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# Differentiated Evolutionary Conservatism and Lack of Polymorphism of Crucial Sex Determination Genes (*SRY* and *SOX9*) in Four Species of the Family Canidae

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The sex determination process is under the control of several genes of which two (*SRY* and *SOX9*), encoding transcription factors, play a crucial role. It is well-known that mutations at these genes may cause the development of an intersexual phenotype. The aim of this study was to conduct a comparative analysis of the coding sequence and 5'-flanking regions of both genes in four species of the family Canidae (the dog, red fox, arctic fox and Chinese raccoon dog). Similarity of the coding sequence of the *SOX9* gene among the studied species was higher (99.7-99.9%) than in the case of the *SRY* gene (96.7-97.3%). Only single nucleotide changes were found in the compared coding sequences, whereas in the 5'-flanking region of both genes nucleotide substitutions, as well as insertions and deletions were observed. None of the changes detected in the 5'-flanking region occurred within the potential consensus sequences for transcription factors. No polymorphism was found for either of these genes in any of the analyzed species.

Key words: *SRY*, *SOX9*, dog, red fox, arctic fox, Chinese raccoon dog.

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Mammalian sex determination is controlled by dozens of genes (COTINOT *et al.* 2002) of which two (*SRY* and *SOX9*) play a crucial role in this process. *SRY* (sex determining region Y) is a single-exon gene localized on the Y chromosome and encodes a transcription factor composed of three domains: the N-terminal, HMG (high mobility group) and C-terminal. At the N- and C-terminal ends of the HMG domain nuclear localization signals (NLSs) are located which facilitate the transport of the SRY protein to the nucleus of pre-Sertoli cells (KNOWER *et al.* 2003). The highly conserved HMG domain recognizes the A/TAACAAT/A motif and causes DNA bending in regulatory elements of the target gene. This leads to chromatin conformation changes and facilitates the binding of other proteins regulating the expression of target genes (LIM *et al.* 1998).

The *SOX9* gene is expressed at a very low level in male and female early fetuses, but the occurrence of SRY protein up-regulates *SOX9* gene expression in male and down-regulates it in female fetal gonads. The *SOX9* gene, belonging to the *SOX* gene family, is composed of 3 exons. Similarly to SRY, the SOX9 protein is also a transcriptional factor and contains the HMG-like domain which is flanked by two NLSs. This domain (encoded by the 3'part of exon 1 and the 5' part of exon 2) has a strong affinity to the AACAAT and AACAAAG motifs. In addition, the SOX9 protein contains two other domains encoded by exon 3, namely PQA (rich in proline, glutamine and alanine) and PQS (rich in proline, glutamine and serine). The SOX9 protein, besides sex determination, is also involved in the chondrogenesis process (KUCINSKAS & JUST 2005).

Mutations of the human *SRY* gene result in gonadal dysgenesis, referred to as the Swyer syndrome. The majority of such mutations have been localized in the HMG domain (VEITIA *et al.* 2001). It is estimated that approx. 15-20% of sex reversal cases with the XY chromosome complement are caused by *SRY* gene mutations (KNOWER *et al.* 2003). Mutations in the *SOX9* gene are frequently associated in humans with campomelic dysplasia – CD (skeletal malformations). An additional male-to-female sex reversal syndrome is observed in 75% of CD patients with XY chromosomes

(MEYER *et al*. 1997; NINOMIYA *et al*. 2000). Not only point mutations within the open reading frame, but also translocations, insertions and deletions outside this gene may result in campomelic dyspalsia. In mice, regulatory elements are localized approx. 1Mbp upstream of the *SOX9* gene – their disruption in XX mice can result in sex reversal – *Odd sex – Ods* mice (QIN *et al*. 2004). Moreover, incorrect expression and normal male development occurs in XY mice with a *SRY* gene knockout and a disruption in regulatory elements upstream of the *SOX9* gene. This confirms that the *SOX9* gene may activate testis development without the SRY protein (QIN & BISHOP 2005).

The dog genome is relatively well known since both the genome sequence (LINDBLAD-TOH *et al.* 2005) and dense marker maps (BREEN *et al.* 2004; HITTE *et al.* 2005) are available. Moreover, comparative genomic studies of the family Canidae, including so-called comparative chromosome painting (for a review see GRAPHODATSKY *et al.* 2008) and comparative single-locus mapping (for a review see SZCZERBAL *et al.* 2007), are in an advanced state. Recently the chromosome localization of three sex determination genes (*SOX9*, anti-Müllerian hormone – *AMH* and androgen receptor – *AR*) was established in the dog, foxes and Chinese raccoon dog (NOWACKA-WOSZUK & SWITONSKI unpublished). On the other hand, molecular knowledge on mutations responsible for the quite frequently diagnosed intersexual phenotype in dogs is very limited. The only known and very recently identified mutation concerns the Müllerian Inhibiting Substance Receptor gene which causes the Persistent Müllerian Duct Syndrome (WU *et al.* 2009). Thus, it seems clear that studies of the *SRY* and *SOX9* gene sequence and polymorphism may prove useful in molecular studies of intersex dogs and three other species (the red fox, arctic fox and Chinese raccoon dog) which to some extent are also considered as farm animals. Until now, no comparative data on the *SRY* and *SOX9* gene sequences in the family Canidae have been available.

The aim of this study was to conduct a comparative analysis of the coding sequence and 5'-flanking regions, as well as search for polymorphism, in the *SRY* and *SOX9* genes in four species of the family Canidae, namely the dog (*Canis lupus f. familiaris*), red fox (*Vulpes vulpes*), arctic fox (*Alopex lagopus*) and Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*).

## Material and Methods

Blood samples were collected from 36 dogs representing 18 breeds, 24 red foxes, 22 arctic foxes and 24 Chinese raccoon dogs. The samples originated from dogs submitted for parentage control and foxes and raccoon dogs kept at a local fur-bearing animal farm.

Genomic DNA was extracted from peripheral blood with the use of a commercial "Blood DNA Prep Plus" kit (A&A Biotechnology, Gdansk, Poland). The entire coding sequence and 157 bp of the 5'-flanking region of the *SRY* gene were amplified with the use of primers described earlier by NOWACKA-WOSZUK *et al.* (2007). For the *SOX9* gene 10 primer pairs described by NOWACKA *et al.* (2005) were applied for the amplification of the coding sequence and a part of the 5'-flanking region (545bp). All the amplifications were performed under standard PCR conditions using a T-gradient thermocycler (Biometra, Germany). The SSCP or MSSCP techniques were applied for polymorphism screening. The SSCP electrophoresis was run for 20 hours at 20°C (130V) or at 10°C (180V). Conditions for the MSSCP technique were as follows: 1.5 hour at 30°C (180V); 2 hours at 20°C (300V) and 2 hours at 10°C (430V). A 9% polyacrylamide gel was stained with the use of a 0.2% silver nitrate solution. One animal for each fragment representing each species was sequenced at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. All obtained sequences were compared with the reference canine sequence for the *SRY* (GenBank, AF107021) and *SOX9* (EU371732 and NW876330) genes.

The *in silico* comparative analyses of nucleotide sequences were performed by the Clustal W method with the use of MegAling (DNAStar) software. The TESS software was applied to search for potential transcription binding sites in the 5'-flanking region of the studied genes.

## Results and Discussion

The lengths of the *SRY* gene coding sequences were identical in all studied species (663bp); however, numerous single nucleotide differences between the species were observed (Table 1), but no polymorphism was found in any species. Interspecies differences (single nucleotide changes) in the coding sequence were found in all three domains, including the HMG domain, which is encoded between nucleotide positions 151 and 387. The highest number of changes in the entire coding sequence, as compared to the dog sequence, was found in the arctic fox (22) and the lowest in the red fox (18). However, the number of changes in the fragment encoding the HMG domain varied from 2 (red fox) to 5 (Chinese raccoon dog). Some of the differences altered the amino acid sequence and the numbers of such differences, as compared to

Table 1

Nucleotide differences in the coding sequence of the *SRY* and *SOX9* genes (compared with the dog; *SRY*: GenBank AF107021; *SOX9*: GenBank EU371732); * - differences localized in fragment encoding the HMG domain

| Gene | Red fox | | Arctic fox | | Chinese raccoon dog | |
|---|---|---|---|---|---|---|
| | nucleotide | amino acid | nucleotide | amino acid | nucleotide | amino acid |
| *SRY* | c.12A>C | no change | c.12A>C | no change | c.14T>C | p.Leu5Ser |
| | c.41T>A | p.Val14Glu | c.57C>T | p.Ala18Val | c.57C>T | p.Ala18Val |
| | c.57C>T | p.Ala18Val | c.66G>T | no change | c.69G>A | no change |
| | c.66G>T | no change | c.69G>A | no change | c.83G>T | p.Arg28Leu |
| | c.69G>A | no change | c.82C>T | p.Arg28Phe | c.105G>C | no change |
| | c.83G>T | p.Arg28Leu | c.83G>T | | c.150C>G | no change |
| | c.109T>G | p.Tyr36Asp | c.148C>A | no change | c.219A>G* | no change |
| | c.148C>A | no change | c.150C>G | | c.310G>C* | p.Glu104Arg |
| | c.150C>G | | c.243A>G* | no change | c.311A>G* | |
| | c.243A>G* | no change | c.348A>G* | no change | c.343G>C* | pGlu115Gln |
| | c.386C>T* | p.Thr129Met | c.386C>T* | p.Thr129Met | c.386C>T* | p.Thr129Met |
| | c.451G>A> | p.Val151Ile | c.431T>C | p.Met144Thr | c.430A>C | p.Met144Leu |
| | c.464T>C | p.Phe155Ser | c.433C>G | p.Leu145Val | c.451G>A | p.Val151Ile |
| | c.469C>T | p.Pro157Ser | c.451G>A | p.Val151Ile | c.459G>C | p.Glu153Asp |
| | c.477C>T | no change | c.464T>C | p.Phe155Ser | c.464T>G | p.Phe155Cys |
| | c.481C>G | p.Thr161Arg | c.469C>T | p.Pro157Ser | c.481C>G | p.Thr161Arg |
| | c.533G>T | p.Cys178Phe | c.474C>T | no change | c.524A>G | p.Gln175Arg |
| | c.624C>T | no change | c.477C>T | no change | c.533G>T | p.Cys178Phe |
| | | | c.495T>G | p.Ser165Arg | c.624C>T | no change |
| | | | c.533G>T | p.Cys178Phe | | |
| | | | c.567G>A | no change | | |
| | | | c.624C>T | no change | | |
| *SOX9* | c.633G>T | no change | c.633G>T | no change | c.648C>G | no change |
| | c.648C>G | no change | c.648C>G | no change | | |
| | c.792A>G | no change | c.1356C>G | no change | | |
| | c.984G>T | no change | | | | |
| | c.1356C>G | no change | | | | |

the canine reference sequence, were 10 (for the red fox), 11 (arctic fox) and 14 (Chinese raccoon dog), respectively. The entire coding sequences of the *SRY* gene of the red fox (EU371736), arctic fox (EU371737) and Chinese raccoon dog (EU371738) were deposited in GenBank.

A comparison of the *SRY* gene coding sequences of the foxes and the Chinese raccoon dog with the canine sequence showed a high similarity reaching 96.7% for the arctic fox, 97.1% for the Chinese raccoon dog and 97.3% for the red fox. An analysis of the 157 bp fragment of the 5'-flanking region, apart from single nucleotide changes, also revealed insertions and deletions (Table 2). The *in silico* analysis of this fragment showed that these changes did not affect potential consensus sites for the GATA4 and WT1 transcriptional factors.

The comparative analysis of the *SRY* gene sequence included, apart from the studied species, also the human (NM003140), mouse (NM011564) and cat (DQ095188) sequences – Table 3. The similarity between the dog and cat, belonging to the same order (*Carnivora*), was the highest (80.7%). A complex, comparative analysis of the *SRY* gene, including its 5'- and 3'-flanking regions in the family Felidae, was recently presented by KING *et al.* (2007). The authors found 85 (12%) variable sites in a comparison of nucleotide sequences from 36 analyzed species. They observed higher conservatism in the region encoding the

Table 2

Nucleotide differences identified in the 5'-flanking region (compared with the dog, *SRY*: GenBank AF107021; *SOX9*: GenBank NW876330)

| Gene | Red fox | Arctic fox | Chinese raccoon dog |
|---|---|---|---|
| *SRY* | c.-30delC | c.-21CG | c.-34_-30delTTTTC |
| | c.-33TC | c.-30delC | c.-50_-49insTG |
| | c.-35TC | c.-33TC | c.-60GT |
| | c.-36TC | c.-35TC | c.-79delA |
| | c.-40CA | c.-36TC | c.-108GA |
| | c.-44CG | c.-40CA | |
| | c.-47_-46insA | c.-44CG | |
| | c.-49_-48insAG | c.-47_-46insA | |
| | c.-54CG | c.-49_-48insAG | |
| | c.-60GT | c.-54CG | |
| | c.-87AG | c.-60GT | |
| | c.-93CG | c.-87AG | |
| | c.-132_-131insA | | |
| *SOX9* | c.-36CT | c.-36CT | |
| | c.-64AC | c.-220AT | |
| | c.-337TA | c.-249CT | |
| | c.-418AG | c.-352AG | |
| | c.-420GT | c.-416TG | |
| | c.-420GT | c.-437CG | |
| | c.-439GC | c.-439GC | |
| | c.-450GC | c.-450GC | |
| | c.-493delT | c.-456CA | |
| | c.-212_-211 insCCCCCCA | c.-493delT | |
| | | c.-212_-211 insCCCCCCA | |

Table 3

Similarity of the coding sequence of the *SRY* and *SOX9* gene in seven mammalian species (below the diagonal percentage similarities refer to the *SRY* gene, and above the diagonal the *SOX9* gene)

| X | Dog | Red fox | Arctic fox | Chinese raccoon dog | Cat* | Mouse | Human |
|---|---|---|---|---|---|---|---|
| Dog | X | 99.7 | 99.8 | 99.9 | no data | 91.0 | 94.7 |
| Red fox | 97.3 | X | 99.9 | 99.7 | no data | 90.8 | 94.4 |
| Arctic fox | 96.7 | 98.5 | X | 99.9 | no data | 90.8 | 94.5 |
| Chinese raccoon dog | 97.1 | 97.3 | 96.4 | X | no data | 90.9 | 94.6 |
| Cat | 80.7 | 79.8 | 79.8 | 79.6 | X | no data | no data |
| Mouse | 44.9 | 45.2 | 44.3 | 45.7 | 36.3 | X | 91.7 |
| Human | 72.8 | 72.2 | 72.0 | 72.7 | 71.7 | 41.3 | X |

* data on the coding sequence of the feline *SOX9* gene was not available.

HMG domain (8.5% variable sites) when compared with the amino (15.9%) and carboxyl (12.7%) ends of the gene. In the regions outside the gene, 17.6% and 19.3% variable sites were found in the 5'- and 3'-flanking regions, respectively. Moreover, insertions and deletions, resulting in different lengths of the encoded protein, were observed in three species (the ocelot, clouded leopard and jungle cat). Phylogenetic analysis showed that the rate of evolution in the Felidae family (average substitution rate) amounted to 0.07% per site per million years.

The length of the coding sequence of the *SOX9* gene (1542bp) was identical in the studied species and the level of its evolutionary conservatism was much higher. Only a few nucleotide changes (outside the fragment encoding the HMG-like domain) were found in the coding sequence of this gene. Similarly to the *SRY* gene, no polymorphism was identified in any of the studied species. A comparison of the three canid sequences with the dog reference sequence (GenBank EU371732) revealed the following numbers of single nucleotide differences: 5 in the red fox (two in exon 2 and three in exon 3), 3 in the arctic fox (two in exon 2 and one in exon 3) and only 1 change in the Chinese raccoon dog (exon 2) (Table 1). These substitutions did not alter the amino acid sequence of the encoded protein. The complete coding sequences of the *SOX9* gene were deposited in GenBank for the red fox (EU371733), arctic fox (EU371734) and Chinese raccoon dog (EU371735).

A comparative analysis of the *SOX9* coding sequence between the dog and the other studied canids showed very high levels of identity: 99.7% (red fox), 99.8% (arctic fox) and 99.9% (Chinese raccoon dog), respectively. Moreover, we compared the obtained coding sequences with the human (NM000346) and mouse (NM011448) sequences deposited in GenBank (tab.3). Unfortunately, the complete cat *SOX9* gene coding sequence is still unavailable. Again, as it was already observed in case of the *SRY* gene, identity between human and mouse orthologues was lower (91.7%) than between human and dog (94.7%), red fox (94.4%), arctic fox (94.5%) and Chinese raccoon dog (94.6%), respectively. In contrast to the coding sequence, in the 5'-flanking region numerous differences (substitutions, insertions and one deletion) were found between the species (Table 2). The largest number of changes was observed in the arctic fox, while the smallest in the Chinese raccoon dog. Using TESS software a potential WT1 binding site was found within the analyzed fragment, but none of the observed nucleotide differences in any species was located at this site.

The obtained results show that the evolutionary conservatism of the *SRY* gene is lower than in case of the *SOX9* gene. This is in agreement with other published data. PATEL *et al.* (2001) analyzed both genes in six primates and calculated that the similarities of the nucleotide and amino acid sequences were 92.5% and 85.8%, respectively, for the *SRY* gene, and 98.9% and 99.8%, in the case of the *SOX9* gene. Comparative studies of 141 sequences, encoding 80 amino acids of the HMG domain of the *SRY* and *SOX* genes in different species were presented by NAGAI (2001). The author concluded that the *SRY* gene evolved very quickly (28% changes per 100 million years) when compared to the *SOX* genes (1.8% per 100 millions years). In the *SOX* gene family the most conserved is the *SOX9* gene (from 0% changes in the mouse and alligators to 1.8% in birds) and the *SOX3* gene (from 0.7% in fishes to 1.3% in birds).

Generally, *SRY* gene conservatism in placental mammals is high in the fragment encoding the HMG domain and low elsewhere (WATERS *et al.* 2007). On the other hand, the 5'-flanking region of the *SRY* gene, besides the short fragment (a potential binding site for the Sp1 protein) upstream of the transcription start site, shows poor evolutionary conservatism, as revealed in a study of ten mammalian species originating from different orders. However, within taxonomic groups (e.g. primates, rodents or bovids) the 5'-flanking sequence presents high conservatism (MARGARIT *et al.* 1998). In our study in the 5'-flanking region we found substitutions, insertions and deletions in close vicinity (between 30 and 132 nucleotide) to the translation start site, but they were not located within consensus sequences for transcription factors. A comparative analysis of the 5'-flanking region of the *SRY* gene was recently conducted by ROSS *et al.* (2008). The authors analyzed human, cattle, sheep, pig and mouse sequences and described conserved sequences and potential binding sites for transcription factors. They recognized four regions spanning from immediately upstream of the *SRY* gene up to 8.3kb from the transcription start site. Within these sequences, the authors specified 210 conserved sites for 38 transcription factor families.

The comparative analysis of the SOX9 amino acid sequence in human and mouse showed 71% similarity (BAGHERI-FAM *et al.* 2001). Moreover, the analysis of approx. 2.7 million base pairs upstream of the *SOX9* gene in human, mouse and a fish, *Fugu rubripes*, indicated the existence of 5 conserved elements (E1-E5). These elements were composed of about 100 nucleotides and their similarity varied from 67% to 80%. In human they span 290 kbp upstream of the *SOX9* gene. Moreover, conserved elements (E6-E8) were also found in the 3'-flanking region. It is thought that mutations in these fragments can disturb the expression of the *SOX9* gene. In our study only a few changes in the

open reading frame were found, confirming that the *SOX9* gene is highly conserved in mammals. However, many differences were observed in the 5'-flanking region, including substitutions, insertions and a deletion, but these changes did not alter consensus sequences for transcription factors.

In conclusion, our study shows that in the family Canidae the coding sequence of the *SRY* gene is less evolutionary conserved than that of the *SOX9* gene. In case of the 5-flanking sequences, this difference is not as distinct. Moreover, a lack of polymorphism indicates high conservatism within the species.

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