Clone and Sequence Analysis of Sox Genes in *Rana tientaiensis*

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Abstract.- The *Sox* genes of *Rana tientaiensis* were amplified and cloned using highly degenerate primers designed from the conservative motif (HMG-box) of the human SRY gene. The SSCP technique was used to detect different clones. Seven distinct *Sox* gene fragments were obtained from both male and female *R. tientaiensis*; no sexual differences were observed. Seven of these fragments (named *RtSox3a, RtSox3b, RtSox3c, RtSox4, RtSox11, RtSox12,* and *RtSox14*) exhibited 95%, 95%, 95%, 97%, 98%, 97%, and 97% similarity (respectively) to the corresponding homologous human SOX genes. The eighth fragment showed 79% and 77% similarity to the human *SOX21* and *SOX14* genes, as well as varying levels of similarity to other group B Sox genes. The eighth gene, provisionally named Rt*SoxB*14, may be a new member of the Sox gene family or a derivative of an existing *Sox* gene. Phylogenetic analysis suggests that the *RtSox* genes are highly conserved members of the *SoxB, SoxC* and *SoxD* gene groups. Sequence analysis further illustrates that the gene *Sox3* found in *R. tientaiensis* are duplicates of those seen in the mammalian *Sox* gene family. Amino acid positions 15–19 are characteristic of each group in the *Sox* family.

Keywords.- *Sox* genes, SSCP, *Rana tientaiensis*, subgroup diagnosis.

Introduction

The Y chromosome-linked gene SRY is a dominant inducer of testis development in mammals (Sinclair et al., 1990) and a founding member of a gene family with sequence homology to the High Mobility Group (HMG) domain (Fawcett and Klymkowsky, 2004). Since discovery of the *SRY* gene, many members of the *SOX/Sox* (SRY-related HMG box) gene family have been found throughout the vertebrates, showing at least 60% protein similarity to the SRY HMG domain. Genes in each subgroup show over 80% similarity. These *SOX/Sox* genes have also been found to be involved in physiological processes such as sex determination and the development of the CNS, neural crest and endoderm (Bowles et al., 2000). *Sox1, Sox2, Sox3* and *Sox11*, for instance, are expressed mainly in the developing nervous system (Collignon et al., 1996; Pevny et al., 1998), and *Sox*4 is essential for heart and lymphocyte development (Schilham et al., 1996). The *SOX/Sox* genes have been divided between ten subgroups, named A to J (Table 1), not all of which occur in the same taxa (Bowles et al., 2000); groups I and J (containing *sox31, sox32* and *sox33*), for instance, are only found in Zebrafish (Girard et al., 2001; Lunde et al., 2004).

Some amphibians have ZZ/ZW or XX/XY modes of sex determination, but most species do not have heteromorphic chromosomes, making the study of evolution and the mechanism of sex determination in these organisms interesting. *Rana tientaiensis* (2n = 26) is one of these species without identifiable sex chromosomes (Guo et al., 1991). In this paper, we describe the cloning and sequencing of the eight Sox genes in *Rana tientaiensis* with the aim of researching the diversity and evolution of this gene family. Sequence analysis indicates that some of these genes in *R. tientaiensis* are duplicated.

Materials and Methods

Two male and two female *Rana tientaiensis* were captured from Taolin and Tingxi Anhui Provinces, China. Genomic DNA was isolated from muscle tissues using routine protocols. A pair of degenerate primers (sn1: ATGAAYGCNTTYATGGTNTGG; sn2: GGNCGRTAYTTRTARTCNGG) were designed using multiple alignments of the HGM-box sequence of *SRY*, corresponding to the MNAFMVW and PDYKYRP motifs found in the HMG boxes of a wide range of *Sox* proteins.

PCR reactions were 30 µl in volume, including 18.75 µl ddH_2O and approximately 100 ng of genomic DNA, 1.5mM Mg^{2+}, 120 µM dNTP, 0.3 µM/primer and 1.25 µl Taq polymerase. PCR cycling conditions were 1 cycle for 5 minutes at 97°C, followed by 35 cycles with 40 sec. at 94°C, 40 sec. at 53°C, 1 min. at 72°C, and finally 72°C for 10 minutes to complete the final reaction.

Clones were genetically sequenced to detect the positive clones with *Sox* DNA insertions. PCR products were detected by 1.5% agarose gels and cloned by pMD 18-T Vector (purchased from TAKARA). Positive clones were screened by SSCP (single-strand conforma-

Table 1. Classification of the *Sox* gene family.

A	B	C	D	E	F	G	H	I	J
SRY	*Sox1*	*Sox4*	*Sox5*	*Sox8*	*Sox7*	*Sox15*	*Sox30*	*Sox31*	*Sox32*
	Sox2	*Sox11*	*Sox6*	*Sox9*	*Sox17*	*Sox16*			*Sox33*
	Sox3	*Sox24*	*Sox12*	*Sox10*	*Sox18*	*Sox20*			
	Sox14	*Sox22*	*Sox13*						
	Sox21		*Sox23*						
	Sox25								

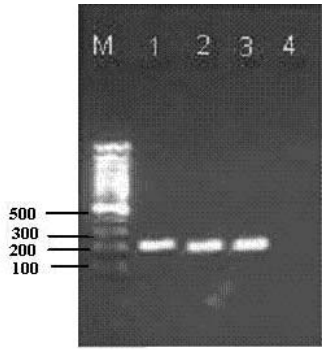

Figure 1. Amplified *Sox* gene fragments from 1: male *Rana tientaiensis*, 2: female *R. tientaiensis*,3: Human; 4: negative control; M: DL2000 marker (TaKaRa).

tion polymorphism) analysis and sequenced with the universal sequencing primers on an ABI377 auto-sequencer. DNA sequences were analyzed using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTALX programs. Molecular Evolutionary Genetic Analysis (MEGA) software was used to construct the phylogenetic tree.

Results and Discussion

A 203 bp fragment of *Rana tientaiensis* genomic DNA was obtained using the degenerate PCR primers listed above. The fragment was identical to that found in the human genome (Fig. 1), indicating that these fragments belong to homologous genes.

Of the 113 white clones (i.e., those with insertions), 64 were positive for the *Sox* insertion, as confirmed through nucleotide analysis following genomic amplification. Eight distinct positive clones were found in both male and female *Rana tientaiensis*; there were no sexual differences.

Seven of the eight distinct genes were named as follows: *RtSox3a*, *RtSox3b, RtSox3c, RtSox4, RtSox11, RtSox12, and RtSox14.* The amino acid sequences from these genes had 95%, 95%, 95%, 97%, 98%, 97%, 97% and 97% similarity (respectively) to homologous *SOX* genes in Human. These seven genes belonged to the *SoxB*, *SoxC* and *SoxD* subgroups, all of which lack introns (Bowles et al., 2000). The 9th *Sox* gene was provisionally named *RtSoxB14*; the amino acid sequences from this gene had 79% similarity to the Human *SOX21* gene, 77% similarity to the *Human Sox14* gene, as well as varying levels of similarity to other group B *Sox* genes. Nucleotide and putative amino acid sequences for the eight *Sox* genes are listed (Fig. 2)

The amino acid sequences from the eight clones were compared to 44 published *Sox* gene sequences in GenBank, including sequences from Human (*HomoSRY, SOX1, 2, 3, 4, 7, 9, 11, 12, 14, 15, 21, 30*), Mouse (*MusSox1, 2, 3, 4, 7, 9, 11, 12, 14, 15, 21, 30)*, *Gallus gallus* (*GallSox*2, 3, 9, 11 , 14, 21), *Danio rerio* (*DaniSox1, 2, 4, 11*), *Xenopus laevis* (*XenopusSox2, 4, 11*), *Takifugu rubripes* (*TakifuguSox1, 14b*) and *Eremias breuchleyi* (*EbSox2, 4, 11, 12, 14 ,21*) (Table 2). All sequences were analyzed using neighbor-joining (NJ) methods by MEGA 2.0 (Fig. 3).

Amino acid sequences between the *RtSox* genes were highly conserved. Representatives of the *Sox* gene in other species were also highly conserved with much similarity between sequences. Gene duplication has likely caused most of the diversity seen in the HMG box superfamily, for which the *Sox* genes show the highest mutation rate (Laudet et al., 1993). The high similarity seen between the *Rana* and human genes in this study are certainly indicative of gene duplication.

In the case of *Sox12*, amino acid sequences were nearly identical, although the 10th amino acid was N instead of H in Human and Mouse (Fig. 2). In *Sox 4*, the 48th amino acid was R instead of Q in Mouse, and in *Sox11*, the 48th amino acid was D instead of N in Mouse, Human and several other species. The high degree of similarity amongst these genes suggests that they belong to the same gene family, and may perform similar roles amongst taxa. For example, the three highly conserved genes in group C display overlapping expression patterns, and *Sox4* and *Sox11* display overlapping expression patterns in the mouse embryonic pancreas (Lioubinski et al., 2003).

According to Laudet et al. (1993), *Sox4* is considered to be an early offshoot of the *SRY* gene in the *Sox* family phylogeny. The conservative nature of *Sox4* homologues in non-mammalian amniotes is interesting because in mammals, the *SRY* gene exhibits rapid evolu-

Table 2. Sox genes sequence in different species

Sequence	Accession number	Sequence	Accession number
Human sapiens		***Mouse musculus***	
HomoSOX1	NP_005977	*MusSox1*	BAC75667
HomoSOX2	CAA83435	*MusSox2*	NP_035573
HomoSOX3	CAA50465	*MusSox3*	AAH52024
HomoSOX4	NP_003098	*MusSox4*	NP_ 033264
HomoSOX7	NP_113627	*MusSox7*	NP_035576
HomoSOX9	CAA86598	*MusSox9*	AAH04064
HomoSOX11	BAA88122	*MusSox11*	NP_033260
HomoSOX12	CAB81632	*MusSox12*	NP_035568
HomoSOX14	AAI06731	*MusSox14*	XP 284529
HomoSOX15	NP_008873	*MusSox15*	NP_033261
HomoSOX21	NP_009015	*MusSox21*	NP_808421
HomoSOX30	NP_848511	*MusSox30*	AAF99391
HomoSRY	AAT37462	***Danio rerio***	
Gallus gallus		*DaniSox1*	NP_001032751
GallSox2	NP_990519	*DaniSox2*	NP_001002483
GallSox3	NP_989526	*DaniSox4*	BC065354
GallSox9		*DaniSox11*	CAB87379
GallSox11	NP_990518	***Xenopus laevis***	
GallSox14	NP_990092	*XenopusSox2*	AAB62821
GallSox21	BAA77266	*XenopusSox3*	P55863
Eremias breuchleyi		*XenopusSox11*	*Q91731*
EbSox2	DQ067423	***Takifugu rubripes***	
EbSox4	DQ067426	*TakifuguSox1*	AAQ18494
EbSox11	DQ067427	*TakifuguSox14b*	AAQ18499
EbSox12	DQ067428		
EbSox14	DQ067430		
EbSox21	DQ067433		

tion, possibly caused by Y-linked inheritance (Tucker and Lundrigan, 1993). The limited diversity of this gene in non-mammalian taxa may be due to the retention of an ancient conserved function.

It is likely that *Sox3* is the closest homologue to the *Sry* gene based on nucleotide sequence data. Furthermore, *Sox3* is located on the mammalian X chromosome, and is highly similar to SRY (Sinclair et al., 1990), suggesting that they arose through duplication of their common ancestral during differentiation of the sex chromosomes (Collignon et al., 1996; Foster and Graves, 1994; Stevanovic et al., 1993). This is significant because the X and the Y chromosomes are thought to have arisen from a common "autosome" ancestor in the lineage that gave rise to mammals (Wright et al., 1993). Further examination of *Sox* genes in lower vertebrates, Prototheria (monotremes) and Metatheria (marsupials) will be necessary to establish the evolutionary origins of *Sry*. The three conservative *Sox3* genes (sometimes found within the same species or individual) can be identified by the following variations in amino acid sequence: *RtSox3a* has an F at position 46 and an H at position 58; *RtSox3b* has an F at position 46 and an M at position 58; *RtSox3c* has an I on position 46 and an M on position 58.

The *RtSoxB14* is unique among the Sox genes in having the amino acid sequence VITEH at positions 15–19, a K at position 44 and an S at position 50. In the phylogenetic tree (Fig. 3), although *RtSoxB14* was more closely related to subgroup B than other groups, the origin and classification of this gene is ambiguous.

Previously, genes encoding proteins with more than 60% similarity to the *SRY* HMG domain have been named *Sox* (*SRY* box) genes, and *Sox* genes with at least

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RtSox12         ATGAATGCGTTTATGGTATGGTCCCAGAACGAGCGGCGGAAGATCATGGACCAGTGGCCG
RtSox11         ATGAATGCTTTTATGGTATGGTCCAAGATCGAGCGGAGAAAAATCATGGAGCAGTCGCCC
RtSox4          ATGAACGCGTTTATGGTTTGGTCGCAGATCGAGCGGCGCAAGATCATGGAGCAGTCGCCC
RtSox3a         ATGAATGCTTTTATGGTTTGGTCGCGGGGGCAGCGGCGCAAGATGGCTCAGGAAAACCCC
RtSox3c         ATGAATGCGTTTATGGTTTGGTCGCGGGGGCAGCGGCGCAAGATGGCTCAGGAAAACCCC
RtSox3b         ATGAATGCGTTTATGGTTTGGTCGCGGGGGCAGCGGCGCAAGATGGCTCAGGAAAACCCC
RtSoxB14        ATGAACGCGTTTATGGTCTGGTCCAGAGTACAGAGGAGGAAGGTGATTACAGAACATCCT
RtSOX14         ATGAATGCGTTCATGGTGTGGTCCAGGGGGCAGAGGAGGAAGATGGCCCAGGACAATCCC
                ***** ** ** ***** *****       ** ** * **  *       *    **

RtSox12         GACATGCACAACGCTGAGATCTCCAAGCGCCTCGGCCGTCGCTGGCAGCTCCTGCAGGAC
RtSox11         GACATGCACGACGCCGAGATCTCCAAGCGCCTGGGCAAGCGGTGGAAAATGCTGAAGGAC
RtSox4          GACATGTACAACGCCGAGATCTCCAAGCGGCTAGGCAAACGCTGGAAGCTGCTCAAGGAC
RtSox3a         AAGATGCACAACTCGGAGATCTCCAAGCGCCTGGGCGCGGACTGGAAGCTGCTGAGCGAC
RtSox3c         AAGATGCACAACTCGGAGATCTCCAAGCGCCTGGGCGCGGACTGGAAGCTGCTGAGCGAC
RtSox3b         AAGATGCACAACTCGGAGATCTCCAAGCGCCTGGGCGCGGACTGGAAGCTGCTGAGCGAC
RtSoxB14        AAAATGCACAACTCTGAAATTAGCAAAAAGTTGGGGGCACAGTGGAAGATCCTTGGCGAT
RtSOX14         AAGATGCACAATTCGGAGATCAGTAAAAGACTTGGGGCTGAGTGGAAACTTCTGTCTGAA
                 * *** ** *  * ** **    **     * **       *** *  * **    **

RtSox12         TCGGAGAAGATCCCCTTTGTGAAGGAGGCTGAGCGGCTGCGACTCAAGCACATGGCTGAC
RtSox11         AGCGAGAAGATCCCCTTCATCCGCGAGGCCGAGCGGCTGCGACTCAAGCACATGGCTGAC
RtSox4          AGCGACAAGATTCCGTTCATCCAGGAGGCGGAGCGACTGCGCCTCAAGCACATGGCTGAC
RtSox3a         GCGGAGAAGCGCCCTTTCATCGACGAGGCCAAGCGGCTCCGCGCCGTCCACACGAAGGAA
RtSox3c         GCGGAGAAGCGCCCTTTTATCGACGAGGCCAAGCGGCTCCGCGCCGTCCACATGAAGGAA
RtSox3b         GCGGAGAAGCGCCCTATTATCGACGAGGCCAAGCGGCTCCGCGCCGTCCACATGAAGGAA
RtSoxB14        TCAGAGAAGAAGCCTTTTATAGACGAATCAAAAAGGCTGAGAGCTCAGCATATGGTTGAG
RtSOX14         GTCGAGAAAAGACCCTACATTGACGAAGCCAAAAGGTTGAGGGCTCAACACATGAAGGAA
                   ** **    **     *    **  *  *  *  *  *       ** * *   **


RtSox12         TACCCTGACTACAAATATCGCCC
RtSox11         TACCCCGATTACAAATACCGCCC
RtSox4          TACCCTGACTACAAATACCGCCC
RtSox3a         TACCCGGATTACAAATACCGCCC
RtSox3c         TACCCCGATTACAAATACCGCCC
RtSox3b         TACCCTGACTATAAATACCGCCC
RtSoxB14        CATCCCGACTACAAATACCGCCC
RtSOX14         CACCCTGACTACAAATATCGCCC
                 * ** ** ** ** ** *****
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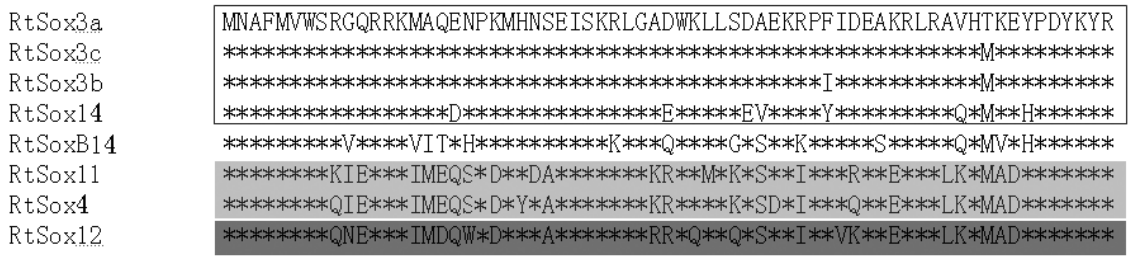
Figure 2. (A) Alignment of nucleotide sequences (above), (B) Alignment of amino acid sequences (Opposite page, top), (C) Percentage amino acid similarity between *Rana tientaiensis Sox* clones as determined by the sequence identity matrix function in Bioedit (Opposite page, bottom).



	HomoSRY	RtSox3a	RtSox3b	RtSox3c	RtSox14	RtSoxB14	RtSox11	RtSox4	RtSox12
HomoSRY	100	67.6	66.1	67.6	63.2	60.2	54.4	54.4	52.9
RtSox3a	---	100	97.0	98.5	88.2	76.4	64.7	64.7	64.7
RtSox3b	---	---	100	98.5	89.7	76.4	64.7	64.7	64.7
RtSox3c	---	---	---	100	89.7	77.9	66.1	66.1	66.1
RtSox14	---	---	---	---	100	76.4	61.7	61.7	61.7
RtSoxB14	---	---	---	---	---	100	63.2	61.7	61.7
RtSox11	---	---	---	---	---	---	100	91.1	83.8
RtSox4	---	---	---	---	---	---	---	100	85.2
RtSox12	---	---	---	---	---	---	---	---	100

Figure 2 (continued).

80% similarity have been placed in the same subgroup. However, as more and more *Sox* genes are identified, the ability to accurately classify these genes decreases. For example, the genes *sox30* and *Ce-soxj* have only 46% and 48% similarity to *Human SRY* HMG. Bowles (2000) attempted to alternatively diagnose the gene family by possession of the amino acid sequence "RPMNAF", which is highly conserved across genes, however, this sequence was also found in the taxonomically ubiquitous gene *cic*, so the sequence "RPMNAFMVW" was provided as a replacement (Bowles et al., 2000).

Now that a sequence identifying the *Sox* gene family has been identified, can sequences be found to characterize the *Sox* subgroups? Following analysis of the available sequences (Figure 4), it appears that positions 15–19 may have be useful for this purpose. The sequence "MAQE(D)N" may work for group B (except for *HomoSOX3* and *MusSox3*), "IMEQS" for group C, "IMEQW" (for *Sox12*) or "ILQAF" (for *Sox5, Sox6* and *Sox13*) for group D, "LADQY" for group E, "LAVQN" (for *Sox7*) or "LAQQN" (for *Sox17* and *Sox18*) for group F, "MAQQN" for group G and "LAKAN" for group H. *RtSoxB14* can be separated from the remaining Sox genes by the unique sequence VITEH, although in mammals (*HomoSOX3* and *MusSox3*) it is changed to "MALEN", like the sequence for *SRY*. This further supports a close relationship between the *SOX3* and *SRY* genes.

Several Sox genes appear to have been duplicated in *Rana tientaiensis*: *RTSox3a, RTSox3b* and *RTSox3c*. Similar duplications in amphibians are uncommon, but they are more frequently encountered in teleosts: Sox1, 4, 9 and 14 has been duplicated in the *sea bass* (Malyka et al., 2003) and Sox21 has been duplicated in the *Zebrafish* (Argenton et al., 2004). The "duplication-degeneration-complementation" model developed by Force et al. (1999) suggests that the partition of ancestral subfunctions is an important mechanism leading to the preservation of multiple gene copies; this model predicts that the probability of gene conservation will be higher in more complex genes with a larger number of subfunctions (Force et al. 1999). Most duplicate genes in *Rana tientaiensis* have silent mutations (except *RtSox3a*, which has an encoded amino acid mutation), but it would appear that the sequences are under selective pressure and may indeed perform separate subfunctions. Future studies investigating *Sox* genes in *Rana tientaiensis* will likely provide much insight into duplicate genes.

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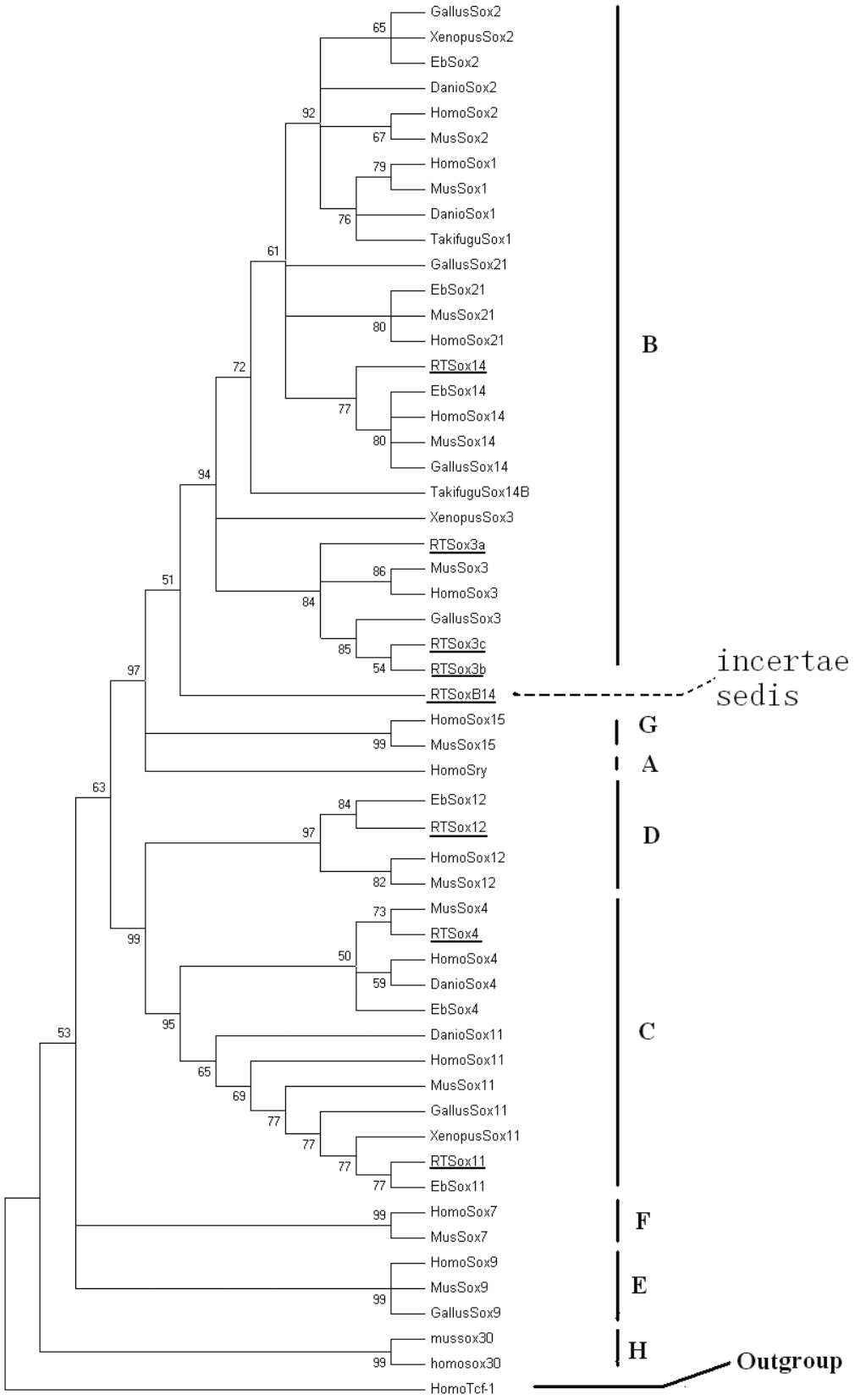

Figure 3. Phylogenetic analysis of Sox/SOX gene family

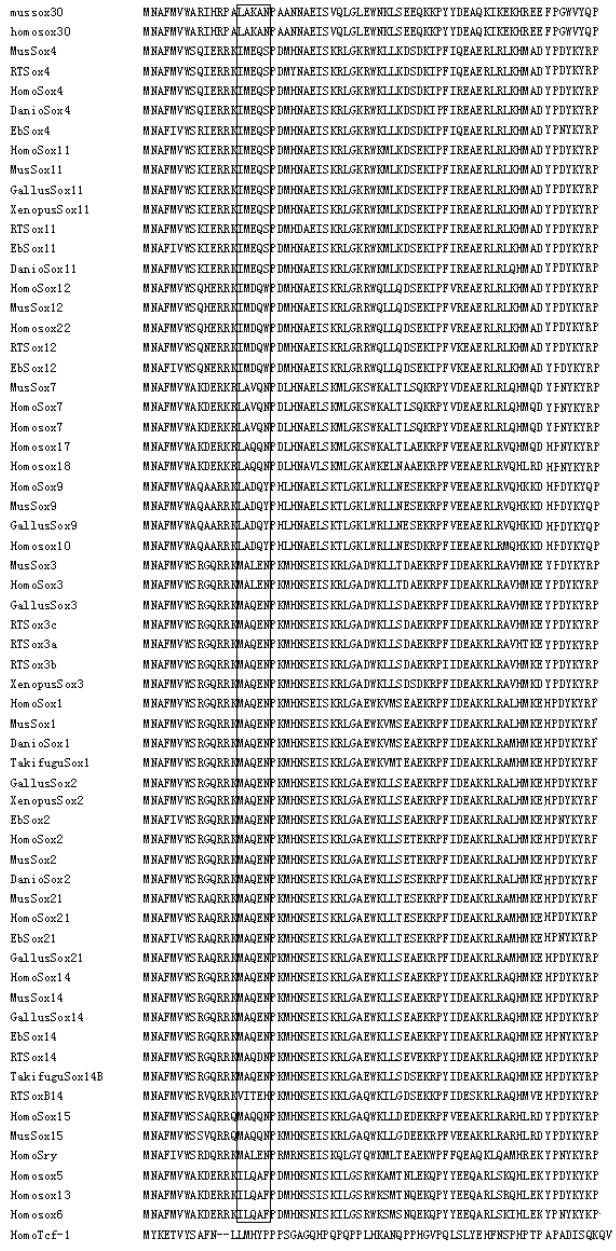

Figure 4. Characteristic Sox amino acid sequences.

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