GYS GHAPPA ILL FE EANFAOTLECT TINN TAKAGYT KLTPVOKYS IP IVL AGREDUNG AQTGSGKTAAFLLPILOV MAN BOGT AS AFRKELOEPPE PEDED SIF AFYOTG INFORVID GINF GKYD GYS GHAPPA ILL FE EANFAOTLECT TINN TAKAGYT KLTPVOKYS IP IIL GREDUNG AQTGSGKTAAFLLPILOV MAN BOGT AS AFRKELOEPPE PEDED SIF AFYOTG INFORVID GYS GYNEN GYS GYS GHOTE IC GCONVLC OT GRILDVIG GREVALNKLRVI	335 283 287 294 275 328 353 315 366 333 IV 451 399 403 410 391
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Larimichthys crocea (79.5%) proteins, while the lowest identity was found with Rcvasa amino acid sequence in *Danio rerio* (62.6%) as shown in Figure 1.

Figure 1. Cont.

	Motif V M	otif VI	
568	AARGLD <mark>I</mark> P <mark>D</mark> VQHVVNFDLP <mark>N</mark> N <mark>IDE</mark> YVH	RIGRTGR <mark>CGNTGRAV</mark> SFYDPDAD <mark>GQL<mark>ARS</mark>LVTILSKA<mark>QQEV</mark>PSWLEECAFSG</mark> PGS <mark>SGVNP</mark> PRRTFASTDSRKGPH <mark>GGSFQD</mark> SI	677
516	AARGLD <mark>IPDVQHVVNFDLPN</mark> N <mark>IDE</mark> YVH	RIGRTGR <mark>CGNT</mark> GRA <mark>v</mark> SFYDPDA <mark>dgqlarSlv</mark> Tvlska <mark>qqe</mark> vPS <mark>wlee</mark> S <mark>AFSg</mark> PAV <mark>T</mark> SF <mark>NP</mark> SRKT <mark>FASTDSRK</mark> G <mark>GSFq</mark> DNS	622
520	A <mark>A</mark> RGLD <mark>I</mark> PDVQHVVNFDLPNN <mark>IDE</mark> YVH	RIGRTGRCGNTGRANSFYDPDADGQL <mark>ARSLVTVLSKAQQEVPSWLEE</mark> SAFSGPAT <mark>T</mark> GFNPPRKNFASTDSRKRGSFQDNS	626
527	A <mark>A</mark> RGLD <mark>I</mark> PDVQHVVNFDLP <mark>N</mark> NIDEYVH	RIGRTGRCGN <mark>T</mark> GRANSFYDPDNDGQLAGSLVSILSKAQQEVPSWLEECVF <mark>SG</mark> <mark>S</mark> GVNPSRRTFASTDSRKGPQ <mark>GSSF</mark> QDSS	633
508	AARGLDIPDVQHVVNFDLPNNIDEYVH	RIGRTGRCGNIGRANSFYDPDADGQLARSLVTILSKAQQEVPSWLEESAFSGPGATGFNPSMRNFATSDSRKGQHGGSFQDNG	617
561	AARGLDIEQVQHVVNFDMPSSIDEYVH	RIGRTGRCGNTGRAVSFFNPESDTPLARSLVKVLSGAQQVVPKWLEEVAFSAHGT <mark>T</mark> GFNPRGKVFASTDSRKGGSFKSDEPPP	670
586	AARGLDIENVQHVINYDVPKEVDEYVH	RIGRTGRCGNTGKATSFFNVQDDHVIARPLVKILTDAHQEVPAWLEEIAFGGHGALNSTYAADSMGEQAGGNAVTTP	689
548	ASRGLDIENVQHVINFDLPNTIEDYVH	RIGRIGREGNIGRAVSFFDDQSDGHLVQSLLKVLSEAQQEVPVWLEEMAVQRINIVASLGAQRNNAGRGRMNPR	648
599	AARGLDIENVQHVINFDLPSTIDEYVH	(IGRIGREGNIGRAISFEDIDSDNHEAQPEVKVESDAQQDVFAWLEEIAFSTYVPPSFSSSTRGGAVFASVDIRKNYQGKHIENIA	711
566	AAKGLUIENVQHVINFULPSIIDEYVH	<pre>(IGKIGKCGNIGKAISFFDLESDNHLAQPLVKVLIDAQQDVPAWLEEIAFSIYIP=GFSGSIKG=NVFASVDIKK===GKSILNIA===</pre>	6/3
	length	aa species identity	
678	-VR <mark>T</mark> QQSALT <mark>AADD</mark> -EE <mark>WE</mark> : 694	aa species identity Rachycentron canadum 100%	
678 623	length -VRTQQSALTAADD-EEWE : 694 -VKSQPAVHTAADDEEEWE : 640	aa species identity Rachycentron canadum 100% Euthynnus affinis 82.6%	
678 623 627	length –VRTQQSALTAADD–EEWE : 694 –VKSQPAVHTAADDEEEWE : 640 –VKSQPAVQTAADDDEEWE : 644	aa species identity Rachycentron canadum 100% Euthynnus affinis 82.6% Thunnus maccoyii 84.4%	
678 623 627 634	length –VRTQQSALTAADD–EEWE : 694 –VKSQPAVHTAADDEEEWE : 640 –VKSQPAVQTAADDDEEWE : 644 –MTSQPAVPAAADN–EDWE : 650	laa species identity Rachycentron canadum 100% Euthynnus affinis 82.6% Thunnus maccoyii 84.4% Seriola quinqueradiata 86.0%	
678 623 627 634 618	length -VRTQQSALTAADD-EEVE : 694 -VKSQPAVHTAADDEEEVE : 640 -VKSQPAVQTAADDDEEVE : 644 -MTSQPAVPAAADN-EDVE : 650 -VTSQPAAQAAADD-DDVE : 634	aaspeciesidentityRachycentron canadum100%Euthynnus affinis82.6%Thunnus maccoyii84.4%Seriola quinqueradiata86.0%Larimichthys crocea79.5%	
678 623 627 634 618 671	length -VRTQQSALTAADD-EEWE : 694 -VKSQPAVHTAADDEEEWE : 640 -VKSQPAVQTAADDDEEWE : 644 -MTSQPAVPAAADA-EDWE : 654 SQISAPSAAAAADD-EEWE : 688	'aaspeciesidentityRachycentron canadum100%Euthynnus affinis82.6%Thunnus maccoyii84.4%Seriola quinqueradiata86.0%Larimichthys crocea79.5%Danio rerio62.6%	
678 623 627 634 618 671 690	length -VRTQQSALTAADD-EEVE : 694 -VKSQPAVHTAADDEEEVE : 640 -VKSQPAVQTAADDDEEVE : 644 -MTSQPAVPAAADD-EDVE : 654 -VTSQPAAQAAADD-DDVE : 634 SQISAPSAAAAADD-EEVE : 688 SFAQEEEASVD : 700	'aaspeciesidentityRachycentron canadum100%Euthynnus affinis82.6%Thunnus maccoyii84.4%Seriola quinqueradiata86.0%Larimichhys crocea79.5%Danio rerio62.6%Xenopus laevis48.7%	
678 623 627 634 618 671 690 649	length -VRTQQSALTAADD-EEVE : 694 -VKSQPAVHTAADDEEEVE : 640 -VKSQPAVQTAADDDEEVE : 644 -MTSQPAVPAAAAAD-EDVE : 650 -VTSQPAAQAAAADD-EDVE : 654 SQTSAPSAAAAADD-EDVE : 688 SFAQEEEASVD : 700 EURMSYSETTFKSVE : 663	'aaspeciesidentityRachycentron canadum100%Euthynnus affinis82.6%Thunnus maccoyii84.4%Seriola quinqueradiata86.0%Larimichthys crocea79.5%Danio rerio62.6%Xenopus laevis48.7%Gallus gallus49.1%	
678 623 627 634 618 671 690 649 712	length -VRTQQSALTAADD-EEVE : 694 -VKSQPAVHTAADDEEEVE : 640 -VKSQPAVQTAADDDEEVE : 644 -MTSQPAVPAAAADD-EDVE : 650 -VTSQPAAQAAADD-DDVE : 654 SQTSAPAAAAADD-EDVE : 658SFAQEEEASWD : 700EMRMSYSETTFKSVE : 663SSFAQEEASWD : 728 CEDSCRUPTUPDESWD	'aaspeciesidentityRachycentron canadum100%Euthynnus affinis82.6%Thumus maccoyii84.4%Seriola quinqueradiata86.0%Larimichthys crocea79.5%Danio rerio62.6%Xenopus laevis48.7%Gallus gallus49.1%Mus musculus52.7%	

Figure 1. Multiple alignment of *Rcvasa* deduced amino acid sequences. The framed regions indicate the eight conserved functional motifs.

Based on the genetic distances calculated with the Poisson correction model, a phylogenetic tree was constructed by the Neighbor-Joining method to investigate the phylogenetic relationship among different species (Figure 2). The results showed that all the teleost species fall into a lineage, the higher vertebrates, including amphibians, birds, and mammals, fall into another lineage, and *Drosophila melanogaster* forms a single cluster. The teleost cluster comprises 14 species, 7 of which are from the Perciformes order, 3 from the Scombriformes order, and 1 each from the Pleuronectiformes, Scorpaeniformes, Cyprinodontiformes, and Cypriformes orders. Moreover, RcVasa clusters together with *Seriola quinqueradiata* Vasa.



Figure 2. Phylogenetic tree of vasa amino acid sequences based on the Neighbor-Joining (NJ) method. The tree is based on a 1000 bootstrap procedure; the scale bar (0.05 in terms of genetic distance) is indicated below the tree; the asterisk indicates the target species in this study.

3.2. Tissue Distribution Patterns of Rcvasa mRNA

The tissue distribution patterns of *Rcvasa* mRNA in 13 tissues was analyzed by semiquantitative RT-PCR. The results showed that *Rcvasa* was exclusively expressed in the ovary and testis, whereas almost no expression was detected in other somatic tissues (Figure 3).



Figure 3. *Rcvasa* mRNA expression in various tissues of cobia. cDNA from various tissues (liver, spleen, kidney, brain, heart, gill, testis, ovary, stomach, intestines, muscle, skin, and eye) was used. β -*actin* was used as an internal control. MK: DNA marker; control: negative control without cDNA template.

3.3. Expression Patterns of Rcvasa mRNA at Different Gonadal Development Stages

Based on histological observation of gonadal differentiation, the testes collected from 90-to-360 dph cobia could be divided into four stages (stage II, III, IV, V). The testes were in the spermatocyte growth stage (stage II) at 90 dph and in stage II–III at 120 dph. Testes at 150 dph and 185 dph were in the spermatocyte mature stage (stage III), while testes at 210 dph and 360 dph were in the spermatid metamorphosis stage (stage IV) and sperm mature stage (stage V), respectively. The first annual ovarian development of cobia was divided into three stages (Stage I, II, III). The ovaries were in the oogonium proliferation-stage (stage I) at 90 dph and developed to stage I–II at 120 dph. Ovaries at 150 dph, 185 dph and 210 dph were in the oocyte primary growth stage (stage II) and entered the oocyte cortical alveolus stage (stage III) at 360 dph.

The expression of *Rcvasa* mRNA was detected in the gonads of both male and female cobia throughout the first annual development. In the testis, the expression level of *Rcvasa* increased significantly after 120 dph; the expression levels at 185 dph (stage III), 210 dph (Stage IV), and 360 dph (Stage V) showed no significant differences. The expression level at 90 dph (Stage II) was the lowest; it then increased significantly, reaching the maximum at 210 dph (Stage IV), which was 2.78 times that at 90 dph (Figure 4).

During the first annual ovarian development, the expression levels of *Rcvasa* first increased and then decreased. The lowest expression level was detected at 90 dph (stage I). The relative expression reached the maximum at 210 dph (stage II), and its value was about 4.97 times that at 90 dph, while the relative expression at 360 dph (stage III) was significantly lower (Figure 4).



Figure 4. *Rcvasa* mRNA expression patterns during the first annual gonadal development of cobia. The results are presented as the mean \pm SD (n = 3), and the values with different letters (a–d) appeared significantly different in pairwise comparisons (p < 0.05).

3.4. Localization of Rcvasa mRNA in Germ Cells during Gametogenesis

The localization of *Rcvasa* mRNA in the gonads at different developmental stages was investigated by CISH. *Rcvasa* mRNA signals were restricted to germ cells and were not detected in somatic cells. In the testis of 120 dph cobia, *Rcvasa* mRNA was predominantly detected in the periphery of spermatogonia, primary spermatocytes, and secondary spermatocytes. The expression level of *Rcvasa* was strong in spermatogonia and scarcely detected in spermatids (Figure 5A1). Similar expression patterns were observed in the testis at 210 dph, when *Rcvasa* mRNA signals were also mainly distributed in the periphery of spermatogonia and in spermatocytes (Figure 5B1). In the testis at 360 dph, *Rcvasa* mRNA signal was detected in all germ cells throughout spermatogenesis and were concentrated in spermatocytes; it appeared significantly weaker in spermatids and spermatozoa (Figure 5C1).



Figure 5. Cont.



Figure 5. Distribution of *Rcvasa* mRNA at different developmental stages in cobia gonads, analyzed by CISH. (**A1,B1,C1**), sections of testes at 120 dph, 210 dph, and 360 dph were hybridized with an *Rcvasa* oligonucleotides probe; (**A2,B2,C2**), sections of ovaries at 120 dph, 210 dph, and 360 dph were hybridized with an *Rcvasa* oligonucleotides probe. (**D1,D2**), negative control, sections of testes and ovaries at 210 dph, hybridized with a sense probe. SG: spermatogonia; PSC: primary spermatocyte; SSC: secondary spermatocyte; ST: spermatid; SP: spermatozoa; OG: oogonium (chromatin nucleolar oocyte); I: oocyte at stage I (perinucleolar oocyte); II: oocyte at stage II (vitellogenic oocyte).

During ovarian development, *Rcvasa* mRNA was specifically expressed in germ cells and evenly distributed in the cytoplasm and nucleoli. In the ovary of 120 dph cobia, *Rcvasa* mRNA was localized in oogonium (chromatin nucleolar stage), stage I and II oocytes (perinucleolar and previtellogenic stage) (Figure 5A2). In the ovary at 210 dph, *Rcvasa* mRNA signal was concentrated in the cytoplasm and nucleoli of stage II oocytes (previtellogenic stage); with the accumulation of oocyte protoplasm, the signals gradually became stronger (Figure 5B2). In the ovary at 360 dph, *Rcvasa* mRNA signal detected in the cytoplasm of stage III oocytes (vitellogenic stage) was significantly weakened (Figure 5C2).

4. Discussion

The present study reported the isolation and characterization of cobia *vasa* homologue. The deduced amino acid sequence of the protein encoded by *Rcvasa* contains eight conserved motifs of the DEAD protein family [27]. RcVasa showed an appreciable identity to the Vasa protein from other marine teleosts, as well as to the Vasa homologue of *Seriola quinqueradiata* (86.0%). In addition, a glycine-rich region in the N-terminal region of RcVasa containing 10 arginine–glycine (RG) repeats and 7 arginine–glycine–glycine (RGG) repeats was observed. This glycine-rich region is regarded as a characteristic of single-stranded nucleic acid-binding proteins, such as RNA helicase, which may regulate the activity of different *vasa* transcripts [28]. Phylogenetic analysis revealed that RcVasa closely aligned to its teleost counterparts. These findings illustrate that the *vasa* gene has been highly conserved in the evolution of teleost, and the conserved motifs of the Vasa protein potentially play an important role in sustaining protein structure and function [29].

The tissue distribution patterns of *vasa* mRNA in 150 dph juvenile cobia showed that *Rcvasa* was specifically and abundantly expressed in testis and ovary. Previous reports

also showed that *Rcvasa* is a gonad-specific gene and may be involved in the regulation of gonadal development [19,20,30,31].

The expression of *Rcvasa* increased significantly when the testis developed from stage I to III, but no significant difference was detected after the late stage III. It has been reported that in the early stage of the testicular development of fish, spermatogonia undergo mitosis and gradually transforms into primary spermatocytes; then, primary spermatocytes divide into secondary spermatocytes [32]. As a result, the expression pattern of *Rcvasa* is positively correlated with the number of spermatocytes, suggesting that Rcvasa might play an important role in the regulation of spermatogenesis. Previous studies have reported that the expression levels of catfish (*Clarias gariepinus*) vasa gradually increased during testis development from stage I to VI and decreased significantly after stage V [33]. Mu et al. found that the expression of Korean rockfish *vasa* maintained a high level in testicular stages I to III, but *vasa* expression decreased significantly when spermatids started to mature [31]. These results indicate that there are significant interspecies differences in the expression patterns of vasa during the testicular development of teleost. The expression levels of Rcvasa in the early stage of ovarian development tended to increase gradually. As the ovaries developed to stage II, the oocytes became larger by accumulating protoplasm consisting of carbohydrates, proteins, and nucleic acids [32], and the expression of Rcvasa significantly increased. The oocytes began to produce egg yolk in ovarian stage III, and the expression of Rcvasa decreased significantly. These results suggest that Rcvasa might participate in the accumulation of oocyte protoplasm and in the formation of ovum yolk.

Furthermore, Rcvasa mRNA was exclusively expressed in germ cells and not in somatic cells. During testicular development, Rcvasa mRNA signal was mainly concentrated in the periphery of spermatogonia, primary spermatocytes, and secondary spermatocytes, and it was found to be weak in spermatids and spermatozoa. Similar results were reported in other fish [34,35]. The localization of Revasa mRNA during spermatogenesis suggested that this protein might play a major role in the transformation of spermatogonia into primary spermatocytes, but not in the development and maturation of spermatids. As reported, bluefin tuna vasa mRNA was only expressed in spermatogonia and not in other testicular cells [19]. This indicated that vasa might have a species-specific role in regulating the transformation of spermatogenic cells. The expression pattern of *Rcvasa* during oogenesis was similar to those observed in several teleost species [3,18,36] and revealed that Rcvasa mRNA was abundant in early primary oocytes (stage I and II, perinucleolar and previtellogenic stage) and deficient in yolk vesicle (cortical alveolus) formation-stage oocytes (stage III, vitellogenic stage). These findings indicate that vasa might regulate oocyte development during the early stage of oogenesis. As for gilthead sea bream (Sparus aurata), the vasa mRNA level was low in early primary oocytes and high when the oocytes developed to the mature gametes [37]. According to a report in tilapia, vitellogenesis was usually accompanied by the accumulation of vasa maternal factors, showing that vasa might be involved in regulating the maturation of oocytes [15]. However, a weak *Rcvasa* mRNA signal was detected in stage III oocytes. The reason for this might be related to the fact that the total protein content of these maturating oocytes increased, and as a result, the relative concentration of Rcvasa mRNA was diluted. Similar to the expression patterns of vasa during testicular development, a certain interspecies difference was also found in the regulation of vasa expression during oogenesis.

5. Conclusions

The *vasa* gene from cobia was cloned and characterized, and further investigation of the expression level and localization of *Rcvasa* mRNA during the first annual gonadal development was performed. The high and exclusive expression of *Rcvasa* mRNA in germ cells indicates the important role of *vasa* in spermatogenesis and oogenesis in this important aquaculture species. The gradually increasing mRNA level along with the growth and development of cobia also provides a basis for clarifying the mechanisms underlying the migration and differentiation of PGCs in teleosts.

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Conflicts of Interest: The authors declare no conflict of interest.

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