



Sex chromosomal abnormalities associated with equine infertility: validation of a simple molecular screening tool in the Purebred Spanish Horse

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Summary

Chromosomal abnormalities in the sex chromosome pair (ECAX and ECAY) are widely associated with reproductive problems in horses. However, a large proportion of these abnormalities remains undiagnosed due to the lack of an affordable diagnostic tool that allows for avoiding karyotyping tests. Hereby, we developed an STR (single-tandem-repeat)-based molecular method to determine the presence of the main sex chromosomal abnormalities in horses in a fast, cheap and reliable way. The frequency of five ECAX-linked (*LEX026*, *LEX003*, *TKY38*, *TKY270* and *UCDEQ502*) and two ECAY-linked (*EcaYH12* and *SRY*) markers was characterized in 261 Purebred Spanish Horses to determine the efficiency of the methodology developed to be used as a chromosomal diagnostic tool. All the microsatellites analyzed were highly polymorphic, with a sizeable number of alleles (polymorphic information content > 0.5). Based on this variability, the methodology showed 100% sensitivity and 99.82% specificity to detect the most important sex chromosomal abnormalities reported in horses (chimerism, Turner's syndrome and sex reversal syndromes). The method was also validated with 100% efficiency in 10 individuals previously diagnosed as chromosomally aberrant. This STR screening panel is an efficient and reliable molecular–cytogenetic tool for the early detection of sex chromosomal abnormalities in equines that could be included in breeding programs to save money, effort and time of veterinary practitioners and breeders.

Keywords chimerism, diagnostic tool, equines, genetic infertility, microsatellites, molecular characterization, mosaicism, sex reversal syndrome, turner syndrome

Introduction

The Purebred Spanish horse (PRE) is the main horse breed in the Iberian Peninsula and one of the most important livestock production systems in Spain, with an active population of 68 971 mares and 42 308 males (Sánchez-Guerrero *et al.* 2016). PRE importance exceeds the

economic benefits, as it is also recognized as an icon and hallmark of the culture and traditions of Spain. Every year, near 10 000 new individuals are evaluated and included in the breeding program. However, sex chromosomal abnormalities are rarely reported in these animals, despite the fact that their prevalence in Polish breeds is close to 2% (Bugno *et al.* 2007). The horse karyotype ($2n = 64$) has 31 pairs of autosomal chromosomes (ECA1–ECA31) and two sex chromosomes (ECAX and ECAY). Its high complexity is evidenced by the presence of a large number of small acrocentric chromosomes (including ECAY) that could be easily mis-identified and by the fact that several metacentric chromosomes (ECA1–ECA6) could be misleadingly taken as ECAX (Bowling *et al.* 1997). The prevalence of sex

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chromosomal abnormalities in horses is more common than in other domestic animals; more than 95% of them are linked to numeric alterations in the sexual pair (Lear & McGee 2012). Historically, karyotype alterations have been diagnosed by cellular cultures and classical (direct karyotyping or banding) or molecular (fluorescence *in situ* hybridization, FISH) cytogenetic techniques (Lear & Bailey 2008). However, these methods are difficult, expensive and time consuming, and the number of laboratories that may perform them is small. This situation, and the fact that most of the horses with sex chromosomal pathologies do not evidence any morphological abnormality or behavioral change until adulthood, has led to a higher percentage of undiagnosed cases (Lear & Bailey 2008).

Three different syndromes are responsible for more than 90% of the aberrations observed in this species. Turner's syndrome, first described in humans in the 1950s (Ford *et al.* 1959) as the absence of a sex chromosome ($2n = 63$, X in horses), is the main pathology diagnosed (Power 1987). It is usually ignored in veterinary practice because most animals bearing this disease have a normal external appearance and only a slight internal dysgenesis and irregular estrous behavior (Lear & McGee 2012). Sex reversal syndrome is the second-most reported sex chromosomal pathology in equines and is characterized by a discordance among phenotype, sexual behavior and karyotype (Villagómez *et al.* 2011). Initially, this disease was reported as having two different presentations: phenotypically male animals bearing a female chromosome complement ($2n = 64$, XX) and vice versa ($2n = 64$, XY mares) (Kent *et al.* 1988). However, the characterization of the *sex determining region Y* gene *SRY* in horses by Pailhoux *et al.* (1995) allowed for the differentiation of four possible sex-reverse syndromes: male and female *SRY*-negative and positive individuals. Chimerism is the third most important sex chromosomal syndrome reported in horses; it has been identified in several species and widely diagnosed as Freemartinism in cattle (Jost *et al.* 1972). This syndrome derives from the exchange of blood tissue and hormones through placental circulation between di-zygotic twin brothers. Although this condition is of great importance in cattle, its prevalence in horses is very low because twin pregnancy is a pathological condition that is normally terminated once detected (Davies Morel *et al.* 2012). All of these syndromes are currently diagnosed by conventional techniques, which as was mentioned previously, are difficult, time consuming and not easily available in several countries.

Molecular markers are widely used as an effective tool for genetic identification, traceability and assessment of parentage relationships among animals (Fernández *et al.* 2013). This highly automated and fast methodology allows for the detection of genetic variability among individuals and can therefore be used in scientific, forensic and legal applications. A few years ago, Kakoi *et al.* (2005) proposed that the

STR (single-tandem-repeat)-based parentage testing could be used to detect sex chromosomal abnormalities in Thoroughbred horses. More recently, using a similar approach, we determined the presence of such abnormalities in two Andalusian horses using a simple DNA sample (Demyda-Peyras *et al.* 2013; Demyda-Peyrás *et al.* 2014). However, these methodologies did not include the detection of *SRY*, a gene involved in gonadal differentiation during embryonic development (Berta *et al.* 1990). This gene has been shown to be extremely important in the differentiation of sex reversal presentations in horses (Lear & McGee 2012), and therefore, is an interesting candidate to be included in a diagnostic tool.

To this end, we developed and characterized a cheap, reliable and fast diagnostic tool to detect the most important sex chromosomal abnormalities described in horses in a single multiplex polymerase chain (PCR) reaction. Furthermore, we comprehensively characterized the genetic variability of STR markers employed in a sample population of PRE horses, determining the sensitivity and specificity of our newly developed screening method.

Material and methods

Animals

A total of 271 animals from 90 different studs belonging to the Asociación Nacional de Criadores de Caballos de Pura Raza Española Stud Book were analyzed. Among the total PRE population of 200 000 available specimens, 261 normal individuals (121 males and 140 females) were selected, minimizing their co-ancestry values, to analyze slightly related animals. Ten extra animals previously karyotyped and diagnosed as chromosomally abnormal by our laboratory (two chimeric, two with Turner's syndrome and six with sex reversal syndromes) were included as positive controls to validate the efficiency of the tool. To increase the accuracy of the validation, 20 extra normal animals (10 mares and 10 horses) included in the genomic characterization were also karyotyped. Blood samples were obtained by jugular venopuncture using Tri-sodium EDTA Vacutainers™ for DNA extraction and Na-heparin Vacutainers™ to perform cell cultures. Hair samples (at least 50 bulbs) were also collected for DNA isolation.

Molecular analyses

DNA was obtained from blood and hair follicles using a Blood Genomic DNA Extraction Kit and a Tissue Genomic DNA Purification Kit (Canvax Biotech) respectively, according to the manufacturer's protocol. Five markers located on the ECAX (*LEX003*, *UCDEQ502*, *TKY38*, *LEX026* and *TKY270*) and two markers located on the ECAY (*EcaYH12* and *SRY*) were amplified by PCR using the primer pairs previously designed by several authors (Table 1).

Table 1 Sequences of primers used in this study.

Marker	Amplicon size	Primers	ECA location	References
LEX026 (STR)	300–318	5'-[6-FAM]TCCAGAGTGAATGGCAAATC-3' 5'- AAGAACTAGAACCTACAACCTAGG-3'	Xp	Coogle <i>et al.</i> (1996b)
TKY38 (STR)	105–141	5'-[6FAM]TAAGTATTCTCATAAACCGGG-3' 5'-GGAATAATAACAGCATCCTC-3'	Xq23	Hirota <i>et al.</i> (2001)
TKY270 (STR)	156–172	5'-[HEX]CTGCTTTAGAGAAACAAACT-3' 5'-CCATGGTGAGAAAAATGAGA-3'	X	Kakoi <i>et al.</i> (2000)
LEX003 (STR)	194–216	5'-[6FAM]ACATCTAACCCAGTGCTGAGACT-3' 5'-AAGACTAGACCTACAACCTAGG-3'	Xq	Coogle <i>et al.</i> (1996a)
UCDEQ502 (STR)	162–176	5'-[6FAM]JAGAGGGCAAAGTCAGAGCTT-3' 5'-AGCACCTGATGCTTCTTGTT-3'	X	Eggleston-Stott <i>et al.</i> (1997)
EcaYH12 (STR)	92	5'-[6FAM]CGAACAGGTGACGAAGCATC-3' 5'-GCAGACATGCACACCAACC-3'	Y	Wallner <i>et al.</i> (2004)
SRY (Gene fragment)	249	5'-[HEX]TACCACCCTCTTCAACG-3' 5'ATCACGAGACCACCCATGA-3'	Y	Bannasch <i>et al.</i> (2007)

The type of marker is mentioned in brackets: STR: single tandem repeat (microsatellite).

Amplification was carried out using 23 μ l of the following mixture: 20–60 ng of genomic DNA, 1.5–7.5 pmol of each primer pair, 0.33 mmol/l dNTPs, 2.5 mmol/l of MgCl₂, 1.5 μ l of 10 \times PCR reaction buffer and 1.5 U of Horse-Power™ Taq DNA polymerase (Canvax Biotech). The thermal protocol was as follows: an initial denaturation at 95 °C for 10 min, 33 cycles at 94 °C for 30 s, 57 °C for 1 min and 72 °C for 30 s, followed by 72 °C for 10 min. The amplification products were genotyped by capillary electrophoresis using an Applied Biosystems 3130 xl DNA sequencer in the Central Service for Research Support of the University of Córdoba. Allele sizes were determined after processing the raw data with the software package GENEMAPPER 4.0 using a LIZ 500 bp internal size standard (Applied Biosystems).

ECAX STR- statistical analysis

Statistical analyses were performed using GENETIX 4.0.1 (Belkhir *et al.* 1996–2004). Allele frequency (A_f) as well as average (X_n) and total number (A_n) of alleles were determined for the entire population. Observed (H_{ob}) and expected (H_{ex}) heterozygosity were calculated only for female individuals. Allele variability (polymorphic information content, PIC) was determined for each marker in the whole population using CERVUS 3.0.6. (Kalinowski *et al.* 2007). AF comparisons were determined using the Fisher exact test with SPSS STATISTICS 20 software (IBM). Finally, the sensitivity (proportion of positives that are correctly identified as such) and specificity (proportion of negatives that are correctly identified as such) of the diagnostic tool were determined based on allele variability in this population.

Cell culture and karyotyping

Twenty normal individuals (10 mares and 10 horses) were karyotyped to validate the molecular results.

Cytogenetic analysis was performed on metaphase chromosomes obtained from lymphocytes cultures according to Rodero-Serrano *et al.* (2013). Chromosome spreads were obtained by dropping 100 μ l of the cell suspension onto wet slides. Giemsa-stained karyotypes were analyzed in a Cytovision platform (Leica) using the *Equus caballus* standardized karyotype (Richer *et al.* 1990) and confirmed by C-banding (Sumner 1972) in at least 100 metaphases. The type and number of abnormalities detected were recorded. DNA samples from 10 horses previously diagnosed with chromosomal abnormalities in the sexual pair were used as positive controls.

Results

Genetic characterization of ECAX-linked markers

The A_f of ECAX markers is shown in Table 2. All of them were highly polymorphic, with the number of alleles ranging from six (TKY270) to 11 (LEX003). Interestingly, A_n was higher in males for three ECAX-linked markers, with seven different alleles represented only in males (202 and 216 for LEX003; 302, 304 and 310 for LEX026; and 172 and 176 for UCDEQ502). On the contrary, five different alleles (318 for LEX026; 127 and 139 for TKY38 and 170 for UCDEQ502) were represented only in mares. Three markers (LEX003, LEX026 and UCDEQ502) showed a similar A_f between sexes, whereas it was different in two of them (TKY270 and TKY38) ($P < 0.05$). Finally, X_n per marker (8.4) also demonstrated high genetic variability in the population analyzed.

Results of H_{ex} and $H_{i,b}$, heterozygosity and PIC are shown in Table 3. LEX003 showed the highest H_{ex} (0.802) and $H_{i,b}$ (0.761) values, whereas TKY38 (0.679) and UCDEQ502 (0.575) showed the lowest. PIC was higher than 0.50 in all ECAX-linked markers analyzed.

Table 2 Allelic frequencies obtained in the five ECAX microsatellites used in this study.

STR	Allele	A_f females	A_f males	
LEX003	194	0.1413	0.2137	
	198	0.0326	0.0342	
	200	0.0072	0.0427	
	202	0.0000	0.0085	
	204	0.0254	0.0256	
	206	0.3225	0.2479	
	208	0.1014	0.0940	
	210	0.2065	0.1709	
	212	0.0290	0.0171	
	214	0.1341	0.1368	
	216	0.0000	0.0085	
	LEX026	300	0.1522	0.1849
		302	0.0000	0.0252
		304	0.0000	0.0084
306		0.0833	0.0840	
308		0.0543	0.0336	
310		0.0000	0.0084	
312		0.3152	0.2857	
314		0.3913	0.3697	
318		0.0036	0.0000	
TKY270		156	0.1728	0.2783
	164	0.0074	0.0348	
	166	0.1176	0.0609	
	168	0.4449	0.3826	
	170	0.2537	0.2261	
	172	0.0037	0.0174	
	TKY38	105	0.0906	0.1441
107		0.0725	0.1695	
109		0.3949	0.3432	
127		0.0036	0.0000	
129		0.3877	0.3008	
131		0.0217	0.0085	
139		0.0072	0.0000	
141		0.0217	0.0339	
UCDEQ502		162	0.0531	0.0787
	164	0.4735	0.4607	
	166	0.0398	0.0112	
	168	0.2434	0.3090	
	170	0.0177	0.0000	
	172	0.0000	0.0112	
	174	0.1726	0.1180	
	176	0.0000	0.0112	

STR, single tandem repeat; A_f , allele frequency. Frequencies per allele are characterized as the fraction of total determinations segregated by sex of the individuals.

Table 3 Observed (H_{ob}) and expected (H_{ex}) heterozygosity and polymorphic information content (PIC) of the ECAX microsatellites calculated in the entire population.

STR	H_{ob}	H_{ex}	PIC
LEX003	0.761	0.802	0.796
LEX026	0.659	0.714	0.682
TKY270	0.698	0.694	0.663
TKY38	0.674	0.679	0.665
UCDEQ502	0.575	0.682	0.631

STR, single tandem repeat.

Screening capability

Specificity in the study sample was 99.82%, estimated by the probability of finding the five STR markers in homozygosity in females with a normal karyotype (other possibilities were covered by the different result combinations). Likewise, the sensitivity of the diagnostic tool was 100%, because the possibility of obtaining a diallelic result in animals with only one ECAX was null.

Validation of the methodology in chromosomally abnormal individuals

The molecular markers used in this study allowed us to determine the presence or absence of ECAX and ECAY and the number of chromosomes with a different genetic background (cell lines) through the number of alleles detected. In the same reaction, we also determined the presence of the SRY gene, which plays a crucial role in the case of sex reversal horses. Based on the combinations of the results of those seven markers, we detected most of the sex chromosomal pathologies previously associated with reproductive failures in horses (90% CI).

All individuals studied using the molecular methodology proposed in this report were diagnosed correctly. The karyotypes and STR profiles in animals used as positive controls are shown in Table 4. Mares with a normal karyotype (64,XX) showed non-amplified ECAY markers and at least one ECAX marker in heterozygosity (two alleles) (Fig. S1). Males with normal karyotype (64,XY) showed amplification for ECAY markers and only one allele peak for ECAX markers (Fig. S2). Individuals affected by the sex reversal syndrome showed karyotypes and an STR profile opposite to their phenotype, also discriminated by the presence or absence of SRY (Figs S3–S5). Animals affected by Turner's syndrome (63,X) showed monoallelic results in all ECAX markers and no amplification in ECAY markers (Fig. S6). Finally, results from chimeric animals were characterized by a positive amplification for ECAY markers and a multiallelic profile (two or three alleles) for at least one of the ECAX markers (Fig. S7). Differences in DNA results from blood and hair samples indicated blood chimerism. Whereas blood DNA showed the STR chimeric profile, hair DNA showed a normal STR profile according to the sex of the individual (male or female). On the other hand, if the STR chimeric profile was detected in blood and hair DNA samples, the individual was diagnosed as a true (full body) chimeric.

Discussion

Among the main syndromes associated with unexplained infertility in horses, Turner's syndrome (63,X) is probably the most reported one (Lear & McGee 2012). Our results showed that this abnormality could be diagnosed with STR

Table 4 Molecular profiles of ECAX and ECAY STR markers and the SRY gene obtained using the methodology described in this study associated with the most important sex chromosomal abnormalities described in horses.

Phenotype	Molecular genotype		Karyotype	Diagnosis	
	ECAX STR profile	ECAY marker			
		ECAYH12	SRY		
Male	One allele per marker	±	+	64,XY	Normal male
Female	At least one marker in heterozygosis	–	–	64,XX	Normal female
Female	One allele per marker	–	–	63,X0	Turner's syndrome ¹
Male	At least one marker with 2 or 3 alleles	±	+	XX/XY ¹	Male chimerism ²
Female	At least one marker with 2 or 3 alleles	±	+	XX/XY ¹	Female chimerism ²
Male	At least one marker in heterozygosis	–	+	64,XX	Male SRY positive DSD ³
Male	At least one marker in heterozygosis	–	–	64,XX	Male SRY negative DSD
Female	One allele per marker	+	+	64,XY	Female SRY positive DSD
Female	One allele per marker	±	–	64,XY	Female SRY negative DSD

Diagnoses were performed as the combination of the phenotype and molecular genotype of the individuals. The karyotype stated is the one associated with every different syndrome.

¹Results on Turner's syndrome had a 99.82% specificity.

²The proportion of cell lines may change among individuals without affecting the results. Different results obtained from blood and hair DNA samples demonstrated blood chimerism. Similar results demonstrated true chimerism.

³64,XX SRY+ DSD has not yet been reported in horses, but it can be detected with this methodology.

markers with high reliability. Interestingly, it cannot be detected using the molecular markers routinely employed in domestic animals, such as amelogenin (Hasegawa *et al.* 2000) or zinc finger protein genes (Han *et al.* 2010), as there is no discordance between genotypic and phenotypic sex. Therefore, the number of undiagnosed cases could be higher and the prevalence underestimated. In this pathology, the diagnostic confidence obtained using our approach was higher than 98%, as we saturated the ECAX with several highly variable markers that allowed for the detection of the number of chromosomes present.

Concerning the sex reversal syndrome and its presentations, only three have already been reported (Villagómez *et al.* 2009), with the exception of the 64,XX SRY-positive disorder in sex development (DSD). This difference with other species, such as humans (Ergun-Longmire *et al.* 2005), is probably due to the fact that SRY is located in a specific region far from the pseudo-autosomal boundary region, making the gene exchange between ECAY and ECAX unlikely (Raudsepp *et al.* 2010). It has been demonstrated that 64,XY SRY-negative DSD females is the most common presentation in sex reversal horses (Lear & McGee 2012). Its clinical presentation is characterized by the same phenotypic presentation as that in Turner's mares, with the consequential high percentage of undiagnosed cases until adulthood (Anaya *et al.* 2014). Our methodology also provides a simple and reliable alternative for detecting the four possible presentations of the sex reversal syndrome (according to their sex chromosomal complements and presence or absence of SRY) based on a simple blood sample, thus allowing for a reduction in the number of undiagnosed horses. However, this methodology cannot discriminate the cause of such abnormality, which can range from point

mutations (Révay *et al.* 2012) to small or even large chromosomal deletions (Raudsepp *et al.* 2010).

Another advantage of the methodology developed here is the possibility to discriminate between true and blood chimerism. Currently, the effect of male–female blood chimerism on horse fertility is under discussion. Previous reports suggest that blood chimeras are normal (Bouters & Vandeplassche 1972; Juras *et al.* 2010; Demyda-Peyras *et al.* 2013), even when one of the lines has been diagnosed as aberrant (Demyda-Peyras *et al.* 2014). However, Bugno *et al.* (1999) reported idiopathic subfertility in a mare that delivered for the first time after four reproductive seasons. Considering that the twinning rate in horses has been estimated at approximately 3.5% of gestations (Wolc *et al.* 2006) and that about 15% of those pregnancies produce at least one live foal (Miller & Woods 1988), we speculate that the percentage of blood chimeric individuals among horses should be much higher than the reported cases. However, because most of the studies agree with the lack of reproductive symptoms in chimeric mares, it is highly possible that most of those animals remain undiagnosed. On the contrary, true chimerism is a different condition characterized by the fusion of two zygotes or early embryos into a single individual. It has already been reported in horses (Dunn *et al.* 1981) and other domestic species (Batista *et al.* 2000), and there is agreement that true chimerism is associated with major reproductive abnormalities in animals (Padula 2005). The use of the methodology described in the present study would allow veterinary practitioners and breeders to differentiate between these two presentations and to more appropriately manage the individuals affected, given that true chimeric individuals are sterile. On the other hand, the use of molecular

screening could allow for the determination of the real prevalence of blood chimerism in horses.

All diagnostic tests are characterized by their sensitivity and specificity (Van Der Schouw *et al.* 1995). Screening tests, for instance, are used to quickly determine the presence of some disease in a population and therefore must be highly sensitive to avoid by all means the detection of false negatives. In molecular tests based on the combination of individual results of different STRs, sensitivity and specificity are directly related to the number of markers employed and their variability (Garvin *et al.* 1998). We demonstrated that the markers evaluated in our study were highly polymorphic (elevated PIC) with a high number of possible allele combinations, covering a large number of homozygous and heterozygous genotypes. With this information, the probability of a 'false female positive' result (a normal mare diagnosed as having Turner's syndrome) was 0.0017, estimating that only one out of every 588 mares would be erroneously identified as affected by this condition (false positive). In this study, validated in 30 karyotyped animals, the accuracy of the diagnostic tool was 100%. The possibility of a false negative result can be ruled out, as there is no possibility of having more than two alleles in non-chimeric animals (only one or two ECAX and therefore a maximum of one or two alleles) or of obtaining a heterozygous result in a marker in animals with only one ECAX (63,X or sex reversed animals). Although sensitivity remains fixed across horse populations, specificity can vary as a function of the STR A_f used in the different breeds. In this sense, the PIC of several microsatellite markers previously reported in different Japanese (Tozaki *et al.* 2003), European (Achmann *et al.* 2004) and Spanish (Cañon *et al.* 2000) horse breeds were similar to those obtained in our study. This fact strongly suggests that this methodology will remain highly sensitive despite the breed analyzed.

The only existing population study that determined the percentage of sex chromosomal abnormalities in horses reported a prevalence close to 2% in certain Polish autochthonous breeds (Bugno *et al.* 2007). Our results did not show any abnormality in the 261 newly analyzed animals, suggesting that the incidence of sex chromosomal abnormalities in PRE horses is lower. Our DNA samples were collected from animals belonging to the PRE Stud Book and were specifically selected to minimize co-ancestry among individuals, maximizing the genetic differences. With these criteria, the allele variability present in the population was determined more properly. However, all the subjects analyzed showed a normal phenotype, probably because infertile animals are usually excluded from the official registers by breeders, as they could be considered bad publicity for their herds. Yet, the lack of positive results could also be due to the reduced number of individuals screened. Therefore, a solid estimation of the real prevalence of sex chromosomal abnormalities in PRE can be obtained only with a higher number of randomly selected animals.

In conclusion, we demonstrated that the use of molecular markers could be an interesting option for detecting and screening for the most important sex chromosomal abnormalities in the horse in a fast, cheap and robust way. In addition, we estimated the sensitivity and specificity of this methodology in a given population of European horses, obtaining very reliable results (higher than 99.5% in both cases). Nevertheless, a larger number of individuals should be analyzed to obtain a more accurate idea of the prevalence of sex chromosomal abnormalities in these breeds. Overall, the use of this strategy as a screening tool integrated into official breeding programs could systematically detect, in an early and efficient way, the presence of reproductively impaired individuals, saving time and money of veterinarians and breeders.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Figure S1 Electropherogram profile of a normal female (64, XX).

Figure S2 Electropherogram profile of a normal male.

Figure S3 Electropherogram profile of a 64,XY, SRY-negative DSD mare (sex reversal mare).

Figure S4 Electropherogram profile of a 64,XY, SRY-positive DSD mare (sex reversal mare).

Figure S5 Electropherogram profile of a 64 XX, SRY-negative DSD male (sex reversal horse).

Figure S6 Electropherogram profile of Turner's profile ($2n = 63,X$).

Figure S7 Electropherogram profiles of blood and hair DNA of an individual with blood chimerism.