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The use of a novel combination of diagnostic molecular and cytogenetical approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism.

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Summary

Sexual chromosomal aberrations are known to cause congenital abnormalities and unexplained infertility in horses. Most of these anomalies remain undiagnosed because of the complexity of the horse karyotype and the lack of specialized laboratories that can perform such diagnoses. On the other hand, the utilization of microsatellite markers is a technique widely spread in horse breeding, mostly due to their usage in parentage tests. We

studied the usage of a novel combination of diagnostic approaches in the evaluation of a very uncommon case of chromosomal abnormalities in a Spanish Purebred colt, primarily detected using a commercial panel of short tandem repeat (STR) makers. Based on these results we performed a full cytogenetical analysis using conventional and fluorescent *in situ* hybridization (FISH) techniques with individual ECAX (*Equus caballus* chromosome X) and ECAY (*Equus caballus* chromosome Y) painting probes. We also tested the presence of two genes associated with the sexual development in horses and an extra novel panel of 8 microsatellite markers specifically located in the sex chromosome pair. This is the first case reported of a leukocyte chimerism between a chromosomally normal (64,XY) and abnormal (63,X0) cell lines in horses. Our results indicate that the use of the STR markers as a screening technique and as a confirmation utilizing cytogenetic techniques can be used as a very interesting easy and non-expensive diagnostic approach to detect chromosomal abnormalities in the domestic horse.

Keywords: Horse infertility diagnosis; Chromosomal abnormalities; Microsattellitte analysis; *In situ* fluorescent hybridization.

1. Introduction

The association between atypical chromosome constitutions and developmental and phenotypical abnormalities has been widely described in domestic animals with the usage of clinical cytogenetics [1]. In horses, these types of determinations are performed in a low number of laboratories in the entire world, probably due to the complexity of *Equus caballus* karyotype and the lack of commercially available hybridization probes to evaluate each one of their 33 different chromosomes [2]. Because of this, some horses with abnormal karyotypes could be misdiagnosed due to the inability of conventional and molecular cytogenetic techniques to precisely determine aberations such as mosaicism or chimerism [3]. Aditionally, the number of misdiagnoses could be higher if we consider that a great percentage of horses with this type of chromosomal aberrations are associated with normal phenotypes [4].

On the other hand, short tandem repeat (STR) markers, a fast molecular analysis widely employed in parentage tests, could be used as a powerful screening technique to detect the existence of most of the karyotype anomalies present in certain chromosomes and even more, to detect some particular abnormalities that cannot be diagnosed using other cytogenetic techniques [5].

Currently, to perform a parentage test, the International Society for Animal Genetics (ISAG) recommends the use of a set of 17 standardized STR markers. As a disadvantage, this set of microsattellittes usually has only one marker (*LEX003*) linked to ECAX (*Equus caballus* chromosome X) and none linked to ECAY (*Equus caballus* chromosome Y). This may be problematic because most of the chromosome abnormalities reported in horses are those related to the sex chromosomes, such as the equine Turner Syndrome (X chromosome monosomy) and XY sex reversal syndrome [6, 7]. For this reason, the use of extra specific

65 microsattellite markers located in the X and Y chromosomes will make it possible to detect
66 this kind of chromosomal disorder.
67 Due to this, we developed a novel diagnostic approach combining the use of cytogenetic
68 and molecular biology techniques in order to detect horse chromosomal abnormalities in a
69 way which is quick, simple and much cheaper [3]. In this study we investigated the case of
70 a Spanish Purebred horse showing an aberrant karyotype detected by using a specific set of
71 sex chromosomes-related STR markers and molecular cytogenetic techniques. We also
72 compared the results obtained in the analysis of different tissue samples to determine the
73 existence of blood or true chimerism.
74

75 **2. Material and methods**

76 **2.1. Physical examination**

77 The colt was physically examined at the age of 14 mo. It showed a normal external
78 morphology according to the standards of the Spanish Purebred Breeders Association and it
79 was approved to enter the official register book of the breed. External genitalia of the colt
80 appeared to be morphologically normal without any signs of dysfunction. To date, the
81 animal has not shown any abnormality in its behaviour. Seminal parameters were not yet
82 evaluated due to the colt sperm not being collected. However, the animal showed a normal
83 sexual behaviour in the presence of a mare in heat.
84 The pregnancy of which the colt was conceived was reported to the database of the Spanish
85 Purebred Association as a single pregnancy and also the foal was delivered as a single
86 offspring. Moreover, the mother of the colt was examined by a veterinarian. It did not show
87 any symptoms of phenotypic or physiological abnormalities.
88

89 **2.2 Animal samples**

90 Hair and blood samples were collected from the studied animal. Blood samples were
91 obtained by jugular venopunction using Tri-sodium ETDA BD Vacutainers™ (MBL,
92 Cordoba, Spain) for DNA isolation and sodium heparin BD Vacutainers™ (MBL) for cell
93 culture. At the same time, 50 hair bulbs were collected for DNA isolation.

94

95 **2.3. Cell Cultures and Chromosome Analysis**

96 The chromosome slides were made from peripheral blood lymphocyte cultures using our
97 standard protocol [8]. After centrifugation white cells were cultured in 7 mL of RPMI 1640
98 medium (R4130, Sigma Aldrich, Madrid, Spain) supplemented with 5 µg/mL of Pokeweed
99 Lectin (L9379, Sigma Aldrich) 10mL per liter of antibiotic antimicotic 100X solution
100 (A5955, Sigma Aldrich,) and 0.7 mL of autologous plasma. Cultures were incubated in 12
101 mL sterile culture tubes (Techno Plastic Products, Trasadingen, Switzerland), in horizontal
102 position at 38ºC for 72 h. One hour before harvesting, 1 µg /mL of colcemid™ (N-
103 Deacetyl-N-methylcolchicine, D1925, Sigma Aldrich) was added to each tube. After that,
104 cells were re-centrifuged, harvested and incubated for 25 min in 0.075 M KCl (Scharlau,
105 Barcelona, Spain) hypotonic solution. Finally, cells were fixed twice in a cold methanol:
106 acetic acid (3:1) (Qemical, Esparraguera, Spain) solution and stored for 24h at 4ºC.
107 Chromosome spreads were obtained by cell dropping and air drying.
108 Giemsa stained metaphases [9] were assessed using a Polyvar microscope (Reichert Jung,
109 Austria) with a x1250 magnification. A total of 100 analyzable metaphases (those with
110 intact and non-overlapping chromosomes) were counted.

111

112 **2.4. *In situ* hybridization**

Metaphase spreads were analyzed by fluorescent *in situ* hybridization with two whole chromosome painting probes (WCPPs) specific to ECAX and ECAY. Probes were prepared by chromosome microdissection and degenerated oligonucleotide primers (DOP) - polymerase chain reaction (PCR) amplification using our routine protocols and labeled for double color fluorescence simultaneous hybridization [10]. The ECAX probe was DOP-labeled by biotin-16- deoxyuridine triphosphate (dUTP) (Roche Applied Science, Penzberg, Germany) and the ECAY probe by Cyanine 3 dye (PerkinElmer, Waltham, USA). A standard FISH protocol [11] with some minor modifications was applied. The labeled probes were denatured at 70ºC for 10 min. Metaphase spreads were digested in: RNAse (10mg/mL) at room temperature for 60 min and then in 0.02% pepsin solution at 37ºC for 10 min. After RNAse and pepsin digestion, target metaphase spreads were denatured in a hybridization solution containing 30% of 2x saline-sodium citrate (SSC) and 70% formamide (Sigma-Aldrich) at 70ºC for 2.5 min. Thereafter, the probes were applied onto the metaphase spreads, covered, sealed with rubber cement and hybridized overnight in a dark moisture chamber at 37ºC. The post-hybridization washes were as follows: twice in 50% formamide in 2xSSC and twice in 1xSSC at 42°C. Signals were detected and amplified using avidin-Fluorescein isothiocyanate (FITC) and anti-avidin antibodies (Sigma-Aldrich). Chromosome staining was performed with 4',6-diamidino-2-phenylindole (DAPI) (Cambio, Cambridge, UK). The slides were analyzed under the fluorescence microscope (Axiophot, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a digital CCD camera driven by Lucia software (Laboratory Imaging LTD, Prague, Czech Republic). A 365 nm filter (UV light was used to detect the DAPI staining and a 450/490 nm filter (Blue light) was used to detect the FITC signal.

2.5. Molecular analyses

2.5.1. ECAX and ECAY linked genes

DNA was obtained from blood and hair follicles using the QIAamp DNA mini kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer protocol. DNA from a stallion and a mare served as a positive and a negative control. DNA was amplified by PCR using primers for two genes strongly related to sex development in mammals (sex-determining region Y gene (SRY); amplified according to Hasegawa et al. [12] and zinc finger X-chromosomal protein gene (ZFX) and zinc finger Y-chromosomal protein gene (ZFY); amplified according to Han et al. [13]). The amplified products were assessed in a 2% agarose gel (80v, 70min run).

2.5.2. Microsatellite genotyping

A total of 24 STR markers were genotyped for blood and hair DNA samples. Initially, a set of 16 standard STR markers used for paternity tests in horses was assessed according to our previous studies [14]. *Loci* were amplified using a commercially available fluorescently-labeled primers (StockMarks® kit for horses, PE Applied Biosystems, Foster City, CA, USA). PCR reactions were performed following the manufacturer's instructions.

Five extra X-linked (*LEX003* [15], *LEX0026* [16], *TKY38* [17], *TKY270* [18] and *UCEDQ502* [19] and three extra Y linked (*ECAYH12*, *ECAYA16* and *ECAYM2;* [20]) additional markers were amplified according to Kakoi et al. [21]. Primers for all markers were labeled using 6-carboxyfluorescein (FAM^{TM}, Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or HEX Phosphoramidite dye (HEX^{TM}, Applied Byosystems) and amplified in two multiplex PCRs: one containing all the ECAY linked markers (EcaYH12, 6-FAM; EcaYA16, HEX and EcaYM2, HEX) and the second containing all the ECAX markers (*LEX003*, 6-FAM; *LEX026*, 6-FAM; *TKY38*, 6-FAM; *TKY270*, HEX and

UCDEQ502, 6-FAM). Each PCR was performed in a 23 µL reaction mixture containing: 20–60 ng of genomic DNA, 1.5–7.5 pmol of each primer pair, 0.33 mmol/L of dNTPs, 2.5 mmol/L of $MgCl_2$, 1x PCR reaction buffer and 1.5 U of MBL Taq 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. The final elongation step was performed at 72˚C for 10 m. Amplification products were frozen and genotyped afterwards by capillary electrophoresis using an Applied Biosystems 3130 *xl* DNA sequencer (SCAI genomics core, University of Cordoba, Spain). Allele sizes were determined after processing the raw data with the Genotyper 4.0 software package using a LIZ 500 bp internal size standard (Applied Biosystems).

3. Results

3.1. Cytogenetic analysis.

Two different karyotype lines were detected in the metaphases derived from the blood cultures. A high percentage of the spreads (98%) showed 64 chromosomes, 27 of which were diagnosed as bi-armed chromosomes. These metaphases were diagnosed as 64, XY. The remaining 2% of the analyzed spreads showed 63 chromosomes with the same number of bi-armed chromosomes. In these metaphases we detected the absence of one unidentified small chromosome (probably the ECAY). The first presumptive diagnose was a presence of a sex chromosomal blood mosaicism 63,X0?/64,XY.

3.2. *In situ* hybridization

The preliminary diagnosis was confirmed by using *in situ* hybridization in a total of 96 lymphocyte spreads. The high percentage of the analyzed metaphases (95.8%) have shown

a large X chromosome with a green signal and a smaller Y-chromosome with a red signal (Figure 1). The remaining metaphases (4.2%) have shown only one X-chromosome with a green signal.

3.3. ECAX and ECAY linked genes

PCR analysis performed on the DNA obtained from blood and hair samples showed the same results. ECAY specific sequences (SRY; 429bp fragment; Figure 2a and ZFY; 553bp fragment; Figure 2b) were detected in the DNA samples obtained from the studied colt and male positive control, but were not detected in the female positive control. Conversely, the specific sequence linked to ECAX (ZFX; 604 bp) was detected only in the DNA samples obtained from the studied colt and female positive control.

3.4. STR analysis.

The results obtained from parentage tests of STRs markers in blood and hair DNA samples are shown in Table 1. Two markers (*AHT5* and *ASB17*) showed three different alleles compatible with abnormal chromosomal complements only in DNA from blood samples (Figure 3). The remaining 14 markers were normal with 11 heterozygous and 3 homozygous loci detected. The genotype obtained from DNA acquired from hair follicles showed 16 loci with normal results (13 heterozygous and 3 homozygous loci).

The results obtained from the extra ECAX and ECAY linked microsatellites showed a similar pattern (Table 2). In DNA obtained from blood samples two of the ECAX-linked markers (*LEX026* and *UCDEQ502*) showed two different alleles (Figure 4). This result is only compatible with the existence of two X chromosomes originating from different

cellular lines. The remaining markers (three ECAX-linked and 3 ECAY-linked markers) and all results of DNA obtained from hair follicles showed only one allele.

4. Discussion

To our knowledge, this is the first reported and proved case of a 64,X0/XY blood chimerism in horses in which the different cell lines derived from twin siblings of different sex in which one of the fetuses had also an abnormal karyotype 63,X0.

There are few reports estimating the incidence of chromosomal abnormalities in horses and only one was performed on a large population, estimating an overall rate of individuals with karyotype abnormalities at 1.6% [22]. However, in this study the rate of aberrant karyotypes was 0% for males and 3,7% for females. We suggest that these results could be influenced by the fact that chromosomal abnormalities in horses are normally linked with reproductive problems [2]. In this sense, the number of males that are reproductively active is much lower than in mares. Furthermore, some stallions could be not diagnosed as carriers of a chromosomal abnormalities because they often remain asymptomatic.

This is the fourth reported case with a 63,X0/64,XY karyotype, all of which were diagnosed as mosaicisms. The previous three reports were only diagnosed using conventional cytogenetic techniques in lymphocyte cultures [23-25]. With these techniques it is not possible to determine if an individual was a chimera or a mosaic or if the abnormality was present in the blood or in the whole individual. It is noteworthy that one of these previous studies speculates about the possibility of a cellular chimerism or mosaicism [24]. However, at that particular time (1981), there were no available methodologies (such as PCR, FISH, etc.) to perform such determinations.

The most probable cytogenetic explanation of our results would be that the individual was carrying a cellular mosaicism in which one of the cell lines had an abnormal chromosomal complement, similar to the case previously described by Bugno et al. [26]. In both cases, the most likely hypothesis was that the two different cell lines originated from the same embryo due to an abnormal mitotic segregation. During that, the ECAY would be lost producing a different cell line with an aberrant karyotype (63,X0). However, this case is incompatible with the two different alleles observed in the *LEX026* and *UCDEQ* ECAX-linked loci and with the three different alleles observed in the autosomal loci *AHT5* and *ASB17* in the DNA obtained from blood samples. This observed microsatellite pattern and the fact that STR results from hair follicle DNA were normal, can be only compatible with the mixture of blood cells belonging to two different fetuses in the maternal womb. Therefore, our case could only be derived from a blood cellular chimerism, in which one of the embryos had additionally an abnormal 63,X0 karyotype. Thus, the combination of these two chromosomal abnormalities in a single individual is extremely rare and to our knowledge, has not been yet reported in horses.

Another interesting finding was that the foal evaluated in this study did not show any morphological or physiological abnormality in both the external and internal physical examination. This is in agreement with previous findings observed in horses carrying a 64,XX/64,XY chimeric karyotype. These animals are normally derived from twin heterosexual pregnancies and the fetuses and foals were completely normal [14, 27, 28]. This occurs because the vascular anastomosis responsible for the Freemartin syndrome in other species is developed after the sexual differentiation of the horse [29], without without causing the typical female sterility observed in cattle [30].

In the present study, both ZFX/ZFY and SRY fragments were amplified in the blood and hair DNA samples. These results (ZFY (+), ZFX (+) and SRY (+)) are in agreement with the observed phenotypic sex of the colt, confirming that the majority of the analyzed cells had ECAY present, presumptively derived from the 64,XY normal male embryo.

The total absence of physical abnormalities is noteworthy suggesting that there was no influence of the 4% of blood cells carrying the abnormal karyotype (63,X0) on the foal's health. These findings are totally opposite to those observed in the previously reported cases of horses carrying this type of karyotype abnormality: one horse with bilateral ovotestes, an underdeveloped penis, bilateral seminal vesicles and uterine tissue [24]; one externally phenotypically normal mare with small, firm ovaries consisted of undifferentiated ovarian stroma [23] and more recently a Thoroughbred colt with ambiguous external genitalia and a short penis that faced backward between its hind limbs [25]. These three cases were reported as true hermaphrodites. We suggest that the absence of phenotypical abnormalities observed in the studied foal was probably originated because, unlike the others, this was an individual carrying a blood cell chimerism. As in the case of leukocyte chimerism 64,XX/64,XY previously described in horses, individuals carrying this chromosomal aberration have a normally developed genital organs.

Chromosomal abnormalities in livestock animals are routinely diagnosed using conventionally available cytogenetic techniques (chromosome banding), fluorescent *in situ* hybridization (FISH) [10] and more recently comparative genomic hybridization [31]. The conventional techniques are often inaccurate in identifying individual chromosomes [32]. On the contrary, FISH techniques can determine precisely chromosomes involved in an

aberration; however, it is a method routinely applied only in a few laboratories around the world encountering obstacles due to the complexity of the horse karyotype [2]. More recently, Holl et al. [33] performed the first aneuploidy detection in horses using SNP array comparative genome hybridization (SNP-CGH). This robust diagnostic tool, increasingly used in prenatal genetic diagnostic (PGD) in humans [34] is based on allelic ratios from genotyping arrays what allows an estimation of chromosome copy numbers. However, this expensive technique is currently being used commercially only in human embryos [35] and was only reported as a research tool in a very few domestic animals cases [36]. We, therefore, propose the use of this combination of diagnostic molecular and cytogenetical approaches that allow for a rapid screen of the presence of chromosomal abnormalities in the sex chromosomes of the horse based on the extended set of STR markers. This is a fast and accurate technique that could be utilized in a standard genetic laboratory, thus providing the possibility of being an interesting diagnostic tool in places in which other techniques may not be performed.

5. Conclusion

In present study the use of molecular markers widely employed for routine parentage analysis has been demonstrated to be a simple and efficient tool to detect the presence of chromosome abnormalities among horses. With the use of this method, we were able to report for the first time the ocurrence of a chromosomal chimerism between a chromosomally normal and chromosomally aberrant cell line in horses. The use of this combined molecular technique will allow for detection and a more precise diagnosis of sex chromosome abnormalities which are known to be a severe and usually undiagnosed problem connected with reproductive failures in horses.

6. Acknowledgements

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7. Conflict of Interest

The authors does not have any conflict of interest.

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411
412

413 Table 1: Results of Short Tandem Repeats (STR) parentage test using DNA obtained from
414 blood and hair samples from a Spanish purebred colt.

415

Microsatellite loci	Size Range (bp)	Blood	Hair	ECA
AHT4	140-166	**HJ**	**HJ**	**24**
AHT5	126-147	**KLN**	**LN**	**8**
ASB17	104-116	**HMR**	**HR**	**2**
ASB2	237-268	**KN**	**KN**	**15**
ASB23	176-212	**IJ**	**IJ**	**3**
CA425	224-247	**N**	**N**	**28**
HMS1	166-178	**IL**	**IL**	**15**
HMS2	215-236	**J**	**J**	**10**
HMS3	146-170	**MN**	**MN**	**3**
HMS6	154-170	**KN**	**KN**	**4**
HMS7	167-187	**LO**	**LO**	**1**
HTG10	83-110	**OS**	**OS**	**21**
HTG4	116-137	**KM**	**KM**	**9**
HTG6	74-103	**JO**	**JO**	**15**
HTG7	114-128	**O**	**O**	**4**
VHL20	83-102	**LQ**	**LQ**	**30**

416

417 Footnote

418 Results obtained from a set of 16 standard STR markers used for paternity tests in horses,
419 standardized by the International Society for Animal Genetics (ISAG). Loci were amplified
420 using a commercially available fluorescently-labeled primers (StockMarks® kit for horses,
421 PE Applied Biosystems, Foster City, CA, USA). Bp: base pair; blood: DNA obtained from
422 blood tissue; Hair: DNA obtained from hair follicles; ECA: *Equus caballus* chromosome
423 number

424

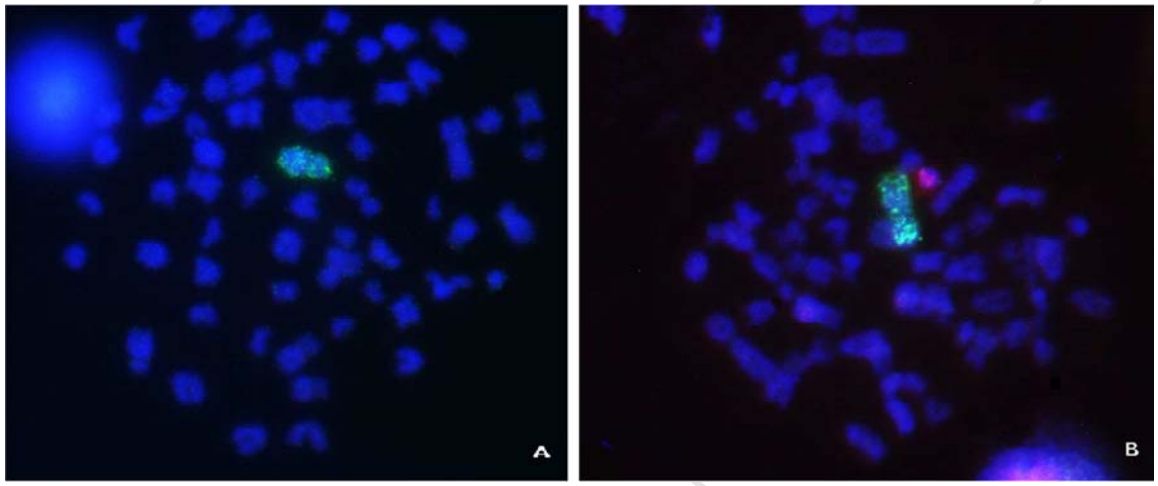
Table 2: Results of Short Tandem Repeats (STR) markers linked to the sexual chromosome pair using DNA obtained from blood and hair samples from a Spanish purebred colt.

Microsatellite loci	Size Range (bp)	Blood	Hair	ECA
LEX003	**194-214**	**210**	**210**	**X**
LEX026	**300-314**	**300 / 314**	**300**	**X**
TKY38	**105-131**	**129**	**129**	**X**
TKY270	**154-172**	**154**	**154**	**X**
UCDEQ502	**164-176**	**166 / 170**	**166**	**X**
EcaYH12	**95**	**95**	**95**	**Y**
EcaYA16	**154**	**154**	**154**	**Y**
EcaYM2	**118**	**118**	**118**	**Y**

Footnote

Results obtained from a set of 8 specific ECAY and ECAX linked STR markers (X-linked (*LEX003*, described by Coogle et al. [15] *LEX0026,* described by Coogle et al. [16]; *TKY38,* described by Hirota et al. [17], *TKY270,* described by Kakoi et al. [18]; *UCEDQ502*, described by Eggleston-Stott et al. [19] and three extra Y linked (*ECAYH12*, *ECAYA16* and *ECAYM2,* described by Wallner et al. [20]. Bp: base pair; blood: DNA obtained from blood tissue; Hair: DNA obtained from hair follicles; ECA: *Equus caballus* chromosome number

Figure 1: Fluorescent in situ hybridization of horse metaphases showing 63,X0 and 64,XY karyotypes.



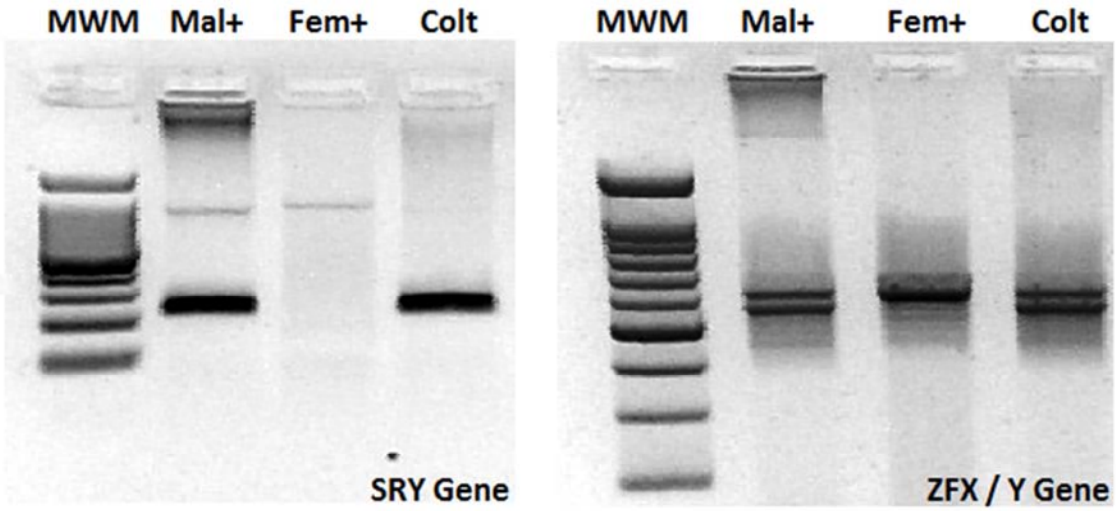
Footnote

Horse metaphase spreads hybridized using two specific *Equus caballus Y chromosome (*ECAY) and *Equus caballus X chromosome* (ECAX) fluorescent labelled probes according to Bugno et al. [10].

Fig 1A: 63,X0 metaphase showing 62 autosomal chromosomes (Blue stained) and a single large green signal from the hybridization of the ECAX.

Fig 1B: 64,XY metaphase showing 62 autosomal chromosomes (Blue stained), a single large green signal from the hybridization of the ECAX and a small red signal from the hybridization of ECAY. 1250X magnification.

454 Figure 2: Molecular analysis of the zinc finger X and Y protein (ZFX/Y) and sex-
455 determining region Y (SRY) genes amplified using blood and hair follicle DNA obtained
456 from the studied Spanish Purebred colt.



457

458

459 Footnote:

460 Electrophoretic gels from SRY (Fig 2a) and ZFX/Y (Fig 2b) genes.

461 A positive SRY single fragment (429 bp) was observed in the male control and in the
462 sample horse (Left figure). A positive ZFX fragment (604 bp) was observed in the three
463 tested animals. The ZFY fragment (553 bp) was only observed in the male control and in
464 the evaluated horse (right figure).

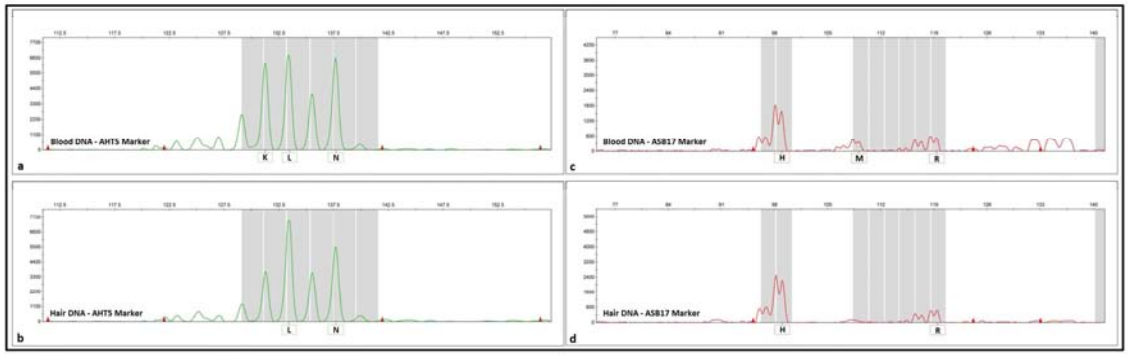
465 MWM: molecular weight marker; Mal+: male positive control; Fem+: female positive
466 control; Colt: studied colt

467

468 Figure 3:

469 Short Tandem Repeats (STR) markers from paternity test obtained from hair and blood

470 DNA samples from the studied Spanish Purebred colt..



471

472 Footnote

473 STRs electropherograms obtained from AHT5 and ASB17 markers acquired from blood

474 and hair follicle DNA. Three alleles were observed in AHT5 blood samples (K, L and N;

475 Fig 3a) and only two were observed in AHT5 hair samples (L and N; Fig. 3b). The same

476 pattern was observed in ASB17 loci: Three alleles in blood samples (H, M and R; Fig 3c)

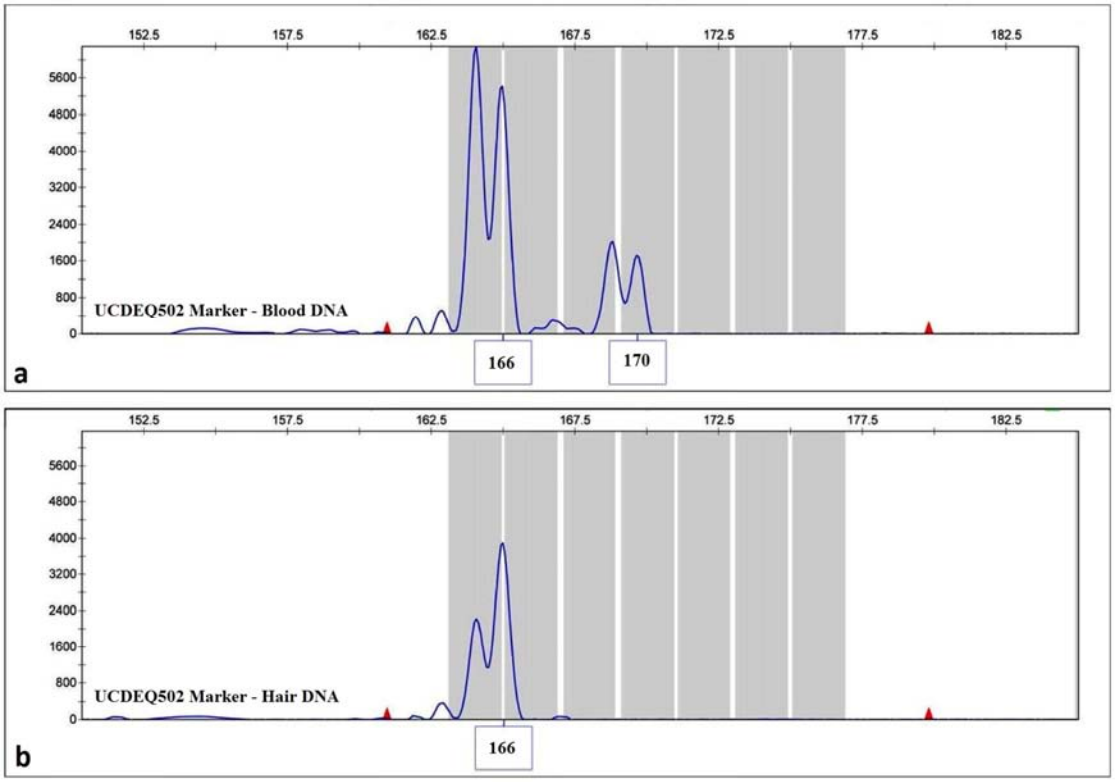
477 and only two alleles in hair samples (H and R; Fig 3d).

478

479 Figure 4:

480 Result obtained from an expanded Short Tandem Repeats (STR) panel linked to the sexual

481 chromosome pair of hair and blood DNA samples from the studied Spanish Purebred colt..



482

483

484 Footnote

485 STRs electropherograms of X-linked UCDEQ502 locus acquired from blood (a) and hair

486 (b) DNA samples. Two alleles were observed in blood DNA samples (166 and 170; Fig 4a)

487 and only one was observed in hair DNA samples (166; Fig. 4b).

488