Molecular cloning, characterization, and expression profiles of androgen receptors in spotted scat (*Scatophagus argus*)

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ABSTRACT. Androgen plays critical roles in vertebrate reproductive systems via androgen receptors (ARs). In the present study, the full-length spotted scat (*Scatophagus argus*) androgen receptor (sAR) cDNA sequence was cloned from testis. The sAR cDNA measured 2448 bp in length with an open-reading frame of 2289 bp, encoding 763 amino acids. Amino acid alignment analyses showed that the sARs exhibited highly evolutionary conserved functional domains. Phylogenetically, the sARs clustered within the ARβ common vertebrate group. Real-time polymerase chain reaction (RT-PCR) revealed that sAR expression varied in level and distribution throughout the tissues of both females and males. sAR expression was detected during testicular development by quantitative RT-PCR. The results showed that the highest transcription of sARs was observed in the mid-testicular stage, and remained at a high expression level until the late-testicular stage. In addition, the effects of 17α-methyltestosterone (MT) and estrogen (E_2) on the expression of sARs in ovaries were determined using quantitative RT-PCR. sAR expression increased at 12 and 24 h post-

MT treatment and decreased with E_2 treatment. The present study provides preliminary evidence indicating gonadal plasticity of spotted scat under exogenous steroidal hormone treatments. It also provides a theoretical basis for sex reversal and production of artificial pseudo-males for female monosex breeding.

Key words: *Sarcophagus argus*; Androgen receptor; Steroid hormones; Testicular development

INTRODUCTION

Androgen is well known for its important roles in controlling reproduction by binding and activating androgen receptors (ARs). These receptors belong to the nuclear receptor superfamily (Apostolinas et al., 1999) and share a similar structure exhibiting three typical functional domains; the transactivation domain (TAD), the DNA binding domain (DBD), and the ligand binding domain (LBD). The TAD shows low-sequence identity, while the DBD is highly conserved among different species. The LBD contains conserved binding sites for ligands including testosterone (T), 11-ketotestosterone (11-KT), methyldihydrotestosterone (MDHT), 17α-methyltestosterone (MT), and other pharmaceutical androgens (Brinkmann et al., 1999). As ligand-activated transcription factors, ARs mainly exert their genomic influences by binding to androgen-response elements to promote or repress the transcription of target genes.

To date, ARs have been identified and characterized in many fish species. In most fish, two AR subtypes (ARα and ARβ) have been identified. These subtypes have been found in various species, including rainbow trout (*Oncorhynchus mykiss*) (Takeo and Yamashita, 1999), kelp bass (*Paralabrax clathratus*) (Sperry and Thomas, 1999), Japanese eel (*Anguilla japonica*) (Ikeuchi et al., 1999), Nile tilapia (*Oreochromis niloticus*) (Todo et al., 1999), Atlantic croaker (*Micropogonias undulatus*) (Sperry and Thomas, 2000), and the western mosquitofish (*Gambusia affinis*) (Ogino et al., 2004). However, this is not true for all teleosts. For example, there is only one AR subtype in goldfish (Pasmanik and Callard, 1988), fathead minnow (*Pimephales promelas*) (Wilson et al., 2004), sea bass (*Dicentrarchus labrax*) (Blázquez and Piferrer, 2005), zebrafish (Jørgensen et al., 2007), *Spinibarbus denticulatus* (Liu et al., 2009), and orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010). This indicates a difference in genome duplication between fishes. During evolution, one of the duplicated AR genes might have been lost in a lineage-specific manner (Thornton, 2001).

Androgen is the key steroid hormone for both male and female reproduction (Yeh et al., 2003; Chakraborty et al., 2009). In *S. denticulatus*, AR expression was detected during testicular and ovarian recrudescence. AR mRNA expression in *S. denticulatus* was elevated during the early-recrudescing phase, and then decreased significantly at the late- and full-recrudescing stages (Liu et al., 2009). However, a weak transcript signal was also observed in the ovary, suggesting a potential AR function during early-recrudescing testicular development (Liu et al., 2009). In zebrafish, AR expression increased gradually, and reached a peak at late-testicular development stages (Jørgensen et al., 2007). A similar trend was observed in orange-spotted grouper (Shi et al., 2010). These reports suggest important AR roles in testicular development. ARs act as mediators in the androgen signal pathway and have been demonstrated to be affected by steroid hormones (e.g., androgen and estrogen). A number of studies have shown that exogenous androgens such as MT, MDHT, 11-KT, and T could promote AR expression in teleosts (He et al., 2003; Hossain et al., 2008; Shi et al., 2010; Gao et al., 2015), and play a role in the sex reversal process. However,

estrogen (E_2) potentially also increases AR expression in Atlantic croaker (Sperry and Thomas, 2000) and black porgy (He et al., 2003). Therefore, the physiological role of steroids in AR expression regulation is still unclear, and needs further investigation.

The spotted scat, *Scatophagus argus*, an euryhaline subtropical fish, is widely cultured in Indo-Pacific waters (Barry et al., 1993). The sexes differ markedly in growth rate: at 1 year of age, the growth rate of female fish is two times greater than that of males (Sivan and Radhakrishnan, 2011). For this reason, female monosex farming has been suggested to be beneficial in this species. However, there are few studies on the sex reversal of *S. argus*. At present, sex reversal induced by exogenous steroid hormones is considered as an effective method that has been widely used in many fishes (He et al., 2003; Shi et al., 2010; Rivero-Wendt et al., 2013a; Gao et al., 2015). As the key factor mediating the effect of androgens, ARs have been shown to be involved in female to male sex change. Therefore, it is necessary to obtain further information concerning the effects of exogenous steroids on ARs in spotted scat (sARs). In the present study, full-length sAR cDNA sequences were cloned, followed by sequence characterization and tissue distribution analyses. Furthermore, the expression profiles of gonadal sARs during testicular development and sAR transcription levels in ovaries treated with MT and E_2 were determined.

MATERIAL AND METHODS

Animal husbandry and tissue collection

Spotted scat was obtained from Zhuhai Yucheng Fry Cultivation Base (Zhuhai, Guangdong, China). Fish were reared at temperatures of 24.5°-29.2°C in indoor tanks. Fish were measured and sacrificed by decapitation following MS222 anesthetization. Their fresh tissues such as brain, pituitary, liver, gonad, kidney, heart, intestine, spleen, gill, and muscle were obtained and put in liquid nitrogen immediately after euthanasia and subsequently stored at -80°C. In order to confirm the gonadal stage, a piece of gonadal tissue from each fish was fixed in Bouin's fluid for histology. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Guangdong Ocean University.

sAR cloning and sequence analysis

Total RNA from adult *S. argus* (body weight ca. 250 g) testes was prepared using Trizol reagent (Life Technologies, Carlsbad, CA, USA). The concentration of total RNA was measured using UV-spectrophotometry (Nanodrop 2000c, Thermo Scientific, Wilmington, DE, USA) and the quality was tested using $OD_{260/280}$ (1.8-2.0) and 0.8% agarose gel with ethidium bromide (EB) (the 28S and 18S ribosomal RNA bands were clear and without smears). The first-strand cDNA was synthesized using the SMART-RACE cDNA Amplification Kit (Clontech, Takara, Shiga, Japan). Partial sARs were obtained using degenerate primer pairs and the full-length cDNA was cloned using the RACE-cloning methods following the SMART-RACE protocol. All primers used in this study are listed in Table 1. The polymerase chain reaction (PCR) program was as follows: 2 min at 94°C; 40 cycles of 30 s at 94°C, 1 min at 55°-58°C, 1 min at 72°C; and 10 min at 72°C for adenylation in the Bio-Rad thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR amplification products were examined on 1.5% EB-stained agarose gel. The potential bands were collected and purified using the E.Z.N.A. Gel Extraction Kit (Omega, Guangzhou, China) and subcloned into the pTZ57R/T-cloned vector (Thermo Scientific). Positive clones were sequenced by BGI Company (Shenzhen, China).

Table 1. Sequences of PCR primers used.

Gene	Purpose	Primer	5' to 3' sequence
AR	Partail cDNA PCR	AR-F1	GAGCACATGGATCCGGACAC
		AR-R1	TCCTYCTACTTRTGRAACAAGAT
	5'-RACE PCR	AR-5R1 (first)	GCTYTGCASAGCTCYCTGGCTGT
		AR-5R2 (nest)	GCTGTYTCTGAGATTGTGGCGCA
	3'-RACE PCR	AF-3F1 (first)	GAACATCGGATGCACATATCCAC
		AF-3F2 (nest)	GATGAGACATCTTTCACAGGAG
	Quantitative RT-PCR and tissue distribution PCR	Q-F	ATGAGCCAAACTAGCCGACAGC
		Q-R	TCATGAAACAAAATGGGTTTA
β-actin	Quantitative RT-PCR and tissue distribution PCR	F	CAGACAGCACAGTGTTGGCGT
		R	CAGACAGCACAGTGTTGGCGT

Mixed bases: Y: C/T; R: A/G; M: A/C; S: G/C; H: A/C/T; K: G/T.

sAR tissue distribution

Total RNA was extracted from brain, pituitary, liver, gonad, kidney, heart, intestine, spleen, gill, and muscle of adult male and female *S. argus* (body weight ca. 250 g). One microgram of total RNA from each tissue sample was treated with DNase I (Fermentas) and the first-strand cDNA was synthesized using the ReverTra Ace-α First-Strand cDNA Synthesis Kit (TOYOBO, Saitama, Japan). The sAR primers used for the tissue distribution and quantitative RT-PCR analyses are listed in Table 1. Mock reactions without the reverse transcriptase as negative controls were done at the same time. The amplification regime consisted of 40 cycles of 15 s at 95°C, 55°C for 15 s, and 72°C for 30 s; followed by further amplification at 72°C for 5 min. β-actin (Li et al., 2015) was used as an internal control. PCR products were separated on a 1.5% agarose gel and visualized with EB.

Histological procedures

Following 24 h in Bouin's fluid treatment, the gonadal tissue samples were transferred to 70% ethanol. Briefly, the fixed tissue samples were embedded in paraffin and were serially sectioned at 10 μm. The sections were stained with hematoxylin and eosin, and the classifications of testicular stage were determined under IQ50 light microscopy (Nikon, Tokyo, Japan). Five samples (N = 5) of each testicular stage were chosen to examine the sAR expressions.

In vitro effect of exogenous steroid hormones on sAR expression

The *in vitro* incubation of gonadal fragments was conducted following the methods of Zhang et al. (2007). Briefly, the ovaries from five adult female *S. argus* (body weight ca. 250 g) were removed and washed three times with Hank's balanced salt solution. The ovaries were cut into small pieces, and placed onto a 24-well culture dish and pre-incubated with L15 medium (Life Technologies) for 2 h at 25°C in a humidified incubator under 5% CO_2. Subsequently, the pre-incubation medium was removed and a new medium containing either E_2 or MT was added to treat the ovarian fragments and to reach a final concentration of 10^{-6} M. And the control groups were treated with the same medium without E_2 and MT at the same time. After incubation for 6, 12 and 24 h, the ovarian fragments were collected and stored at -80°C.

Quantitative RT-PCR analysis for sAR expression

The first-strand cDNA of each gonad was prepared following the manufacturer protocol of the ReverTra Ace-α First-Strand cDNA Synthesis Kit (TOYOBO). Quantitative RT-PCR was performed with the Roche LightCycler 480 (Roche, Bromma, Sweden) RT-PCR system using SYBR(R) Premix Ex Taq™ (Clontech Takara). Negative controls without templates were run simultaneously with the sARs. The quantitative RT-PCR conditions were as follows: denaturation at 94°C for 1 min; 40 cycles of 5 s at 94°C, 57°C for 10 s, 72°C for 20 s, 84°C for 10 s (fluorescent data collection); and termination with the melt curve analysis. The specificity of each primer pair and the quantitative RT-PCR data were evaluated using the melt curve of each gene. The threshold cycle values (Ct) for sAR and β-actin were obtained using quantitative RT-PCR and data analysis using the $2^{\Delta Ct}$ method.

Statistical analysis

Multiple-amino acid sequence alignments were performed using Clustal X (v. 1.81) (Thompson et al., 1994) and a protein phylogenetic tree was created using MEGA4.0 using the neighbor-joining method (Kumar et al., 2004). The data in the present study are reported as means ± SE. Statistical differences were analyzed by one-way ANOVA of SPSS 13.0 (SPSS, Chicago, IL, USA). A significance level of 0.05 ($P < 0.05$) was used to denote statistical significance.

RESULTS

sAR cloning and sequence analysis

The different sAR cDNA were isolated from the spotted scat testes. The full-length sAR cDNA consisted of 2448 nucleotides with an open-read frame of 2289 bp, encoding 763 amino acid residues and exhibiting a similar sequence length as that found in other species (Figure 1). The spotted scat sARs belonged to the typical nuclear receptor family, containing the three typical functional domains TAD, DBD, and LBD (Figure 2). Multiple sAR alignments showed that the TAD domains of the sARs were the most variable regions with very low identity. The DBD domains possessed the DNA binding sites and the conserved P-box and D-box motifs. The LBD domain contained five conserved leucines, which constituted the zipper motif.

Phylogenetic analysis showed that the ARs could be divided into two evident teleost subclades. The sAR from *S. argus* clustered with the ARb subclade, similar to the ARs of sea bass and Atlantic croaker (Figure 3).

sAR tissue distributions

sAR expression was detected in the tissues of both male and female individuals (Figure 4). A strong expression signal was observed in both male and female pituitaries. Moderate sAR expression was found in the brain, ovary, gill, heart, muscle, and spleen in females, and in the testis in males. Low expression levels were detected in the liver and intestine in females. Similarly, low transcription signals were observed in the male brain, liver, heart, intestine, spleen, gill, and muscle. No expression signals were observed in male or female kidneys.

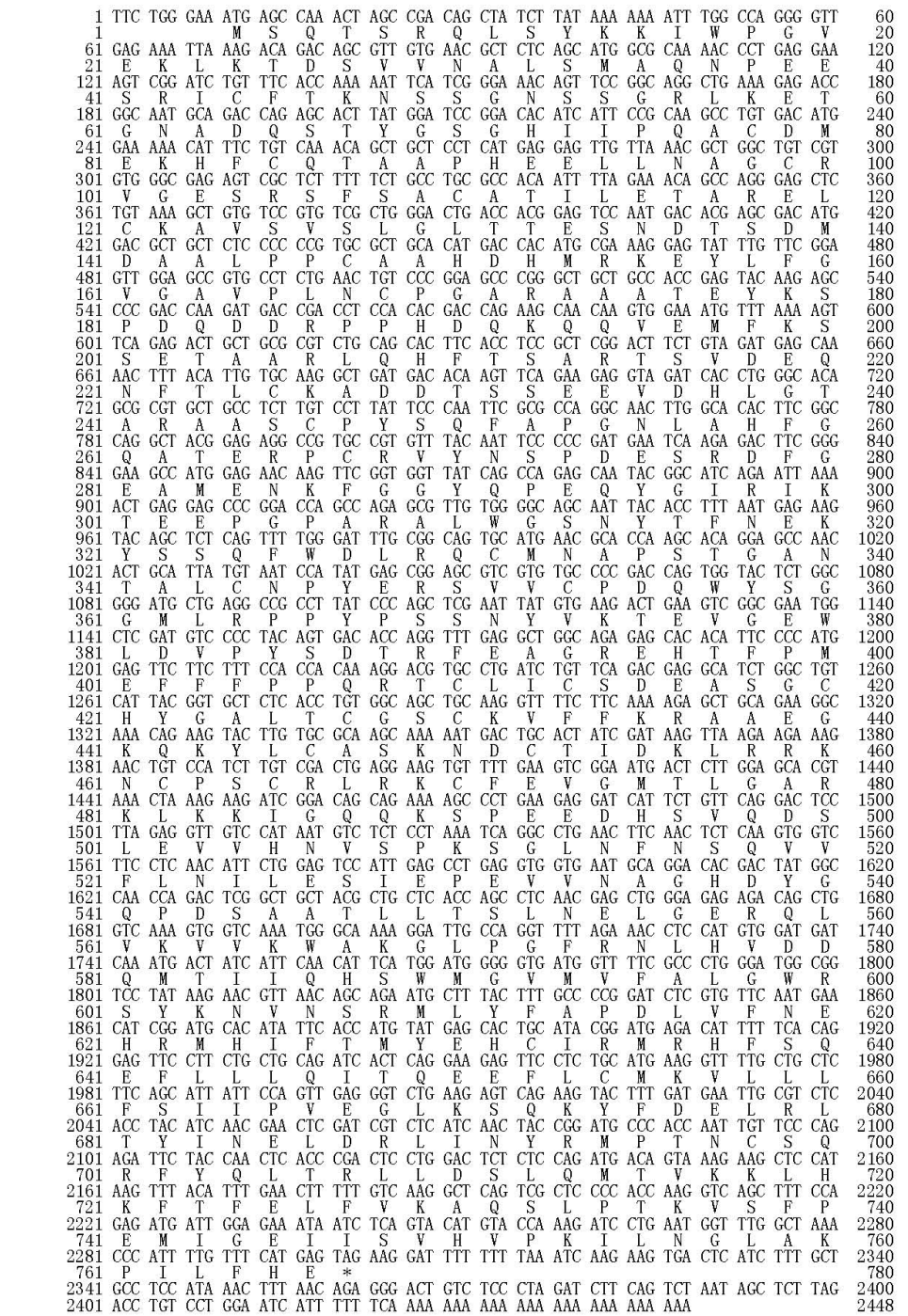

Figure 1. Nucleotide and deduced amino acid sequence of the sARs. The translation stop codon is designated with an asterisk.

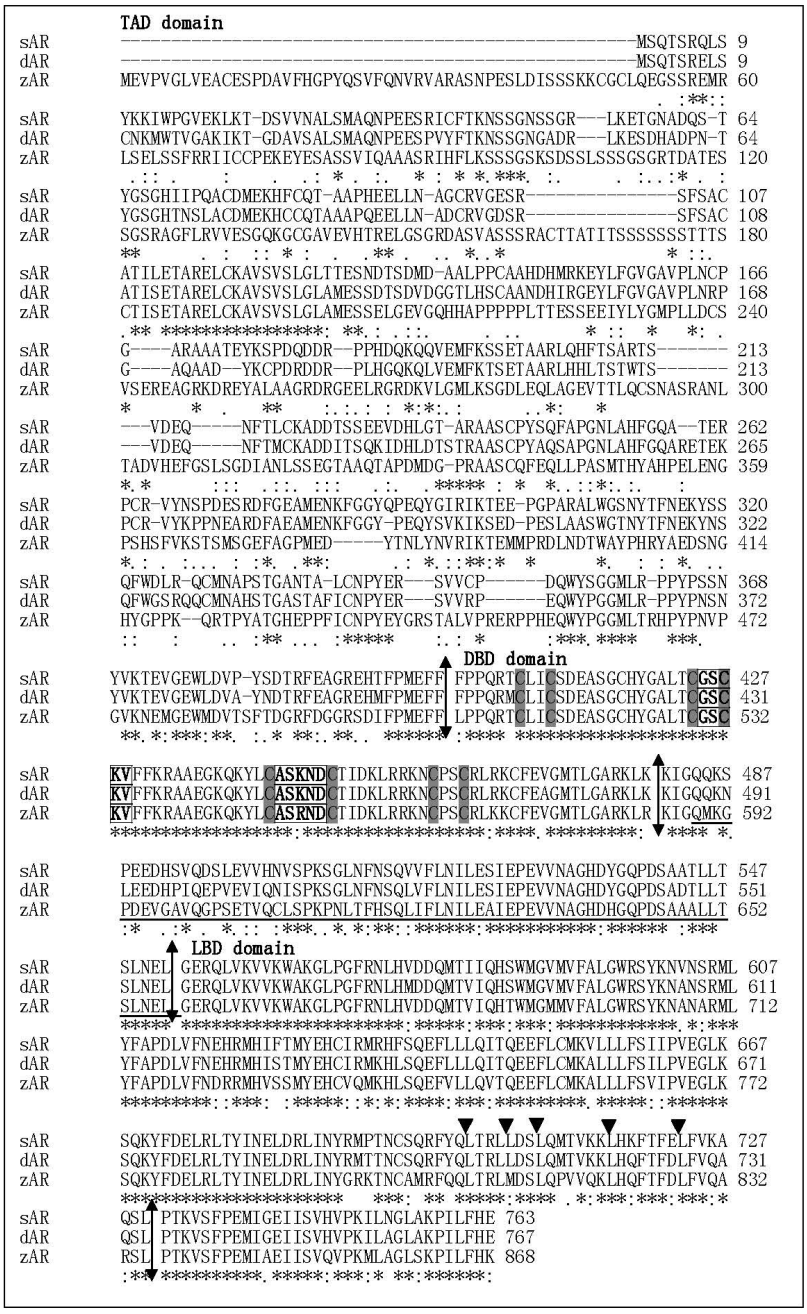

Figure 2. Amino acid alignment of the ARs. The amino acid sequences of the ARs are divided into different functional domains with vertical double arrows and the conserved amino acids are marked with asterisks. In the DNA binding-domain (C domain), the eight conserved cysteine residues are highlighted in gray and the functional elements, including the P-box and D-box motifs, are boxed. The nuclear localization signal is underlined and the leucine zipper motif in the LBD domain is indicated by triangles.

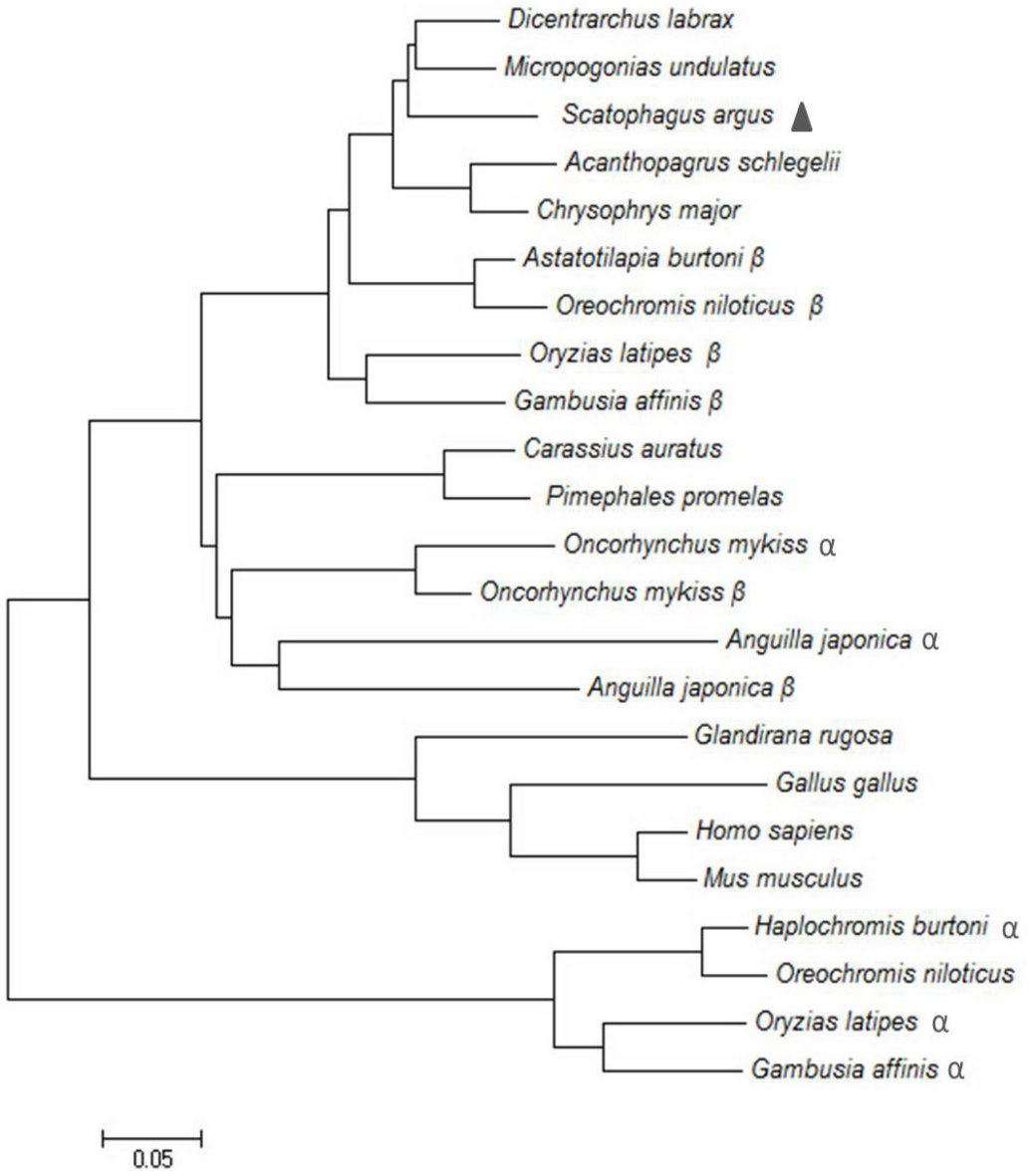

Figure 3. Phylogenetic analysis of AR in vertebrates. AR sequences used for analysis and their GenBank accession Nos. were *Micropogonias undulatus* (AAU09477.1); *Oncorhynchus mykiss* ARα (NP_001117656.1); *Haplochromis burtoni* ARα (AAD25074.2); *Oreochromis niloticus* ARα (BAB20081.1); *Oryzias latipesi* ARα (BAI58983.1); *Anguilla japonica* ARα (BAA75464.1); *Gambusia affinis* ARα (BAD81045.1); *Oncorhynchus mykiss* AR (NP_001117657.1); *Astatotilapia burtoni* ARβ (AAL92878.2); *Oreochromis niloticus* ARβ (BAB20082.1); *Oryzias latipes* ARβ (NP_001098151.1); *Anguilla japonica* ARβ (BAA83805.1); *Acanthopagrus schlegelii* AR (AAO61694.1); *Carassius auratus* AR (AAM09278.1); *Dicentrarchus labrax* AR (AAT764 33.1); *Pimephales promelas* AR (AAF88138.2); *Chrysophrys major* AR (BAA33451.1); *Gambusia affinis* ARβ (AAL92878.2); *Gallus gallus* ARa (NP_001035179.1); *Glandirana rugosa* AR (AB491724.1); *Homo sapiens* AR (AH002607.1); *Mus musculus* AR (CAA42160.1). The sAR is denoted by a triangle. The phylogenetic tree was constructed using MEGA4.0 by a neighbor-joining method.

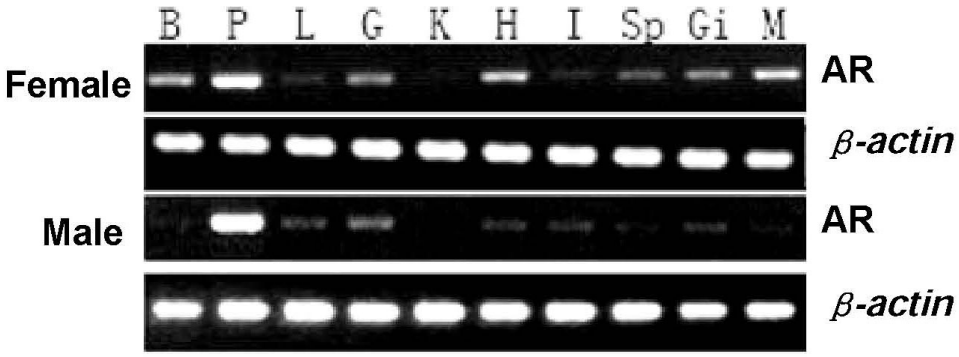

Figure 4. Tissue distributions of sAR in various tissues by RT-PCR. The sAR mRNA expressions for females and males are shown in the top and bottom panels, respectively. β-actin was used as an internal control for the relative quantity of cDNA used in the PCRs. The tissues analyzed were: B, brain; P, pituitary; L, liver; G, gonad; K, kidney; H, heart; I, intestine; Sp, spleen; Gi, gill; M, muscle.

sAR expression patterns during testicular development

The gonadal morphology of testicular development in *S. argus* is shown in Figure 5. According to the testicular histology characteristics, the testicular development was divided into three stages. Testes with spermatogonia (SG) and spermatocytes (SC I) were considered to be in early-testicular stage (Figure 5A). The testes were assigned to the mid-testicular developmental stage once the spermatocytes (SC II) and spermatid (ST) appeared (Figure 5B). In the late-developmental stage, testes were filled with spermatozoa (SZ) (Figure 5C).

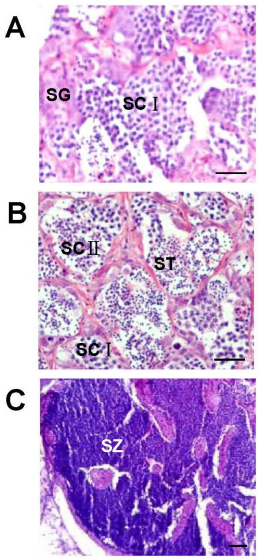

Figure 5. Gonadal histological sections of spotted scat during testicular development. **A.** Early-testicular stage with spermatogonia (SG) and spermatocytes (SC I); **B.** mid testicular stage with the spermatocytes (SC II) and spermatids (ST); **C.** late-testicular stage with the spermatozoa (SZ); Scale bar = 100 μm.

Gonadal sAR expression was low in the early-testicular stage, reached peak transcription at the mid-testicular developmental stage, and decreased in the late-testicular developmental stage while remaining high compared to the early-testicular stage (Figure 6).

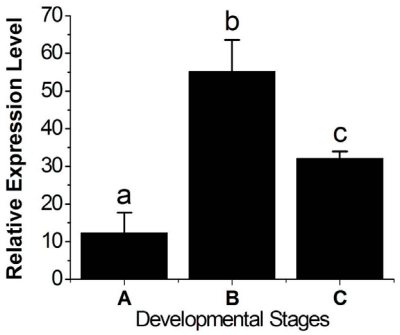

Figure 6. Expression profiles of sARs in the gonads of spotted scat during the early- (**A**), mid- (**B**), and late- (**C**) testicular stages. The data are reported as means ± SE (N = 5) and values denoted with different lower case letters are significantly different at the probability level of 0.05 ($P < 0.05$).

sAR expression patterns under exogenous steroid hormone treatments

No significant differences between the control group and the MT and E_2 treatment groups were found in 6-h treatment. The sAR mRNA expression was significantly higher at 12 and 24 h in the MT treatment group than in the control group (Figure 7A). In the E_2 treatment group, the expression of sAR was significantly suppressed at 12 and 24 h time points (Figure 7B).

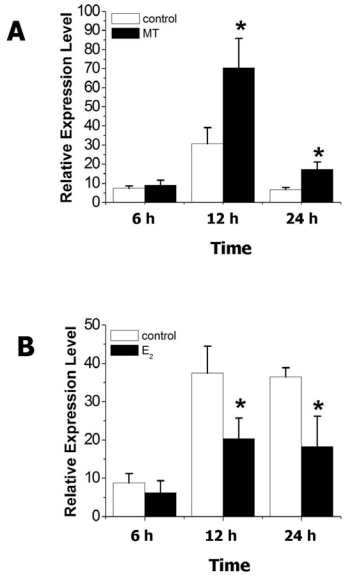

Figure 7. *In vitro* effect of MT and E_2 on ovarian sAR expression. The relative expression patterns of sAR following MT (**A**) and E_2 (**B**) treatments, respectively. The data are reported as means ± SE (N = 5 for all treatments) and the values marked with an asterisk are significantly different from the control treatment at $P < 0.05$.

DISCUSSION

In the present study, *S. argus* AR cDNA was characterized and sequence analysis revealed that the sequences shared the typical AR structures found in other species. Low domain similarities and variable lengths of the TAD domains in the ARs endowed their variable transactivation for specific physiological responses (Apostolinas et al., 1999). The ARs' DNA-binding domains exhibited the highest identities. They also contained conserved motifs and elements, such as the zinc-finger motifs, P-box, and D-box, which are essential to ensure precise reorganization (Shi et al., 2010). The sAR LBD had conserved leucine residues and shared high-sequence identity, which suggests ligand-specificity.

The RT-PCR analysis revealed that sARs were expressed in both male and female *S. argus*, and showed obvious tissue-specific expression patterns, implying multifunctionality of sARs in different tissues. This agrees with the results found in other teleosts (Huang et al., 2011). sAR expression was moderate in ovaries. Based on previous studies on fathead minnow (Wilson et al., 2004), Atlantic croaker (Sperry and Thomas, 2000), and kelp bass (Sperry and Thomas, 1999), AR signal blockage by antiandrogen would cause a significant decrease in aromatase expression in ovaries, and, thus, disrupt the normal endocrine function. Therefore, appropriate sAR expression in the ovaries might be associated with proper ovarian development (Rivero-Wendt et al., 2013b; Liu et al., 2014). Interestingly, sARs were observed to be most abundant in the pituitary of both males and females. This result differs from what has been found in other studies and may involve the positive and negative feedback regulations for the synthesis and release of gonadotropins (Breton and Sambroni, 1996; Huggard et al., 1996).

The androgen signal has been demonstrated to play a critical role during testicular development (Apostolinas et al., 1999; Chakraborty et al., 2009). In the present study, the sAR expression profile was determined during testicular development. A high mRNA level was observed in the mid-developmental stage, and remained high during the late-developmental stage. Similar results have been found in zebrafish (Jørgensen et al., 2007), orange-spotted grouper (Shi et al., 2010), and wrasse (Kim et al., 2002). In in *S. denticulatus*, serum T and 11-KT concentrations increased during the mid-testicular developmental stage (Liu et al., 2009; Huang et al., 2011), which is consistent with the sAR expression levels observed in the present study. This indicates that the androgen signal mediated by sARs mainly contributed during the mid-testicular developmental stage.

Gonadal development was triggered and maintained by the steroid hormones, especially E_2 and MT (Yeh et al., 2003; Hossain et al., 2008). ARs have been shown to play critical roles during gonad development. ARs, as mediators in the gonads, would be affected by steroid hormones. According to previous studies, AR mRNA expression could be increased or suppressed by androgen treatment (Hillier et al., 1997; Larsson et al., 2002). Furthermore, E_2 could also regulate AR transcription not only *in vitro* (Fujimoto et al., 1999; Yeh et al., 2003), but also *in vivo* (Miyamoto et al., 1998). Therefore, the role of E_2 and androgens in AR regulation remains unclear. In the present study, sAR expression increased in ovaries *in vitro* following MT treatment, which agrees with the results found in orange-spotted grouper (Shi et al., 2010) and in wrasse (Kim et al., 2002). sAR expression was significantly inhibited under E_2 treatment, also in agreement with previous studies (Chang et al., 1997; He et al., 2003). Thus far, several studies have demonstrated that ARs likely constitute important factors associated with sex reversal that may be used in seeding and farming applications (Kim et al., 2002; Yeh et al., 2003; Hossain et al., 2008). As we know, androgens stimulate spermatogenesis and promote the development of testicular tissues via ARs. During female to male sex change, AR expression increases significantly in order to increase the

androgen signal, thus contributing to stimulation of the atresia and apoptosis of ovarian tissues (Drummond, 2006; Katoh et al., 2006; Shi et al., 2010). In contrast, during male to female sex change, the AR mRNA levels must be decreased (He et al., 2003). Sex reversal is a complicated physiological process with ovarian degradation and testicular development, which is controlled by gonadal steroid hormones (Yeh et al., 2003). In the present study, sAR expression could be stimulated by MT and inhibited by E_2, suggesting gonadal plasticity produced by the exposure to exogenous steroid hormones in *S. argus*.

In conclusion, AR cDNA sequences from spotted scat were cloned and their tissue distributions were investigated. Furthermore, the expression levels, in relation to testicular development, were measured and the expression of ARs in ovaries in response to exogenous steroid hormone treatment was examined. The results suggest that sARs might play a dominant role in testicular development. Thus, we have preliminary evidence indicating gonadal plasticity of spotted scat under exogenous steroid hormone treatments. This may indicate a potential for sex reversal induction and female monosex farming.

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