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Original article

# *SRY*-related genes in the genome of the rice field eel (*Monopterus albus*)

Rongjia ZHOU[a,*], Hanhua CHENG[a], Quiyang ZHANG[b], Yiqing GUO[a], Richard K. COOPER[b], Terrence R. TIERSCH[c]

[a] Department of Genetics and Center for Developmental Biology, College of Life Sciences, Wuhan University, Wuhan 430072, PR China
[b] Department of Veterinary Science, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, USA
[c] Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70803, USA

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**Abstract –** The mammalian sex determining gene, *SRY*, is the founding member of the new growing family of *Sox* (*SRY*-like HMG-box gene) genes. *Sox* genes encode transcription factors with diverse roles in development, and a few of them are involved in sex determination and differentiation. We report here the existence of *Sox* genes in the rice field eel, *Monopterus albus*, and DNA sequence information of the HMG box region of five *Sox* genes. The *Sox*1, *Sox*4 and *Sox*14 genes do not have introns in the HMG box region. The *Sox*9 gene and *Sox*17 gene, which each have an intron in the conserved region, show strong identity at the amino acid level with the corresponding genes of mammals and chickens. Similar structure and identity of the *Sox*9 and *Sox*17 genes among mammals, chickens and fish suggest that these genes have evolutionarily conserved roles, potentially including sex determination and differentiation.

**fish / *Sox* / cloning / sex determination**

## 1. INTRODUCTION

The identification of the testis-determining gene on the mammalian Y chromosome has been one of the recent breakthroughs of developmental biology. This gene, named "sex-determining region Y" (*SRY*) is responsible for initiating testis development during mammalian embryogenesis [1,19,20,25,27]. Sequence analysis of *SRY* demonstrated that it contains a 79 amino acid HMG-box which binds to DNA and bends it in a sequence-specific manner. Mutations in the HMG-box region, which alter the abilities of binding and bending, are

* Correspondence and reprints
E-mail: rjzhou@public.wh.hb.cn

associated with sex reversal in XY females [1,10,16]. This suggests that *SRY* is involved in transcriptional regulation. The *SRY* gene belongs to a rapidly growing family of genes that are related by sequence homology to the HMG box, named *Sox* genes (*SRY*-like HMG-box gene). The members of the *Sox* gene family have been conserved through evolution, and have been found in a wide variety of species including humans [7], mice [36], marsupials [11], birds [14], turtles [26], *Xenopus* [23], alligators, lizards, *Drosophila* [6] and fishes [12,15,29,30,32]. Although recent studies show that some *Sox* genes have important developmental roles, many of them have not been identified. In the *Sox* gene family, besides *SRY* as the sex-determining gene in mammals, the *Sox*9 gene is another candidate for the sex-determining gene, and also for cartilage formation, in mammals and chickens, and perhaps in some fishes [28] and alligators [35], although several rodent species do not possess these genes for their sex determination [2]. The *Sox*3 gene would be a candidate as an ancestor for the sex-determining gene *SRY* [13].

In contrast to those of mammals, the sex determining mechanisms of fishes are poorly characterized. Most species of fish lack heteromorphic sex chromosomes. Genes responsible for sex determination have not been identified, and little is known about the molecular genetics of sex determination. The rice field eel, *Monopterus albus*, which undergoes natural sex reversal from the female to male, could be informative for research of the labile sex-determining mechanisms of fishes. The rice field eel is also one of the most economically important freshwater fishes in East Asia. Fish producers desire all-male populations because the males grow faster and larger than females, and the males are also considered to taste better. We therefore investigated the existence, and DNA sequences of *SRY*-related genes in the rice field eel to assist in developing methods for understanding and controlling sex phenotype in this species.

## 2. MATERIALS AND METHODS

### 2.1. Experimental fish and DNAs

Rice field eels were obtained from markets in the Wuhan area in China. Genomic DNA was isolated from whole blood cells, testis and ovaries by routine methods.

### 2.2. Southern blot hybridization

DNA of rice field eels was digested with the *Eco*RI restriction enzyme, electrophoresed on 0.8% agarose-TBE gels and transferred to Hybond-N filters in 10× SSC buffer. The probe, an 800 bp fragment of the human *SRY* gene (including the HMG-box) was labeled with $^{32}$P, added to filters in hybridization buffer (5 mM EDTA, 0.25 M $Na_2HPO4$ pH 7.2, 7% SDS) and hybridized for

16 h at 55 °C. The filters were washed using 2× SSC and 0.1% SDS at 55 °C and using 0.5× SSC and 0.5% SDS at 65 °C before autoradiography was performed. Band sizes were estimated by using a λDNA *Hin*dIII size Marker.

### 2.3. Degenerate PCR, cloning and sequencing analysis

The primers for degenerate PCR were:

5′GATGGATCCATGAA(C/T)GC(A/T/C)TT
(C/T)AT(G/A/T)GT(A/G/T/C)GG3′

and

5′GCGCGAATTCGG(A/G/T/C)(C/T)(G/T)(A/G)TA
(C/T)TT(A/G)TA(A/G)T(C/T)(G/A/T)GG3′

which are the same as those reported by Denny *et al.* [7] but with our addition of restriction site sequences at the 5′ end of the primers. The genomic DNA from blood cells was used as template for PCR, and products from male DNA were cloned into pBluescript (Stratagene, La Jolla, CA) and sequenced using the Ready-Reaction Cycle Sequencing kit (Perkin Elmer) and an automated DNA sequencer (ABI 310 Genetic Analyzer, Perkin Elmer, CA). All nucleotide sequences were analyzed using the Sequence Navigator software (version 1.0.1, Perkin Elmer) to determine similarity with other *Sox* genes listed by the National Center for Biotechnology Information (http/www.ncbi.nih.gov). A phylogenetic tree was constructed with DNASIS software.

## 3. RESULTS AND DISCUSSION

### 3.1. Southern blot analysis

To determine whether genes homologous to *SRY* were present in the genome of the rice field eel, a probe containing an 800 bp fragment of human *SRY* including the conserved HMG box domain was used in this study. The probe was hybridized to the *Eco*RI-digested genomic DNA from blood cells of male and female rice field eels. The probe identified a 3.2 kb fragment in both sexes, although a small gel shift in lane 9 was observed since different amounts of DNA loaded in the lane (Fig. 1a). At low stringency, another five bands were observed, but sex-related differences were not found. Since different chromosomal constitution between gonad and other tissues were observed in some species of the Peramelidae, Southern blot of rice field eel genomic DNA isolated from testis and ovaries was analyzed. Similar, 3.2 kb fragments were identified (Fig. 1a), which suggested that there was not a blockage of recombination in the *SRY*-related genes during meiosis, or there would be the same genetic constitution between germinal and somatic cells.

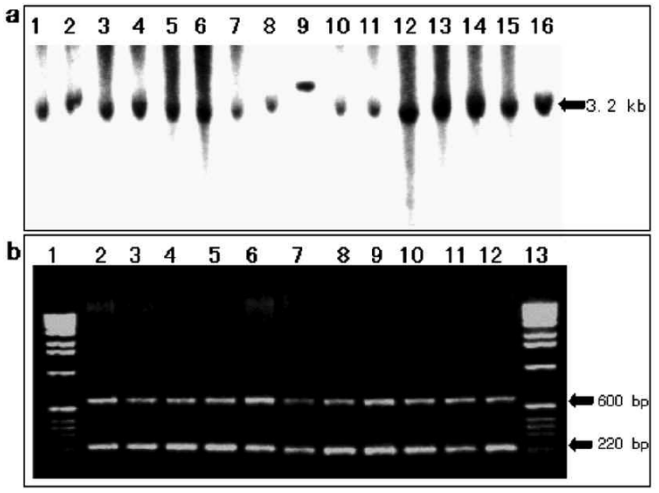


**Figure 1.** (a) Southern blot of genomic DNA from blood, testis and ovaries of 6 male and 5 female rice field eels after hybridization with a 800 bp human *SRY* probe including the conserved HMG box. Lanes 1–6, male, blood; 7–11, female, blood; 12–15, testis; 16, ovary. (b) DNA fragments amplified by PCR from genomic DNA of both sexes of the rice field eel with degenerate primers targeting *Sox* genes. Lanes 1 and 13, 1 kb DNA ladder; 2–6, male; 7–12, female.

### 3.2. Isolation of *SRY*-related genes

To gain more information about the *SRY*-related genes, especially *Sox*9 and *Sox*17, potentially involved in sexual development in rice field eels, genomic DNA was used as a target for PCR amplification using degenerate primers designed to target the conserved HMG box of *SRY* and *Sox* genes. The *SOX*4 to –15 genes were obtained by using these pairs of primers [7]. Two different sizes of bands, 220 bp and 600 bp, were observed in males and females (Fig. 1b). These two fragments from males were cloned and sequenced separately. For the 220 bp fragment, three different *Sox* genes were found, which were designated *Sox*1, *Sox*4 and *Sox*14 (Fig. 2a) because they showed 96%, 96% and 94% identities at the amino acid level of the HMG box region with the corresponding *Sox* genes of the mouse by Blast search [31, 36]. *Sox*4 of the rice field eel also showed 98% agreement with the amino acid sequence of the human *SOX*4 gene [9]. *Sox* genes play a variety of roles in development. Mouse *Sox*1 is associated with the developing nervous system and urogenital ridge [5], and *Sox*4 has been shown to have a role in the regulation of lymphoid differentiation [9], while *Sox*14 is expressed in 15-day-old mouse embryos [36], which suggests important roles for these *Sox* genes in development.

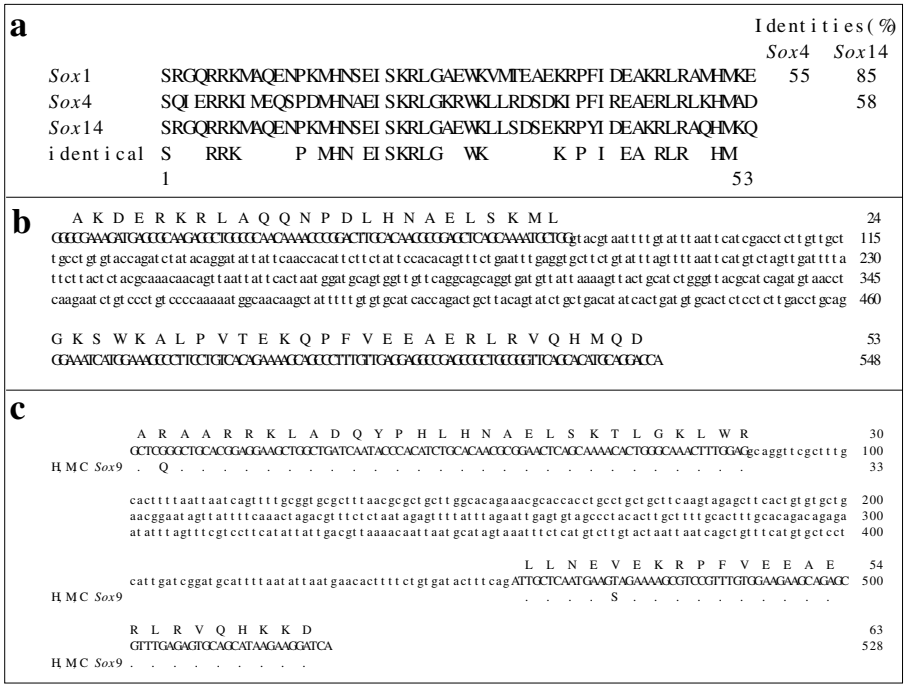


**Figure 2.** (a) Amino acid sequence comparison of the HMG-box region of *Sox*1, *Sox*4, and *Sox*14 of the rice field eel. The numbers on the right show the identities (%) among the *Sox* genes of the rice field eel. The GenBank accession numbers are *Sox*1, AF001043; *Sox*4, AF001044, and *Sox*14, AF001045. (b) The nucleotide sequence and deduced amino acid sequence of rice field eel *Sox*17. The intron is represented by the lower case. The GenBank accession number is AF001047. (c) The nucleotide sequence and deduced amino acid sequence of the rice field eel *Sox*9 and comparison with *Sox*9 of humans, mice and chickens. Use of "." indicates the sharing of an amino acid among rice field eels, humans, mice and chickens; H, human; M, mice; C, chickens. The GenBank accession number is AF001046.

Because there is an intron in the HMG box region of both *Sox*9 and *Sox*17 of mammals, we cloned the 600 bp fragment in order to search the orthologues of these genes of the rice field eel. Two different *Sox* genes were identified, which were more similar to *Sox*9 and *Sox*17 of mammals and chickens. The Blast search showed that the amino acid sequence of *Sox*17 of the rice field eel was most close to (93% identical) the sequence of the HMG box of the mouse *Sox*17 gene [8, 17]. Interestingly, there was also an intron found in the HMG box of *Sox*17 of rice field eel similar to the intron found in *Sox*17 of the mouse at the same splicing site (Fig. 2b). The finding of similar structure between *Sox*17 genes of the rice field eel and mammals suggests that this gene has conserved functions. Recent studies show that the mouse *Sox*17 gene is expressed in

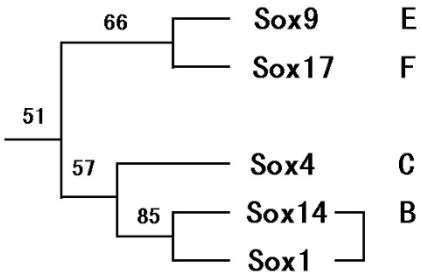


**Figure 3.** Phylogenetic tree of the rice field eel *Sox* genes. The number on the lines of roots show the amino acids identities (%) among the *Sox* genes. Groups B, C, E and F are shown on the right.

spermatogonia and may function as a transcriptional activator in the premeiotic germ cells [17]. As in human *Sox*9, there was an intron in the HMG box region of *Sox*9 of the rice field eel, and this gene showed 96% agreement in the amino acid sequence of the HMG box region with the *Sox*9 of humans, mice and chickens by Blast search (Fig. 2c). In the HMG box region, there were only two amino acids which were different from the *Sox*9 gene of these species. The homologues of *SOX*9 from humans [11,33], mice [37], chickens [18,24], alligators, puffer fish [6,24], and rice field eels show a high level of protein conservation, which suggests that *Sox*9 has conserved functions, potentially including sex determination.

These *Sox* genes of the rice field eel were organized in a phylogenetic tree based on their amino acid identities (Fig. 3). All *Sox* genes have been divided into seven A-G groups [34]. The *Sox*1 and *Sox*14 of the rice field eel belong to group B, *Sox*4 to group C, while the related genes *Sox*9 and *Sox*17 fall into groups E and F respectively, which contain one intron interrupting their HMG domain encoding regions. The organization of the *Sox* family into seven groups suggests that each of these groups may have distinct and specific functions. To date, few reports have analyzed the specific function of individual *Sox* genes. Further studies will allow us to identify the functional differences that may exist between these *Sox* gene groups.

Natural sex reversal from females to males has been demonstrated in the rice field eel [3,4,21,22]. The successive events of natural sex reversal in the species were found to be genetically governed, although appropriate environmental factors also influenced the events. The genetic switch mechanism whereby the phenotype of the rice field eel is shifted from females to males must involve the expression of regulatory genes. Elucidation of this mechanism in this species could cast new light on the field of vertebrate sex determination and differentiation. There has not been any report concerning gene sequences involved in sex determination and differentiation in this species before the

present work. The genes *Sox*9 and *Sox*17 could be candidates for regulatory genes in natural sex reversal in the rice field eel, since the homologues of *Sox*9 and *Sox*17 from a variety of species have conserved functions in sexual development, although they have other roles in development. It would be informative to further characterize these two genes and to clone the other genes involved in sex determination, such as *DMRT*1, for exploration of the sex determination and differentiation of this species.

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