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First case of sterility associated with sex chromosomal abnormalities in a jenny

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Contents

Chromosomal abnormalities are one of the main causes of genetic infertility in horses. Currently, their detection rate is rising due to the use of new diagnostic tools employing molecular markers linked to the sex chromosome pair. Despite genetic similarities, there are no previous reports of sterility associated with chromosomal abnormalities in the domestic donkey (*Equus asinus*). Hereby, we determined the presence of a chromosomal mosaicism in a female donkey with reproductive problems using molecular methodologies developed for horses. A two-and-a-half-year-old jenny characterized by morphological abnormalities of the reproductive tract was cytogenetically analysed using conventional and fluorescent techniques and a group of microsatellite markers (short tandem repeat, STR). At the same time, five ultrasound measures of the reproductive tract were taken and compared with eight contemporary jennies of the same breed. After slaughter, morphological examinations showed that the case study had a blind vaginal vestibule defining an empty pouch that covered the entrance of the cervical os. Histopathological studies demonstrated that this abnormal structure was compatible with a remnant hymen. Molecular markers, STR and fluorescent in situ hybridization determinations revealed that the animal was a 62, XX/61,X mosaic and, therefore, the first case of chromosomal abnormalities in the sex pair reported in donkeys.

1 | INTRODUCTION

Domestic donkeys (*Equus asinus*) are one of the most important draft animals used in several regions of the world for transport labours. It has been proposed that donkey (*Equus asinus* [EAS]; 2*n* = 62) and horse (*Equus caballus* [ECA]; 2*n* = 64) karyotypes have shared a common ancestor for 4.45 million years now (Orlando et al., 2013). This fact may explain the high percentage of similarities between species, thus allowing the use of comparative cytogenetic analysis (cross-species fluorescent in situ hybridization, ZOO-FISH) (Raudsepp & Chowdhary, 1999) and allowing the determination of chromosomal abnormalities in donkeys using horse probes (Bugno-Poniewierska, Wnuk, Witarski, & Słota,

2009). Before the standardization of the donkey GTG-banded karyotype (Raudsepp, Christensen, & Chowdhary, 2000), only three cases of chromosomal abnormalities had been reported (Darré et al., 1998; Houck, Kumamoto, Cabrera, & Benirschke, 1998; Trommershausen-Bowling & Millon, 1988). All of them included some kind of centric fusion, modifying the chromosomal number in minus one or two. However, due to the lack of agreement in the nomenclature, the identification of the chromosomes involved should be acknowledged carefully. After standardization, a few more cases were reported (Alaoui, Jordana, & Ponsa, 2004; Rank et al., 2003), but none of them included the sex pair or any reproductive failure or infertility. On the contrary, and despite their evolutionary proximity, chromosomal aberrations are a common cause of sex developmental abnormalities in horses, and responsible for a higher percentage of unexplained reproductive failures (Lear & Bailey, 2008). This species showed a highly increased prevalence of aberrant chromosome complements compared with any other domestic species. In particular, Turner's syndrome (2*n* = 63,X) and disorders of sexual development (DSD) such as sex reversal syndrome accounted for more than 70% of all the reported cases (Lear & McGee, 2012). Molecular methodologies became a primary tool for clinical cytogenetics in domestic animals, particularly equines (Lear & Bailey, 2008). In our days, the use of combined methods including traditional karyotyping, FISH and molecular markers helped to overcome the difficulties faced in equine clinical cytogenetics, thus increasing the number of cases reported (Demyda-Peyras, Bugno-Poniewierska, Pawlina, Anaya, & Moreno-Millán, 2013; Lear & McGee, 2012). Furthermore, next-generation genomic procedures such as array-based comparative genomic hybridization (CGH), a routine but expensive techniques used in human cytogenetics, are also being used in this species (Bugno-Poniewierska, Staroń, Potocki, Gurgul, & Wnuk, 2016; Holl, Lear, Nolen-Walston, Slack, & Brooks, 2013). In donkeys, however, there are no cytogenetic studies using molecular tools, probably due to the lack of specific molecular probes and the absence of a genomic reference, as the donkey genome sequencing project is still in the process of development. To overcome this situation, the molecular tools previously developed for horses could be an interesting option to be used in donkey cytogenetics considering that this species followed a closely related evolutionary pathway (Carbone et al., 2006).

Therefore, we genetically characterized a jenny by classical cytogenetic techniques, ZOO-FISH and a set of molecular markers previously developed in horses as well as the reproductive organs of the individual by ultrasonography examination and post-mortem dissection.

2 | MATERIAL AND METHODS

2.1 | Animal, physical examination and samples

A two-and-a-half-year-old jenny belonging to the Andalusian breed (Fig. S1) and showing absence of sexual behaviour, cyclicity or oestrous signs during all her first reproductive season was studied. An initial ocular inspection revealed a normal external morphology and a regularly sized and shaped vulva. The internal reproductive organs were measured using an Aloka SSD 500 ultrasound (ALOKA Co. Ltd., Tokyo, Japan) to determine the size of the body of the uterus, uterine horns and ovaries. During the reproductive season, the same determinations and a classification of the sexual cycle status were performed, according to Brinsko et al. (2011), in eight contemporary and cycling jennies belonging to the same herd and then statistically compared with our case by confidence interval test.

At the same time, blood samples were collected by jugular venipuncture using BD Vacutainers™ (MBL, Cordoba, Spain) for DNA isolation (Tri-sodium EDTA) and cell culture (sodium heparin). At the same time, 100 hair bulbs were collected for DNA isolation.

2.2 | Anatomical, pathological and histological determinations

After performing the clinical and genetic assessments, the owner decided to slaughter the animal. In that opportunity, all the reproductive organs were harvested directly from the slaughterhouse for further anatomical and histopathological determinations. The entire reproductive tract was visually inspected and evaluated immediately after slaughter and subsequently fixed in 10% formalin for 48 hr. Thin samples (1 cm^2) of vagina, the abnormal membrane, uterus and both ovaries were cut and processed with routine histological methods: dehydration through ascending grades of ethanol (70%, 90% and 100%), two washing steps with xylol, final embedding in paraffin and freezing at −20°C. Histological sections (3–5 μm) were cut with a microtome (ACCU-CUT SRM 200, Sakura, Leyden, Netherlands), mounted on glass slides and stained with haematoxylin–eosin (HE). Determinations were analysed in a DP20 bright field microscope (OLYMPUS, Madrid, Spain) at 100× and 200× magnification.

2.3 | Cell cultures and chromosome analysis

Cytogenetic analysis was performed on microscope preparations of metaphase chromosomes obtained after a routine culture of peripheral blood lymphocytes according to our standard procedure (Rodero-Serrano, Demyda-Peyrás, González-Martinez, Rodero-Franganillo, & Moreno-Millán, 2013). Chromosome spreads were achieved by dropping 100 μl of the cell suspension onto wet slides. Giemsa-stained karyotypes were analysed in a Cytovision™ platform (Leica, Madrid, Spain) using the *Equus asinus* chromosome standard proposed by Di Meo et al. (2009). The percentage of numerical abnormalities (metaphases different to 2*n* = 62) and normal metaphases was recorded. Subsequently, chromosome analysis was performed by ZOO-FISH using two distinct whole chromosome painting probes designed for the horse sex chromosome pair (ECAX; *Equus caballus* chromosome X and ECAY; *Equus caballus* chromosome Y) (Bugno & Słota, 2007; Pieńkowska-Schelling, Bugno, Owczarek-Lipska, Schelling, & Słota, 2006). Template DNA was obtained by chromosome microdissection and amplified by degenerate oligonucleotide primed–polymerase chain reaction (DOP-PCR). Probes were labelled with two different DOP-modified nucleotides. The

EASX probe was indirectly labelled using biotin-16-deoxyuridine triphosphate (Roche Applied Science, Penzberg, Germany) and detected using avidin–fluorescein isothiocyanate (FITC; green signal; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). On the other hand, the EASY probe was directly labelled using the cyanine 3 dye (Cy3-dUTP; red signal; PerkinElmer, Waltham, USA). Hybridization on donkey spreads was performed according to our standard protocols (Bugno-Poniewierska et al., 2009). Metaphases were scored on an Axiophot epifluorescence microscope (Carl Zeiss Microscopy, Poznań, Poland) equipped with appropriate filters. Captures were made in a stabilized camera driven by LUCIA software (Laboratory Imaging LTD, Prague, Czech Republic). The percentage of different cell lines was recorded in 205 metaphases. Images showing two green signals were scored as 62,XX, and images showing only one green signal were scored as 61,X.

2.4 | Chromosome-linked marker analysis

DNA was isolated from hair and blood samples using the Tissue Genomic DNA Purification Kit and the Blood Genomic DNA Extraction Kit (Canvax Biotech. SL, Cordoba, Spain), respectively, following the manufacturer's instructions. DNA was quantified using a Nanodrop™ 2000 (Thermo Scientific, Madrid, Spain) and stored at −20°C until use.

Five different sex-linked fragments were determined by PCR using primers designed for horses. Sex-determining region gene (*SRY*, ECAY-linked) and amelogenin ECAX (*AMX*)- and ECAY (*AMY*)-linked genes were amplified according to Hasegawa, Sato, Ishida, Fukushima, and Mukoyama (2000). The exon 21 of the *KIT* gene was used as positive control for the PCR during the *SRY* amplification as described by Haase et al. (2007). Zinc finger protein ECAX-linked (*ZFX*) and ECAY-linked (*ZFY*) genes were amplified according to Han, Yang, Ko, Oh, and Lee (2010). The amplified products were assessed on a 2% (*ZFXY* and *SRY*) and 3% agarose gels (*AMXY*) at 80 v for 70 and 180 min runs, respectively, using a male and a female donkey, horse DNA and pure water as concurrent controls.

Six additional microsatellite markers, five X-linked (*LEX003*, *LEX0026*, *TKY38*, *TKY270* and *UCEDQ502*) and one Y-linked (*ECAYM2*), were analysed (Demyda-Peyrás et al., 2014). Primers were labelled using 6-carboxyfluorescein (FAM™, Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or HEX Phosphoramidite dye (HEX™, Applied Biosystems) and amplified in a multiplex single PCR as follows: 23-µl reaction mixture containing 20–60 ng genomic DNA, 1.5–7.5 pmol of each primer pair, 0.33 mmol/L dNTPs, 2.5 mmol/L $MgCl_2$, 1× PCR buffer and 1.5 U Horse-Power™ Taq DNA polymerase (Canvax Biotech, Cordoba, Spain). Samples were initially denatured at 95°C for 10 min, followed by 33 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 30 s and a final elongation step at 72°C for 10 min. Amplified products were genotyped by capillary electrophoresis using an Applied Biosystems 3130 xl DNA sequencer (SCAI genomics core, University of Cordoba, Spain). Allele allocation was performed using GENOTYPER 4.0 software package using a LIZ 500-bp internal size standard (Applied Biosystems, Madrid Spain). Additional information regarding markers is provided in Table S1.

2.5 | Statistical analysis

The statistical comparisons between the case study and the population of normal jennies analysed were performed by estimating the exact probability of each parameter using the Probability Distribution Calculator of Statistica™ for Windows (v8.0, StatSoft Inc.). The normal distribution of each parameter was defined by the mean and standard deviation values of the eight normal jennies.

3 | RESULTS

3.1 | Physical examination

An initial ocular inspection revealed a normal external morphology and a regularly sized and shaped vulva (Figure 1). A further examination of the jenny revealed a short blind vaginal vestibule (only 13.5 cm long) with no cervical os and an abnormal ball-shaped structure inside (8 cm diameter approximately) not solid to palpation, which naturally protruded from the vulva (Figure 1B, C). Ultrasonography showed an abnormal development of the internal organs. Both ovaries were hypoplastic without any sign of follicular activity. The uterus body was small, and it had an unusual consistency (Figure S2). After sacrifice, the anatomical examination showed that the ball-shaped structure was a membrane that protruded caudally forming an empty pouch (Figure 2). Surprisingly, the cervix was

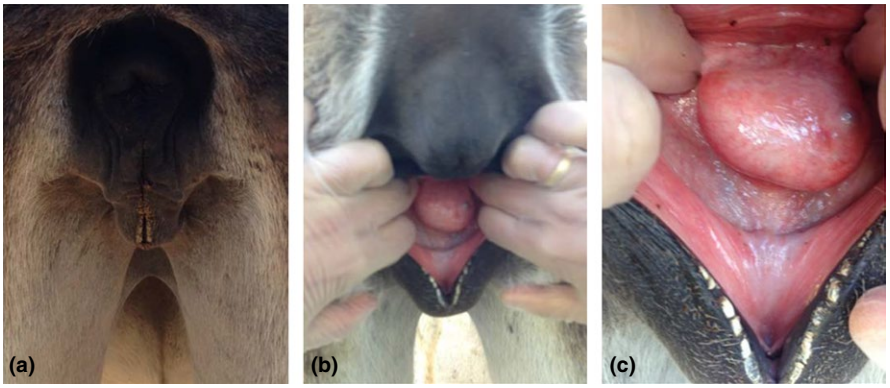

FIGURE 1 External reproductive organs of the analyzed case. (a) Normal-sized vulva. (b) and (c) Abnormal ball-shaped structure located at the end of the vaginal vestibule which protruded spontaneously. Note the absence of cervical os

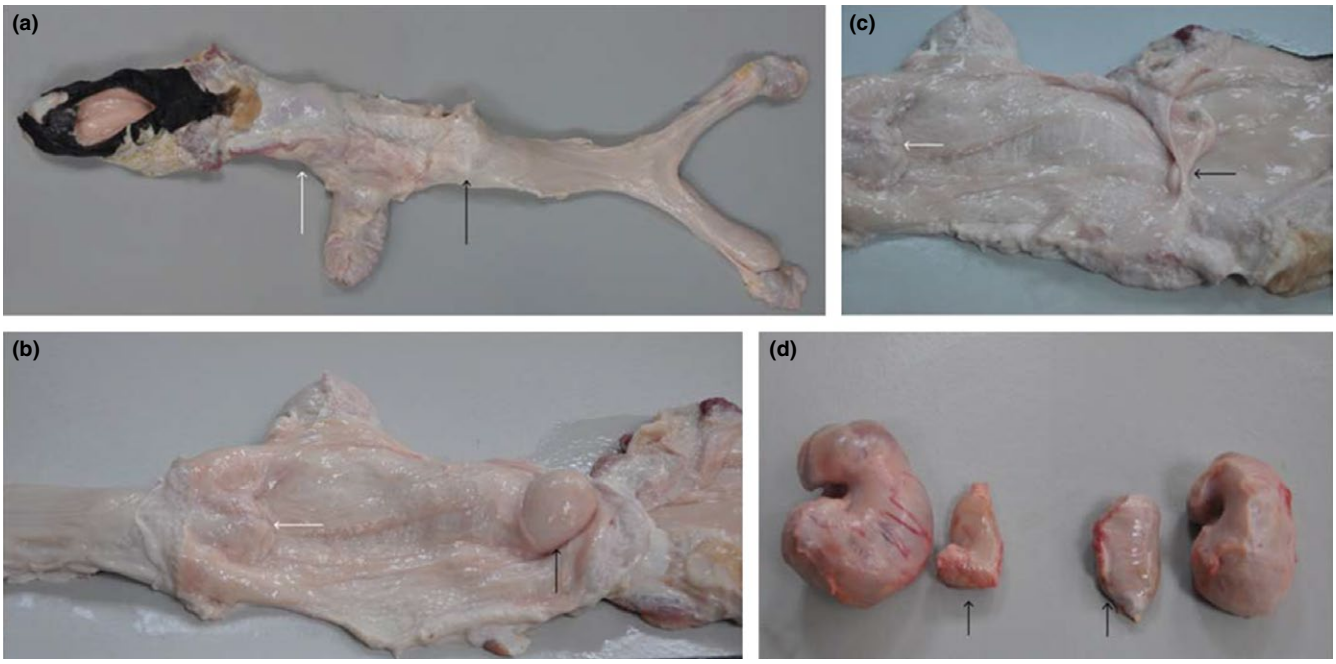

FIGURE 2 Reproductive tract of the study animal recovered after slaughter. (a) Complete reproductive tract. White arrow, abnormal ball-shaped pouch. Black arrow, hidden cervix. (b) Dissection of the blind pouch delimited by the cranial part of the vaginal vestibule and the cervical os. Black arrow, unusual membrane protruding in craneal direction. White arrow, cervical os. (c) Dissection of the vaginal vestibule; it can be seen that the abnormal structure was a membrane (black arrow). White arrow, cervical os. (d) Comparison between the hipoplastic ovaries of our case (black arrows) and another pair obtained from a coeval and fertile jenny

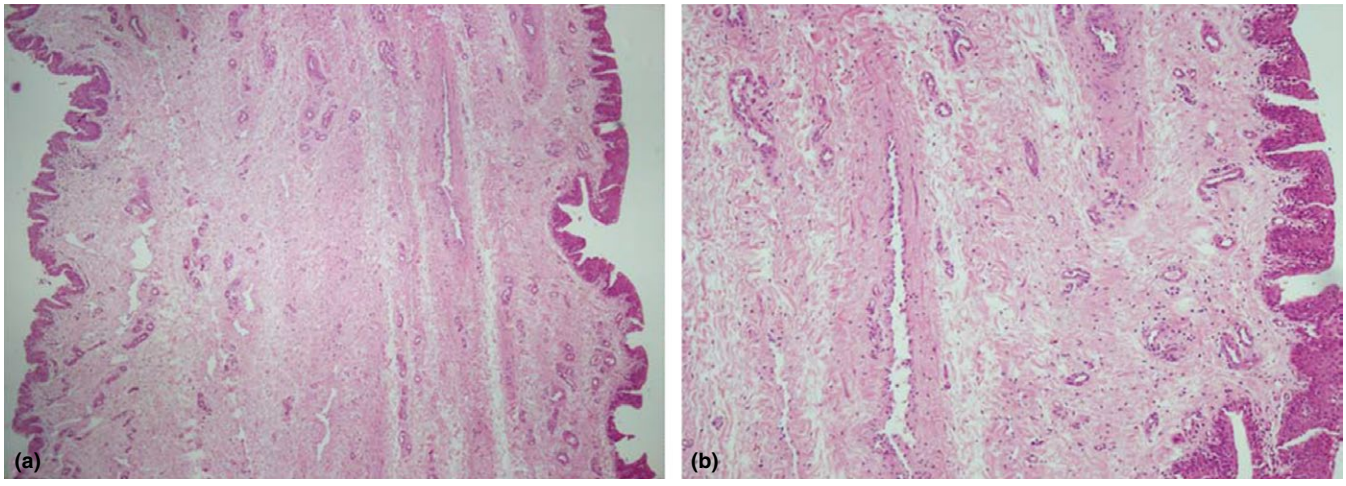

FIGURE 3 Histological analysis of the abnormal blind vaginal pouch. As described in the Results section, the blind vestibule was cranially closed by a membrane consistent with vaginal tissue histology (blind vagina). Histopathological studies showed a stratified squamous epithelium surrounding a lamina, composed of abundant blood vessels and connective tissue. (a) 100× magnification. (b) 200× magnification

present, but the entrance was obstructed by the membrane which was located approximately 10 cm before/caudal the cervical os. Histopathological studies showed that this membrane had a stratified squamous epithelium in both sides (cranial and caudal) surrounding a lamina composed of abundant blood vessels, both arterial and venous, and connective tissue which extended to the opposite side of the membrane (Figure 3). No abnormal tissues or signs of infection were observed. Histological samples from the vagina and uterus were normal (Figure S3). The ovaries size was also analysed and compared with those obtained from another fertile coeval jenny and resulted at least two-thirds smaller (Figure 2). Histologically, they showed a normal parenchyma predominantly composed of connective tissue with a few poorly developed primordial follicles and the absence of secondary or developing follicles (Figure S3). The ultrasonographic determinations obtained from the case study and eight contemporary normal animals are shown in Table 1.

TABLE 1 Size of the internal reproductive organs of eight normal jennies and the sterile case study

Jenny	Right ovary	Left ovary	Right horn	Left horn	Uterus body	Oestrous phase
1	61	56	32	43	58	Dioestrous
2	89	46	57	46	67	Oestrous
3	45	48	45	40	57	Dioestrous
4	73	62	28	28	36	Dioestrous
5	59	60	43	56	55	Dioestrous
6	54	67	32	36	46	Dioestrous
7	39	73	27	33	52	Oestrous
8	55	60	47	31	51	Dioestrous
Mean ± STD	59 ± 15	59 ± 9	38 ± 10	39 ± 9	52 ± 9	
Case	25*	34*	31	35	20*	Anoestrous

All measurements were performed in the longer diameter of each organ and are expressed in millimetres. Mean and STD were determined among the eight normal jennies. Asterisks in the same column show statistical difference $^*p < .001$.

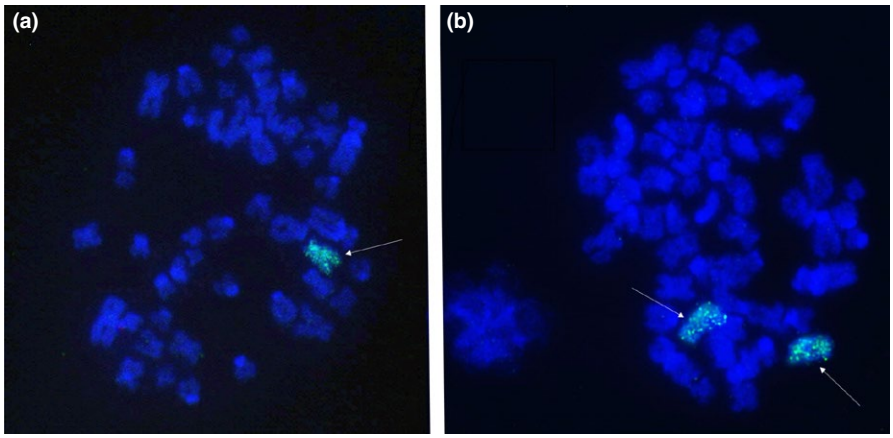

FIGURE 4 ZOO-FISH metaphases of *Equus asinus* (donkey) hybridized using *Equus caballus* (horse) probes. (a) 61,X metaphase. (b) 62, XX metaphase. Arrows show the EASX (X chromosome)

3.2 | Chromosomal assessment

Giemsa-stained karyotypes presented two cellular lines: (i) a prevalent line with a standard shape and number of female karyotype (62,XX), and (ii) a minority line (less than 3% of the metaphases determined with a monosomy presumably in the sex pair 61,X?). ZOO-FISH analysis confirmed a 62,XX/61,X karyotype (Figure 4). The percentage of each cell line was 96.6% (198 cells) and 3.4% (7 cells), respectively. There were no EASY positive cells (red signal).

3.3 | Molecular determinations

All the molecular markers employed were correctly amplified in both male and female donkeys and in the horses used as controls.

Our case study showed the presence of *ZFX* and *AMX* and the absence of *ZFY*, *AMY* and *SRY* amplified fragments, in agreement with the results expected in a healthy female (Figure 5).

STR results were compatible with an average female individual. One X-linked marker was diallelic (*LEX003*), and four were monoallelic (*LEX026*, *TKY38*, *TKY270* and *UCDEQ502*), while the Y-linked marker was not amplified. Results of blood and hair DNA were identical in all the molecular determinations.

4 | DISCUSSION

To our knowledge, there are no previous reports of sex chromosomal abnormalities in donkeys, and even less associated with abnormalities in the reproductive tract or sterility. Our individual showed a karyotype with a predominant (normal) and a minority (Turner-like) cell line. Molecular analysis confirmed the presence of two different EASX lines with an identical genetic background as there were no tri-allelic markers and blood and hair DNA results were identical, defining our case as a mosaicism. This methodology proved to be highly efficient in horses (Demyda-Peyrás et al., 2014), and the present results show that it could also be used successfully in donkeys.

Mosaicism in horses is usually associated with anomalies in the reproductive tract (Paget et al., 2001; Pawlak, Rogalska-Niznik, Cholewiński, & Świtoński, 2000; Sato et al., 2012). These individuals showed an ample range of morphological abnormalities, from gonadal

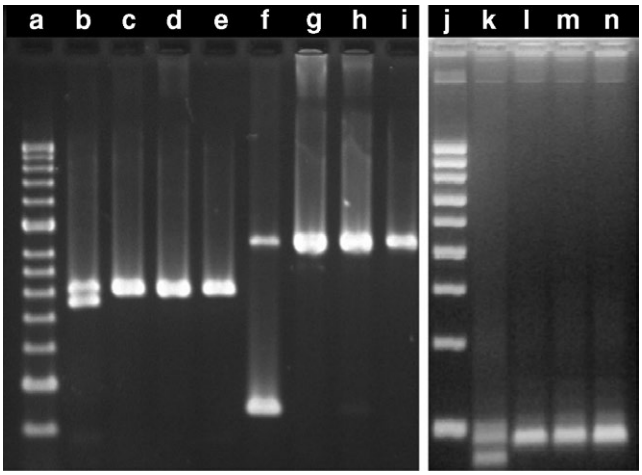

FIGURE 5 PCR results of zinc finger protein X and Y, sex determination region and amelogenin X and Y genes in the case study. Samples were checked in a 2% agarose gel (80 v; 70 min run) a–i and in a 3% agarose gel (80 v; 180 min run) j–n and stained with ethidium bromide using 100–1,500 bp Standard Size Ladder (a and j). b, f and k: Jack positive control. c, g, and l: Jenny positive control. d, h and m: Case study blood DNA. e, I and n: Case study hair DNA. b–e: Zinc finger protein X- and Y-linked genes (*ZFX* and *ZFY*). ZFX (604 bp) was positive in all the animals (b–e). ZFY (553 bp) was only positive in b. f–i: *SRY* + *Kit* genes. The *KIT* (785 bp) was positive in all the animals (f–i). SRY (249) was only positive in f. k–n: Amelogenin X and Y genes (*AMX* and *AMY*). *AMX* (184 bp) was positive in all the animals (k–n). *AMY* (164 bp) was only positive in k (lower band)

dysgenesis to pseudohermaphroditism. It has been suggested that the cause of such differences is the complexity of the horse chromosomal complement (Villagómez et al., 2009). Another possibility, as it has been suggested in humans, is that the relative percentage of normal/abnormal cell lines (degree of mosaicism) could modulate the extension of the reproductive abnormalities observed (Sarkar & Marimuthu, 1983). Such degree of mosaicism could explain the diversity found in 63,X/64,XX equines reported (Bugno, Słota, & Kościelny, 2007; Lear & McGee, 2012; Power, 1990), which varied from sterile acyclic animals to individuals showing a brief window of oestrous cycling or even a case of a phenotypically normal mare. In our study, and despite the extremely low percentage of Turner-like cells detected, the morphological abnormalities were important, suggesting that the regulatory mechanism of sexual development could be different in donkeys.

An important advantage of the use of molecular markers in cytogenetic diagnostics is their ability to discriminate the genetic background of different cell lines, which is not possible by direct karyotyping. Due to that, mosaicism and chimerism cannot be distinguished from each other. Such differentiation is important because blood chimerism is not associated with reproductive failures in horses, contrarily to that described in the freemartin syndrome of cows (Padula, 2005). We have previously reported a similar case in which a colt showed 96.4% normal (64,XY) and only 3.6% abnormal (63,X; Turner-like) cells, without presenting any morphological abnormality (Demyda-Peyrás et al., 2014). In our jenny, the percentage of abnormal cells was identical to the populations observed is such previous case and several abnormalities were found. However, the individual was a male, in which case the effect of the chromosomal abnormality could have been attenuated (Lear & Bailey, 2008), and it also presented blood chimerism, while reproductive problems associated with this syndrome in horses are still under discussion.

The ultrasonographic anatomical determinations performed in the jenny showed that both ovaries were significantly smaller and that the uterus body was shorter in comparison with the coeval animals. On the contrary, both uterus horns were normal, but the consistency on the rectal palpation was pasty. These findings, together with the lack of reproductive behaviour, fully agree with similar cases reported in horses, including Turner-like/female mosaicisms (Bugno, Słota, & Zabek, 2001), Turner's syndrome cases (Power, 1990) and 64,XY *SRY*-negative DSD horses (Anaya et al., 2014; Villagómez et al., 2011). All of them showed an external morphology without abnormalities, different extents of gonadal dysgenesis or abnormalities in the internal reproductive organs, and the absence of oestrous behaviour.

The morphological evaluation showed a blind vaginal sack only. To our surprise, although the cervical os was present, it was enclosed in a closed pouch defined by the cranial part of the blind vagina, and inaccessible from the vaginal entrance. Curiously, histological analyses showed that the vagina was normal. The same results were obtained in uterus and ovary tissues, despite the lack of folliculogenesis in both ovaries. This abnormality had been previously reported in horses with chromosomal abnormalities, in agreement with our findings (Card, 2012).

One of the primary impairments in the clinical cytogenetics of equids is the lack of reliable and affordable diagnostic tools and specialized laboratories available for veterinary practitioners. Furthermore, karyotype results are not always definitive, especially when the smaller autosomes or the Y chromosome are involved (Lear & Bailey, 2008). In horses, C-banding could be utilized to determine the presence of the sex chromosome pair in an individual, as ECA11, ECAX and ECAY are the only chromosomes with a particular staining pattern (Richer, Power, Klunder, McFeely, & Kent, 1990). This is an interesting option as more than 90% of cases of individuals carrying chromosomal abnormalities are precisely related with ECAX and ECAY. However, despite the higher prevalences reported (up to 2% of individuals of some populations (Bugno et al., 2007)), it is very usual that horse breeders and veterinarians do not associate unexplained infertility with chromosomal abnormalities. This trend could be worse in donkeys, in which sex chromosomal abnormalities could not be determined by C-banding as the sexual pair (EASX and EASY) does not show any differential staining pattern (Raimondi et al., 2011) and, therefore, conventional cytogenetic techniques are even more complicated. This fact, the lack of commercial FISH probes and the absence of the donkey genome reference sequence make the diagnosis of this kind of genetic diseases limited to few and high specialized laboratories.

In our study, we successfully used in donkeys the molecular probes and markers previously developed for horses. All the molecular markers amplified correctly, also showing an adequate variability in a broader Andalusian donkey population analysed simultaneously,

potentially able to detect the most important chromosomal abnormalities associated with the sexual pair in this species. This methodology is currently being used as a screening tool in an ongoing study to determine whether the number of individuals with chromosomal abnormalities in donkeys is lower than in horses or whether the difference previously observed is due to the lack of chromosomal diagnoses in this species.

In conclusion, we present the first case of sex chromosomal abnormalities in the domestic donkey. This cellular mosaicism produced reproductive abnormalities and sterility in an Andalusian jenny. We also demonstrated that already developed horse cytogenetic probes and molecular markers could be used as a diagnostic cross-species methodology in donkeys with a high degree of certainty. Further studies are necessary to determine whether the low prevalence of chromosomal abnormalities reported in donkeys is due to a real difference between species, or to a high number of animals that remain undiagnosed.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

JD, IO and MH performed all the reproductive determinations. MBP performed FISH determinations. GA, AM and SDP performed the molecular determinations. MM performed karyotyping. JD, AM, MM, PPG and SDP write and reviewed the manuscript.

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