

Missense mutation in the ligand-binding domain of the horse androgen receptor gene in a Thoroughbred family with inherited 64, XY (SRY+) Disorder of Sex Development

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Abstract

Disorders of sex development (DSD) have long been documented in domestic animal species including horses. However, there is only a single report of an androgen receptor mutation causative of such a DSD syndrome in a horse pedigree. Here, we present a new familial AR mutation in horses. A missense mutation (c.2042G>C) at AR exon 4 explains the segregation of the DSD in a Thoroughbred horse pedigree. The mutation, expected to affect the ligand-binding domain of the androgen receptor protein, led to complete androgen insensitivity of XY SRY+, testicular DSD individuals. Additionally, design of a PCR-RFLP technique provided an accurate molecular test for identification of horses carrying the mutation.

Introduction

Androgenic functions lead to the development of a male phenotype during gestation of the mammalian XY embryo, in addition to the secondary sexual characteristics that appear after puberty in an individual [Dohle et al., 2003]. Androgen hormones elicit their effects on target cells by binding a cytosolic androgen receptor (AR) that is a member of the nuclear receptor superfamily [Brinkmann et al., 1989]. The AR protein acts as a transcription factor at sites of the androgen response DNA elements [Koochekpour, 2010]. A recent study identified hundreds of AR target genes by transcriptome analysis of AR knockout mice, demonstrating a wide variety of functions regulated by AR in Sertoli cells [De Gendt et al., 2014]. The receptor itself is encoded by the AR-gene - a 186 kbp DNA region of the human X chromosome - that contains eight exons and translates into a 99 kD protein. These exons code for three specific and conserved regions of the receptor across mammalian species [Brinkmann et al., 1989; Jääskeläinen, 2012]. Exon 1, when translated is implicated in the regulation of AR functions through the presence of multiple phosphorylation and co-activator binding sites. Moreover, exons 2-3 encode its DNA binding domain (DBD), which contains a zinc finger-dimer responsible for DNA binding and specificity. The remaining exons 4-8 translate into the region responsible for the ligand-binding domain (LBD), a hinge-like region that links the LBD to the DBD, and provides more regulatory control as well as translocation and transactivation capabilities of the receptor protein [Brinkmann et al., 1989; Jääskeläinen, 2012].

When the function of the AR is impaired, several different conditions may result [Singh et al., 2007]. The most noteworthy of these conditions is the spectrum of the so-called Androgen Insensitivity Syndrome (AIS), as well as its association with neoplastic diseases including prostate and testicular cancers [Holterhus et al., 2005]. In humans, the androgen receptor gene mutation database (<http://androgendb.mcgill.ca>, [Gottlieb et al., 2012]) records over 1100 known mutations resulting in varying degrees of AIS or other conditions. Currently, AIS is classified as a disorder of sex development (DSD) and occurs when the AR is unable to produce the downstream transcriptional regulatory effects or alternatively these effects are insufficient to lead to a normal male phenotype. This could result in XY individuals with underdeveloped masculine features such as a micropenis, in the case of partial androgen insensitivity (PAIS), or XY individuals with a female phenotype in the case of complete androgen insensitivity (CAIS) [Holterhus et al., 2005; Hughes et al., 2012]. The frequencies of these conditions in humans are

reported at 1:20,000 to 1:99,000 for those identified genetically as males [Mazen et al., 2010; Oakes et al., 2008].

Intersexuality has been reported in a wide array of domestic animal species including dogs, buffalo, and horses [Poth et al., 2010; Iannuzzi et al., 2004; Howden, 2004; Villagómez et al., 2009a]. The first molecular link between a 64, XY DSD condition and the androgen receptor was documented by Révay et al. [2012], who identified a start codon mutation in the androgen receptor gene of a American Quarter Horse pedigree that resulted in CAIS for three members of the horse family.

Here we report on the findings of a novel equine AR mutation that is segregated amongst closely related family members of the Thoroughbred breed.

Materials and methods:

This study was performed in accordance with the animal care guidelines of the University of Pretoria.

Clinical description of the proband

A four year-old Thoroughbred mare that showed elevated urinary testosterone levels during routine in-competition testing by the South African National Horseracing Authority Laboratory in Johannesburg, was presented to the Onderstepoort Veterinary Academic Hospital, University of Pretoria, for gender verification. Externally, the horse resembled a mare, with a normal vulva and perineum. Manual eversion of the clitoris revealed this structure to be normal. The udder was non-lactating and appeared normal on inspection and palpation. On vaginoscopy, the vagina was found to be blind ending with no apparent cervix. Trans-rectal palpation failed to identify tubular structures consistent with either an uterus or a cervix. An ovoid structure, approximately 8x5x4 cm in size, with a smooth profile and without an obvious ovulation fossa, was palpated in the region approximating to the expected site of the left ovary. Trans-rectal and trans-abdominal ultrasound (SonoScape A5V, SonoScape Co. Ltd., Shenzhen, China) of this structure was performed using both a 7.5 mHz linear array and a 5mHz curvilinear transducer, respectively. The structure was found to be homogeneously echogenic with a central core of increased echogenicity, resembling a cryptorchid testis ultrasonographically. Neither an

epididymis nor features typical of ovarian structures were identified. A second structure of approximately 6x6x4 cm located dorsally in the abdominal cavity, to the right of the midline and just cranial to the pelvic inlet, was identified on further trans-rectal examination. This structure's palpable and ultrasonographic features similarly resembled a cryptorchid testis.

The horse was challenged with an intravenously-administered bolus of 6000 I.U. human chorionic gonadotrophin (hCG) (Chorulon; Zoetis, South Africa), a luteinising hormone (LH) analog, to observe potential testosterone production by radioimmunoassay (#PITKTT-8, Coat-A-Count Testosterone; Diagnostic Product Corporation, Los Angeles, USA; Cox et al., 1973). Serial serum samples, including a pre-treatment sample, were collected by jugular venipuncture. The pre-treatment serum testosterone level was 3576.37 pg/ml (reference values for a mature, intact stallion are 800-2000 pg/ml). Testosterone levels remained high post-hCG administration: 30 min (3 554.67 pg/ml), 4 h (3 478.39pg/ml) and 24 h (3 778.10 pg/ml).

At the owner's request, bilateral gonadectomy via standing flank laparoscopy was performed, as previously described [Rubio-Martínez, 2012]. The excised gonads resembled testes macroscopically. Histopathology of these structures revealed hypoplastic seminiferous tubules, containing Sertoli cells without germ cells, surrounded by connective tissue containing Leydig cells. No features of ovarian tissue were identified histologically in the sections examined.

Karyotyping performed by Unistel Medical Laboratories, Tygerberg, South Africa revealed a 64, XY chromosome constitution, consistent with gender assignment as a male.

Sampling of additional pedigree members

Peripheral blood samples (with EDTA) were drawn from the DSD clinical case along with eight additional maternal relatives: the dam (CM), a full sister (FC), two aunts (AU and CV), two half-sisters (CM14 and WS), a female cousin (VS) and a half-brother (HT). The pedigree of the animals, drawn by Pedigree Draw software (Genial Inc.), is represented in **Figure 1**. An unrelated male and a female were used as controls. All samples underwent DNA extraction using the PrepFiler Forensic DNA Extraction Kit (Thermo Fisher Scientific), as per the manufacturer's instructions [Brevnov et al., 2009].

Figure 1. Pedigree of the investigated Thoroughbred horse family. The proband DSD is marked with an arrow, * denotes animals tested with PCR-RFLP only. The causative exon 4 mutation genotypes are marked for each sequenced animals.

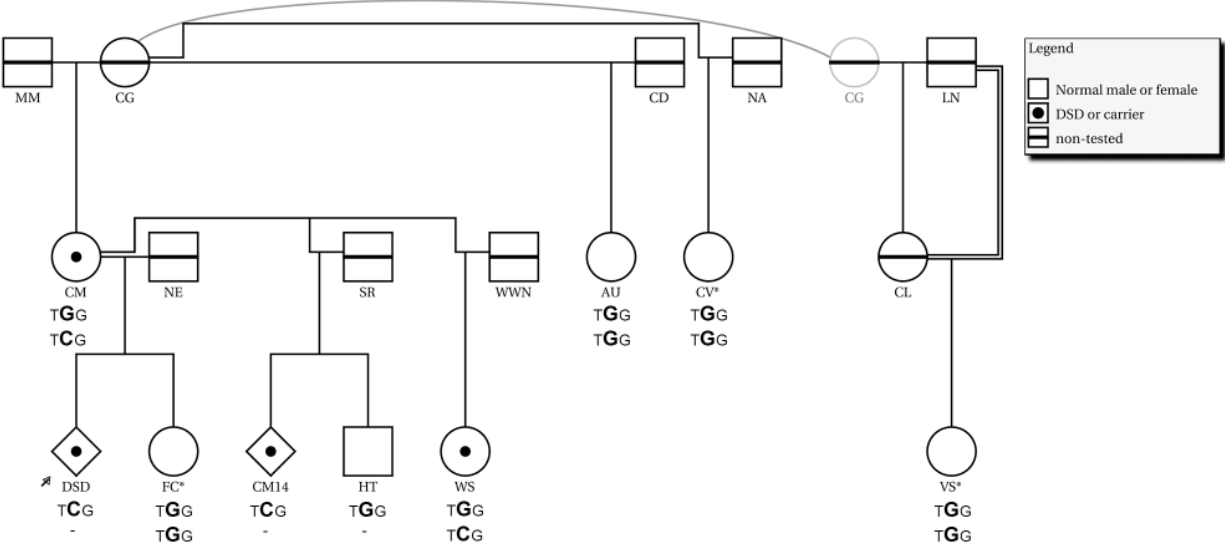


Table 1: Primers used to amplify 5 Y-chromosome genes and sequence the AR exons.

AR: Androgen receptor, EIF1AY: Eukaryotic translation initiation factor 1A Y, SRY: Sex determining region Y, TSPY: Testis specific protein Y, USP9Y: Ubiquitin-specific protease 9 Y, ZFY: Zinc finger Y. * Marks the primers used for PCR-RFLP.

Primer name	Sequence (5'-3')	size (bp)	qPCR Efficiency	GenBank Acc #	Ref
AR-E1BF	GCTAGCTGCAAGGACTACCG	514	-	NM_001163891	Revay et al., 2012
AR-E1BR	GACGAGGATGACTCAGCTGC				
AR-E1CF	CGTCCCAGAGGCCAGA	491	-		
AR-E1CR	GCAGAGAACCTTTGCATTCC				
AR-E1DF	AACAGCTTCGGGGAGATTG	530	-		
AR-E1DR	GAGAGTGTGCCAGGAAGAGG				
AR-E1EF	AGAACCCGCTGGACTATGG	518	-		
AR-E1ER	GAAACCCTGTTGAGCTCTCG				
AR-E2F	CCACATGACAGGAAACCCTA	459	-		
AR-E2R	GCCCGCTAGTGACTIONTGTCT				
AR-E2-2F	GGAGACTGCCAGGGATCAT	85	1.97		
AR-E2-2R	AGAAGCTTCGTCCCCACAG				
AR-E3F	TTCGTCCCCATGAAAGACTC	494	-		
AR-E3R	GGCCATGTTGGATATGAAGG				
AR-E4F	CAGGGTAGCTGTGCTGTGTG	451	-		
AR-E4R*	TGAGCAGGAAAGGATTGGAC				
AR-E4-F2*	GAAGGGGAGGCTTCCAG	343			
AR-E5F	GTTTGTCTCCCCCTGCTCT	456	-		
AR-E5R	GGAAATGCACCTCAGATTGG				
AR-E6F	CTTGTCCTGAAACACCAGCA	475	-		
AR-E6R	GCAAAGAAATTGGTCTCTAGCC				
AR-E7F	CCCAGGCATACAGACTTCAAT	450	-		
AR-E7R	TCTCCTGGGCCACACTC				
AR-E8F	TCAGTGAGTCCCTGGAGACC	494	-		
AR-E8R	AAGAGAAAAAGCCCAGCAAA				
EIF1AY-F	GATCGTGGCCTTCTGACATT	187	1.96	ET052957	Paria et al.,

EIF1AY-R	TTATTTTTGGGCATGGTGGT				2011
SRY-F	TGCATTCATGGTGTGGTCTC	200	2	EU687565	
SRY-R	ATGGCAATTTTTCGGCTTC				
TSPY-F	GAAGTCAGGCACACCAGTGA	189	1.85	EU687567	
TSPY-R	TAAGGCTGCAGTTGTCATGC				
USP9Y-F	GGTTATGAAATGGTCTCTGC	228	1.82	EU687569	
USP9Y-R	CGAGTCTGTCCATCAGGAGTC				
ZFY-F	TGAGCTATGCTGACAAAAGGTG	186	1.87	EU687571	
ZFY-R	TCTTCCCTTGCTTGCTTGA				

Detection of sex chromosomes by qPCR

Quantitative polymerase chain reaction (qPCR) was used to determine the presence of X and Y-chromosomes in six members of the family (CM, AU, DSD, HT, CM14 and WS). Five Y-chromosomal genes (SRY, ZFY, EIF1AY, TSPY, USP9Y) and a single copy X-chromosomal gene (AR) were amplified in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) under the following thermal profile: 98°C, 2 min; 40×(98°C, 10 s; 59°C, 15 s). A melting curve was then generated between 72°C and 95°C, in 0.5°C/s increments. The 10µl reaction mix consisted of 1× SsoFast EvaGreen Supermix (Bio-Rad), 2.25 mM primers (sequences listed in Table 1) and 5ng genomic DNA. Samples were run in triplicate and runs repeated twice. Specificity of the amplicons was tested against normal female and male control DNA. The ratio of Y-chromosome to X-chromosome in the proband and one half-sister (DSD and CM14) was also calculated relative to the half-brother (HT) after normalization against the single copy X-chromosomal AR reference gene using the efficiency corrected $\Delta\Delta Cq$ formula [Pfaffl, 2001] in CFX Manager (Bio-Rad). Amplification efficiency values were calculated by averaging the individual well efficiencies using the LinReg software (Table 1, [Ramakers et al., 2003]). The one sample t-test was applied to test differences to the expected Y/X ratio value of 1.0 (Graphpad). The specificity of PCR products was tested by 1.5% agarose gel electrophoresis.

Sequencing of AR gene

Primer sets previously designed to anneal at the surrounding introns [Révay et al., 2012] (Table 1) were used to sequence all 8 exons of the horse AR gene in six pedigree members (CM, AU, DSD, HT, CM14 and WS). Due to the size of exon 1 (1501 bp) four overlapping primer pairs were designed. The PCR mixture was a 20µL reaction per primer per sample containing: 20 ng DNA, 0.5 µM primers, 1× Amplitaq Gold 360 master mix (Thermo). Amplification was done in an MJ Research PTC-200 Thermo Cycler with the following thermal profile: 95°C, 10 min; 30×(94°C, 45 s; 59°C, 45 s; 72°C, 45 s), 72°C, 10 min. PCR products were visualized for quality control using gel electrophoresis. Sequencing of the resulting PCR products was conducted using fluorescent dye-terminator cycle sequencing and capillary electrophoresis at the Laboratory Services Division of the University of Guelph. The sequences were checked for quality and aligned to the GenBank horse AR reference sequence (NM_001163891) using Geneious Pro software v.7.1.7 (Geneious Inc). Sequences were deposited to the NCBI GenBank (accession numbers KU158182-158187).

Mutation identification by PCR-RFLP analysis

The PCR composed as for the above-mentioned sequencing reaction, but using the E4F2-E4R primer pair to amplify a part of exon 4 and intron 4 (Table 1). A 5 μ L PCR product was digested with 1 μ L FastDigest Sau 96I (Cfr13I, ThermoFisher) in 1 \times FastDigest Green Buffer at 37°C for 12 minutes. The results of digestion were visualized by gel electrophoresis.

Results

Sex chromosome testing by qPCR

The qPCR revealed signals from all 5 Y-chromosomal genes (SRY, ZFY, EIF1AY, TSPY, and USP9Y) in HT, DSD, the control stallion and also identified XY chromosome constitution in one half-sister (CM14). This animal was a previously unidentified case of XY DSD. The X-chromosomal qPCR signal was observed in all samples. The qPCR products were also visualized by gel electrophoresis and revealed single amplicons of the expected product sizes (**Figure 2**). The Y-chromosome: X-chromosome ratio was calculated for DSD and CM14 after normalization against the stallion HT to exclude sex-chromosome mosaicism and found to be the following for the 4 genes: SRY: 1.09, 0.99; EIF1AY: 1.15, 0.85; USP9Y: 1.12, 0.95; ZFY: 0.84, 1.23, respectively (**Figure 3**). None of these values differed statistically from the expected value of 1, thus confirming a normal XY chromosome constitution. The ratio was not calculated for TSPY, as it is multicopy in horses.

Sequencing the AR Gene

All eight exons of the AR gene were sequenced using the primer sets listed in Table 1. Their design ensured full and accurate coverage of the exonic regions and insights into the surrounding introns. We compared the bi-directional sequences of the family members to each other, as well as to the horse AR reference sequence (Gene ID: 100033980). Among the ~5000bp individual AR sequences generated in six members of the family we identified three variable positions (**Figure 4**). These three substitutions occurred in exons 1, 4 and intron 7. The T allele of the previously described exon 1 c.322C>T transition was observed in all six horses, as compared to the reference C allele. The second substitution, a transversion in exon 4 (c.2042G>C) resulted in the TGG codon being changed to TCG, thus encoding for serine instead of the wild type tryptophan (p.Trp681Ser). Both DSD and CM14 were found to be hemizygous, while the proband's mother and half-sister (CM and WS) were heterozygous carriers and an additional two family members (HT and AU) carried only the wild type G allele. Another transversion was discovered in the intronic region just downstream of exon 7 (2493+27G>T) with CM being heterozygous, DSD and CM14 hemizygous and WS homozygous carriers respectively, while HT and AU were both homozygous normal. An independent sequencing run from a second DNA isolate confirmed the genotypes of this latter position.

Figure 2. Y chromosomal genes visualized on agarose gel. Sample order for each gene is: 100bp DNA ladder (with intense 600bp band), CM (XX), AU (XX), DSD (XY), HT (XY), CM14 (XX), WS (XX), ES (control female, XX), BA (control male, XY).

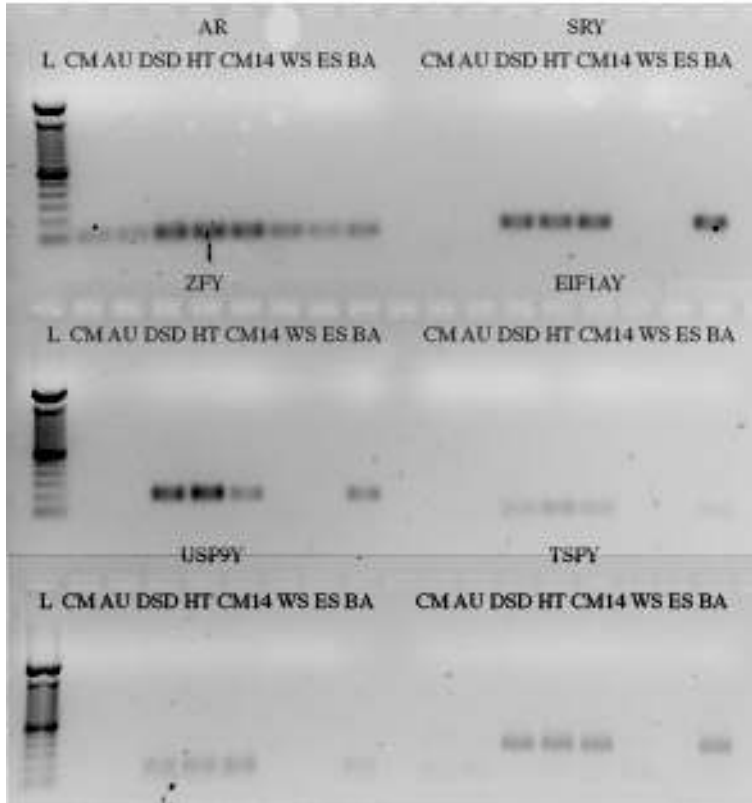


Figure 3. Y chromosomal gene copy number for 2 horses identified with ambiguous genetic and phenotypic sex (DSD and CM14) compared to a healthy stallion and normalized to the androgen receptor gene. Both animals show Y/X ratios at 4 loci that could be interpreted as equal copies of X- and Y-chromosomes.

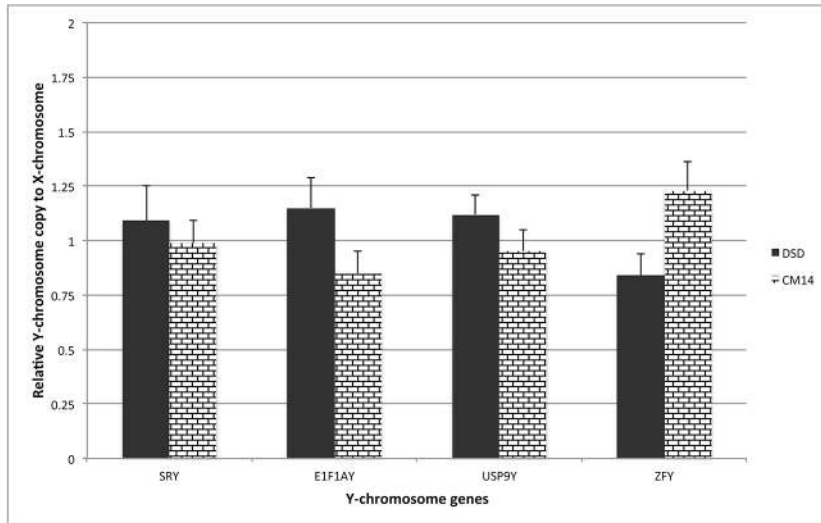


Figure 4. Comparison of family member's genotypes at the 3 identified polymorphic sites. When compared to the reference sequence (NM_001163891) all animals carried the variant T allele at c.322C>T locus. The genotypes segregated according to the observed phenotypes at c.2042G>C, thus CM and WS are heterozygous carriers of the substitution; DSD and CM14 are hemizygous, while AU and HT are homozygous wild type. Segregation of the genotypes at the intronic c.2493+27G>T locus did not match with the phenotypes.

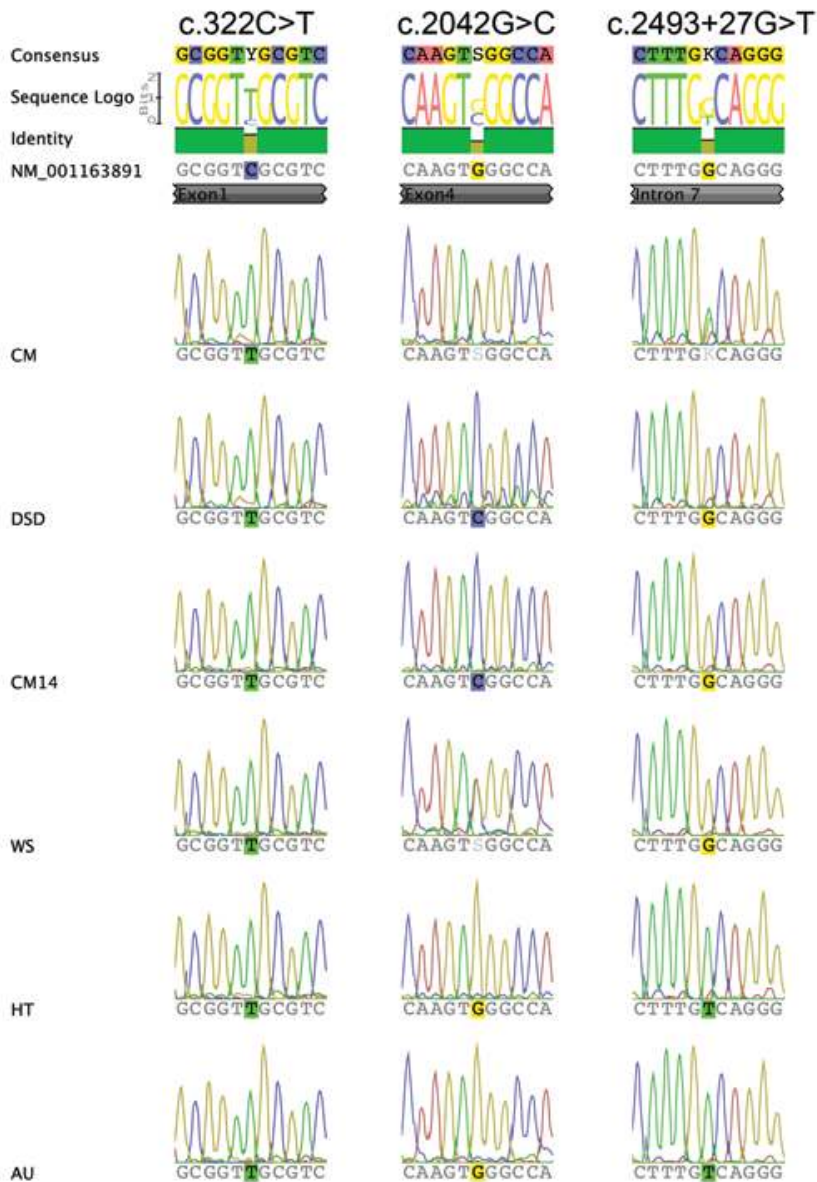
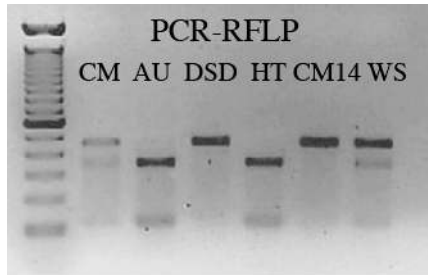


Figure 5. PCR-RFLP of AR exon 4. Digestion shows two hemizygous mutants (DSD and CM14) with one uncut band, two heterozygous carriers (CM and WS) with three bands (one uncut and two from the digested allele) and two homozygous wild type animals (HT and AU) with two bands from the cleavage.



AR PCR-RFLP testing

As only the exon 4 (c.2042G>C) mutation segregated in the pedigree according to the phenotypes, a PCR-RFLP test was designed for this locus to provide a simple way of testing further pedigree members. The Cfr13I restriction endonuclease recognizes the GGNCC cleavage sites, thus carriers of the TGG wild type codon would exhibit a cleavage while the TGC carriers remain uncut. After visualizing the digestion on a 1.5% agarose gel (**Figure 5**) the expected pattern was observed: the hemizygotes DSD and CM14 displayed one uncut PCR product (343 bp). Contrastingly, the two wild type HT and AU had two bands (230 and 113 bp), whereas the heterozygous carriers CM and WS had three bands (113, 230, 343 bp), showing one cut and one uncut allele. The three additional family members (CV, FC, VS) were found to be wild type.

Discussion:

In horses, as in other mammals, sex reversal syndrome refers to cases of DSD where there is discrepancy between the genetic and phenotypic sex of the animal [Villagómez et al., 2009b]. This manifests in a variety of disorders including androgen insensitivity [Holterhus et al., 2005]. Here, a single Thoroughbred DSD horse was observed with typical symptoms of AIS syndrome including a female phenotype with a male genotype constitution (XY, SRY+), intra-abdominal hypoplastic testes, lacking spermatogenesis and elevated serum testosterone levels. Various cases of presumed testicular feminization have been described previously in horses [reviewed by Villagómez et al., 2009b], however most are lacking investigations at the molecular level. The identification of a start codon mutation (c.1A>G) in the AR was the first molecular link between the clinical findings and AIS in horses [Révay et al., 2012]. This is the only mutation of the horse AR currently listed in the Online Mendelian Inheritance in Animals database (OMIA 000991-9796). Moreover, this mutation is currently the only one reported for the AR in domesticated animals.

The current study described a second SNP variant in AR exon 4 (c.2042G>C) that we postulate as a causative missense mutation. This type of transversion is expected to lead to a radical amino acid substitution where tryptophan would be replaced by serine (p.Trp681Ser). These two amino acids are indeed very different in chemical properties and their predicted role in protein structure or function (NCBI Amino Acid Explorer,

http://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi). Tryptophan contains an indole side chain and is thus the largest, non-polar, non-charged, hydrophobic amino acid; while serine is small, polar, non-charged and hydrophilic. Tryptophan is the least plentiful building block in proteins and its evolutionary stability is among the highest [Grantham, 1974; Miyata et al., 1979]; thus tryptophan mutations are very rare and carry the highest risk for causing disease [Vitkup et al., 2003]. Consequently the Trp>Ser replacement is predicted to result in an adverse effect on protein function as reflected by the small or negative values in the various pairwise amino acid substitution matrices [Henikoff, and Henikoff, 1992; Gonnet et al., 1992]. The expression of the region of exon 4 where the mutation occurred contributes to the formation of the ligand-binding domain [Hughes et al., 2012]. This highly complex domain is built from 11 α -helices and 2 β -sheets to form the ligand-binding pocket, in which hydrophobic interactions through tryptophan residues, amongst others, play a critical role [Pereira de Jésus-Tran et al., 2006]. The Trp681 position of the horse AR corresponds to Trp719 in the human protein. There is a paucity of information about the function of Trp719, however essential aromatic stacking function is well documented for tryptophan at position 741 [Pereira de Jésus-Tran et al., 2006]. Interestingly, there are three cases of Trp719 and five cases of Trp741 mutation reported in the human ARDB [Gottlieb et al., 2012] with all of them resulting in AIS. Although none of these mutations are actual Trp>Ser substitutions, these examples support our hypothesis that this exon 4 mutation is AIS-causative in the current horse pedigree.

With a candidate causative mutation successfully identified, the secondary objective was to develop a molecular test for identification of potential carriers among additional family members within the affected pedigree. The designed PCR-RFLP test was shown to be a valuable molecular tool for identifying the causative mutation in potential horse carriers. The horse family studied here was drawn from the South African Thoroughbred population. As Thoroughbreds are primarily selected for breeding based on their prior athletic performance, AIS may only be detectable in apparently normal adult mares that persistently fail to show cyclic reproductive activity. Application of PCR-RFLP testing within families related to the identified DSD horses could provide a rapid and affordable method to screen for affected horses. This would prevent the propagation of this mutation and the associated increased costs and management efforts in the international Thoroughbred breeding industry.

Another silent mutation in exon 1 (c.322C>T) described by Révay et al. [2012] in an American Quarter Horse family, was also observed in the current study. However, while the two alleles (C>T) segregated among the animals in the previous report, all sequenced animals in the current (Thoroughbred) family invariably carried the T allele as homozygous or hemizygous genotypes (**Figure 4**) and therefore the causative status of this SNP was excluded. A third nucleotide substitution was identified in Intron 7 (2493+27G>T) that did not segregate according to the DSD-phenotype and the observed zygosity status of the exon 4 mutation. Nevertheless, these variants expand the currently limited information available on the population variability of AR in horses.

Our results showed that a previously undescribed missense mutation (c.2042G>C) for the horse AR was causative of familial AIS in the proband Thoroughbred's pedigree. While 64, XY (SRY-positive) DSD individuals in the family were hemizygous mutation carriers; phenotypically normal horse mares were either heterozygous carriers or homozygous wild types. It was hypothesized that the mutation leads to a substantial change from a large, non-polar, hydrophobic tryptophan to a small, polar, hydrophilic serine, which interferes with the AR protein to interact with its steroid ligands. Additionally, this study outlined a rapid, efficient and cost-effective molecular test that allows for the identification of this mutation in the Thoroughbred horse population.

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References:

- Brevnov MG, Pawar HS, Mundt J, Calandro LM, Furtado MR, Shewale JG: Developmental Validation of the PrepFiler™ Forensic DNA Extraction Kit for Extraction of Genomic DNA from Biological Samples. *J Forensic Sci* 54:599–607 (2009).
- Brinkmann A, Klaasen P, Kuiper G, Korput J, Bolt J, Boer W, et al.: Structure and function of the androgen receptor. *Urol Res* 17:87–93 (1989).
- De Gendt K, Verhoeven G, Amieux PS, Wilkinson MF: Research Resource: Genome-Wide Identification of AR-Regulated Genes Translated in Sertoli Cells In Vivo Using the RiboTag Approach. *Mol Endocrinol* 28:575–591 (2014).
- Dohle GR, Smit M, Weber RFA: Androgens and male fertility. *World J Urol* 21:341–345 (2003).
- Gonnet GH, Cohen MA, Benner SA: Exhaustive matching of the entire protein sequence database. *Science* 256:1443–1445 (1992).
- Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M: The androgen receptor gene mutations database: 2012 update. *Hum Mutat* 33:887–894 (2012).
- Grantham R: Amino acid difference formula to help explain protein evolution. *Science* 185:862–864 (1974).
- Henikoff S, Henikoff JG: Amino acid substitution matrices from protein blocks. *Proc Natl Acad Sci U S A* 89:10915–10919 (1992).
- Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroiannopoulou K, Macdougall J: Androgen insensitivity syndrome. *Lancet* 380:1419–1428 (2012).
- Holterhus PM, Werner R, Hoppe U, Bassler J, Korsch E, Ranke MB, et al.: Molecular features and clinical phenotypes in androgen insensitivity syndrome in the absence and presence of androgen receptor gene mutations. *J Mol Med* 83:1005–1013 (2005).
- Howden KJ: Androgen insensitivity syndrome in a thoroughbred mare (64, XY—testicular feminization). *Can Vet J* 45:501–503. (2004).
- Iannuzzi L, Di Meo GP, Perucatti a, Incarnato D, Di Palo R, Zicarelli L: Reproductive disturbances and sex chromosome abnormalities in two female river buffaloes. *Vet Rec*

- 154:823–824 (2004).
- Jääskeläinen J: Molecular biology of androgen insensitivity. *Mol Cell Endocrinol* 352:4–12 (2012).
- Koochekpour S: Androgen receptor signaling and mutations in prostate cancer. *Asian J Androl* 12:639–657 (2010).
- Mazen I, El-Ruby M, Kamal R, El-Nekhely I, El-Ghandour M, Tantawy S, et al.: Screening of genital anomalies in newborns and infants in two egyptian governorates. *Horm Res Paediatr* 73:438–442. (2010).
- Miyata T, Miyazawa S, Yasunaga T: Two types of amino acid substitutions in protein evolution. *J Mol Evol* 12:219–236 (1979).
- Oakes MB, Eyvazzadeh AD, Quint E, Smith YR: Complete androgen insensitivity syndrome--a review. *J Pediatr Adolesc Gynecol* 21:305–310 (2008).
- Paria N, Raudsepp T, Pearks Wilkerson A, O'brien P, Ferguson-Smith M, Love C, et al.: A Gene Catalogue of the Euchromatic Male-Specific Region of the Horse Y Chromosome: Comparison with Human and Other Mammals. *PLoS One* 6:e21374 (2011).
- Pereira de Jésus-Tran K, Côté P-L, Cantin L, Blanchet J, Labrie F, Breton R: Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. *Protein Sci* 15:987–999 (2006).
- Pfaffl MW: A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* 29:e45 (2001).
- Poth T, Breuer W, Walter B, Hecht W, Hermanns W: Disorders of sex development in the dog- Adoption of a new nomenclature and reclassification of reported cases. *Anim Reprod Sci* 121:197–207 (2010).
- Ramakers C, Ruijter JM, Deprez RHL, Moorman AF.: Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62–66 (2003).
- Révay T, Villagómez DAF, Brewer D, Chenier T, King WA: GTG mutation in the start codon of

- the androgen receptor gene in a family of horses with 64,XY disorder of sex development. *Sex Dev* 6:108–116 (2012).
- Rubio-Martínez LM: Standing laparoscopic castration in an equine male pseudohermaphrodite. *Equine Vet Educ* 24:507–510 (2012).
- Singh R, Singh L, Thangaraj K: Phenotypic heterogeneity of mutations in androgen receptor gene. *Asian J Androl* 9:147–179 (2007).
- Villagómez DAF, Lear T, Chenier T, Lee S, McGee RB, Cahill J, et al.: Equine Disorders of Sexual Development in 17 Mares Including XX, SRY-Negative, XY, SRY-Negative and XY, SRY-Positive Genotypes. *Sex Dev* 5:16–25. (2009a).
- Villagómez D, Parma P, Radi O, Di Meo GP, Pinton A, Iannuzzi L, et al.: Classical and Molecular Cytogenetics of Disorders of Sex Development in Domestic Animals. *Cytogenet Genome Res* 126:110–131 (2009b).
- Vitkup D, Sander C, Church GM: The amino-acid mutational spectrum of human genetic disease. *Genome Biol* 4:R72 (2003).