



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

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Genet. Mol. Res. 15 (2): gmr.15027838

Received October 10, 2015

Accepted December 29, 2015

Published April 7, 2016

DOI <http://dx.doi.org/10.4238/gmr.15027838>

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 AR β 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 17 α -methyltestosterone (MT) and estrogen (E₂) 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 E₂ 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), methyl dihydrotestosterone (MDHT), 17 α -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 (AR α and AR β) 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	TCCTYCTACTTTRTGRAACAAGAT
	5'-RACE PCR	AR-5R1 (first)	GCTYTGASAGCTCYCTGGCTGT
		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	CAGACAGCACAGTGTGGCGT
		R	CAGACAGCACAGTGTGGCGT

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 μ m. 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% CO₂. Subsequently, the pre-incubation medium was removed and a new medium containing either E₂ 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 E₂ 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^(TM) (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 \pm 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.

1	TTC	TGG	GAA	ATG	AGC	CAA	ACT	AGC	CGA	CAG	CTA	TCT	TAT	AAA	AAA	ATT	TGG	CCA	GGG	GTT	60
1	S	Q	M	S	Q	T	S	R	Q	L	S	Y	K	K	I	W	P	G	V	E	20
61	GAG	AAA	TTA	AAG	ACA	GAC	AGC	GTT	GTG	AAC	GCT	CTC	AGC	ATG	GCG	CAA	AAC	CCT	GAG	GAA	120
21	E	K	L	K	T	D	S	V	N	A	L	S	M	A	Q	N	P	E	E	E	40
121	AGT	CGG	ATC	TGT	TTC	ACC	AAA	AAT	TCA	TGG	GGA	AAC	AGT	TCC	GCG	AGG	CTG	AAA	GAG	ACC	180
41	S	R	I	C	F	T	K	N	S	S	G	N	S	S	G	R	L	K	E	T	60
181	GGC	AAT	GCA	GAC	CAG	AGC	ACT	TAT	GGA	TCC	GGA	CAC	ATC	ATT	CCG	CAA	GCC	TGT	GAC	ATG	240
61	G	N	A	D	Q	S	T	Y	G	S	G	H	I	I	P	Q	A	C	D	M	80
241	GAA	AAA	CAT	TTC	TGT	CAA	ACA	GCT	GCT	CCT	CAT	GAG	GAG	TTG	TTA	AAC	GCT	GGC	TGT	CGT	300
81	E	K	H	F	C	T	A	A	P	H	E	E	L	L	N	A	G	C	R	100	
301	GTG	GGC	GAG	AGT	CGC	TCT	TCT	TCT	GCC	TGC	GCC	ACA	ATT	TTA	GAA	ACA	GCC	AGG	GAG	CTC	360
101	V	G	E	S	R	S	F	S	A	C	A	T	I	L	E	T	A	R	E	L	120
361	TGT	GAA	GCT	GTG	TCC	GTG	TCG	CTG	GGA	ACC	ACG	GAG	TCC	AAT	GAC	ACG	AGC	GAC	ATG	140	
121	C	K	A	V	S	V	S	L	G	L	T	T	E	S	N	D	T	S	D	M	140
421	GAC	GCT	GCT	CTC	CCC	CCG	TGC	GCT	GCA	CAT	GAC	CAC	ATG	CGA	AAG	GAG	TAT	TTG	TTC	GGA	480
141	D	A	A	L	P	P	C	A	H	D	H	M	R	K	E	Y	L	F	G	S	160
481	GTT	GGA	GCC	GTG	CCT	CTG	AAC	TGT	CCC	GGA	GCC	CGG	GCT	GCT	GCC	ACC	GAG	TAC	AAG	AGC	540
161	V	G	A	V	P	L	N	C	P	G	A	R	A	A	A	T	E	Y	K	S	180
541	CCC	GAC	CAA	GAT	GAC	CGA	CCT	CCA	CAC	GAC	CAG	AAG	CAA	CAA	GTG	GAA	ATG	TTT	AAA	AGT	600
181	P	D	Q	D	R	P	P	H	D	Q	K	Q	V	E	M	F	K	S	T	200	
601	TCA	GAG	ACT	GCT	GCG	CGT	CTG	CAG	CAC	TTC	ACC	TCC	GCT	CGG	ACT	TCT	GTA	GAT	GAG	CAA	660
201	S	E	T	A	A	R	L	Q	H	F	T	S	A	R	T	S	V	D	E	Q	220
661	AAC	TTT	ACA	TTG	TGC	AAG	GCT	GAT	GAC	ACA	AGT	TCA	GAA	GAG	GTA	GAT	CAC	CTG	GGC	ACA	720
221	N	F	T	L	C	K	A	D	T	S	S	E	E	V	D	H	L	G	T	240	
721	GGG	CGT	GCT	GCC	TCT	TGT	CCT	TAT	TCC	CAA	TTC	GCG	CCA	GGC	AAC	TTG	GCA	CAC	TTC	GGC	780
241	A	R	A	A	S	C	P	Y	S	Q	F	A	P	G	N	L	A	H	F	G	260
781	CAG	GCT	ACG	GAG	AGG	CCG	TGC	CGT	GTT	TAC	AAT	TCC	CCC	GAT	GAA	TCA	AGA	GAC	TTC	GGG	840
261	Q	A	T	E	R	P	C	R	V	Y	N	S	P	D	E	S	R	D	F	G	280
841	GAA	GCC	GAG	GAG	AAC	AAG	TTC	GGT	TAT	CAG	CCA	GAG	CAA	TAC	GGC	ATC	AGA	ATT	AAA	900	
281	E	A	M	E	N	K	F	G	G	Y	Q	P	E	Q	Y	G	I	R	I	K	300
901	ACT	GAG	GAG	CCC	GGA	CCA	GCC	AGA	GCG	TTG	TGG	GGC	AGC	AAT	TAC	ACC	TTT	AAT	GAG	AAG	960
301	T	E	E	P	G	P	A	R	A	L	W	G	S	N	Y	T	F	N	E	K	320
961	TAC	AGC	TCT	CAG	TTT	TGG	GAT	TTG	CGG	CAG	TGC	ATG	AAC	GCA	ACA	AGC	ACA	GGA	GCC	AAC	1020
321	Y	S	S	Q	F	W	D	L	R	Q	C	M	N	A	P	S	T	G	A	N	340
1021	ACT	GCA	TTA	TGT	AAT	CCA	TAT	GAG	CGG	AGC	GTC	GTG	TGC	CCC	GAC	CAG	TGG	TAC	TCT	GCC	1080
341	T	A	L	C	N	P	Y	E	R	S	V	V	C	P	D	Q	W	Y	S	G	360
1081	GGG	ATG	CTG	AGG	CCG	CCT	TAT	CCC	AGC	TCG	AAT	TAT	GTG	AAG	ACT	GAA	GTG	GGC	GAA	TGG	1140
361	G	M	L	R	P	P	Y	P	S	S	N	Y	V	K	T	E	V	G	E	W	380
1141	CTC	GAT	GTC	CCC	TAC	AGT	GAC	ACC	AGG	TTT	GAG	GCT	GGC	AGA	GAG	CAC	ACA	TTC	CCC	ATG	1200
381	L	D	V	P	Y	S	D	T	R	F	E	A	G	R	E	H	T	F	P	M	400
1201	GAG	TTC	TTT	CCA	CAA	AGG	ACG	TGC	CTG	ATC	TGT	TCA	GAC	GAG	GCA	TCT	GGC	TGT	1260		
401	E	F	F	P	P	Q	R	T	C	L	I	C	S	D	E	A	S	G	C	420	
1261	CAT	TAC	GGT	GCT	CTC	ACC	TGT	GGC	AGC	TGC	AAG	GTT	TTC	TTC	AAA	AGA	GCT	GCA	GAA	GGC	1320
421	H	Y	G	A	L	T	C	G	S	C	K	V	F	F	K	R	A	A	E	G	440
1321	AAA	CAG	AAG	TAC	TTG	TGC	GCA	AGC	AAA	AAT	GAC	TGC	ACT	ATC	GAT	AAG	TTA	AGA	AGA	AAG	1380
441	K	Q	K	Y	L	C	A	S	K	N	D	C	T	I	D	K	L	R	R	K	460
1381	AAC	TGT	CCA	TCT	TGT	CGA	CTG	AGG	AAG	TGT	TTT	GAA	GTC	GGA	ATG	ACT	CTT	GGA	GCA	CGT	1440
461	N	C	P	S	C	R	L	R	K	C	F	E	V	G	M	T	L	G	A	R	480
1441	AAA	CTA	AAG	AAG	ATC	GGA	CAG	CAG	AAA	AGC	CCT	GAA	GAG	GAT	CAT	TCT	GTT	CAG	GAC	TCC	1500
481	K	L	K	K	I	G	L	Q	K	S	P	E	E	D	H	S	V	Q	D	S	500
1501	TTA	GAG	GTT	GTC	CAT	AAT	GTC	TCT	CCT	AAA	TCA	GGC	CTG	AAC	TTC	AAC	TCT	CAA	GTC	GTC	1560
501	L	E	V	V	H	N	V	S	P	K	S	G	L	N	F	N	S	Q	V	V	520
1561	TTC	CTC	AAC	ATT	CTG	GAG	TCC	ATT	GAG	CCT	GAG	GTG	GTG	AAT	GCA	GGA	CAC	GAC	TAT	GGC	1620
521	F	L	N	I	L	E	S	I	E	P	E	V	V	N	A	G	H	D	Y	G	540
1621	CAA	CCA	GAC	TCG	GCT	ACT	ACG	CTG	CTC	ACC	AGC	CTC	AAC	GAG	CTG	GGA	GAG	AGA	CAG	CTG	1680
541	Q	P	D	S	A	A	T	L	L	T	S	L	N	E	L	G	E	R	Q	L	560
1681	GTC	AAA	GTG	GTC	AAA	TGG	GCA	AAA	GGA	TTG	CCA	GGT	TTT	AGA	AGC	CTC	CAT	GTG	GAT	GAT	1740
561	V	K	V	V	K	W	A	K	G	L	P	G	F	R	N	L	H	V	D	D	580
1741	CAA	ATG	ACT	ATC	ATT	CAA	ATC	TCA	TGG	ATG	GGG	GTG	ATG	GTT	TTC	GCC	TTC	GGA	TGG	CGG	1800
581	Q	M	T	I	I	Q	H	S	W	M	G	V	M	V	F	A	L	G	W	R	600
1801	TCC	TAT	AAG	AAC	GTT	AAC	AGC	AGA	ATG	CTT	TAC	TTT	GCC	COG	GAT	CTC	GTG	TTC	AAT	GAA	1860
601	S	Y	K	N	V	N	S	R	M	L	Y	F	A	P	D	L	V	F	N	E	620
1861	CAT	CGG	ATG	CAC	ATA	TTC	ACC	ATG	TAT	GAG	CAC	TGC	ATA	CGG	ATG	AGA	CAT	TTT	TCA	CAG	1920
621	H	R	M	H	I	F	T	M	Y	E	H	C	I	R	M	R	H	F	S	Q	640
1921	GAG	TTC	CTT	CTG	CTG	CAG	ATC	ACT	CAG	GAA	GAG	TTC	CTC	TGC	ATG	AAG	GTT	TTG	CTG	CTC	1980
641	E	F	L	L	L	Q	I	T	Q	E	E	F	L	C	M	K	V	L	L	L	660
1981	TTC	AGC	ATT	ATT	CCA	GTT	GAG	GGT	CTG	AAG	AGT	CAG	AAG	TAC	TTT	GAT	GAA	TTG	CGT	CTC	2040
661	F	S	I	I	P	V	E	G	L	K	S	Q	K	Y	F	D	E	L	R	L	680
2041	ACC	TAC	ATC	AAC	GAA	CTC	GAT	CGT	CTC	ATC	AAC	TAC	CGG	ATG	CCC	ACC	AAT	TGT	TCC	CAG	2100
681	T	Y	I	N	E	L	D	R	L	I	N	Y	R	M	P	T	N	C	S	Q	700
2101	AGA	TTC	TAC	CAA	CTC	ACC	CGA	CTC	CTG	GAC	TCT	CTC	CAG	ATG	ACA	GTA	AAG	AAG	CTC	CAT	2160
701	R	F	Y	Q	L	T	R	L	L	D	S	L	Q	M	T	V	K	K	L	H	720
2161	AAG	TTT	ACA	TTT	GAA	CTT	TTT	GTC	AAG	GCT	CAG	TGC	CTC	CCC	ACC	AAG	GTC	AGC	TTT	CCA	2220
721	K	F	T	F	E	L	F	V	K	A	Q	S	L	P	T	K	V	S	F	P	740
2221	GAG	ATG	ATT	GGA	GAA	ATA	ATC	TCA	GTA	CAT	GTA	CCA	AAG	ATC	CTG	AAT	GGT	TTG	GCT	AAA	2280
741	E	M	I	G	E	I	I	S	V	H	V	P	K	I	L	N	G	L	A	K	760
2281	CCC	ATT	TTG	TTT	CAT	GAG	TAG	AAG	GAT	TTT	TTT	TAA	ATC	AAG	AAG	TGA	CTC	ATC	TTT	GCT	2340
761	P	I	L	F	H	E	*														780
2341	GCC	TCC	ATA	AAC	TTT	AAC	AGA	GGG	ACT	GTC	TCC	CTA	GAT	CTT	CAG	TCT	AAT	AGC	TCT	TAG	2400
2401	ACC	TGT	CCT	GGA	ATC	ATT	TTT	TCA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	2448

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

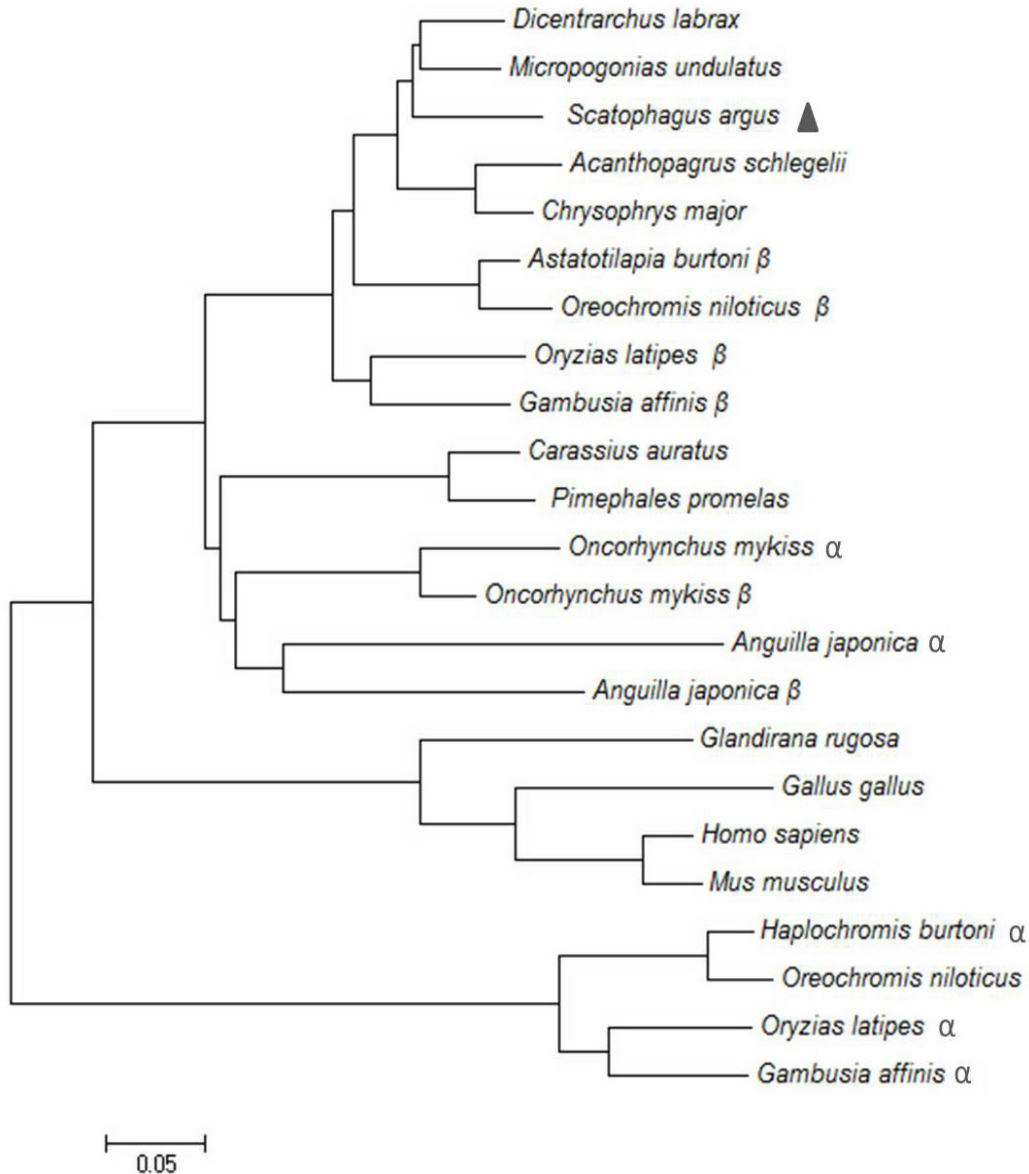


Figure 3. Phylogenetic analysis of AR in vertebrates. AR sequences used for analysis and their GenBank accession Nos. were *Micropogonias undulatus* (AAU09477.1); *Oncorhynchus mykiss* AR α (NP_001117656.1); *Haplochromis burtoni* AR α (AAD25074.2); *Oreochromis niloticus* AR α (BAB20081.1); *Oryzias latipes* AR α (BAI58983.1); *Anguilla japonica* AR α (BAA75464.1); *Gambusia affinis* AR α (BAD81045.1); *Oncorhynchus mykiss* AR (NP_001117657.1); *Astatotilapia burtoni* AR β (AAL92878.2); *Oreochromis niloticus* AR β (BAB20082.1); *Oryzias latipes* AR β (NP_001098151.1); *Anguilla japonica* AR β (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* AR β (AAL92878.2); *Gallus gallus* AR α (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.

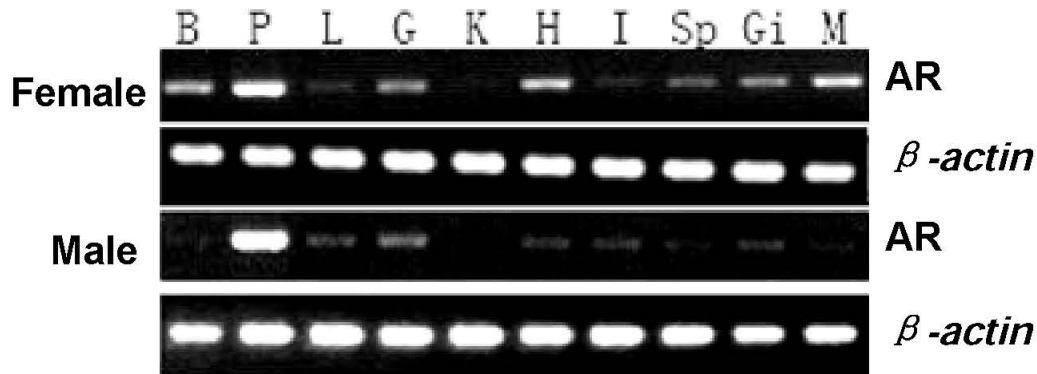


Figure 4. Tissue distributions of sAR in various tissues by RT-PCR. The sAR mRNA expressions for females and males are shown in the top and bottom panels, respectively. β -actin was used as an internal control for the relative quantity of cDNA used in the PCRs. The tissues analyzed were: B, brain; P, 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).

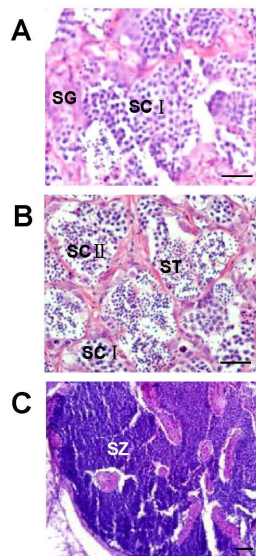


Figure 5. Gonadal histological sections of spotted scat during testicular development. **A.** Early-testicular stage with spermatogonia (SG) and spermatocytes (SC I); **B.** mid testicular stage with the spermatocytes (SC II) and spermatids (ST); **C.** late-testicular stage with the spermatozoa (SZ);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

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).

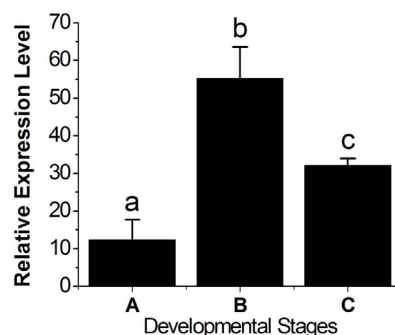


Figure 6. Expression profiles of sARs in the gonads of spotted scat during the early- (A), mid- (B), and late- (C) testicular stages. The data are reported as means \pm SE (N = 5) and values denoted with different lower case letters are significantly different at the probability level of 0.05 ($P < 0.05$).

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).

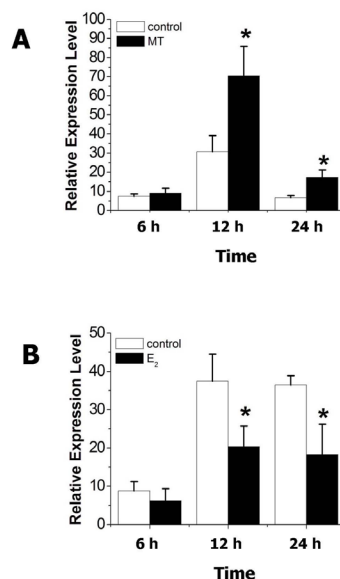


Figure 7. *In vitro* effect of MT and E_2 on ovarian sAR expression. The relative expression patterns of sAR following MT (A) and E_2 (B) treatments, respectively. The data are reported as means \pm SE (N = 5 for all treatments) and the values marked with an asterisk are significantly different from 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 *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 E₂, 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.

ACKNOWLEDGMENTS

Research supported by the Guangdong Province Marine Fishery Science and Technology Promotion Project (#A201408A06), the "Sail Project" of Guangdong Province, the Hainan International Science and Technology Cooperation project (#KJHZ2015-08), and the Guangdong Ocean University Science Projects (#E15181 and #HDYQ2015002).

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