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SOX9 Gene Variants in 27 French Bulldogs with Disorder of Sex Development (XX, SRY-Negative): Identification of First Case of Skeletal Abnormalities Associated with SOX9 Triplication

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Keywords

Copy number variation · Dog · Droplet digital PCR · Disorder of sex development · Intersexuality · Ovotestis · Sex reversal

Abstract

Introduction: The *SOX9* gene encodes a transcription factor that acts downstream of the Y-linked *SRY* gene and plays a pivotal role in fetal testis development. Duplication of *SOX9* or its regulatory sequences is a known cause of testicular or ovotesticular disorder of sex development (DSD) in chromosomal females (XX DSD). Numerous reports have described canine XX DSD, characterized by virilization (e.g., enlarged clitoris) and the presence of testes or ovotestes. This study aimed to identify *SOX9* variants in a cohort of

French bulldogs with XX (*SRY*-negative) DSD. **Methods:** In total, 27 DSD dogs were studied, including 20 with abdominal, spermatogenetically inactive testes, three with inactive testis and ovotestis, one with inactive testis and ovary, and one with ovotestes; in two dogs, analysis of gonads could not be performed. Moreover, 24 control females of the same breed, all with normal external female genitalia, were included. **Results:** Three known DNA variants were identified in *SOX9*: a 3 bp insertion/deletion (CCT/---, rs852828782), a T>C SNP (rs22704771) in the 5' UTR, and an intronic T>G SNP (rs9183825). These variants were rare, and their distribution was similar in both cohorts. Additionally, the number of *SOX9* gene copies was assessed using ddPCR. A single XX DSD case with additional skeletal malformations carried three copies of *SOX9*, while all other cases and control females had two copies. **Conclusion:** We

conclude that *SOX9* duplication is a rare cause of XX DSD in French bulldogs, and that the identified sequence variants in this gene are not associated with the disorder.

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Introduction

Testicular or ovotesticular disorder of sex development (DSD) in chromosomal females (XX) is a congenital malformation diagnosed in numerous dog breeds [1], with French bulldogs among the most frequently affected [2–4]. Unfortunately, the molecular basis of this DSD remains poorly understood. However, several reports suggest that causative or associated DNA variants may be located in the chromosomal region containing the *SOX9* gene. This gene, whose expression is triggered by the sex-determining region Y protein (SRY), plays a crucial role in directing the development of undifferentiated fetal gonads into testes [5].

In human patients with XX DSD (*SRY*-negative), various DNA variants have been identified, including duplications or multiplications of sequences containing regulatory elements of the *SOX9* gene, as well as duplications of the entire *SOX9* locus [6, 7]. Studies of XX (*SRY*-negative) DSD dogs have also revealed an association with an increased number of repeats in the copy number variation region (CNVR1) located upstream of *SOX9* [8]. Targeted sequencing of a 1.5-Mb region on canine chromosome 9, which includes the *SOX9* gene, revealed sequence similarity between CNVR1 and the 5' flanking region of the *MAGI2* gene, located on a different canine chromosome and encoding a protein involved in ovarian development. These findings suggested that CNVR1 contains regulatory sequences of *MAGI2*, which, if duplicated, may potentially induce ectopic expression of *SOX9* in XX DSD individuals, resulting in the repression of female sex development pathway [9]. Further studies confirmed the association of CNVR1 with XX DSD; however, this relationship was not observed in French bulldogs or American Staffordshire Terriers [10]. Conversely, whole genome sequencing has suggested an association between the XX DSD phenotype and a missense single-nucleotide polymorphism (SNP) in the *PADI6* gene in both breeds [4]. Another study on the distribution of copy number variation regions (CNVRs) in XX DSD dogs was performed using a comparative genome hybridization approach. One of the 11 identified CNVRs included the promoter of the *SOX9* gene. This CNVR, a duplication of a 458-kb segment, was present in three out of the four studied DSD cases [11]. Addi-

tionally, duplications of *SOX9* have also been reported in XX DSD dogs [10, 12].

Analyses of the human *SOX9* sequence have revealed that mutations altering the amino acid sequence of the encoded protein cause skeletal abnormalities (campomelic dysplasia), and in patients with XY sex chromosomes, DSD is also observed [13]. There is also a single report describing a gain-of-function missense mutation in *SOX9* in an XX DSD patient [14]. Potential importance of *SOX9* gene variants in the pathogenesis of testicular or ovotesticular XX (*SRY*-negative) was also shown in pigs [15]. The *SOX9* sequence has previously been studied in XX DSD dogs, including French bulldogs, but no associated DNA variants were detected [4, 16]. The aim of the present study was to search for causative or associated DNA variants in the coding sequence of the *SOX9* gene, as well as to assess its copy number variation, in two cohorts: 27 XX (*SRY*-negative) DSD French bulldogs and 24 unaffected control females of the same breed.

Material and Methods

Animals

A total of 27 French bulldog females with an enlarged clitoris (DSD cases) were included in the study, comprising 19 cases reported previously [2–4, 8, 10, 17] and 8 newly analyzed cases (online suppl. Table S1; for all online suppl. material, see <https://doi.org/10.1159/000548218>; Fig. 1; Table 1). A single DSD case (ID_7568) presented with skeletal malformations. Moreover, molecular studies of the *SOX9* gene were performed in 24 control female dogs of the same breed (online suppl. Table S3).

Methods

Cytogenetic, Molecular, Histological, and Radiographic Analyses of New DSD Cases

Chromosome preparations obtained from short-term lymphocyte cultures were stained with Giemsa. Sex chromosomes were identified based on their banded morphology, in contrast to the single-armed morphology of all autosomes. Microscopic evaluation was performed using a Nikon Eclipse E600 Fluorescence Microscope (Melville, NY, USA) equipped with a cooled CCD digital camera and Lucia software.

Molecular detection of Y-linked genes (*SRY* and *ZFY*) and the X-linked *ZFX* gene was carried out as previously described [18]. Histological examination of the gonads was possible in five out of the eight newly described

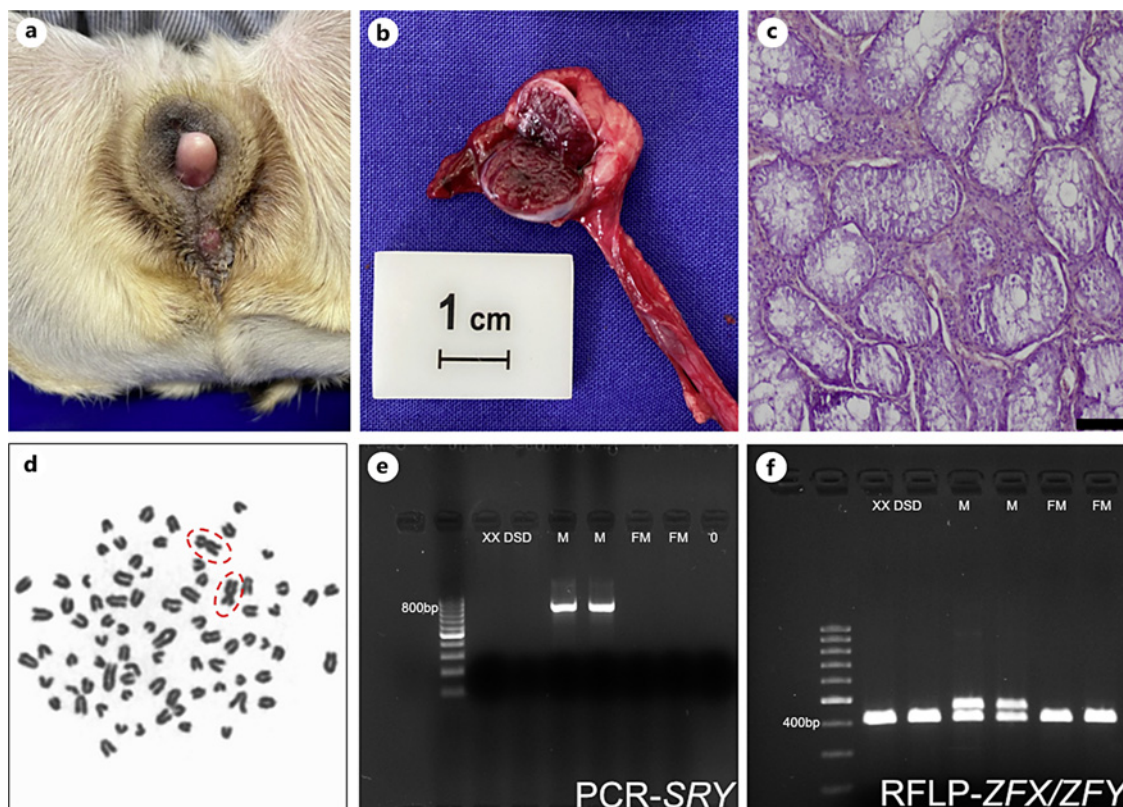


Fig. 1. Characteristics of DSD cases. **a** Enlarged clitoris – animal ID_7507. **b** Surgically removed testis – animal ID_7507. **c** Histology of inactive testis – animal ID_7507 (scale bar = 100 μ m). **d** XX metaphase spread – animal ID_7707. **e** Absence of the *SRY* gene – animal ID_7813. **f** *ZFY*-negative and *ZFX*-positive – animal ID_7813. M, control males; FM, control females; 0, negative control (sample with no DNA template).

cases. The gonads were fixed in 10% formalin, and tissue sections were stained with hematoxylin and eosin (Masson-Goldner method). Radiographic (X-ray) examination was performed for one case with suspected skeletal malformations.

Sequencing of *SOX9* Gene

The entire coding sequence of the *SOX9* gene was amplified by PCR using primers reported previously [16]. In addition, a 515 bp fragment of the 5' untranslated region (5'UTR) was amplified using the following primers: forward (F): 5'-AGCCTTACCCGCAAAATCA; reverse (R): 5'-CTGCTCGTCGGTCATCTTCA.

The annealing temperature was set at 58°C. PCR products were sequenced using the Sanger method, beginning with enzymatic purification using exonuclease I and alkaline phosphatase (Thermo Fisher Scientific), according to the manufacturer's protocol. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), followed

by fragment purification with Sephadex G-50 columns (Sigma). The final products were analyzed by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems). Sequence data were analyzed using DNASTar software, and the resulting chromatograms were compared with the reference genomic sequence (NC_051813).

Detection of *SOX9* Copy Number by Droplet Digital PCR

The copy number of the *SOX9* gene was estimated using droplet digital PCR (ddPCR), following the protocol described previously [10]. Five assays were designed to amplify sequences specific to the 5' flanking region, 5'UTR, exon 1, exon 2, and exon 3 of the gene. *HSD17B7* or *C7orf28B3* was used as reference gene. All probes targeting *SOX9* were labeled with the FAM fluorophore, while probes targeting the reference genes were labeled with the HEX fluorescent dye. Primer and probe sequences are listed in online supplementary Table S2. Genomic DNA was digested with either *HaeIII* or *HindIII*

Table 1. Gonadal status and genotypes at three investigated sites in *SOX9* gene of XX (*SRY*-negative) DSD dogs

Animal ID	Gonads	rs852828782	rs22704771	rs9183825	References
7461	T	ins/del	TT	TG	This study
7507	T	ins/ins	CC	TT	This study
7522	NA	ins/ins	CC	TT	This study
7548	T	ins/ins	CC	TT	This study
7567	T	ins/ins	CC	TT	This study
7568 ^a	T	ins/del	TC	TG	This study
7707	NA	ins/ins	TT	GG	This study
7813	T + OT	ins/ins	TC	TT	This study
6916	T	ins/ins	TT	TT	10
6619	T	ins/ins	TC	TT	2
6692	T	ins/ins	TC	TT	8
6717	T	ins/ins	CC	TT	17
6780	T	ins/ins	CC	TT	10
6795	OT	ins/ins	CC	TT	10
6882	T	ins/ins	TC	TT	10
6888	T	ins/ins	CC	TT	10
6889	T	ins/ins	CC	TT	10
6894	T + OT	ins/ins	CC	TT	10
6909	T	ins/ins	TC	TT	10
6910	T + O	ins/ins	TC	TT	10
7004	T	ins/ins	CC	TT	3
7075	T	ins/ins	TC	TT	3
7077	T + OT	ins/ins	CC	TT	3
7119	T	ins/ins	TC	TT	3
7127	T	ins/ins	CC	TT	3
7303	T	ins/ins	CC	TT	4
7323	T	ins/ins	CC	TT	4

T, testis; OT, ovotestis; O, ovary; NA, not analyzed. ^aPresence of skeletal malformations.

restriction enzymes prior to ddPCR. The ddPCR was performed according to the manufacturer's instructions (Bio-Rad). Fluorescence detection was carried out using a QX200 Droplet Reader (Bio-Rad), and the results were analyzed with QuantaSoft software (Bio-Rad).

Results

Among the newly diagnosed XX DSD cases, the following gonadal statuses were observed: (i) spermatogenetically inactive testes in four cases, and one case

with a testis and an ovotestis, as determined by histological analysis; (ii) morphologically confirmed testes observed post-gonadectomy without histological examination (one case); and (iii) two cases with abdominal gonads that were not removed (Table 1, online suppl. Table S1). In previously reported DSD cases, gonadal histology was examined in all 19 dogs: 15 had spermatogenetically inactive abdominal testes, two had a testis and an ovotestis, one had an inactive testis and an ovary, and one had ovotestes (Table 1). All newly diagnosed DSD cases had a normal female karyotype (78,XX) and, as expected in normal females, lacked the

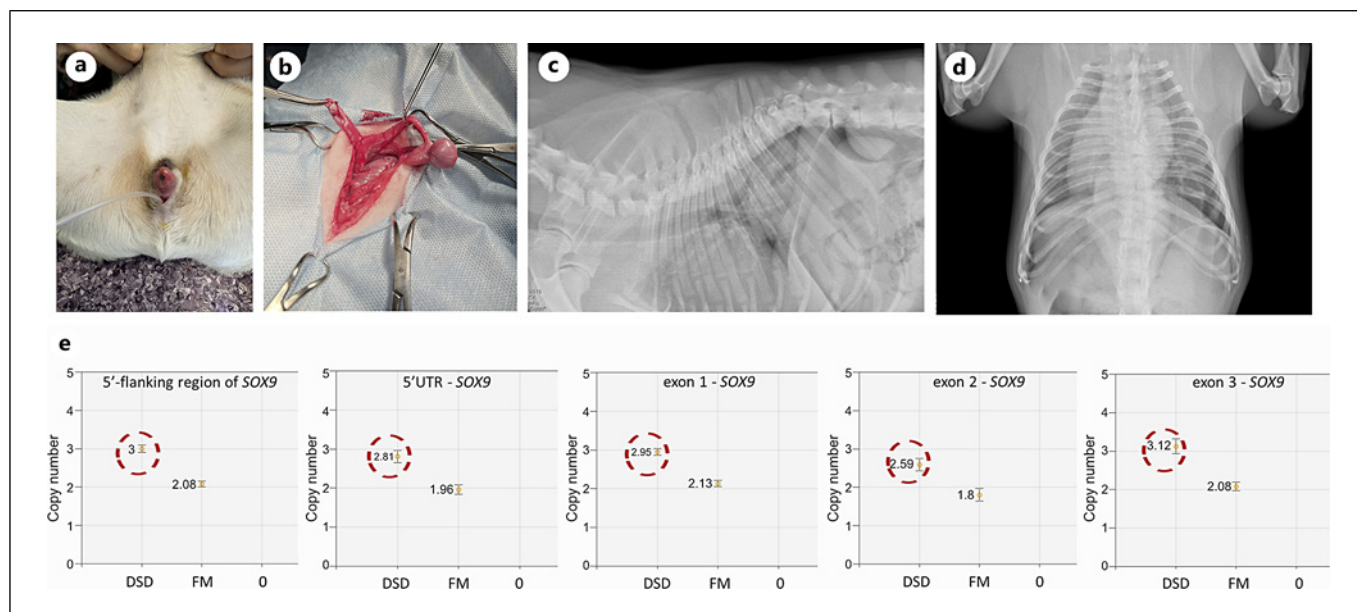


Fig. 2. XX DSD dog with skeletal malformations (ID_7568). **a** Enlarged clitoris. **b** Internal genitalia during surgery. **c, d** X-ray images of the thoracic spine showing vertebral deformities. **e** Detection of three copies of the 5'-flanking region and exons of SOX9 by ddPCR. FM, control female; 0, negative control.

Y-linked genes *SRY* and *ZFY* (Fig. 1). In a single case of testicular XX DSD (ID_7568), skeletal malformations were also observed.

No DNA variants were found within the coding sequence of the *SOX9* gene. However, three known variants were identified in the 5'UTR and intron 2. In the 5'UTR, a 3 bp insertion/deletion (CCT/---, rs852828782) and a T>C SNP (rs22704771) were detected, while the intron 2 variant was a T>G SNP (rs9183825). The distribution of genotypes at the three studied sites in DSD dogs is presented in Table 1, and that for control females in online supplementary Table S3. At the indel site (c.-226_-224del), two DSD dogs were heterozygous (ins/del), and the remaining cases were homozygous for the insertion (ins/ins) (online suppl. Fig. S1). Among control females, three were heterozygous (ins/del) and 21 were homozygous for the insertion (ins/ins). For the 5'UTR SNP (c.-36T>C), genotype distribution in the DSD cohort was as follows: CC = 15, TC = 9, and TT = 3 (online suppl. Fig. S2). In the control group, the distribution was: CC = 10, TC = 11, TT = 3. For the intronic SNP (c.685 + 13T>G), the following genotype distribution was observed among DSD cases: TT = 24, TG = 2, GG = 1 (online suppl. Fig. S3); in the control group, genotypes were distributed as follows: TT = 19, TG = 5, GG = 0.

Analysis of *SOX9* copy number revealed the presence of two copies in 26 XX DSD cases and in all control females. However, in one dog with testicular XX DSD (ID_7568) three copies of this gene were found. Interestingly, this dog also exhibited skeletal malformations, including advanced and numerous hemivertebrae (deformation and shortening) affecting nearly the entire thoracic spine; adhesions of multiple spinous processes; markedly narrowed intervertebral spaces at C2/C3 and L1/L2; and slight deformation and shortening of the L1 vertebra (Fig. 2).

Discussion

The molecular background of testicular or ovotesticular XX DSD has been extensively studied in humans, with causative DNA variants identified in several genes, including *SOX3*, *SOX9*, *SOX10*, *RSPO1*, and *WNT4* [6]. It is worth mentioning that the DSD phenotype can also be caused by altered epigenetic mechanisms, e.g., abnormal modification of lysine 9 in histone H3, such as its demethylation, which leads to male-to-female sex reversal in mice [19], or its acetylation, which is associated with an increased risk of cryptorchidism in dogs [20].

In domestic animals, a causative mutation – a large deletion affecting the expression of the *FOXL2* gene – has been identified only in goats [21]. In the present study, we performed a molecular analysis of *SOX9* in French bulldogs, a breed in which XX (SRY-negative) testicular or ovotesticular DSD is relatively common. To the best of our knowledge, this is the largest cohort of unrelated XX DSD dogs from a single breed in which a molecular study of the *SOX9* gene has been conducted. Eight newly diagnosed XX DSD cases were included in this study, further supporting the notion that French bulldogs are predisposed to this disorder. In one XX DSD case, three copies of the *SOX9* gene were detected. This finding is consistent with previous reports indicating that this phenomenon is a rare cause of XX (SRY-negative) DSD in both humans [22, 23] and dogs [10, 12].

The *SOX9* gene encodes a transcription factor involved in the regulation of organogenesis in various organs, including bone and testis [24]. It consists of three exons encoding a highly conserved amino acid sequence across mammalian species. The *SOX9* polypeptide comprises 509 amino acids in humans and 513 in dogs. The amino acid sequence similarity between humans and dogs is 94.75%, while for other mammals (e.g., pig, horse, cat, mouse, and rat), the similarity is slightly higher (>96%) [7]. High conservation of this gene has also been observed within the Canidae family, including dog, red fox, arctic fox, and Chinese raccoon dog [25]. In these species, the similarity of the coding sequence ranges from 99.7% to 99.9%.

Data on polymorphisms in the 5'UTR of the canine *SOX9* gene remain limited. However, a study of this region in XX DSD pigs indicated that an 18 bp indel affects *SOX9* expression in vitro [26]. It was suggested that this variant might be associated with the DSD phenotype. Subsequent studies, however, did not confirm this association [27].

Loss-of-function DNA variants in *SOX9* are known to cause campomelic dysplasia in humans, a syndrome characterized by skeletal abnormalities. In affected human males (46,XY), a DSD phenotype is also observed. This condition is rare and in most cases is caused by de novo dominant mutations in *SOX9* [13]. Interestingly, in the XX DSD French bulldog examined in this study – who carried three copies of *SOX9* and exhibited skeletal malformations – no coding sequence variants affecting the amino acid sequence of the *SOX9* protein were found. This dog was heterozygous at all three polymorphic sites: two located in the 5'UTR and one in intron 2. However, the same heterozygous genotype was also observed in

control females. It is worth noting that the spinal malformations observed in this dog were similar to those reported in a human 46,XX patient with a missense mutation in *SOX9* [28].

In conclusion, our findings support earlier observations that French bulldogs are predisposed to XX (SRY-negative) testicular or ovotesticular DSD. Three copies of the *SOX9* gene appear to be a rare variant associated with this condition, while nucleotide variants in this gene were not linked to the DSD phenotype or skeletal malformations. To the best of our knowledge, this study presents the first reported case of a testicular XX (SRY-negative) DSD dog with skeletal abnormalities carrying three copies of the *SOX9* gene.

Statement of Ethics

Blood and gonad samples were collected in veterinary clinics during routine neutering procedures, following standard veterinary protocols. Sample collection was carried out with the owners' consent and with the approval of the local Bioethical Commission for Animal Care and Use in Poznan, Poland (certificate no. 1/2019). All procedures were conducted in accordance with relevant guidelines and regulations, including Directive 2010/63/EU.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.N.W.: molecular studies and manuscript preparation; I.S.: cytogenetic and molecular analyses, and manuscript preparation; S.A.: cytogenetic and molecular analyses, and sample collection; B.S., D.R., W.N., N.S., N.T., M.M., and E.D'.A.: clinical investigation and material collection; S.D.: histological analysis; M.So. and Z.S.: molecular analysis; M.Sw.: study supervision and manuscript preparation.

Data Availability Statement

All data are presented in this article.

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