1 # The use of molecular and cytogenetic methods as a valuable tool in
2 the detection of chromosomal abnormalities in horses: A Case of sex
3 chromosome chimerism in a Spanish Purebred colt.

4 Demyda-Peyrás S.[a*]; Membrillo A.[a]; Bugno-Poniewierska, M[b]; Pawlina K[b]; Anaya G[a];
5 and Moreno-Millán M.[a]
6 a Laboratory of Applied and Molecular Animal Cytogenetic, MERAGEM (AGR-158)
7 Research Group, Department of Genetic, Faculty of Veterinary Sciences, University of
8 Cordoba, Spain.
9 b Laboratory of Genomics, National Research Institute of Animal Production, Krakowska
10 1, 32-083, Balice, Poland.
11
12 *Corresponding author: Sebastián Demyda-Peyrás. Email: sebass@uco.es
13 Address: CN IV KM 396, Campus Rabanales CP 14071. Gregor Mendel Building,
14 Cordoba, Spain. Phone Number: +34- 957-218509
15
16 **Summary**

17 Chromosomal abnormalities associated to the sexual pair are reported as a problem more
18 common than thought in horses. Most of them remains undiagnosed due to the difficulty
19 observed in the horse karyotype and the lack of interest of breeders and veterinarians in this
20 type of diagnoses. Approximately 10 years ago, the Spanish Purebred Breeders Association
21 has implemented a DNA paternity test to evaluate the pedigree of every newborn foal. All
22 candidates who showed abnormal or uncertain results are routinely submitted to
23 cytogenetical analysis to evaluate the presence of chromosomal abnormalities. We study

the case of a foal showing three and even four different alleles in several loci in the Short Tandem Repeat (STR) based DNA parentage test. To confirm these results, filiation test was repeated using follicular hair DNA showing normal results. A complete set of conventional and molecular cytogenetic analysis were performed to determine their chromosomal complements. C banding and *in situ* fluorescent hybridization had shown that foal presents a blood sex chimerism 64,XX/64,XY with a cellular percentage of 70/30 approximately. The use of a diagnostic approach combining routine parentage QF-PCR based STR screening tested with classical or molecular cytogenetic analysis could be a powerful tool that allows early detection of foals that will have a poor or even nil reproductive performance due to chromosomal abnormalities saving time, efforts and breeders' resources.

36 **Introduction**

37 The Spanish Purebred Horse (SPB) is the most important equine breed reared in the Iberian
38 Peninsula (Gómez et al. 2009). Its importance exceeds the productive interest, being
39 recognized as a hallmark of Spanish image and traditions around the world (Valera et al.
40 2005). To prevent errors in the allocation of parentage, Spanish Purebred Breeders
41 Association has implemented a mandatory DNA paternity test prior to allowing the
42 entrance of any foal in the Association´s records. A set of 17 Short Tandem Repeat markers
43 (STR) recommended for paternity tests and individual identification by the International
44 Society for Animal Genetics (ISAG), is used to evaluate the pedigree of every newborn
45 candidate. Foals showing abnormal results (three or more alleles or two or more alleles
46 with highly unequal areas in several loci or disagreement between phenotypic and
47 genotypic sex) are routinely submitted to chromosomal analysis to evaluate the presence of
48 chromosomal abnormalities.

49 Horse karyotype was finally standardized in 1996 (Bowling et al. 1997) and it consists of
50 31 pairs of autosomal chromosomes and one pair of sexual chromosomes, which varies
51 upon the sex of an animal. However, cytogenetic evaluation in horses remains very scarce,
52 usually due to the lack of interest by owners and clinical field veterinarians in these kinds
53 of studies (Lear and Bailey 2008) or due to the difficulty to find qualified laboratories to
54 conduct this type of analysis (Moreno-Millan et al. 2012). Normally, only infertile or sub-
55 fertile high-valued mares or stallions that develop some kind of phenotypic alteration are
56 submitted to cytogenetic studies. In this sense, there is a single large-scale cytogenetic
57 screening performed to date in horses (Bugno et al. 2007). This lack of studies occurs
58 despite the fact that primary infertility in phenotypically normal mares had repeatedly been
59 associated with several sex chromosome abnormalities (Villagómez et al. 2009). Among

60 horses, both true or blood chimerism reports are very unusual (Padula 2005).This may be
61 due to twin pregnancy, mainly cause of these kind of abnormalities in mammals, is
62 diagnosed as pathological and is normally removed at an early stage of gestation in the
63 horse (Anne 2009). To our knowledge, previous cases of this condition were reported only
64 in mares (Bowling et al. 1993; Bugno et al. 2007; Moreno-Millan et al. 1991) or dizygotic
65 twins (Juras et al. 2010; Miyake et al. 1982). However, it has not be reported yet in a foal
66 born in a single birth. This could be because a high number of cases remain undiagnosed.
67 But even more if we consider that, a high percentage of the foals which have chromosomal
68 aberrations show no phenotypic or clinical signs until they reach adulthood (Kakoi et al.
69 2005). Therefore the aim of this study was: 1) to determine the karyotype of a SPB foal by
70 classical and molecular cytogenetic methods; 2) to compare chromosome analysis with
71 results of QF-PCR analysis performed on hair follicles and blood DNA and 3) to evaluate
72 the use of complementary molecular methods in the cytogenetic analyses in the horse as a
73 fast and reliable screening technique.
74
75 **Material and methods**
76 **Animal**
77 Samples from a registered Spanish Purebred foal were submitted to a routine blood DNA
78 parentage test and cytogenetical analysis. Ten ml of peripheral blood were obtained by
79 jugular venopunction using Tri-sodium ETDA Vacutainers™ for DNA isolation and
80 sodium heparin Vacutainers™, for cell culture. At the same time, 100 hair follicles were
81 individually collected for DNA isolation. The foal was declared as the result of a single
82 birth without evidence of a twin pregnancy. Internal veterinary examination was not

83 performed because the foal was too young. External revision of the animal was made
84 without any sign of phenotypic abnormalities.
85
86 **Cell Cultures and Chromosome Analysis**
87 Chromosome preparations were made from peripheral blood lymphocyte cultures using our
88 standard protocol (Rodero-Serrano et al. 2013). Briefly, 10 ml blood samples were
89 centrifuged at 800 g for 10 min. White cell interphase and 1 ml of autologous plasma were
90 inoculated into 10 ml sterile tubes with 8 ml of RPMI 1640 medium supplemented with 5
91 μgr/ml Pokeweed Lectin, 100 IU Penicillin/ml, 100 μg/ml Streptomycin and 250 ng/ml of
92 amphotericin B. Cultures were incubated at 38ºC for 72 h. One hour before harvesting, 1
93 μgr /ml colcemid was added to each tube. After that, cells were re-centrifuged, harvested
94 and incubated for 25 min in 0.075 M KCl hypotonic solution. Finally, cells were fixed in a
95 cold methanol : acetic acid (3:1) solution twice and stored for 24h at 4ºC. Chromosome
96 spreads were obtained by dropping 120μl of the cell suspension onto pre-frozen wet slides.
97 Slides were air dried for 20 min and stained for 15 min in a 10% Giemsa water solution.
98 Samples were assessed using a Reichert Polyvar microscope with 1250X magnification. A
99 total of 40 analyzable metaphases (those with intact and non-overlapping chromosomes)
100 were evaluated and counted. Sex chromosomes were assessed using C-banding, according
101 to Sumner (1972). Percentage of 64,XY and 64,XX metaphases was assessed in 248
102 metaphases.
103
104 **In situ hybridization**
105 Metaphase spreads were analyzed by fluorescent *in situ* hybridization with two whole
106 chromosome painting probes (WCPPs) specific for ECAX (*Equus caballus* chromosome

107 X) and ECAY (*Equus caballus* chromosome Y). Probes were prepared by chromosome
108 microdissection and DOP-PCR using our routine protocols and labeled for double color
109 fluorescence simultaneous hybridization (Bugno et al. 2009). The X chromosome probe
110 was DOP-labeled by biotin-16-dUTP and the Y chromosome probe by Cy3. A standard
111 FISH protocol (Pinkel et al. 1986) with some minor modifications was applied. Labeled
112 probes were denatured at 70ºC for 10 min. After RNAse and pepsin digestion, target
113 metaphase spreads were denatured in a hybridization solution containing 2xSSC and 70%
114 formamide, at 70ºC for 2.5 min. Thereafter, probes were applied onto the metaphase
115 spreads, covered, sealed with rubber cement and hybridized overnight in a dark moisture
116 chamber at 37ºC. The post-hybridization washes were as follows: twice in 50% formamide
117 in 2xSSC and twice in 1xSSC at 42°C. Signals were detected and amplified using avidin-
118 FITC, and anti-avidin antibodies. Chromosome staining was performed with DAPI. Slides
119 were analyzed under the fluorescence microscope (Axiophot, Carl Zeiss) equipped with a
120 digital CCD camera, driven by Lucia software (Laboratory Imaging LTD, Prague, Czech
121 Republic).
122
123 **DNA isolation and amplification**
124 DNA was isolated separately from blood and hair follicles using QIAamp DNA mini kit
125 (Qiagen, Carlsbad, CA) according to the manufacturer protocol. Samples were genotyped
126 for a set of 17 STR recommended for paternity tests and individual identification by the
127 International Society for Animal Genetics (ISAG). Microsatellites were amplified using
128 fluorescently-labeled primers (StockMarks® for horses, PE Applied Biosystems, Foster
129 City, CA) following the PCR conditions given by (Dimsoski 2003) in a Mastercycler® ep
130 gradient S thermal cycler (Eppendorf, Germany). Activation of the AmpliTaq Gold DNA

131 polymerase was made by a single incubation for 10 min at 95 °C. Thereafter, a total of 30
132 cycles were performed with the following step-cycle profile: denaturation at 95 ºC for 30
133 sec, followed by primer annealing at 60 ºC for 30 sec, and primer extension at 72 ºC for 60
134 sec, with a final extension of 60 min at 72 °C. The PCR products were frozen until they
135 were genotyped by capillary electrophoresis using an Applied Biosystems 3130 *xl* DNA
136 sequencer. Allele sizes were determined after processing the raw data with the software
137 package Genotyper 4.0 using a LIZ 500 bp internal size standard (Applied Biosystems).
138
139 **RESULTS**
140
141 Cytogenetic analysis.
142 Metaphases derived from blood cultures have shown two different cell types. All spreads
143 (n=40) analyzed have shown the correct chromosomal number (2n=64) of the horse
144 karyotype. Fourteen presumably male metaphases (35%) showed 13 pairs of bi-armed
145 chromosomes and a long submetacentric chromosome unpaired. Twenty-six presumably
146 female metaphases (65%) showed 14 pairs of bi-armed chromosomes. The first diagnose
147 was a sex chromosomal blood chimerism 64,XX/64,XY. To confirm the preliminary
148 diagnose, 248 metaphases were C-banded. As a result, 169 (68.12%) were diagnosed as
149 metaphases with two X chromosomes, carrying a positive C-band located in the q arm and
150 79 (31.85%) were diagnosed as metaphases with XY chromosomes with only one
151 chromosome showing the same pattern (Figure 1).
152
153 ***In situ* hybridization**

154 Results of *in situ* hybridization were in agreement with those obtained using conventional
155 cytogenetic techniques. A total of 197 hybridized metaphases were analyzed. 136 (69.03%)
156 have shown two identical X-chromosome green signals and 61 (30.96%) have shown a
157 large X chromosome green signal and a smaller Y-chromosome red signal (Figure 2).
158

159 **STR analysis.**

160 Results obtained from microsatellite analysis from blood and hair follicles DNA are shown
161 in table 1. Among the 17 loci tested in genomic DNA obtained from the blood sample four
162 different alleles were observed in one loci (*ASB17*, ECA2) (Figure 3) and three alleles were
163 observed in four loci (*AHT4,* ECA24; *AHT5*, ECA8; *ASB2*, ECA15 and *HTG4*, ECA9)**.** The
164 remaining 12 markers were normal, with 8 heterozygous and 4 homozygous loci detected.
165 The genotype observed in the DNA obtained from hair follicles has shown 17 loci with
166 normal results, with 10 homozygous and 7 heterozygous loci.
167

168 **Discussion**

169 The freemartin syndrome represents the most frequent form of intersexuality detected in
170 several livestock species (Padula 2005). However, it is an extremely rare condition in the
171 horse, probably due to two primary causes: Firstly, twin pregnancy is a pathological
172 condition in horses, representing a potentially life-threatening condition to the mare and
173 foal (Miller and Woods 1988). Secondly, the vascular anastomosis, which occurs normally
174 in other species, is not as commonly observed in horses. In the first case, twin pregnancies
175 are normally detected early during pregnancy and corrected by manual reduction (Davies
176 Morel et al. 2012), transvaginal ultrasound-guided aspiration (Govaere et al. 2008) and
177 more recently by transabdominal ultrasound-guided cardiac puncture (Sper et al. 2012).

178 Several reports have demonstrated that higher success rates are achieved when this
179 correction is performed before 36$^{th}$ day of gestation, when the embryo is fixing to the
180 endometrium (Govaere et al. 2008; MacPherson and Reimer 2000). At this stage, cellular
181 exchange between the fetuses is impossible since the fusion of chorionic foetal circulation
182 has not yet ocurred. In the second case, Bouters and Vandeplassche (1972) have
183 demonstrated studying placental circulation of 51 horse twins that only half of them has
184 shown vascular anastomoses during pregnancy. None of those cases showed abnormal
185 gonads or genital tracts. It was probably due to the fact that anasotomoses occurred after the
186 critical period of sex differentiation in the horse (Padula 2005).
187 A higher than expected percentage of subfertility cases in horses may be attributed to
188 genetically abnormal individuals (Lear and McGee 2012). However, mostly of these
189 anomalies remains undiagnosed due to cytogenetic studies are not common in this species
190 (Villagómez et al. 2009). Horse karyotype analysis using classical cytogenetic methods is a
191 time-consuming difficult labour, mainly due to the elevated chromosome number and their
192 complex morphology. To overcome this problem, the application of *in situ* hybridization
193 could provide a fast and accurate diagnosis. However, commercial FISH probes for horse
194 chromosomes are barely produced and its cost prevents that can be used for routine
195 cytogenetic analysis. Conversely, the use of STR paternity tests can be considered as a
196 valuable tool to address this issue. These tests are fewer labour-intensive diagnostic testing
197 strategies with a better cost-benefit ratio and a wide range of commercial kits available
198 (Donaghue et al. 2010). Today, they are increansingly used on a regular basis by breeder
199 associations as requirement to allow the registration of a foal in the Stud Book, due to
200 paternal filiation missassigments that usually occurred prior to their use. In this sense, the
201 appearance of an abnormal allele pattern in STRs analysis is already used as a screening

202 technique to detect trisomies (Osborne et al. 2005), chimerisms (Vodicka et al. 2004) and
203 monosomies (Edwards and Waters 2008) in humans pregnancies. In that cases, available
204 commercial sets of STR markers are distributed along the whole genome, existing specific
205 markers for all human chromosomes. In our protocol, there are three STR markers in
206 chromosome ECA15, two markers in ECA3 and ECA4 and only one marker located in 10
207 other different chromosomes. This means that there are 20 chromosomes without any
208 marker present (Table 1). Due this fact, it is possible that some cases remain undiagnosed,
209 typically if the abnormality is located on a chromosome with no microsatellites associated.
210 Most of the chromosomal abnormalities reported in horses were related to the sexual
211 chromosome pair (Villagómez et al. 2011). As a disadvantage, commercial paternity tests
212 have only one marker (LEX003) linked to ECAX, resulting in a higher chance of
213 misdiagnosis. In our results, a "normal" two allele pattern consistent with a heterozygous
214 loci was observed in LEX003 (*M* and *P* alleles) in blood samples, but only a homozygous *P*
215 allele was observed in the hair samples. It only can be explained by the coexistence of two
216 different cell lines in the same animal. Furthermore, the presence of the ECAY was not
217 detected because there is no STR marker on it in the kit we used. To overcome this issue,
218 Kakoi et al. (2005) used a complementary group of STR markers linked to the sexual
219 chromosomes (five ECAX-linked and three ECAY-linked) in horses with abnormal or
220 doubtful results in parentage test obtaining excellent results. The possibility of
221 misdiagnosis with several markers located in the target chromosome is virtually nil. QF-
222 PCR is also used to diagnose the presence of a "di-allelic" trisomy by comparing marker
223 peak area in humans (Vodicka et al. 2004). Two copies of a particular alelle will cause a
224 larger area than those in which one copy of the allele is present. This possibility was not
225 tested in horses yet probably because diagnostic kits are not extremely refined and accurate

226 as in humans. Nevertheless, ECAX monosomy (63,X0), the most common chromosomal
227 abnormality in horses (Bugno et al. 2008), still cannot be confirmed without cytogenetic
228 analysis. STR results will show a single allele in all the X-linked markers, but only
229 karyotyping allows for the definitive determination of the absent chromosome.
230 In our case, we also observed "tri-allelic" loci in four different chromosomes and a "quadri-
231 allelic" loci in ECA15 (marker *ESB17*). This combination can be only derived from two
232 heterozygous progenitors for a particular loci, that segregate two different alleles in each
233 descendant line. For this reason QF-PCR could be suggested as a definitive diagnostic tool
234 for determining cellular chimerism in horses, as previously described in humans (Donaghue
235 et al. 2010) and sheep (Martinez-Royo et al. 2009).
236
237 **Conclusion**
238 Previous studies have demonstrated that sex chromosome abnormalities are a significant
239 problem among equines (Bugno et al. 2007; Kakoi et al. 2005). Actually, more and more
240 horses with fertility disruptions are being the subject of genetic tests. We suggest that the
241 use of a diagnostic approach combining routine parentage STR tests with classical or
242 molecular cytogenetic analysis could be a powerful tool that allows the early detection of
243 foals that will have poor or even nil reproductive performance due to chromosomal
244 abnormalities.. The use of this combined strategy will save time, efforts and breeders'
245 resources.
246

247 **Acknowledgements**

248 The present study was supported by the Laboratory of Molecular and Applied Animal
249 Cytogenetic, MERAGEM (AGR-158) Research Group, University of Cordoba, Spain and
250 by the Laboratory of Genomics, National Research Institute of Animal Production, Poland.
251 We want to thank the Spanish Purebred Horse Association for the samples. Sebastián
252 Demyda Peyrás has a PhD fellowship from MAEC-AECID of Spain.

253

254

255 **references**

256

257 Anne SJ: Diagnosis and management off twinning in mares. In Practice 31:226-231 (2009).
258 Bouters R, Vandeplassche M: Twin gestation in the mare: The incidence of placental
259 vascular anastomoses and their influence on the reproductive performance of heterosexual
260 equine twins. J Reprod Fert 29:149 (1972).
261 Bowling AT, Breen M, Chowdhary BP, Hirota K, Lear T, Millon LV, De Ponce Leon FA,
262 Raudsepp T, Stranzinger G: Report of the third international committee for the
263 standardization of the domestic horse karyotype, davis, ca, USA, 1996. International system
264 for cytogenetic nomenclature of the domestic horse. Chromosome Res 5:433-443 (1997).
265 Bowling AT, Stott ML, Bickel L: Silent blood chimaerism in a mare confirmed by DNA
266 marker analysis of hair bulbs. Anim Genet 24:323-324 (1993).
267 Bugno M, Słota E, Kościelny M: Karyotype evaluation among young horse populations in
268 poland. Schweiz Arch Tierheilkd 149:227-232 (2007).
269 Bugno M, Słota E, Pieńkowska-Schelling A, Schelling C: Identification of chromosome
270 abnormalities in the horse using a panel of chromosome-specific painting probes generated
271 by microdissection. Acta Vet Hung 57:369-381 (2009).
272 Bugno M, Ząbek T, Golonka P, Pieńkowska-Schelling A, Schelling C, Słota E: A case of
273 an intersex horse with 63,x/64,xx/65,xx,del(y)(q?) karyotype. Cytogenet Genome Res
274 120:123-126 (2008).
275 Davies Morel MCG, Newcombe JR, Lauber M: Manual reduction of multiple embryos in
276 the mare: The effect on subsequent pregnancy outcome. Vet J 192:322-325 (2012).
277 Dimsoski P: Development of a 17-plex microsatellite polymerase chain reaction kit for
278 genotyping horses. Croat Med J 44:332-335 (2003).
279 Donaghue C, Mann K, Docherty Z, Mazzaschi R, Fear C, Ogilvie C: Combined qf-pcr and
280 mlpa molecular analysis of miscarriage products: An efficient and robust alternative to
281 karyotype analysis. Prenatal Diag 30:133-137 (2010).
282 Edwards S, Waters JJ: Prenatal diagnosis of monosomy 18p involving a jumping
283 translocation. Prenatal Diag 28:764-766 (2008).
284 Gómez MD, Valera M, Molina A, Gutiérrez JP, Goyache F: Assessment of inbreeding
285 depression for body measurements in spanish purebred (andalusian) horses. Liv Sci
286 122:149-155 (2009).

287 Govaere JLJ, Hoogewijs MK, De Schauwer C, Dewulf J, De Kruif A: Transvaginal
288 ultrasound-guided aspiration of unilateral twin gestation in the mare. Equine Vet J 40:521-
289 522 (2008).
290 Juras R, Raudsepp T, Das PJ, Conant E, Cothran EG: Xx/xy blood lymphocyte chimerism
291 in heterosexual dizygotic twins from an american bashkir curly horse. Case report. J Equine
292 Vet Sci 30:575-580 (2010).
293 Kakoi H, Hirota K, Gawahara H, Kurosawa M, Kuwajima M: Genetic diagnosis of sex
294 chromosome aberrations in horses based on parentage test by microsatellite DNA and
295 analysis of x- and y-linked markers. Equine Vet J 37:143-147 (2005).
296 Lear TL, Bailey E: Equine clinical cytogenetics: The past and future. Cytogenet Genome
297 Res 120:42-49 (2008).
298 Lear TL, McGee RB: Disorders of sexual development in the domestic horse, equus
299 caballus. Sex Dev 6:61-71 (2012).
300 MacPherson ML, Reimer JM: Twin reduction in the mare: Current options. Anim Reprod
301 Sci 60-61:233-244 (2000).
302 Martinez-Royo A, Dervishi E, Alabart JL, Jurado JJ, Folch J, Calvo JH: Freemartinism and
303 fecxr allele determination in replacement ewes of the rasa aragonesa sheep breed by duplex
304 pcr. Theriogenology 72:1148-1152 (2009).
305 Miller A, Woods GL: Diagnosis and correction of twin pregnancy in the mare. The
306 Veterinary clinics of North America Equine practice 4:215-220 (1988).
307 Miyake YI, Inoue T, Kanagawa H, Ishikawa T, Mogi K: Chromosomal analysis and blood
308 type examination of multiple births in equine. Jpn J Vet Res 30:11-18 (1982).
309 Moreno-Millan M, Demyda-Peyrás S, Saleno D: Sex chromosomes abnormalities in
310 purebred spanish horses with reproductive problems: Case reports. Bull Univ Ag Sci Vet
311 Med Cluj-Napoca 69:1-7 (2012).
312 Moreno-Millan M, Rodero A, Alonso FJ, Sanz A: First report of cytogenetic studies in
313 spanish breed horses. Genet Sel Evol 23:176-178 (1991).
314 Osborne EC, Trounson AO, Cram DS: A qf-pcr system to detect chromosome 13
315 aneuploidy from as few as ten cells. Am J Med Genet 134 A:33-38 (2005).
316 Padula AM: The freemartin syndrome: An update. Anim Reprod Sci 87:93-109 (2005).
317 Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity,
318 fluorescence hybridization. Proc Natl Acad Sci U S A 83:2934-2938 (1986).
319 Rodero-Serrano E, Demyda-Peyrás S, Rodero-Franganillo A, Gonzales A, Moreno-Millán
320 M: The chromosome translocation rob(1;29) in andalusian endangered cattle breeds Liv Sci
321 Under Review (2013).
322 Sper RB, Whitacre MD, Bailey CS, Schramme AJ, Orellana DG, Ast CK, Vasgaard JM:
323 Successful reduction of a monozygotic equine twin pregnancy via transabdominal
324 ultrasound-guided cardiac puncture. Equine Vet Ed 24:55-59 (2012).
325 Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell
326 Res 75:304-306 (1972).
327 Valera M, Molina A, Gutiérrez JP, Gómez J, Goyache F: Pedigree analysis in the
328 andalusian horse: Population structure, genetic variability and influence of the carthusian
329 strain. Livest Prod Sci 95:57-66 (2005).
330 Villagómez DAF, Lear TL, Chenier T, Lee S, McGee RB, Cahill J, Foster RA, Reyes E, St
331 John E, King WA: Equine disorders of sexual development in 17 mares including xx, sry-
332 negative, xy, sry-negative and xy, sry-positive genotypes. Sex Dev 5:16-25 (2011).

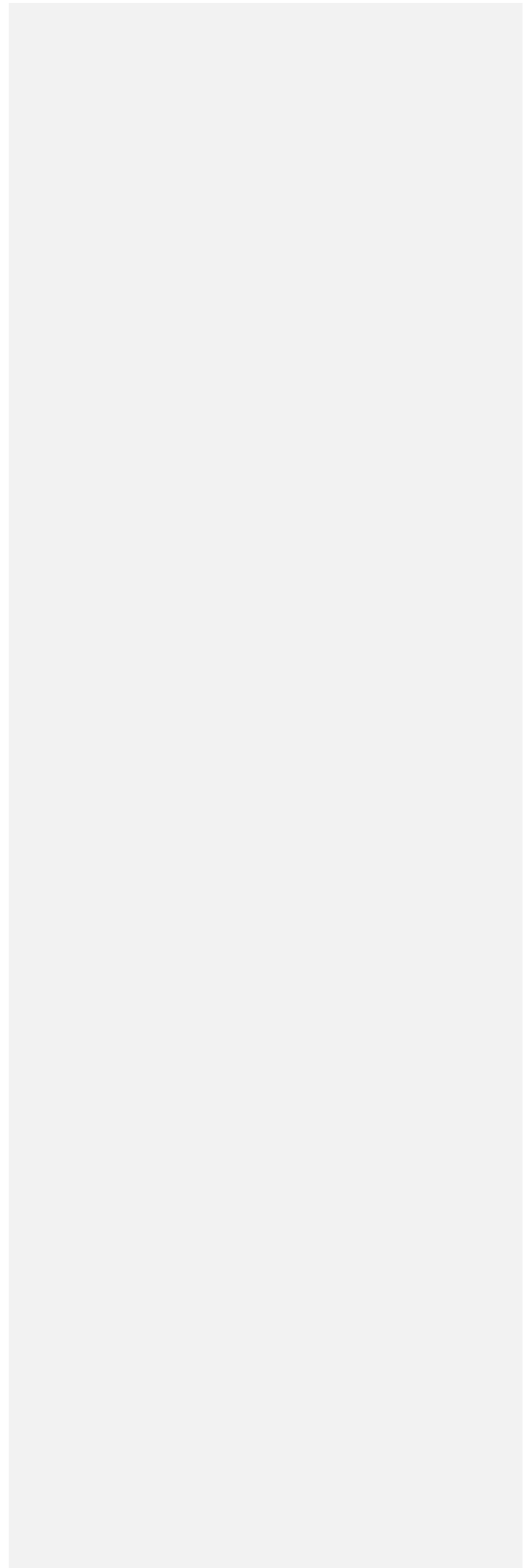
333 Villagómez DAF, Parma P, Radi O, Di Meo G, Pinton A, Iannuzzi L, King WA: Classical
334 and molecular cytogenetics of disorders of sex development in domestic animals.
335 Cytogenet Genome Res 126:110-131 (2009).
336 Vodicka R, Vrtel R, Scheinost O, Zapletalova J, Dusek L, Marie G, Santavy J: Refined
337 quantitative fluorescent pcr of y-chromosome DNA sequences mosaics in turner's
338 syndrome patients - alternative to real-time pcr. J Biochem Biophys Methods 60:151-162
339 (2004).
340
341
342

343 Table 1: Results of Sort Tandem Repeats (STR) using QF-PCR of DNA obtained from
344 blood and hair samples

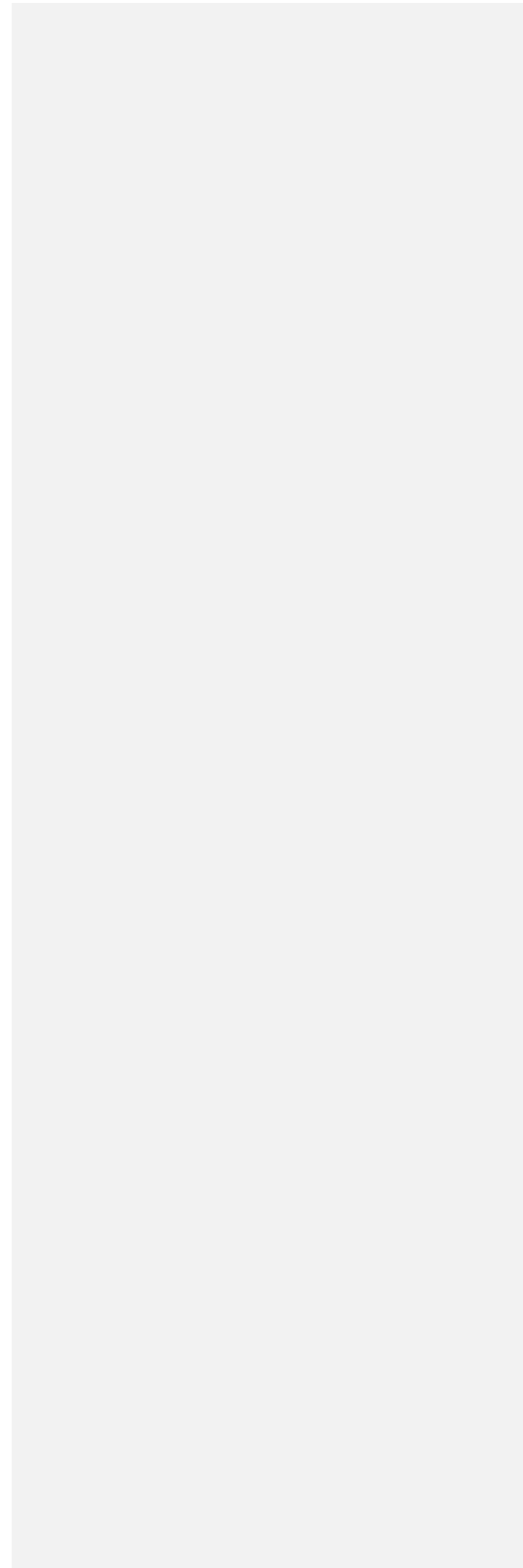
| Microsatellite loci | Size Range (bp) | Blood | Hair | ECA |
|---|---|---|---|---|
| AHT4 | 140-166 | **HJM** | **JM** | **24** |
| AHT5 | 126-147 | **KMN** | **KN** | **8** |
| ASB17 | 104-116 | **FHKM** | **HM** | **2** |
| ASB2 | 237-268 | **KMQ** | **Q** | **15** |
| ASB23 | 176-212 | **L** | **L** | **3** |
| CA425 | 224-247 | **JN** | **JN** | **28** |
| HMS1 | 166-178 | **JM** | **M** | **15** |
| HMS2 | 215-236 | **H** | **H** | **10** |
| HMS3 | 146-170 | **MP** | **MP** | **3** |
| HMS6 | 154-170 | **MO** | **MO** | **4** |
| HMS7 | 167-187 | **N** | **N** | **1** |
| HTG10 | 83-110 | **OR** | **O** | **21** |
| HTG4 | 116-137 | **KLM** | **KM** | **9** |
| HTG6 | 74-103 | **JO** | **O** | **15** |
| HTG7 | 114-128 | **NO** | **NO** | **4** |
| LEX003 | 137-160 | **MP** | **P** | **X** |
| VHL20 | 83-102 | **Q** | **Q** | **30** |

345
346

347 Figure 1: C-Banding of horse metaphases showing 64,XX and 64,XY karyotypes.
348
349
350 Footnote
351 Left metaphase showing a large ECAX with two dark blots in the q arm (long arrow) and a
352 small ECAY with a dark stain over the whole chromosome (short arrow).Left metaphase
353 showing two ECAX with the same staining pattern (long arrows). 1250X magnification.
354
355



356 Figure 2: Fluorescent *in* situ hybridization of horse metaphases showing 64,XX and 64,XY
357 karyotypes.
358
359
360 Footnote
361 A single ECAX large green signal (long arrow) and a ECAY red small signal (short arrow)
362 are observed in the left image and two ECAX large green signals (long arrows) are
363 observed in the right image. It is noteworthy the presence of a 64,XX (female) interphase
364 cell in the lower-right corner on the left image showing two green signals. 1250X
365 magnification.
366



367 Figure 3
368 Electropherograms of microsatellite markers obtained in blood DNA (upper images) and
369 hair follicles DNA (lower images). The number of alleles detected in blood DNA is 4 in
370 ASB17 marker and 3 in AHT5 and ASB2 markers. The number of alleles observed in
371 follicular hair DNA is 2 in AHT5 and ASB17 and only one in ASB2 marker.