A revised genome assembly of the region 5' to canine SOX9 includes the *RevSex* ortholog region

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ABSTRACT

The *SOX* gene family includes many genetic factors that play a determinant role in several developmental pathways. The *SOX9* gene has been identified as a major factor in testis development in mammals after it is activated by the *SRY* gene. However, duplication of the gene itself in some mammalian species, or of a well-delimited upstream '*RevSex'* region in humans, has been shown to result in testis development in the absence of the *SRY* gene. In the current study, we present an accurate analysis of genomic organization of the *SOX9* locus in dogs by both in silico and FISH approaches. Contrary to what is observed in the current dog genome assembly, we found that the genomic organization is quite similar to that reported in humans and other mammalian species, including the position of the *RevSex* region in respect of *SOX9*. The analysis of the conserved sequences within this region in seven mammalian species facilitated the highlighting of a consensus sequence for SRY binding. This new information could help in the identification of evolutionary conserved elements relevant to *SOX9* gene regulation, and could provide valid targets for mutation analysis in XY DSD patients.

INTRODUCTION

The transcription factor *SOX9* belongs to the *SOX* (SRY-related HMG box) gene family and in humans, plays a pivotal role in several embryonic developmental pathways, such as chondrogenesis [Murakami et al., 2000] and testis determination [Eggers et al., 2014]. Conditional gonad-specific knockout of *Sox9* in XY mice embryos results in the development of ovaries [Barrionuevo et al., 2006], whilst its ectopic overexpression in XX embryonic gonads leads to the development of testes [Vidal et al., 2001; Qin et al., 2004]. In humans, *SOX9* haploinsufficiency results in campomelic dwarfism with XY DSD (Disorders of Sexual Development) in around 50% of cases [Wagner et al., 1994]. No case has ever been reported with *SOX9* gain-of-function mutations, or duplications involving only the gene's coding region. On the contrary—both copy number variants (CNVs), and interruptions due to translocations within the more than 1.9 Mb desert region at the 5' of *SOX9*, result in different abnormal phenotypes, including: Pierre Robin sequence, brachydactyly-anonychia, congenital heart defects, and both XY and XX DSD (see Figure 3 in Vetro et al. [2014]). These findings suggest that this desert region contains a series of tissue- and time-specific regulatory elements, responsible for the proper activation of the gene during embryogenesis. In fact, genomic defects of the desert region partly recapitulate the condition of *SOX9* haploinsufficiency with campomelic dwarfism and XY DSD, whilst these might partly represent the effects of its overexpression. In particular, duplications of a minimal overlapping ~70 kb region (*RevSex* region), at 500 kb upstream of *SOX9*, have been shown to result in testis development in more than 10% of the SRY-negative XX individuals [Vetro et al., 2014], suggesting that the duplication leads to *SOX9* overexpression through the increased dosage of gonadal-specific enhancers located within the *RevSex* region. Testis differentiation in absence of *Sry* was also documented in XX transgenic mice [Vidal et al., 2001], in one XX deer [Kropatsch et al., 2013] and two XX dogs [Rossi et al., 2014], all with the duplication of the *SOX9* gene, again suggesting that

its overexpression may vicariate SRY. In our previous study [Rossi et al., 2014], we reported the molecular analysis of seven XX DSD dogs, and showed that two of them carried SOX9 gene duplication. Moreover, we identified—in all the seven dogs—a complex CNV region located at CF9:16,637,884–18,258,282 (CanFam3 genome assembly), already reported by others as polymorphism [Chen et al., 2009; Nicholas et al., 2009; Nicholas et al., 2011; Quilez et al., 2011; Berglung et al., 2012; Molin et al., 2014], appearing either as gain or loss across the whole region, or gain or loss of only one part of the region. This CNV was located at more than 8 Mb from the 3' end of the SOX9 gene (CFA9:8,275,049–8,278,172, plus strains transcription direction), it surprisingly included the orthologous of human *RevSex* region, and it never segregated with the XX DSD condition (Figure 1). These findings cast doubt on the point that this *RevSex* region may contain elements that regulate the expression of SOX9 in the gonad. Since the genomic structure of the entire desert region upstream of SOX9 appears conserved in various mammalian species, we have decided to investigate the correctness of the genomic assembly of the SOX9 locus in dogs by *in silico* and FISH (Fluorescent *In Situ* Hybridization) approaches. Moreover, we have tried to highlight some of the regulation mechanisms dictated by the elements located in the unusual large desert region upstream of *SOX9*, by the comparison of its sequence in seven mammalian species.

MATERIALS AND METHODS

Bioinformatics

Comparison among the considered *RevSex* regions was carried out using the Mulan software (available online for free at mulan.dcode.org) [Ovcharenko et al., 2005]. The parameters considered in this analysis were as follows: ECR (Evolutionary Conserved Region) length: at least 100 bp and ECR similarity: at least 95%. Comparison of human and dog desert regions was carried out with VISTA software [Frazen et al., 2004] (available

online for free at http://genome.lbl.gov/vista/mvista/). The VISTA parameters were minimum conservation identity of 95% and minimum length for a CNS of 50 bp. Other comparisons, including those for the construction of a new assembly of dog chr9 (50 Mb), were performed using BLAST and BLAT tools, available for free at NCBI and UCSC genome browsers, respectively [Altschul et al., 1990; Kent et al., 2002]. In the case of the analysis of the whole dog chromosome 9, a 1,000 bp region for approximately every 100 kb has been utilized to manually carry out the comparison between man and dog.

FISH

FISH experiments were performed, as reported by De Lorenzi et al. [2014]. When three probes were used in the same experiments, one probe was labelled with two different fluorochromes, and the yellow colour was assigned. The BACs (Bacterial Artificial Chromosomes) used as probes belong to CH82 library (Chori), and are reported in Table 1; their schematic positions are also presented in Figure 1.

Dog cell cultures

Peripheral blood samples were collected from several dogs to obtain the prometaphase stage. Chromosome preparations were obtained from a 72 h lymphocyte cell culture in PB-MAX™ Karyotyping Medium (Gibco), according to a standard procedure [Iannuzzi and Di Berardino, 2008].

RESULTS

In silico studies

Using several in silico comparative analyses, we produced a new sequence of the SOX9 locus in dog, which was derived by the combination of CF9 16,475,722–17,962,968 region and CF9 7,937.692–8,275,049 (Figure 2 and Table 2). This 1,824,605 bp long sequence (Supplementary Information 1) is homolog to that present in the other species. Considering this new assembly, the dog region orthologous to the minimal human *RevSex* region (73-

Kb) was found to be located at 662 Kb at the 5' end of the *SOX9* gene, which is comparable with what was observed in humans and the other species considered (Table 3). Conservation analysis performed using Mulan software along the 73-Kb genomic region revealed a different highly conserved region. In this analysis, we discarded the information about gorilla, chimp, and rhesus due their high similitude with the human genome; consequently, only seven species were retained. Supplementary Figure 1 presents the ECRs with a similitude equal to or higher than 95.

A search for the conserved transcription factor evidenced a certain number of conserved sites (not shown). Among these conserved sites, the only one that perfectly conserved the SRY binding site was within the 4461–4472-bp human *RevSex* region, corresponding to 69,531,621–69,531,632 genomic region of the hg19 genome assembly; the positions on the other genome assembly are reported in Table 4. Furthermore, no SNPs were found in this human region, suggesting a specific role for this sequence of bases. Comparative analysis, using VISTA tool, considering the whole desert region of humans and dogs, showed that the *RevSex* region, in its complete length, was not the only well-conserved region in the desert region (Supplementary Figure 2).

We also performed a preliminary bioinformatic analysis, as reported in the Materials and Methods section, to observe the chromosome 9 dog assembly in comparison with the hg19 human assembly. As reported in supplementary Figure 3, many discrepancies were noted, and further experiments, including FISH, re-sequencing or more detailed bioinformatics approaches, are needed to confirm or discard these discrepancies and consequently determine the evolutionary rearrangements.

FISH studies

The assembly of dog chromosome 9 showed the presence of four gaps with no clone bridge; the positions of these gaps are presented in Table 5. As shown in Figure 1, three of these gaps were just around *SOX9*, whereas the last one was found to be very distal (>39

Mb). *SOX9* gene was included between Gaps 1 and 2, which were separated by 1.5 Mb. By using several BACs as probes for FISH experiments, we observed that this assembly was probably incorrect.

By BACs 104H05 and 195H24, we verified that the orientation of the 'blue' contig (Figure 1b) was inverted with respect to that reported in the genome assembly (Figure 3a), with 195H24 that was clearly more centromeric than 104H05. Moreover, the observation of two distinct FISH signals was compatible with the distance reported in the genome assembly, of more than 8 MB. FISH experiments with 297F12 and 195H24 confirmed that the 'blue' contig was inverted, since the two BACs co-localized in the same genomic region, in contrast to what was reported in the genome assembly, where they resulted to be at a distance of 9.3-Mb (Figure 3b). The orientation and position of the 'green' contig (Figure 1b), which included the *SOX9* gene, were more difficult to verify due to its short length (1.5 Mb). To increase the experiment's resolution, we performed interphase FISH, using three BACs: 297F12, 240J07, and 195H24, which showed that the order of the BACs on the dog genome was 240J07-297F12-195H24 (yellow-red-green in Figure 3b). However, it was impossible to identify the centromere and telomere positions; thus, considering the order of the three BACs shown in Figure 3b, two hypotheses were considered (Figure 3e), with the first one appearing more reliable due to the co-localization of 195H24 and 297F12 (Figure 3c). Further experiments to test the orientation of the pink fragment (Figure 1b) by BACs 7K11 and 523D12 (Figure 3d) confirmed the proposed genome assembly.

DISCUSSION

In humans, the *SOX9* gene is preceded by a large region without any protein-coding genes, the so-called *SOX9* desert region; indeed, the closest gene at its 5', *KCNJ2*, is at a distance of 1.9 Mb. This genomic assembly was found to be maintained in several species (Table 2), but not in dogs (canFam2), where the *KCNJ2* gene was determined to be about

8 Mb far from the *SOX9*, and, together to a large part of the human homolog desert region, at its 3' end.

In the present study, we report on a new assembly of this region in dogs. By FISH and *in silico* analysis, we could demonstrate that it has a complete homology with the human region (Figure 2).

The availability of the correct dog desert region assembly allows us to perform comparative analyses to identify the *SOX9* regulatory regions. In this context, the dog is a very interesting species, because it shows abnormal sexual development caused by *SOX9* alterations, comparable with those described in humans. Moreover, in humans, chromosomal rearrangements within the desert region can result in different congenital malformations. Although a clear genotype-phenotype relation is missing, each type of malformation correlates rather well with the localization of CNV/translocations in a specific portion of the desert region (Figure 3 in Vetro et al., [2014]), suggesting that within each portion, regulatory elements are located, switching on and off the gene in a tissue-and time-specific sequence, in order to ensure normal embryo development. The homology of the desert region among different mammalian species, including dogs, suggests that the sequence of these elements obeys a successful programme of differentiation. As presented in Supplementary Figure 2, this genomic region shows the presence of several highly conserved portions, among which the so-called *RevSex* region, as defined by the identification of duplications at 500kb far from the *SOX9* 5' in about 10% of the *SRY*-negative XX males [Vetro et al., 2014]. The minimal overlapping region among these subjects is of 73-Kb. Comparative analysis of this region in seven different mammalian species (Supplementary Figure 1) revealed the presence of several conserved portions, with four of them sharing high homology. This fact could be interpreted as the importance of these regions in different species. Among these conserved sites, one, corresponding to 69,531,621–69,531,632 genomic region of the hg19 genome assembly, showed the same

consensus sequence of the SOX proteins binding sites (Table 4). A specific role for that sequence is also strengthened by the absence of SNPs (Single Nucleotide Polymorphisms) in the human population within this consensus. We might tentatively hypothesize that this consensus sequence represents the DNA binding site for the protein encoded by SRY. In fact, 46,XY DSD-associated deletions, including the RevSex region, have been reported in humans (red bars in Figure 3 of Vetro et al., [2014]). The *Sox9* expression in the Sertoli cell precursors of the developing gonads closely follows that of the *Sry* gene [Sekido et al., 2004] that, at least in mouse, promotes the Sox9 gene expression in synergy with SF1, via a regulatory element—the so-called TESCO, TEstis-Specific enhancer Core Sequence—located upstream of Sox9 [Sekido et al., 2008]. However, the importance of TESCO in other species remains to be demonstrated. Till date, in humans, the analysis of many XY DSD patients did not show any mutation in TESCO [Georg et al., 2010].

Further experiments involving ChIP or mutation analyses in XY sex reversal patients could clarify the relevance of this SOX proteins binding site in the SOX9 activation process.

This hypothetical binding site for SRY, and subsequently for most of the SOX genes, might represent a good target for mutation analysis in DSD subjects. It should be emphasized that the role of the *RevSex* region in gonadal differentiation is attenuated by the finding that in dog, it is contained within a CNV, suggesting that its deletion/duplication does not lead to alterations of embryonic development. This might indicate that the regulatory mechanisms of the expression of *SOX9* are different from those acting in humans, making this region dispensable in the dog. However, it is clear that *RevSex* cannot be the only actor in the activation of *SOX9* in the precursor cells of the gonad. This is suggested by the incomplete penetrance of *RevSex* duplications in human XX subjects that are reported as infertile males, or with different degrees of genital ambiguity, or even as fertile females [Benko et al., 2011]. Also, we should not forget the role of *NR5A1*, well demonstrated by

the many correlations between mutations and abnormalities in the developmental and gonadal function in both the XY and XX backgrounds [Achermann et al., 1999; Lourenco et al., 2009]. It will be also interesting to look for the DNA binding site of other transcription factors, such as *NR5A1*, *DMRT1* or *WT1*. The picture that emerges from all these data is that of a mechanism of expression finely tuned by several factors, which leave ample room for a wide range of phenotypic effects, from a normal development at one extreme to a total sex reversal at the other. The detection of intermediate phenotypes, such as lower fertility, hypospadias, and curved penis, are largely overlooked in the dog, making difficult a reliable genotype-phenotype relationship between humans and dogs. The detection of all the DNA binding sites for all the transcription factors modulating the expression of SOX9 (NR5A1, DMRT1, WT1) will largely clarify how gonadal differentiation occurs.

In conclusion, we have defined a new assembly of the desert region upstream of *SOX9* in dog, which perfectly fits with the one detected in other mammalian species, including humans. We have also highlighted a few conserved sequences that might play a regulatory function, at least in gonadal development, and whose real role may now be investigated in those individuals for which the molecular basis of their abnormal gonadal differentiation/function has not yet been discovered.

ACNOWLEDGEMENTS

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TABLES

Table 1

List and genomic position on CanFam3 genome assembly of used BACs as probes in FISH experiments.

BAC	Start (bp)	End (bp)	Notes
195H24	17,609,124	17,778,444	*RevSex* region
104H05	9,165,409	9,349,831	5’ blue contig
297F12	8,169,940	8,361,578	Sox9 gene
240J07	8,361,178	8,514,176	3’ Sox9 gene
523D12	7,175,358	7,467,663	3’ pink contig
7K11	283,933	491,656	5’ pink contig

Table 2

Sox9 desert region features

Species	Assembly	Chr	KCNJ2 *TGA*	SOX9 *ATG*	Strand	Dimension (bp)
Human	hg19	17	68,172,464	70,117,533	+	1,945,069
Rabbit	oriCun2	19	53,923,203	55,820,838	+	1,897,635
Mouse	mm10	11	111,073,070	112,782,585	+	1,709,515
Chimp	panTro4	17	68,967,727	70,943,563	+	1,975,836
Rhesus	rheMac3	16	67,559,318	69,517,462	+	1,958,144
Cattle	bosTau6	19	61,190,245	59,494,539	-	1,695,706
Pig	susScr2	12	8,647,274	6,871,238	-	1,776,036
Horse	equCab2	11	10,866,408	9,239,727	-	1,626,681
Gorilla	gorGor3	5	13,247,412	11,279,102	-	1,968,310
Dog	canFam3	9	16,475,722	17,962,968	+	1,487,247
			7,937,692	8,275,049	+	337,358
						1,824,605

Table 3

***RevSex* 73 Kb region features**

Species	Assembly	Chr	Start	End	Strain	Length (bp)	SOX9 ATG distance (bp)
Human	hg19	17	69,527,161	69,600,161	+	73,000	517,372
Rabbit	oriCun2	19	55,250,453	55,325,677	+	75,224	495,161
Mouse	mm10	11	112,254,026	112,332,766	+	78,740	449,819
Chimp	panTro4	17	70,339,315	70,413,571	+	74,256	529,992
Rhesus	rheMac3	16	68,920,845	69,001,073	+	80,228	516,389
Cattle	bosTau6	19	60,018,541	59,958,205	-	60,336	463,666
Pig	susScr2	12	7,572,787	7,510,046	-	62,741	638,808
Horse	equCab2	11	9,732,241	9,675,198	-	57,043	435,471
Gorilla	gorGor3	5	11,873,051	11,799,505	-	73,546	520,403
dog	canFam3	9	17,573,722	17,638,252	+	64,531	662,074

Table 4

SRY conserved binding sites

Species	Strand	Start*	End*	Sequence	Assembly	Chr	Start	End
Human	+	4461	4472	acaAACAAtaag	hg19	17	69,531,621	69,531,633
Pig	-	3491	3502	acaAACAAtaag	susScr2	12	7,569,286	7,569,297
Cattle	-	3400	3411	acaAACAAtaag	bosTau6	19	60,015,131	60,015,142
Horse	-	3991	4002	ataAACAAtaag	equCab2	11	9,728,240	9,728,251
Mouse	+	4761	4772	acaAACAAtaag	mm10	11	112,258,786	112,258,797
Rabbit	+	4802	4813	acaAACAAtaag	oriCun2	19	55,255,254	55,255,265
Dog	-	4380	4391	acaAACAAtaag	canFam3	9	17,633,860	17,633,872

* respect to *RevSex* lenght sequence reported in Table 3

Table 5

Position of no bridge gaps in dog chromosome 9 canFam3 genome assembly

Gap	Start	End
Gap 1	7,516,264	7,517,263
Gap 2	8,956,386	8,957,385
Gap 3	18,187,916	18,188,917
Gap 4	47,774,103	47,775,102

FIGURE LEGENDS

FIGURE 1

Schematic position of the gaps and positions of relevant genomic features.

Whole canFam2 chromosome 9, as noted in the UCSC web browser (a) Red arrows show the position of the four gaps. Blue arrow indicates the *SOX9* gene position. The coloured lines represent the contigs presented in (b). (b) Representation of the three involved contigs in FISH analyses (not in scale) cen: centromere; *RevSex*: *RevSex* human homolog region. The position of the BACs used in FISH experiments, as well as those of the considered gaps, are also reported. The three contigs are displayed with three different colours (pink, green and blue) in order to facilitate the interpretation of FISH results. The exact positions of the BAC are reported in Table 1, whereas the positions of the genomic gaps are reported in Table 5.

FIGURE 2

Reconstruction of dog *SOX9* desert region.

The position of the genomic fragment, used to build the dog *SOX9* 5' desert genomic region, is shown. (a) Human hg19 genomic organization; (b) Dog canFam 2 genomic organization; (c) Proposed dog desert region. Gene position (in Mb) in human hg19 assembly (start-end-strain): *KCNJ2* (68,165-68,176-plus), *RevSex* (69,527-69,600-plus), SOX9 (70,117-70,122-plus), and *SLC39A11* (70,642-71,088-minus). Gene position (in Mb) in dog canFam3 assembly (start-end-strain): *KCNJ2* (16,468-16,475-plus), *RevSex* (17,573-17,638-plus), *SOX9* (8,275-8,278-plus), and *SLC39A11* (7,420-7,483-plus).

FIGURE 3

FISH experiments.

The results of the FISH experiments are shown. (a–d) The colour of the BAC name corresponds to the probe colour. (e) Two hypotheses of BAC arrangement on CF9.

Figure 1

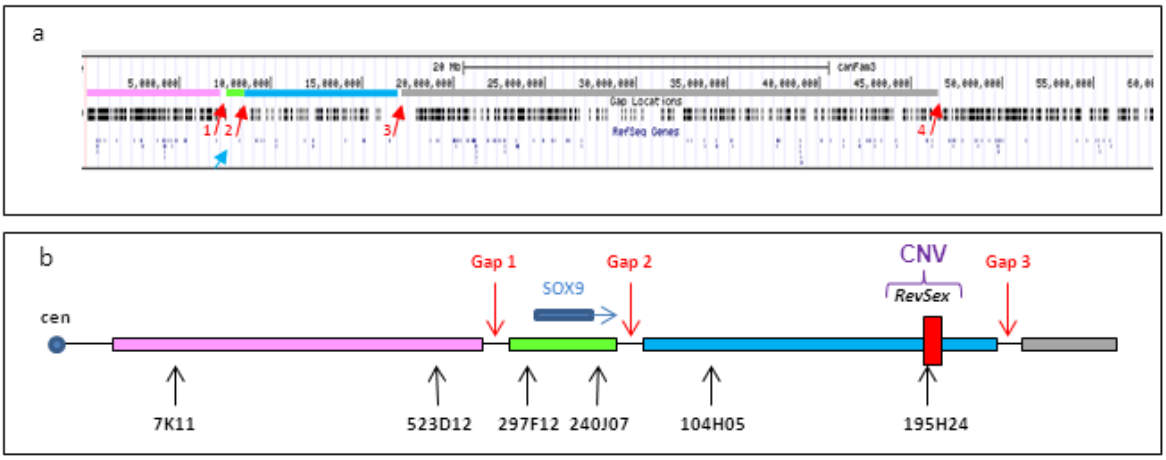

a
1
2
3
4
Gap Locations
RefSeq Genes
b
cen
Gap 1
Gap 2
SOX9
CNV
RevSex
Gap 3
7K11
523D12
297F12
240J07
104H05
195H24

Figure 2

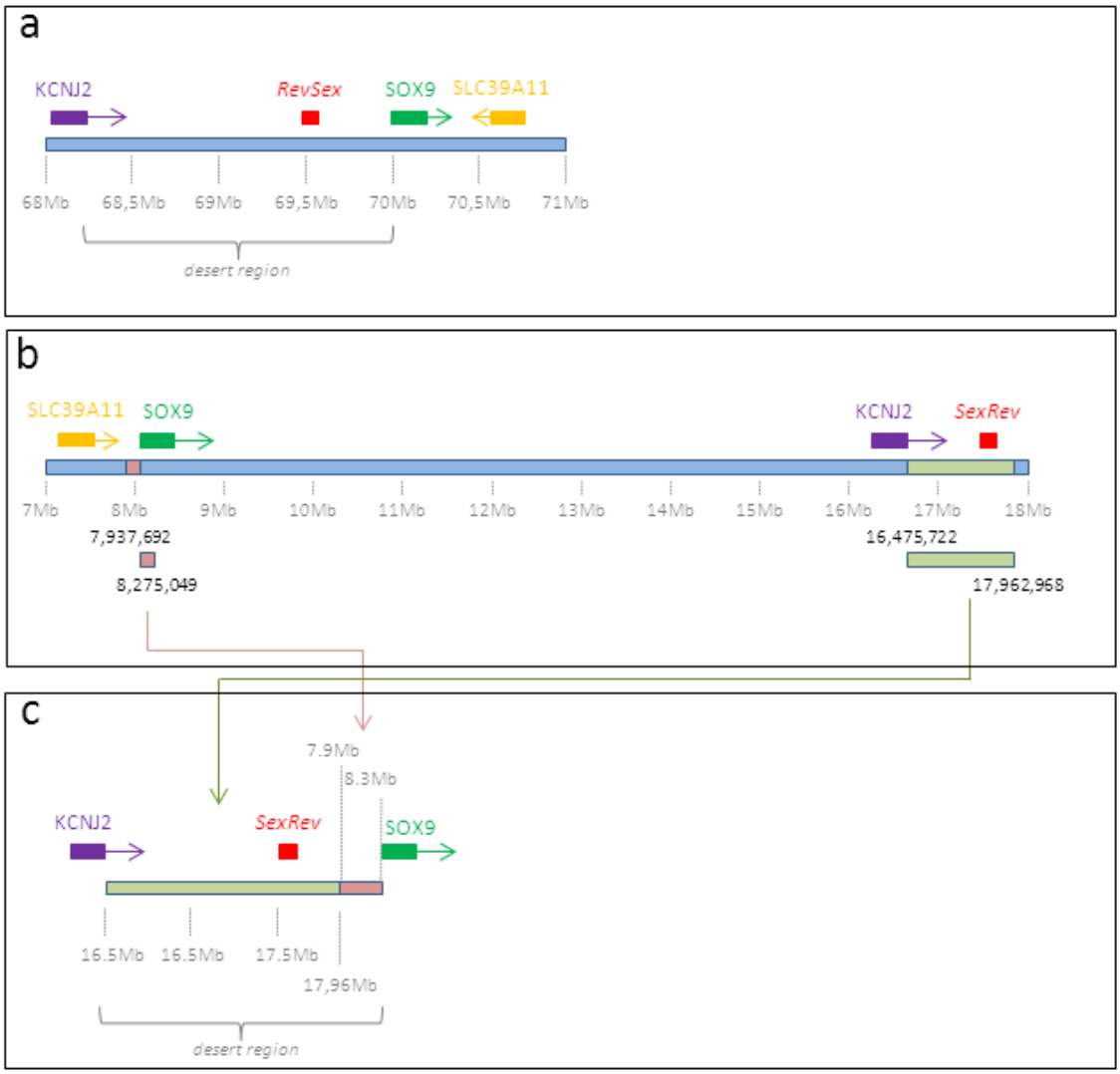
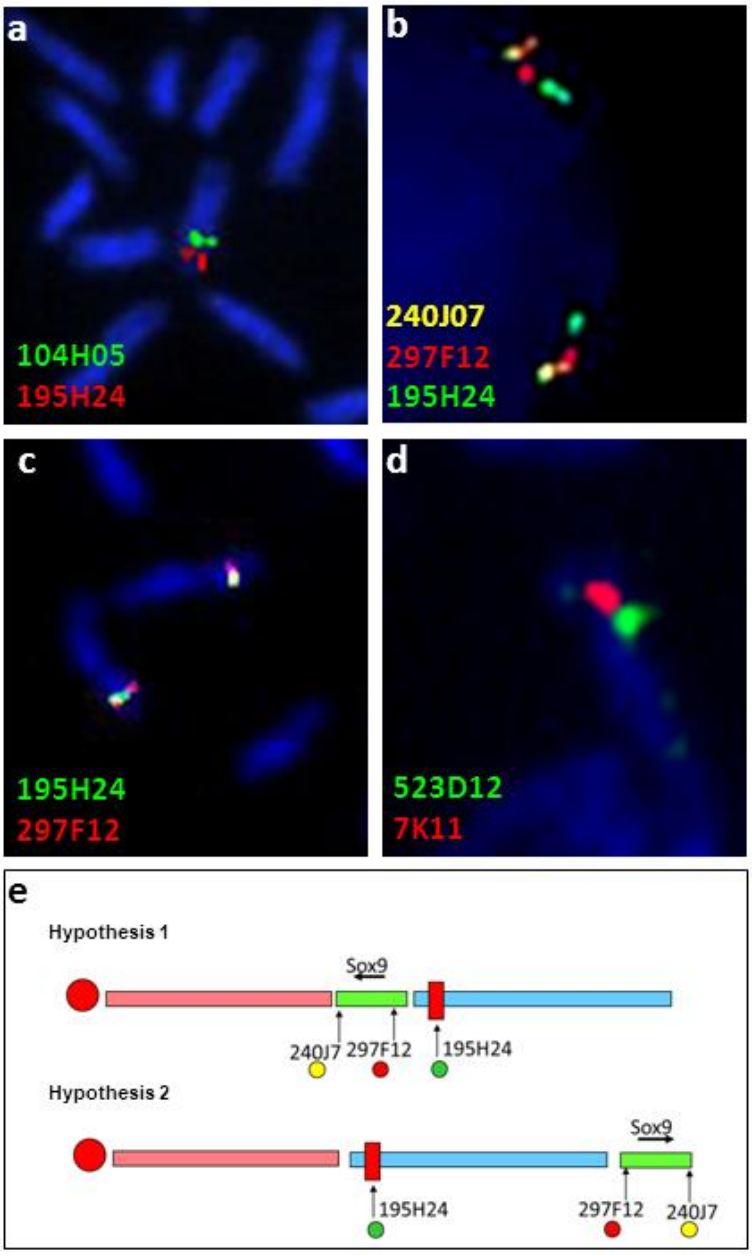

a
KCNJ2
RevSex
SOX9
SLC39A11
68Mb
68,5Mb
69Mb
69,5Mb
70Mb
70,5Mb
71Mb
desert region
b
SLC39A11
SOX9
KCNJ2
SexRev
7Mb
8Mb
9Mb
10Mb
11Mb
12Mb
13Mb
14Mb
15Mb
16Mb
17Mb
18Mb
7,937,692
8,275,049
16,475,722
17,962,968
c
7.9Mb
8.3Mb
KCNJ2
SexRev
SOX9
16.5Mb
16.5Mb
17.5Mb
17,96Mb
desert region

Figure 3


a
104H05
195H24
b
240J07
297F12
195H24
c
195H24
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d
523D12
7K11
e
Hypothesis 1
Sox9
240J7
297F12
195H24
Hypothesis 2
Sox9
195H24
297F12
240J7