Manuscript Details

Manuscript number	YTVJL_2018_13
Title	Diagnosis of XX/XY blood cell chimerism at low percentage in horse.
Article type	Original article

Abstract

Disorders of sexual development (DSDs) are not uncommon in horses and cause economic loss in horse breeding. Thus it is important to develop methods for unambiguous and fast identification of affected horses shortly after birth, as well those who may propagate the condition to the next generation. Genetic causes of DSDs are multivarious and still little known, thus development of diagnostic tests requires accumulating knowledge about individual cases and their aetiologies. In particular it is necessary to perform clinical, ultrasound, surgical, histological, cytogenetic and genetic analyses with close attention in all the affected individuals. This report describe the case of a XX/XY chimeric horse with reproductive apparatus abnormalities and a very low percentage of XY cell in blood highlighting that to avoid undiagnosed case of cell chimeras, above all when studying DSD cases, it is essential to perform both genetic and cytogenetic analyses possibly on more than one tissue.

Keywords	horse; chimerism; diagnosis.
Taxonomy	Animal Genetics, Animal Reproduction, Horse, Large Animal Surgery

Corresponding Author	Sara Albarella
Order of Authors	Sara Albarella, Lisa De Lorenzi, Giuseppe Catone, Gian Enrico Magi, Linda Petrucci, Cecilia Vullo, Emanuele D'Anza, pietro parma, Terje Raudsepp, Francesca Ciotola, Vincenzo Peretti

Submission Files Included in this PDF

File Name [File Type]

CoverLetter_VetJour_Albarella_Rev0.docx [Cover Letter]

Manuscript_VetJour_Albarella_Highlights_rev0.docx [Highlights]

Manuscript_VetJour_Albarella_rev0.docx [Manuscript File]

Fig1.tif [Figure]

Fig2.tif [Figure]

Fig3.tif [Figure]

Fig4.tif [Figure]

Fig5_mod.tif [Figure]

Fig6.tif [Figure]

Suppl_Fig1.tif [Figure]

Suppl_Fig2.tif [Figure]

Suppl_Fig3.tif [Figure]

SupplFig4.tif [Figure]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

Dear Editor,

this is an original paper describing the diagnostic procedure that has been necessary in an unusual case of chimerism in an horse with sexual developmental abnormalities. Interestingly this horse shows only derivatives from male genital apparatus, in opposition to the cases reported up to now in which the reproductive organs where mainly of female origin.
Another peculiarity of this case is that the profiling with microsatellites from ISAG panel on DNA from blood failed to detect both cellular clones. The need of both cytogenetic and genetic analysis for a proper/correct diagnosis in this type of horse DSD is also discussed providing useful guidelines to practising veterinarians to manage this cases.
The paper has not been submitted or published elsewhere, and has the approval of all authors.
We hope that you would consider it for publishing in The Veterinary Journal.
Best regards
Sara Albarella

Highlights

- Most of horses carrying disorders of sexual development are still identified when they have already grown-up.
- To avoid undiagnosed case of chimerism it is essential to perform both genetic and cytogenetic analyses on various tissues.
- Also in horse the proportion of XX/XY cells in the blood does not correlate with the conformation of reproductive organs.

Original article

Diagnosis of XX/XY blood cell chimerism at low percentage in horse.

S. Albarella[a,*], L. De Lorenzi[b,*], G. Catone[c,*], G.E. Magi[c], L. Petrucci[d], C. Vullo[e], E. D'Anza[a], P. Parma[b], T. Raudsepp[f], F. Ciotola[a,*,^], V. Peretti[a]

[a] *Department of Veterinary Medicine and Animal Production, University of Naples Federico II, via Federico Delpino, 1, 80137, Naples (Italy).*
[b] *Department of Agricultural and Environmental Sciences, Milan University, Milan, Italy.*
[c] *School of Biosciences and Veterinary Medicine, University of Camerino, Via Circonvallazione, 62022, Matelica (MC), Italy.*
[d] *School of Veterinary Medicine, University of Perugia, Via San Costanzo, 06126, Perugia, Italy.*
[e] *School of Pharmacy, University of Camerino, Via Madonna delle Carceri, 62032, Camerino (MC), Italy.*
[f] *College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, STX 77843, USA.*

^ **Corresponding author. Tel.: +39 081 2536433**
E-mail address: sara.albarella@unina.it (S. Albarella)

***** *These authors contributed equally to this work*

Abstract

Disorders of sexual development (DSDs) are not uncommon in horses and cause economic loss in horse breeding. Thus it is important to develop methods for unambiguous and fast identification of affected horses shortly after birth, as well those who may propagate the condition to the next generation. Genetic causes of DSDs are multivarious and still little known, thus development of diagnostic tests requires accumulating knowledge about individual cases and their aetiologies. In particular it is necessary to perform clinical, ultrasound, surgical, histological, cytogenetic and genetic analyses with close attention in all the affected individuals. This report describe the case of a XX/XY chimeric horse with reproductive apparatus abnormalities and a very low percentage of XY cell in blood highlighting that to avoid undiagnosed case of cell chimeras, above all when studying DSD cases, it is essential to perform both genetic and cytogenetic analyses possibly on more than one tissue.

Keywords: horse, chimerism, diagnosis

Introduction

Reproduction and fertility are important concerns in horse breeding and early identification of horses with congenital conditions that may lead to reproductive problems will bring a big benefit to horse industry.

Even though cytogenetic and molecular tools have been developed for this purpose, most of horses carrying disorders of sexual development (DSDs) are identified when they have already grown-up, causing economic loss to the breeders and in most cases molecular causes remain unknown (Raudsepp et al., 2010; Ciotola et al., 2012; Peer et al., 2012; Pujar and Meyers-Wallen, 2012). This is because of the limited knowledge about the molecular mechanisms regulating early development and sexual differentiation.

DSDs are among the main causes of horse subfertility or sterility. A variety of phenotypes are associated with this condition ranging from a phenotypically normal mare with gonadal dysgenesis to a horse with ambiguous external genitalia and internal male and female organs (Lear and McGee, 2012). In horses, 4 types of DSDs have been diagnosed up to now: 1) Sex chromosome abnormalities (63,X; 64,XX/64,XY; 65,XXX; 65,XXY; etc.,); 2) 64,XX *SRY*-negative with DSD; 3) 64,XY *SRY*-positive with DSD; 4) 64,XY *SRY*-negative. In horses, XX, *SRY*-positive DSD has never been reported, probably because the *SRY* gene is located far from the pseudoautosomal region and is, thus, less susceptible to meiotic errors between the sex chromosomes compared to, for example, humans where *SRY* translocation to the X chromosome can occasionally occur (Raudsepp and Chowdhary, 2016).

XX/XY chimerism is classified as a chromosome abnormality and, it has been found in the main livestock species and in humans. It is caused either by the exchange of haematopoietic stem cells through placental circulation between dizygotic twins (blood chimaerism) or by the fusion of two zygotes or embryos into a single individual at the very early stages of development (true chimaerism) (Padula, 2005; Anaya et al., 2018). Phenotypic and physiological effects due to this condition are very variable and depend on both the causes and the affected species.

From a scientific point of view a procedure able to detect chimaeras rapidly and early and to differentiate those caused by placental vascular anastomosis in a twin pregnancy rather than an early fusion of two zygotes or embryos would be very useful. In fact, the different phenotypes due to chimaerism, and mainly those XX/XY, are a useful starting point for the understanding of the mechanism of sexual differentiation in mammals, but for this purpose it is necessary to correctly identify affected animals as early as possible in their lifetime so that the development of the reproductive apparatus can be followed during all the growth phases allowing to accumulate new knowledge. Moreover it is necessary to establish the cause of the chimaerism, in twin pregnancy with placental anastomosis between the twins one of them may aborted without breeder's knowledge.

Vascular connections between placentas of heterosexual twins cause in ruminants the so called free-martin syndrome (Padula, 2005; Peretti et al., 2008) in which the female twin is sterile due to malformations of the reproductive apparatus while in equine blood chimeric heterosexual twins are both phenotypically and physiologically healthy and fertile (Juras et al., 2010; Demyda-Peyrás et al., 2014). This difference is probably due to the fact that placental

vascular connections responsible for free-martin syndrome in ruminants and other species occurs after the sexual differentiation of the equine (Demyda-Peyrás et al., 2014).

A different condition is found when chimerism is due to the fusion of two zygotes or embryos. In this last case the phenotype may be normal or ambiguous genitalia may be observed (Malan et al., 2006).

This report describes the diagnosis of the first case of a 64,XX/64,XY chimeric horse, showing a reproductive apparatus in which only male reproductive structures have been developed, with the aim to highlight the need of both cytogenetic and genetic analyses in all animals in which a correct genetic evaluation is required (clinical and DSDs cases, breeders).

Material and Methods

Case

A 15 months old Italian Saddlebred horse registered as filly was submitted to clinical evaluations due to abnormal conformation of external genitalia (Fig. 1) and stallion-like behavior. On physical examination the horse showed a small penis of 11 cm in length in the ventral perineal region without scrotum and an underdeveloped mammary gland (Fig. 1). Urination occurred through a urethral fossa at the distal end of the penis. Transrectal ultrasonography did not allow to visualize internal genitalia. Castration (closed technique) (Supplementary Fig. 1) with primary wound closure was carried out using an inguinal approach. The horse was treated with an intramuscular dose of acepromazine (0.05 mg/kg), and 20 min later was intravenous administered detomidine (20 μg/kg) and butorphanol (0.02 mg/kg) mixed

in the same syringe. Anesthesia was induced with intravenous administration of diazepam (0.05 mg/kg) and ketamine (2.2 mg/kg) intravenous administered. After orotracheal intubation, anaesthesia was maintained with isoflurane vaporised in oxygen and delivered via a large animal circle system. Two symmetrical hypoplastic testis-like structures were found in inguinal rings (Supplementary Fig. 2), removed and processed for histological and genetic evaluation. Blood samples were collected to perform cytogenetic and genetic analyses.

Histopathologic analyses

Pieces of testis like structures samples were fixed in buffered neutral formalin, embedded in paraffin, and sectioned at 3µm for histopathology and immunohistochemistry (IHC). Serial sections were stained with haematoxylin and eosin (HE). For immunohistochemical analysis, sections were mounted on Superfrost®UltraPlus slides and an avidin–biotin–peroxidase-complex (ABC) technique with diaminobenzidine as the chromogen was performed to evaluate the expression of Anti-Mullerian hormone (AMH) or Mullerian inhibiting substance (MIS) using a monoclonal antibody (clone B-11, Santa Cruz Biotechnology, USA) specific for an epitope mapping between amino acids 535-560 at the C-terminus of MIS of human origin. Appropriate negative and positive controls included samples of adult normal horse testis and sections pretreated with blocking peptide were used.

Cytogenetic Analyses

Blood lymphocytes were cultured in RPMI medium with Pokeweed for about 72h at 37.5°C. Two types of cultures, with and without 5-BrdU (20ug/ml) were setup. 5-BrdU and H33258 (40ug/ml) were added to the latter 3.5h before harvesting. Colcemid was added 1h

before harvesting to all cultures and after a hypotonic treatment with 0.075M KCl and three fixations with Carnoy's fixative cell suspensions were used to prepare slides that were allowed to dry and then stained for C- and R-banding or used for FISH-mapping. 84, 400 and 30 metaphases were examined from slides with Giemsa staining, treated for C- and R-banding techniques respectively. Karyotypes were arranged according to the Horse standard karyotype (Iannuzzi et al., 2003). Probes used for FISH experiments were as follows: horse Y-specific BAC clone 147K8 from CHORI-241 library (https://bacpacresources.org/) and horse X-specific BACs 102C09 and 111A23 from INRA library (Milenkovic et al., 2002). BACs were grown overnight at 37 °C in Luria Broth (LB) supplemented with chloramphenicol (12,5µg/ml) and BAC DNA was isolated according to standard protocols described by CHORI (http://bacpac.chori.org/). For each FISH experiment about 250-300 ng of DNA was labeled with biotin by nick translation (Roche Diagnostic kit) or Cy3 (Amersham, Little Chalfont, UK). Biotin-labeled DNA was detected by use of FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) as a green signal; direct Cy3 was detected as a red signal. The probes and the slides were codenatured on a hot plate at 75 ° C for 4 min. Hybridization was performed in a moist chamber at 37 ° C overnight. The chromosomes were identified by means of simultaneous 4′,6′-diaminido-2-phenylindole dihydrochloride (DAPI) staining. The digital images were obtained by use of a Leica DMR epifluorescence microscope (Leica Imaging Systems, Cambridge, UK) equipped with a CCD camera (Cohu, San Diego, CA), and the FITC-avidin, Cy3, and DAPI fluorescence signals were detected with specific filters. The images were recorded, pseudo-colored, and merged by use of QFISH software (Leica Imaging Systems). More over 500 metaphases and nucleus were analyzed. Finally, chromosomes were counterstained with DAPI in Vectashield mounting medium (Vector Lab) antifade solution and

more over than 500 metaphases and nucleus were analyzed using CytoVision® (Leica Biosystems) software.

Molecular Analyses

DNA was extracted from whole blood with Wizard® Genomic DNA purification kit (Promega), and from the testis-like structures with Genelute mammalian Genomic DNA Extraction kit, (Sigma). The DNA extracted from blood was tested by qualitative PCR using primers specific for *SRY*, *ZFY/ZFX* and *EIF* (Table 1). Being all the primers specific for Y regions seems to work less in the investigated horse than in normal male control, PCRs with different number of amplification cycles (from 25 to 35) was performed using the primers SRYQ and HPRT (as control) (see Table 1 for sequences). PCR was performed as recommended by the Taq enzyme supplier (AmlpliTaq Promega) using as start material DNA obtained from blood. The same primers were used to perform a Q-RT-PCR with SYBR®Green (Invitrogen 11733-038) on DNA extracted from blood and from the testis-like structures to evaluate the percentage of XY cells in the case and in a normal, fertile control stallion. The same DNA (from blood and testis-like tissue) was used for genotyping on a panel of 17 microsatellites according to International Society of Animal Genetics (ISAG) guidelines at the laboratory UnireLab srl to establish if the horse was a chimera or a mosaic.

Results

Histopathologic analyses

Both of the testes were composed of low number of small and hypocellular seminiferous tubules that lacked germ cells and spermatozoa and were lined by Sertoli cells, often with frothy,

vacuolated apical cytoplasm (Fig. 2a). Sertoli cells extended from the undulating basement membrane and protruded into the lumen. The interstitial tissue, separating the tubules, was apparently increased due to the reduced number of tubules and was composed by well-developed fibrovascular stroma with embedded many plump oval fibroblast, various macrophages containing abundant, globular, intracytoplasmic, goldenbrown pigment (lipochrome) and few interstitial cells that had small round nuclei and eosinophilic, foamy cytoplasm. The histological findings observed were consistent with severe testicular hypoplasia and Leydig cell atrophy. Sertoli cells showed a diffuse and intense cytoplasmic immunolabeling for AMH (Fig. 2b).

Cytogenetic findings and FISH analyses

The analysis of 84 routinely Giemsa stained karyotypes (without banding), showed only one male (XY) metaphase (1.19%) (Fig. 3). The analysis of 400 C-banded metaphases revealed only one XY metaphase (0.25%) (Fig. 4a) while no XY cells were detected among R-banded metaphases. Karyotyping of an R-banded XX metaphase did not show abnormalities (Fig. 4b), however no information was obtained about the presence or absence of chromosome aberrations for R-banded XY cells. The presence of both the XX and XY cells in blood lymphocytes was further confirmed by FISH with horse Y-specific BAC 147K08 and X specific-BACs 102C09 and 111A23. Analysis of 450 interphase nuclei identified only 4 XY cells (0.8%), while no XY metaphases were observed in this analysis. (Fig. 5)

Molecular analyses

Analysis by PCR with Y-specific markers confirmed the presence of the Y chromosome, though at a low percentage in the case compared to a normal male control. Figure 6 illustrates

qRT-PCR results for the SRY gene, which amplification was analyzed at different cycles. In the case SRY amplification product becomes visible only at cycle 33, clearly indicating the low content of this gene in the case compared to the male control. Q-RT-PCR also allowed to quantify the amount of XY cells in the case. The Ct values for the *SRY* were: 29.15 and 23.28 for the case and the control male respectively, whereas the respective Ct values for the autosomal *HPRT* gene were 21.91 and 23.22. Using the delta-delta Ct method, we calculated the percent of XY cells at 0.68%. These results confirm the low level of blood XX/XY chimerism. The amplification profiles are shown in the Supplementary Fig. 3. The same analyses were performed on testis derived DNA and revealed 13% of XY cells thus almost 20 times more than that observed in blood.

Microsatellite genotyping in blood DNA showed the presence of one or two alleles per each marker. However, the same analysis in testis-derived revealed the presence of three alleles for the microsatellites ASB2, ASB23, CA425, HMS23, HMS6, HMS7, LEX003 indicating that the horse was a chimera, likely originating from the fusion of two zygotes or embryos (see Supplementary Fig. 4).

Discussion

Reproductive apparatus abnormalities observed in a 15 month old horse led to deepen the clinical case by performing clinical, ultrasound, surgical, histological, cytogenetic and genetic analyses with close attention.

Anatomical and histopathological findings of this horse indicate that during embryo development the pathway of formation of the male genital apparatus has been correctly activated. This has led to testes formation and to their migration in inguinal canals. However the genital tubercle has developed in the direction of male external genitalia without reaching a complete and proper conformation. The observed diffuse expression of AMH within Sertoli cells is similar to a previous study where a positive immunostaining of AMH was found in intersex gonad and cryptorchid testis (Ball et al., 2008) and comply with the absence of Mullerian derivatives. This can be due to post-zygotic fusion of two distinct embryos rather than an early anastomosis between the vascular systems of twins (one of which has then be reabsorbed). In this latter case, in fact, typically no abnormalities of the reproductive organs are observed in either twins because when vascular anastomosis are formed sexual differentiation is already undergone (Vanderplassche et al., 1970; Juras et al., 2010; Lear and McGee, 2012). Conversely and in contrast with previously reported cases (Dunn et al., 1981; Moreno-Millan et al., 1991; Bugno et al., 2007), the present case shows no derivatives from female reproductive organs while male organs are almost completely developed.

This phenotype may be due to the prevalence of XX cells over XY cells during critical stages of sex determination and sexual differentiation, so that even though the Y chromosome initiates the SRY-pathway, the low amount of XY cells gene products may not be sufficient for proper and complete male development. On the other hand, the percentage of different cellular clones found in the blood of an animal does not allow to trace back the growth trend of all different cellular clones during embryo development. FISH experiments on metaphases and interphase chromosome confirmed the chimeric condition at a very low level. To our

knowledge, this is the first case of a chimeric horse where such a low percentage of XY cells in the blood (0,68%) is associated with the total absence of female structures. Genome wide microsatellites genotyping performed on DNA from blood failed to reveal the presence of two cellular clones due to the low percentage of XY cells. Instead the same analysis performed on DNA from gonadal tissue revealed the presence of more than 2 alleles for some markers suggesting that this 64,XX/64,XY horse is a chimera likely derived from post zygotic fusion of two distinct embryos (tetragametic chimera) (Malan et al., 2006). This finding shows that when microsatellites genotyping is performed alone in a tissue with a very low percentage (<1%) of a particular cell clone chimerism may remain undiagnosed and eventually discovered only when the affected animal is old enough to show reproductive problems. Routinely Giemsa stained karyotypes (without banding) and CBA tecniques seem to be more sensitives, thus indicating the need to always carry out them in a correct genetic evaluation of a livestock animal or of a clinical case. Moreover early identification of individuals with cell chimerism will allow the improvement of the knowledge about reproductive organs development in particular of molecular mechanism underlying this biological event.

Conclusion

XX/XY chimerism has been rarely diagnosed in horses with only a few cases reported to date (Moreno-Millan et al., 1991; Bugno et al., 1999; Bugno et al., 2007; Juras et al., 2010; Demyda-Peyras et al., 2013). This is probably because twin pregnancy (the main origin of chimeras) causes serious economic loss as a result of a high rate of abortion a tendency for poor postnatal development in the few foals that survive to term thus it is an unwanted condition normally terminated once detected (Juras et al., 2010; Anaya et al., 2017). However, large scale

DNA profiling or cytogenetic survey of horse populations (Bugno et al., 2007; Demyda-Peyras et al., 2013) suggests that the available clinical data underestimate the actual prevalence of these cases. Interestingly to date only a few XX/XY chimeras (Dunn et al., 1981; Moreno-Millan et al., 1991; Bugno et al., 2007) show malformation of genital apparatus, though for all these cases no data were available for the other twin thus the possibility of an early embryonic fusion cannot be excluded. Furthermore, in all the reported cases the proportion of XY cells in the blood was noticeable (>10%). Thus the case described in this study is the first one in which complete regression of Mullerian ducts in favor of the development of male reproductive apparatus is associated with a very low percentage (<1%) of XY cells in the blood. This is in line with the observation in other species (Malan et al., 2006; Peretti et al., 2008) where the proportion of XX/XY cells in the blood does not correlate with the conformation of reproductive organs. It is noteworthy that in this clinical case SRY PCR positivity with 64,XX normal karyotype led to deepen Giemsa stained (without banding) karyotyping and C-banding test allowing to diagnose the chimaerism. Microsatellite genotyping on DNA from tissue allowed to classify the case as a tetragametic chimaera.

Conflict of interest statement

None of the authors has any conflict of interest to state.

Acknowledgments

We wish to thank Dr. Borriello G. and Dr. Riccardi M.G. (Istituto Zooprofilattico Sperimentale del Mezzogiorno); Prof. Chiofalo V. and Dr. Cosenza M. (UnireLab s.r.l.) for their technical support.

Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: …

References

Anaya, G., Fernández, M.E., Valera, M., Molina, A., Azcona, F., Azor, P., Solé, M., Moreno-Millán, M., Demyda-Peyrás, S., 2018. Prevalence of twin foaling and blood chimaerism in purebred Spanish horses. The Veterinary Journal 234, 142-144.

Anaya, G., Molina, A., Valera, M., Moreno-Millán, M., Azor, P., Peral-García, P., Demyda-Peyrás, S., 2017. Sex chromosomal abnormalities associated with equineinfertility: validation of a simple molecular screening tool in the PurebredSpanish Horse. Animal Genetics 48(4), 412-419.

Ball, B.A., Conley, A.J., Grundy, S.A., Sabeur, K., Liu, I.K., 2008 Expression of anti-Müllerian hormone (AMH) in the equine testis. Theriogenology 69(5), 624-31.

Bugno, M., Slota, E., Koscielny, M., 2007. Karyotype evaluation among young horse populations in Poland. Schweiz Arch Tierheilk 149, 227–232.

Bugno, M., Slota, E., Tischner, M., Kozubska-Sobocinska, A., 1999. A case of 64, XX/64, XY leucocytic chimerism in a fertile mare of the Wielkopolska breed. Annals of Animal Sciences 26, 9–16

Ciotola, F., Albarella, S., Pasolini, M.P., Auletta, L., Esposito, L., Iannuzzi, L., Peretti, V., 2012. Molecular and cytogenetic studies in a case of XX SRY-negative sex reversal in an Arabian horse. Sexual Development 6(1-3), 104-7.

Demyda-Peyrás, S., Anaya, G., Bugno-Poniewierska, M., Pawlina, K., Membrillo, A., Valera, M., Moreno-Millán, M., 2014. The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: a rare case with an abnormal cellular chimerism. Theriogenology 81(8), 16-25.

Demyda-Peyras, S., Membrillo, A., Bugno-Poniewierska, M., Pawlina, K., Anaya, G., Moreno-Millán, M., 2013. The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: a case of sex chromosome chimerism in a Spanish purebred colt. Cytogenetic and Genome Research 141, 277–83.

Dunn, H.O., Smiley, D., Duncan, J.R., McEntee, K., 1981. Two equine true hermaphrodites with 64,XX/64,XY and 63,XO/64,XY chimerism. The Cornell Veterinarian 71(2), 123-35.

Han, S.H., Yang, B.C., Ko, M.S., Oh, H.S., Lee, SS., 2010. Length difference between equine ZFX and ZFY genes and its application for molecular sex determination. Journal of Assisted Reproduction and Genetics 27(12), 725-8.

Iannuzzi, L., Di Meo, G.P., Perucatti, A., Incarnato, D., Peretti, V., Ciotola, F., Barbieri, V., 2003. An improved characterizazion of horse (Equus caballus, 2n=64) chromosomes by using replicating G and R banding patterns. Caryologia 56, 201-207.

Juras, R., Raudsepp, T., Das, P.J., Conant, E. and Cothran, E.G., 2010. XX/XY blood Lymphocyte chimerism in heterosexual dizygotic twins from an American Bashkir Curly Horse. Case Report. Journal of Equine Veterinary Science 30, 575-80.

Lear, T.L., and McGee, R.B., 2012. Disorders of sexual development in the domestic horse, Equus caballus. Sexual Development 6, 61–71.

377 Malan, V., Vekemans, M., Turleau, C., 2006. Chimera and other fertilization errors. Clinical
378 Genetics 70(5), 363-73.

379 Milenkovic, D., Oustry-Vaiman, A., Lear, T.L., Billault, A., Mariat, D., Piumi, F., Schibler, L.,
380 Cribiu, E., Guerin, G., 2002. Cytogenetic localization of 136 genes in the horse:
381 Comparative mapping with the human genome. Mammalian Genome 13, 524–34.

382 Moreno-Millan, M., Rodero, A., Alonso, F.J., Sanz, A., 1991. First report of cytogenetic studies
383 in Spanish breed horses. Genetics Selection Evolution 23, S176–S178.

384 Padula, AM., 2005. The freemartin syndrome: an update. Animal Reproduction Science 87(1-2),
385 93-109.

386 Paria, N., Raudsepp, T., Pearks Wilkerson, A.J., O'Brien, P.C., Ferguson-Smith, M.A., Love,
387 C.C., Arnold, C., Rakestraw, P., Murphy, W.J., Chowdhary, B.P., 2011. A gene catalogue
388 of the euchromatic male-specific region of the horse Y chromosome: comparison with
389 human and other mammals. PLoS One 6(7),e21374.

390 Peer, M., Neuhauser, S., Klaus, C., Kuiper, H., Gruber, A.D., Distl, O., Lischer, C., Handler, J.,
391 2012. Laparoscopic gonadectomy in two intersex Warmblood horses. Journal of Equine
392 Veterinary Science 32, 117-122.

393 Peretti, V., Ciotola, F., Albarella, S., Paciello, O., Dario, C., Barbieri, V., Iannuzzi, L., 2008.
394 XX/XY chimerism in cattle: clinical and cytogenetic studies. Sexual Development
395 2(1),24-30.

396 Pujar, S., Meyers-Wallen, V.N., 2012. Sequence variations in equine candidate genes For XX
397 and XY inherited disorders of sexual development. Reproduction in Domestic Animals
398 47(5), 827-34.

399 Raudsepp, T., Chowdhary, B.P., 2016. Chromosome Aberrations and Fertility Disorders in
400 Domestic Animals. Annual Review of Animal Biosciences 4, 15-43.

401 Vandeplassche, M., Podliachouk, L., Beaud, R., 1970. Some aspects of twin-gestation in the
402 mare. Canadian Journal of Comparative Medicine 34, 218–226.

403

404

405

406

407

408

409

Table 1: Primers sequences, Annealing temperatures and, product lengths of the examined genes

Gene	Primer name	Primer sequence	annealing	length
SRY[Han et al., 2010]	SRY-F	TGC TAT GTC CAG AGT ATC CAA CA	58	697bp
	SRY-R	TGA GAA AGT CCG GAG GGT AA		
ZFX/Y [Han et al., 2010]	ZFX/Y-F	AAA TCA AAA CCT TCA TGC CAA T	58	Y 553bp; X 604bp
	ZFX/Y-R	TTC CGG TTT TCA ATT CCA TC		
EIF2s3Y[Paria et al., 2011]	EIF2s3Y_F	GAGCCATCTGTGTGATCGTC	58	223
	EIF2s3Y_R	TATTCCTGGCCCTAAGCACA		
ZFY [Paria et al., 2011]	ZFY_F	TGAGCTATGCTGACAAAAGGTG	58	186
	ZFY_R	TCTTTCCCTTGTCTTGCTTGA		
SRYQ	SRYQ-F	ACAGTCACAAACGGGAGGAG	58	149
	SRYQ-R	AAAGGGAACGTCTGCGTATG		
HPRT	HPRT-F	GAGGCCATCACATTGTAGCA	58	381
	HPRT-R	TCCCCACAGCAATTCTTACA		

Figure Legend

Fig. 1: a) 15 month-old Italian Saddlebred horse with DSD. b) Perineal region of the horse. A = anus; R = raphe, U = urethral opening. c) Inguinal region of the horse in dorsal recumbancy showing the penis (P) and, d) two well developed teats and, the subcutaneous position of the testes.

Fig. 2: (a) Section of the hypoplastic testicles showing small seminiferous tubules lined by a single layer of Sertoli cells (H.E. x10) **(b)** Immunohistochemical stain showing diffuse intense AMH expression of Sertoli cells within seminiferous tubules (IHC, counterstaining with haematoxylin, x 20).

Fig. 3: A male metaphase and the corresponding karyogram of the Italian Saddlebred filly.

Fig. 4: C-banded metaphase plate with 2n=64;XY (a) and, R-banded karyotype with 2n=64;XX (b) of the Italian Sddlebred horse with ambigous genitalia.

Fig. 5: FISH experiments on nuclei and metaphases of the filly. a-b) XY and XX nuclei as revealed by FISH with Y-specific BAC 147K08 (red signal) and X-specific BAC 111A23 (green signal). c) XX metaphase showing signals by X-specific BAC 102C09 (red signal). d) XY nucleus showing signals by Y-BAC 147K08 (green signal) and X-BAC 102C09 (red signal).

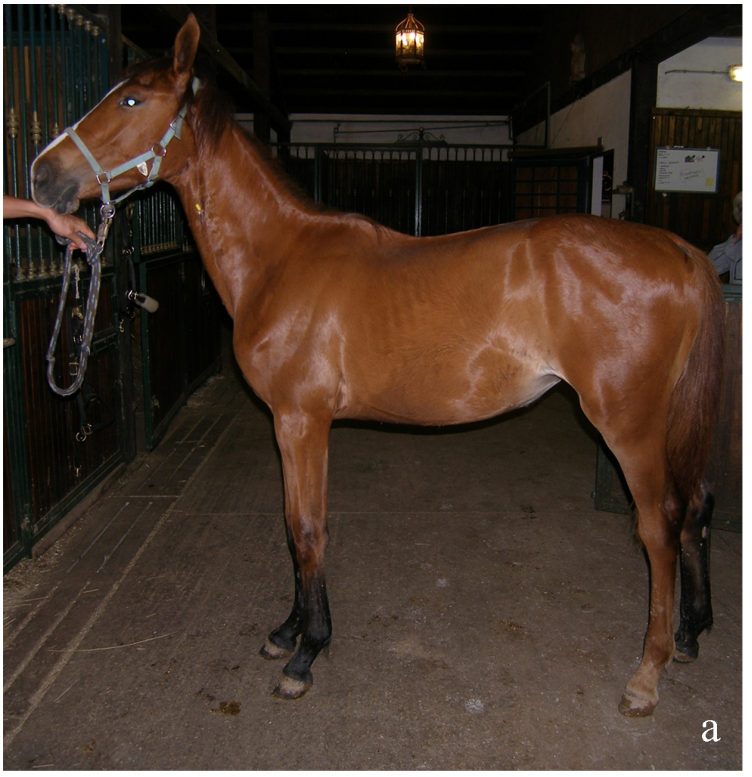
Fig. 6: PCR amplification of a portion of the *SRY* and *HPRT* genes at different cycles. M= 100 bp marker; XY= normal male; Ho: DSD Horse; XX= normal female; H_2O= water. The number reported the amplification cycles performed.

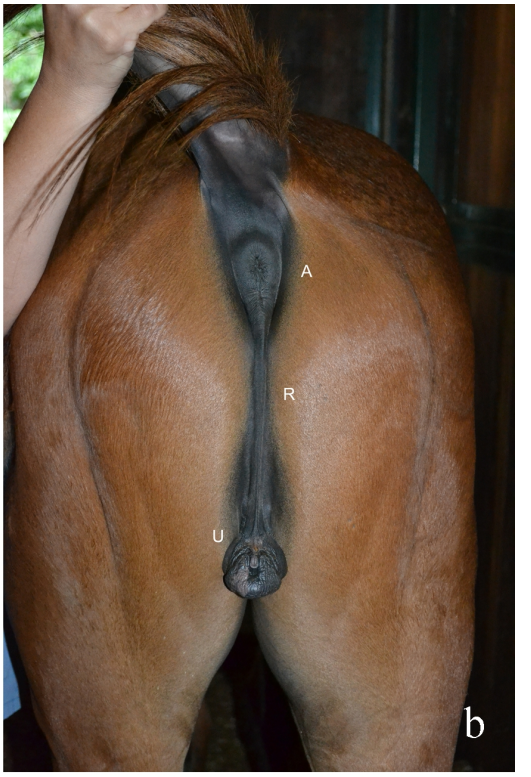
Supplementary Fig. 1: Castration (closed technique) with primary wound closure was carried out using a inguinal approach.

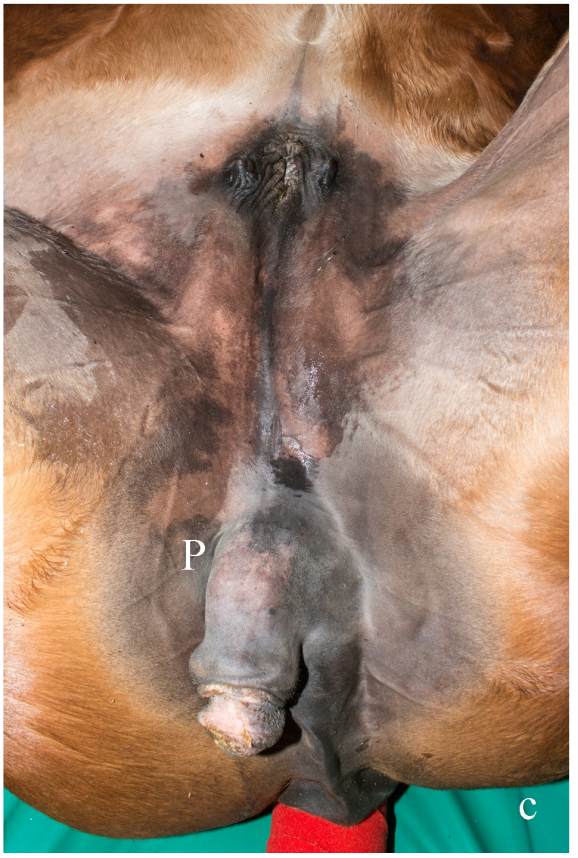
Supplementary Fig. 2: Abnormal hypoplastic testes found in the horse.

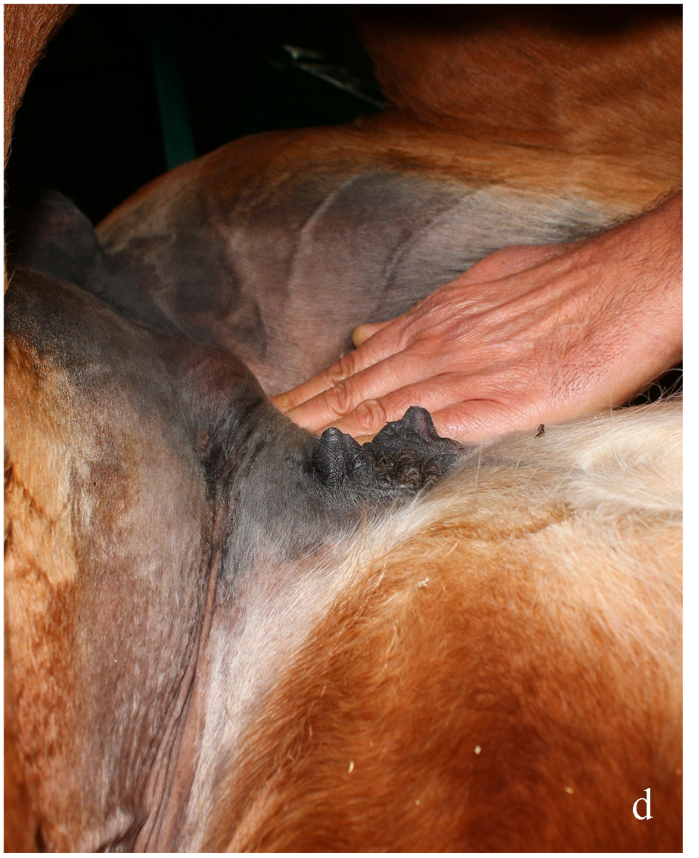
Supplementary Fig. 3: Amplification plot of the Q-RT-PCR. XX/XY= DSD Horse; XY= Normal control male.

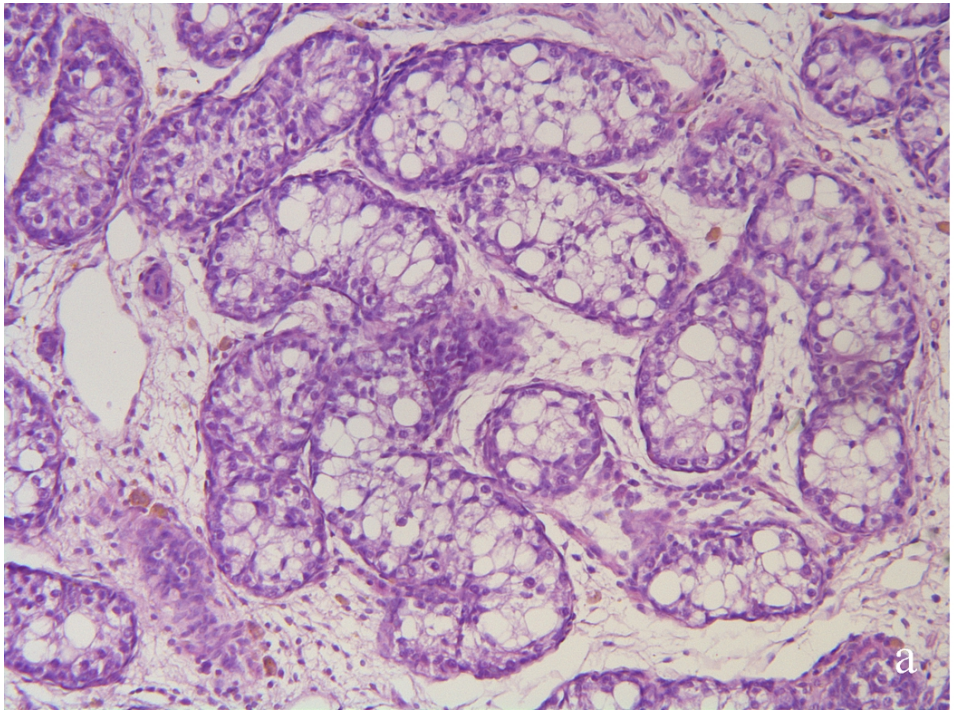
Supplementary Fig. 4: Microsatellite electropherograms obtained from LEX003 markers acquired from blood (a) and testis derived tissue (b) DNA of the analysed horse.

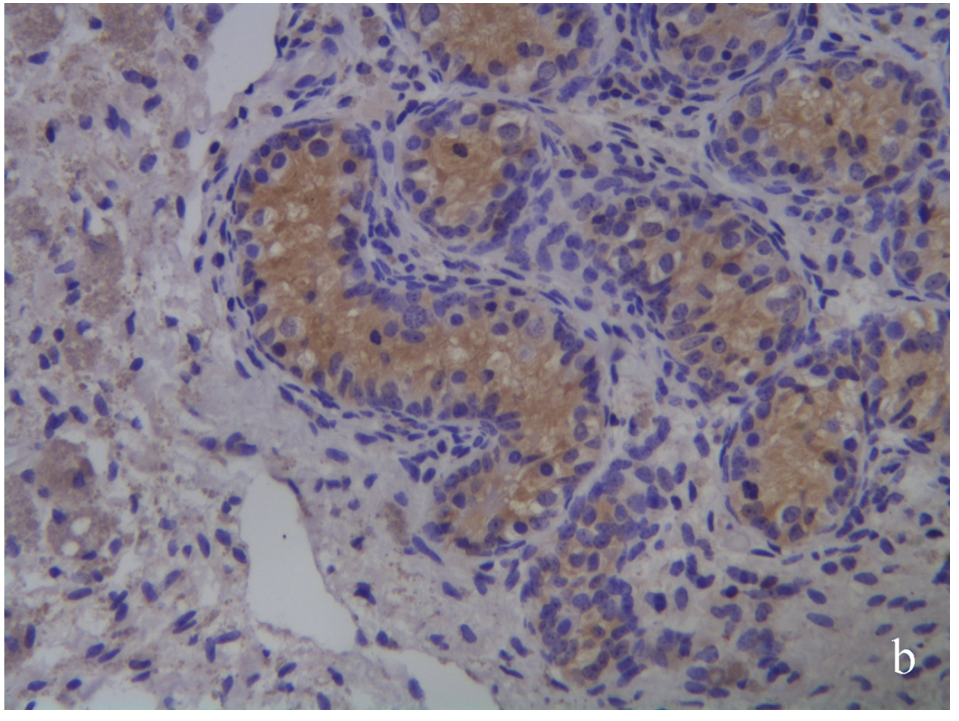

a

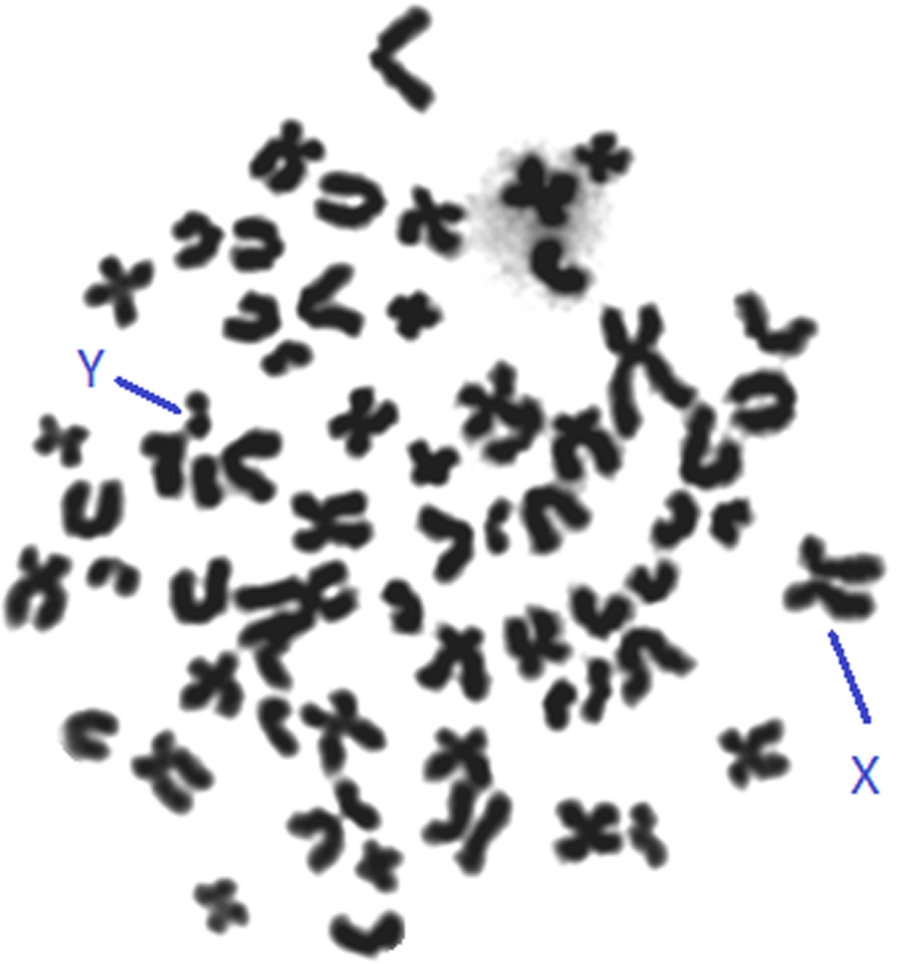

A
R
U
b

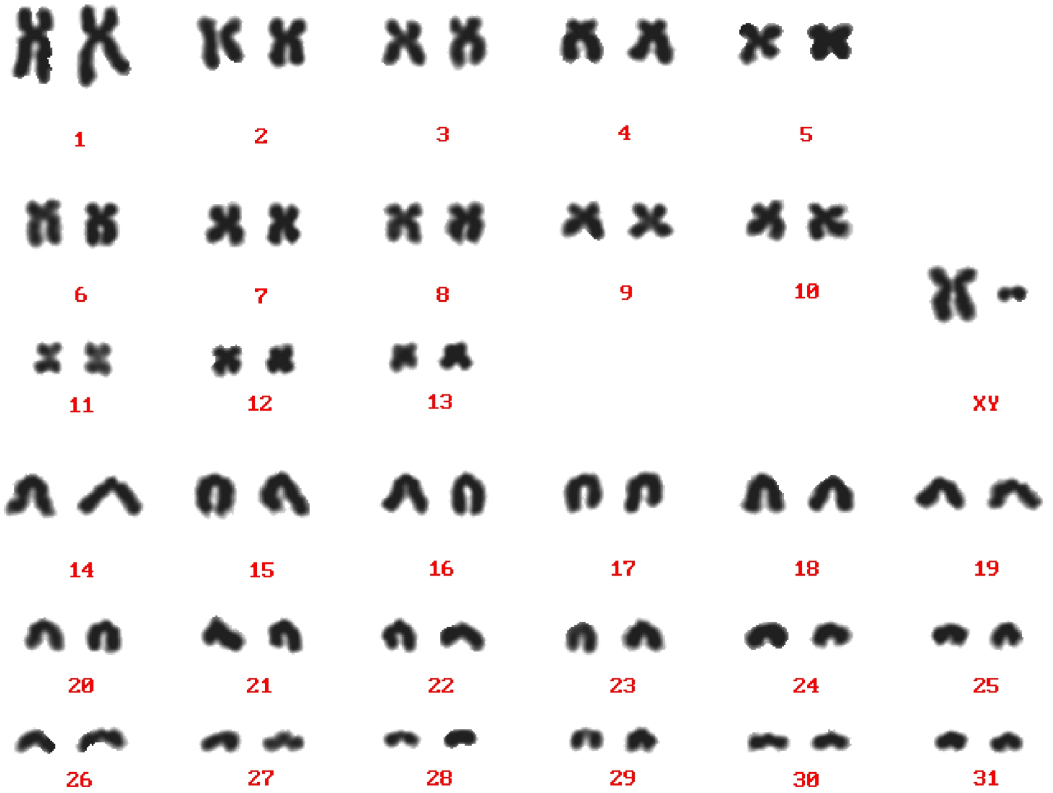

P
c

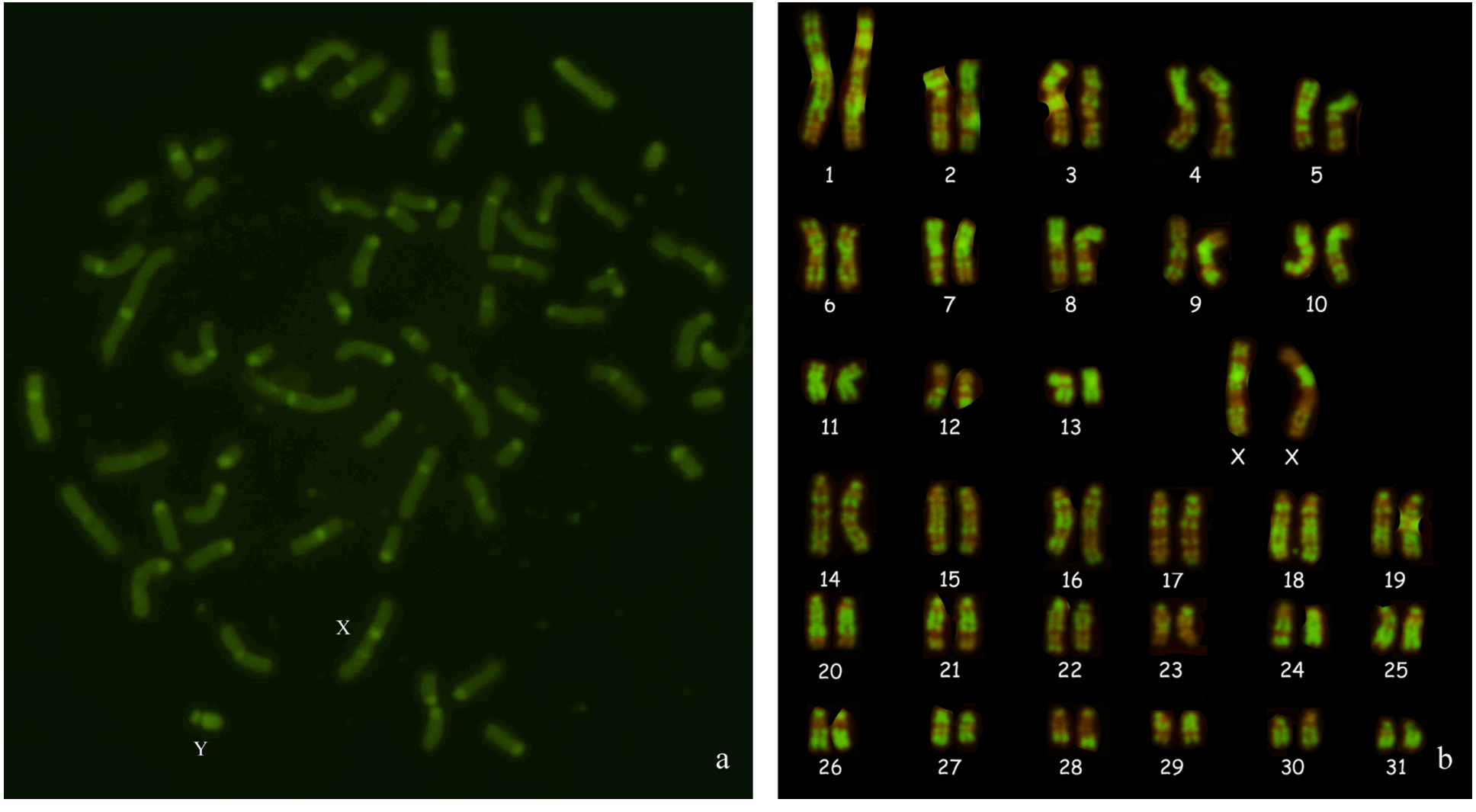

d

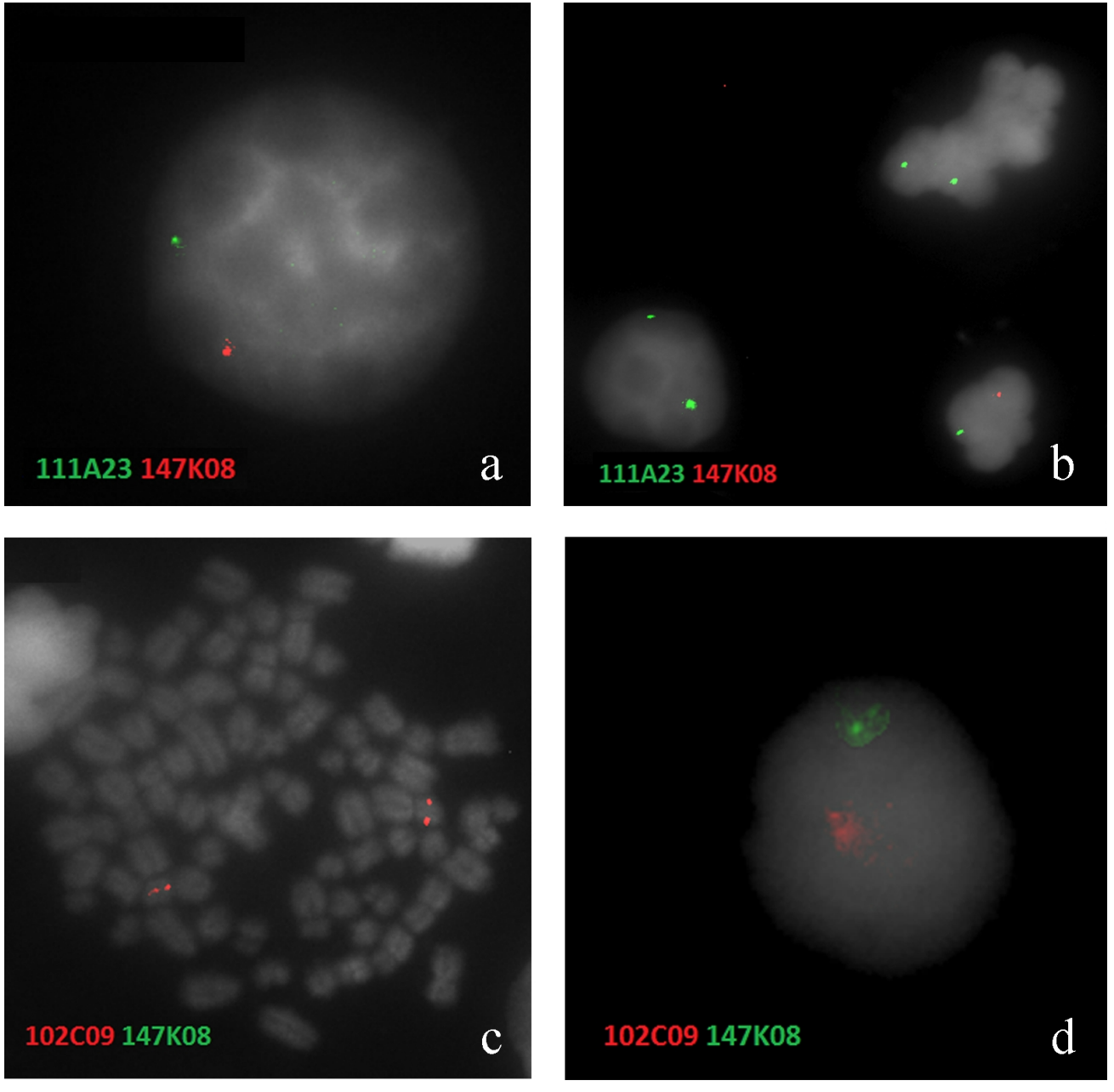

a

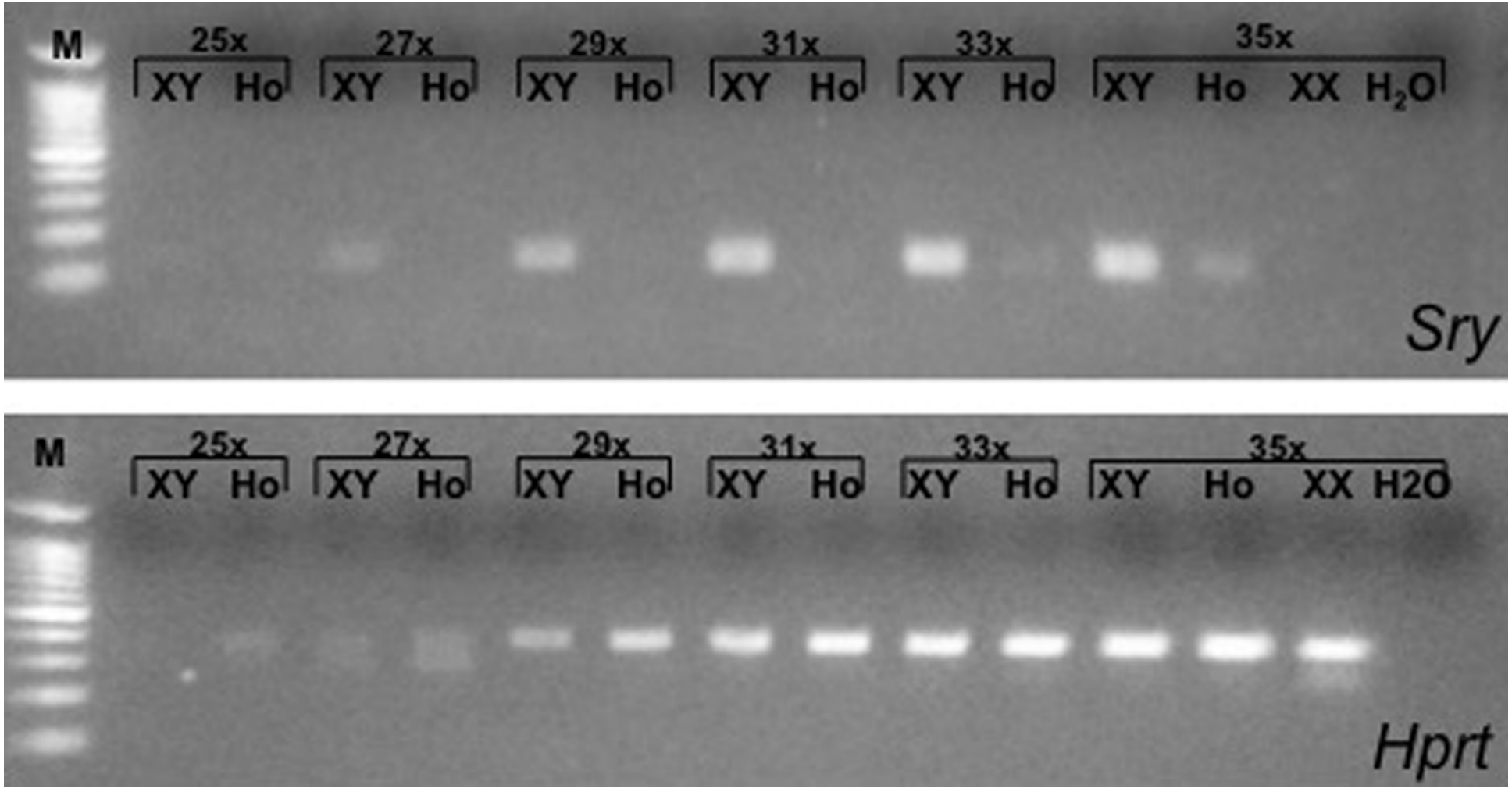

b

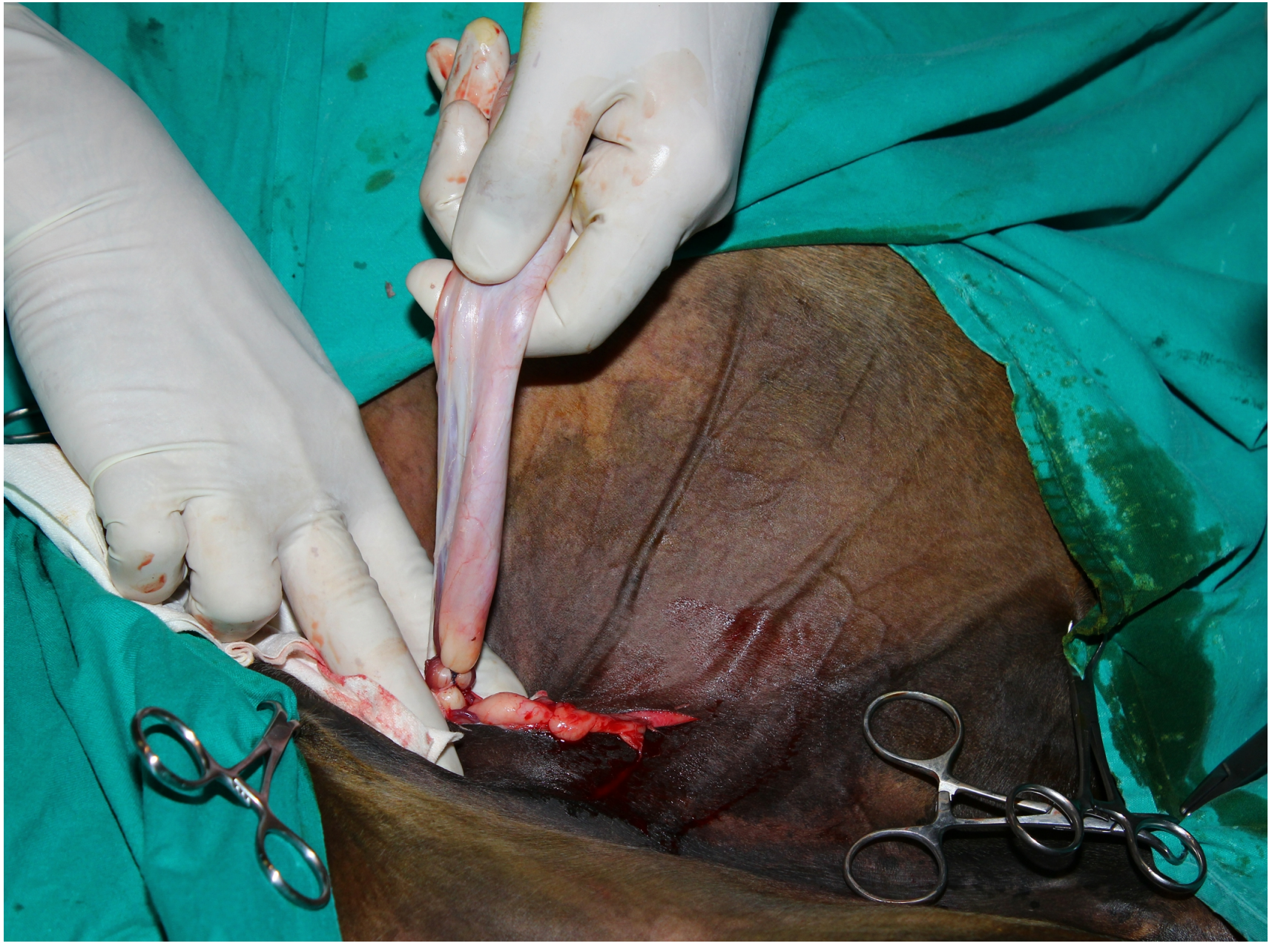

Y
X

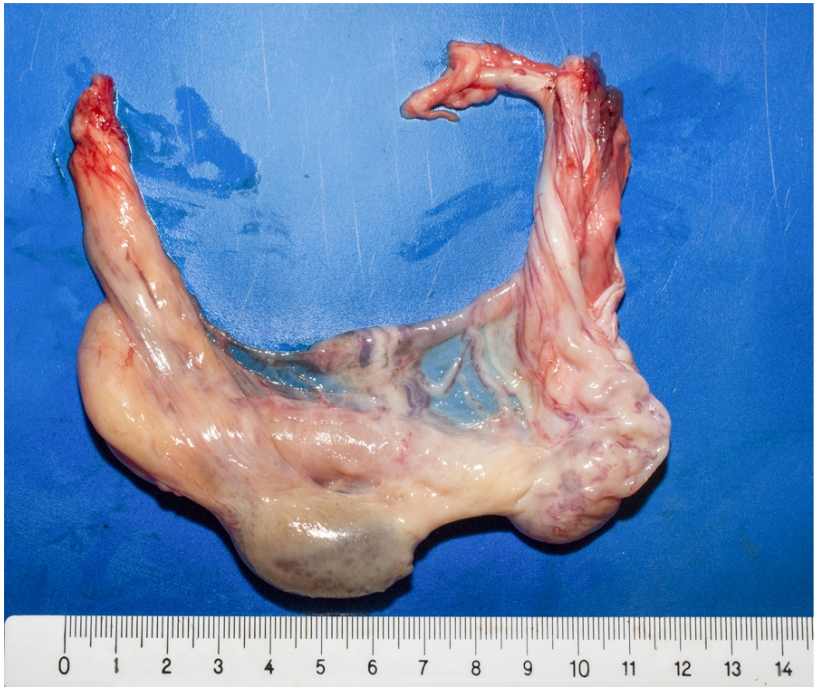

1
2
3
4
5
6
7
8
9
10
11
12
13
XY
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31

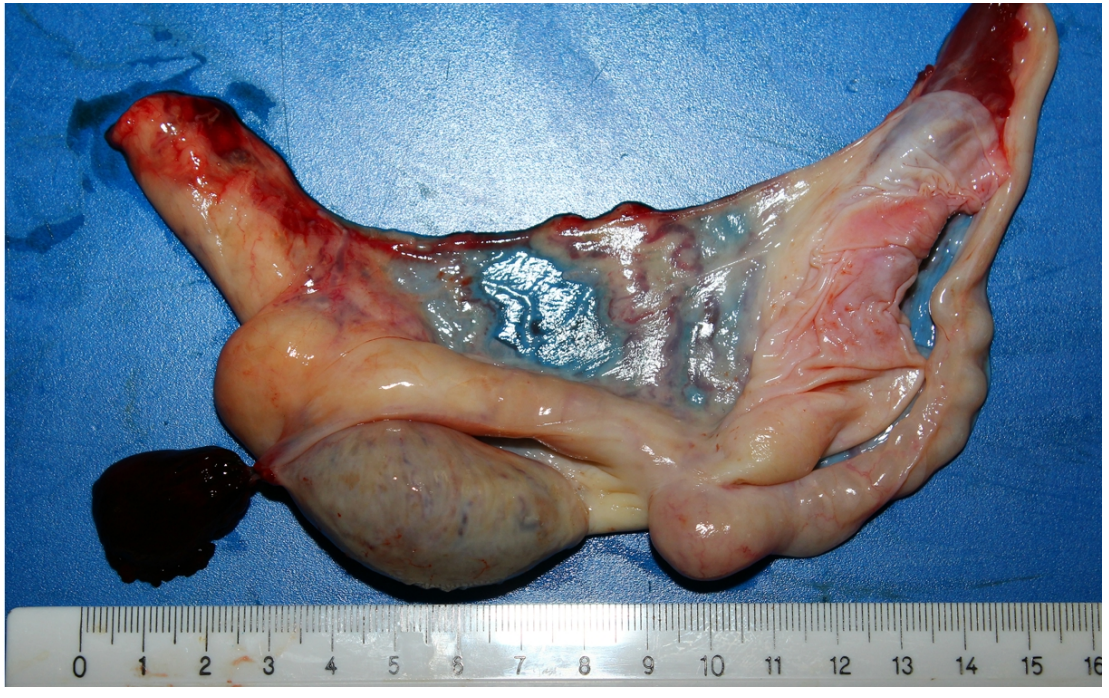

X
Y
a
1
2
3
4
5
6
7
8
9
10
11
12
13
X
X
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
b

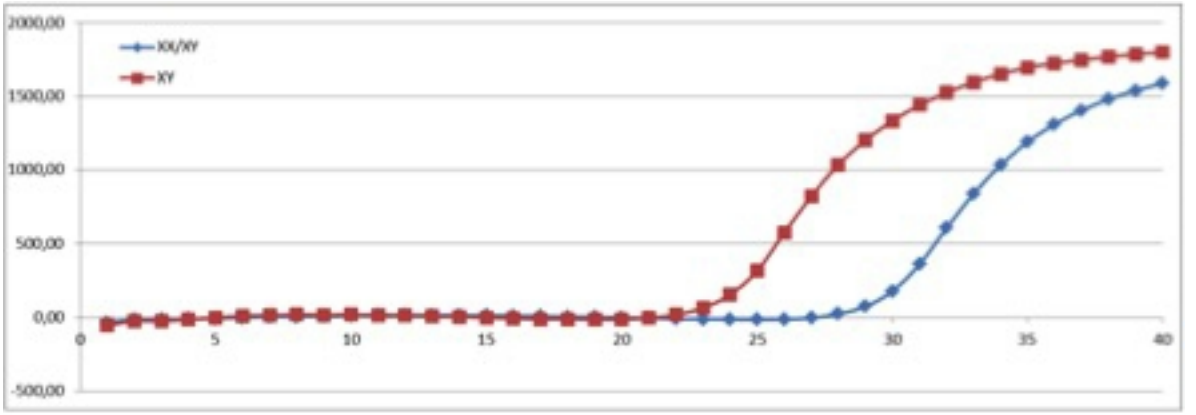

111A23 147K08
a
111A23 147K08
b
102C09 147K08
c
102C09 147K08
d

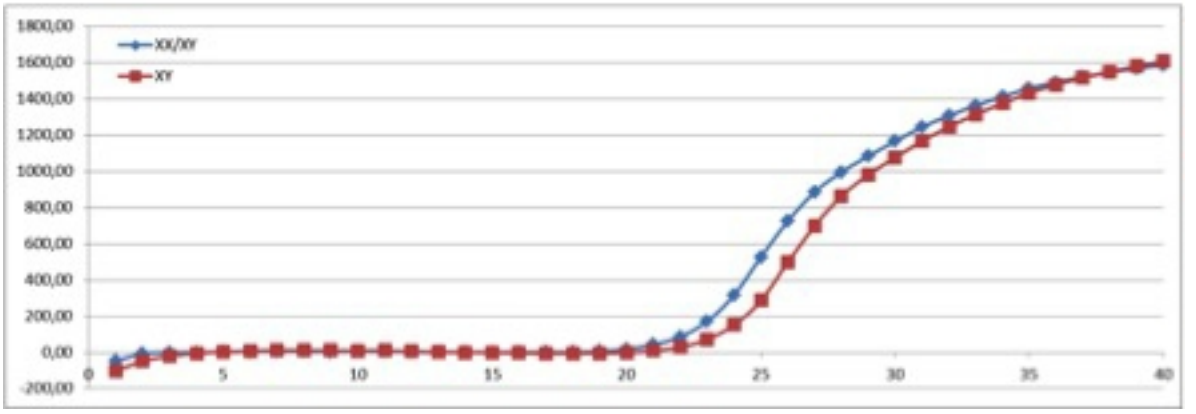

M
25x
XY Ho
27x
XY Ho
29x
XY Ho
31x
XY Ho
33x
XY Ho
35x
XY Ho XX H2O
Sry
M
25x
XY Ho
27x
XY Ho
29x
XY Ho
31x
XY Ho
33x
XY Ho
35x
XY Ho XX H2O
Hprt

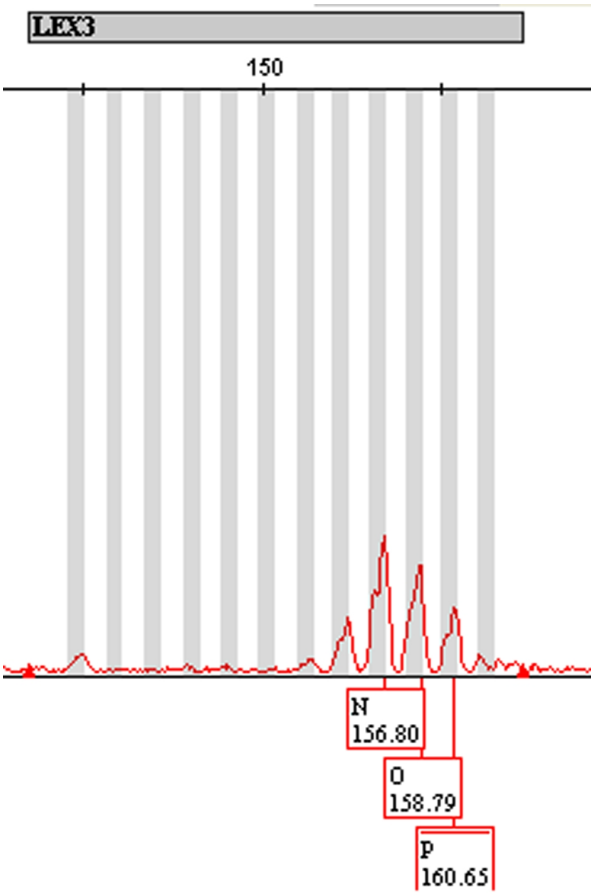



0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

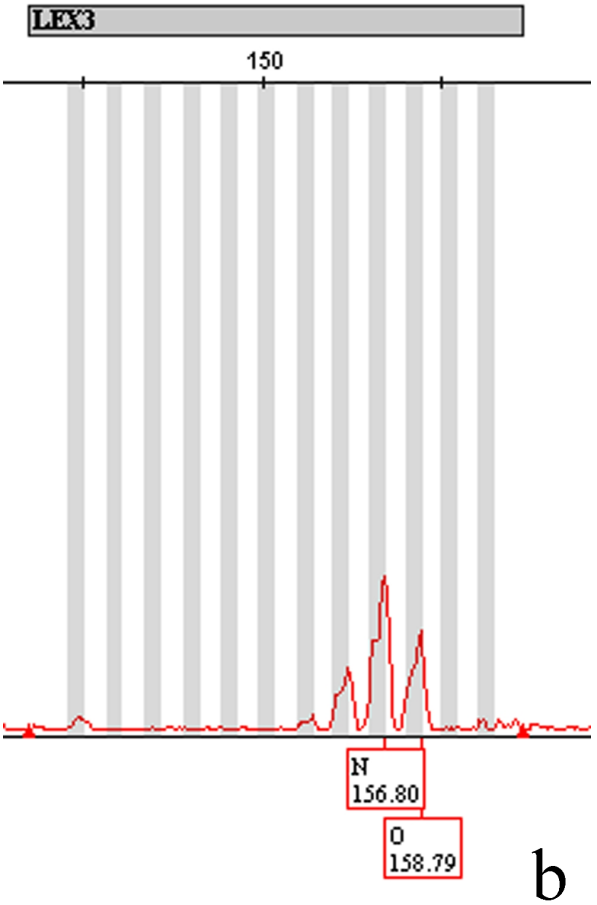

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


2000,00
1500,00
1000,00
500,00
0,00
-500,00
0
5
10
15
20
25
30
35
40
XX/XY
XY


1800,00
1600,00
1400,00
1200,00
1000,00
800,00
600,00
400,00
200,00
0,00
-200,00
0
5
10
15
20
25
30
35
40
XX/XY
XY



a



b