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1 **X-Y aneuploidy rate in sperm of two ‘minor’ breeds of cattle (*Bos taurus*) by**
2 **using dual color fluorescent *in situ* hybridization (FISH)**

3
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1 **Abstract**

2 The present study reports on the frequency of X-Y aneuploidy in the sperm population of
3 two minor cattle breeds reared in Italy, namely Modicana and Agerolese, which are listed in the
4 ‘Anagraphic Register of autochthonous cattle populations with limited distribution’. Totally, more
5 than 50.000 sperm nuclei from 11 subjects (5 and 6, respectively for each breed) have been
6 analyzed by the fluorescent *in situ* hybridization with the Xcen and Y-chromosome specific
7 painting probes. The fraction of X- and Y- bearing sperm was close to the 1:1 ratio in the Modicana
8 breed, whereas in the Agerolese the Y-fraction was significantly higher ($P<0.002$) compared to the
9 X-counterpart. The mean rates of X-Y aneuploidy were 0.510% and 0.466%, respectively, in the
10 two breeds; no significant differences were found among individual bulls within each breed.
11 Average frequencies of disomic and diploid sperm were 0.425% and 0.085% in the former and
12 0.380% and 0.086% in the latter. In both breeds, a) disomy was significantly more frequent than
13 diploidy ($P<0.01$), b) YY disomy was significantly ($P<0.001$) more frequent than XY or XX; (c) MI
14 errors (XY disomy) were significantly ($P<0.01$) less represented than MII (XX+YY disomy).
15 Compared to the dairy (Italian Friesian and Brown) and meat (Podolian and Maremmana) breeds
16 previously analyzed, the ‘minor’ breeds investigated in the present study showed a significantly
17 ($P<0.002$) higher rate of X-Y aneuploidy (0.486% vs 0.159% and 0.190%, respectively).
18 Considering all the breeds analyzed -so far- and assuming no significant inter-chromosomal effect,
19 the baseline level of aneuploidy in the sperm population of the species *Bos taurus* was estimated as
20 5.19%. Establishing the baseline level of aneuploidy in the sperm population of the various
21 livestock species/breeds engaged in animal production could reveal useful for monitoring future
22 trends of their reproductive health, especially in relation to management errors and/or
23 environmental hazards.

24

25 **Keywords:** X-Y aneuploidy; sperm FISH; minor breeds; cattle.

26

1 **1. Introduction**

2 Aneuploidy in germ cells are known to be the most important cause of embryonic mortality,
3 in humans as well as in domestic animals. In fact, it has been associated with infertility,
4 spontaneous abortions, perinatal mortality and mental retardation in humans [1, 2, 3, 4] and with
5 embryonic and fetal mortality in farm animals [5].

6 So far, X-Y aneuploidy in sperm of the main livestock species has been primarily
7 investigated in cattle (*Bos taurus*) [6, 7, 8], pig (*Sus scrofa domestica*) [9], and horse (*Equus*
8 *caballus*) [10], whereas other livestock species, such as river buffalo (*Bubalus bubalis*), sheep (*Ovis*
9 *aries*) and goat (*Capra hircus*), only received little attention [11].

10 In order to expand the actual knowledge upon possible ‘interbreed’ variation in the X-Y
11 aneuploidy in bovine sperm, we recently analyzed two ‘indigenous’ breeds, the Podolian and
12 Maremmana [12] and compared the results with those previously achieved in two highly selected
13 ‘dairy’ breeds, the Friesian and Brown [8].

14 In the present study, we report on the X-Y aneuploidy rates in sperm of two ‘minor’ cattle
15 breeds reared in Italy, namely the Modicana and Agerolese, which are listed in the ‘Anagraphic
16 Register of autochthonous cattle populations with limited distribution’, and attempt to establish the
17 ‘baseline’ level of aneuploidy in the sperm population of the species *Bos taurus*.

18

19 **2. Material and methods**

20 *2.1. The Modicana and Agerolese breeds*

21 The Modicana breed is reared in the Modica area (province of Ragusa, Sicily) mainly for
22 milk production from which a typical Sicilian cheese called ‘Ragusano’ is produced. Actually, 3582
23 heads (550 males and 3032 females) are listed in the ‘Anagraphic Register of autochthonous cattle
24 populations with limited distribution’, active since 1985 in order to rescue the breed from
25 extinction.

1 The Agerolese breed is listed in the same register. Its population is even less represented.
2 Currently, it is estimated only in 400 heads (30 males and 370 females) located in the Regional Park
3 of Monti Lattari, a restricted area between Monti Lattari and Sorrento peninsula (province of
4 Naples). Also this breed is reared mainly for milk production from which a typical cheese called
5 ‘Provolone del Monaco, PDO’ (Protected Denomination of Origin – Reg. EC 121/2010) is
6 produced.

7 Recently, both breeds were involved in protection and development programs for the
8 enhancement of genetic resources and the environmental sustainability. These programs have the
9 aim to increase the population and reduce the inbreeding level.

10

11 2.2. *Semen samples*

12 Frozen semen from 5 and 6 young bulls was provided, respectively, from the Modicana and
13 Agerolese Breeder’s Associations. All bulls examined in this study were previously karyotyped and
14 resulted karyologically normal. Each tested bull belonged to a different herd, whereas their age
15 were in the range 18-24 months.

16

17 2.3. *Chromosome micro-dissection and probes preparations*

18 Metaphase cells for the production of probes via microdissection were prepared according to
19 the standard cytogenetic techniques [13]. For microdissection, the fixed lymphocyte suspension was
20 spread onto a pre-cleaned 24 x 60 mm coverslip, which was then air dried and treated for GTG-
21 banding. The Xcen probe was produced by isolating the pericentromeric region, corresponding with
22 the centromere and with the Xp11-14 region of the standardized GTG-banded karyotype [14]; the
23 probe for chromosome Y was produced by scraping the entire chromosome. Microdissected
24 chromosomes were amplified following the protocol of Engelen et al. [15]. Thermal conditions
25 were: initial denaturation at 96°C for 3 min, 8 cycles performed at 96°C for 1 min, 30°C for 1 min
26 with a 2 min transition from 30°C to 72°C, and 72°C for 2 min. This was followed by 35 cycles of 1

1 min at 94° C, 1 min at 56°C, and 2 min at 72°C. The final extension was carried out at 72°C for 5
2 min.

3 Probes were labeled with digoxigenin-11-dUTP (chromosome Xcen) and biotin-16-dUTP
4 (chromosome Y) (No. 11558706910 and No. 11093070910, respectively) (Roche, Mannheim,
5 Germany) in a second Degenerated Oligonucleotide Primer-Polymerase Chain Reaction (DOP-
6 PCR) using 2 µL of products from the first reaction as template. Cycling parameters were: 3 min at
7 95°C for initial denaturation, 30 cycles of 15 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C, with a
8 5 min final extension at 72°C.

9

10 *2.4. Sperm decondensation*

11 Sperm were decondensed according to the method described by Han et al. [16], slightly
12 modified. Briefly, spermatozoa were washed three times in an equal volume of PBS (pH 7.4)
13 containing 6 mM EDTA(Sigma), then resuspended in PBS containing 5 mM DTT (Sigma), and
14 incubated at room temperature for 20 min. Subsequently, the decondensed spermatozoa were
15 washed twice in PBS and fixed in 3:1 methanol:acetic acid. A 20 µl droplet of the fixed suspension
16 was dropped on a clean microscopic slide and air dried at room temperature.

17

18 *2.5. In Situ Hybridization*

19 Probes for the Y-chromosome and for the centromeric region of the X-chromosome of cattle
20 were hybridized simultaneously on metaphase plates for validation, and subsequently used for
21 sperm analysis. Probes were precipitated in the presence of 10 µg salmon sperm DNA (No. D7656;
22 Sigma) and 10 µg of calf thymus DNA (No. D8661; Sigma) dissolved in 15 µL hybridization
23 solution (50% formamide in 2X SSC + 10% dextran sulfate; No. F7503 and No. D8906,
24 respectively; Sigma) (SSC = Standard Saline Citrate), and finally denatured at 72°C for 10 min, and
25 incubated at 37°C for 90 min.

1 Metaphase preparations were denatured in 70% formamide, 2X SSC (pH 7.0) at 72°C for
2 3 min, whereas sperm preparations for 10 min and successively dehydrated through an ethanol
3 series (70%, 85%, 96% ethanol, 2 min each). The hybridization mixture containing probes was
4 applied on the slides and covered with 24 x 24 mm cover-slips. The slides were hybridized in a
5 moist chamber at 37°C overnight. After hybridization, the slides were washed three times in 50%
6 formamide in 2X SSC (pH 7.0) at 42°C for 4 min, and three times in 2XSSC (pH 7.0) at 42° C for 4
7 min. After post-hybridization washes, the slides were counterstained with DAPI (40,60-diamidino-
8 2-phenylindole, 0.24 mg/mL) (No. D9542; Sigma) in Antifade mounting medium (No. H1000;
9 Vector Laboratories, Burlingame, CA, USA).

10

11 *2.6. Fluorescence Analysis and Scoring*

12 The slides were observed at 100 x magnification with a Leica (Wetzlar, Germany) DMRA
13 fluorescence microscope equipped with DAPI, Fluorescein isothiocyanate (FITC), and Texas Red
14 (TXRD) specific filters, the DAPI/FITC/TXRD triple filter, and phase-contrast optics. Digital
15 images were captured using the Leica Q4000 software. Approximately five thousand sperm nuclei
16 were examined for each animal. The scoring was carried out using strict scoring criteria [17].
17 Briefly, sperm with one signal (green or red) were scored as normal haploid; sperm with two signals
18 were classified as disomic (XX, YY and XY depending on the two signal colors). Diploid sperm
19 were distinguished from disomic sperm on the basis of their size. Because the decondensation
20 process might not be uniform along the slide, size comparison was made strictly within the same
21 microscopic field where the diploid sperm were found. In addition, to verify if this could lead to
22 errors in the estimation of aneuploidy, an additional hybridization experiment was carried out on
23 two samples (one for each breed, previously analyzed with Xcen and Y probes) by using a probe for
24 chromosome 6, as reported in Nicodemo et al. [8].

25

26 *2.7. Statistical analysis*

1 The following statistics were used: the χ^2 test with Yates' corrections for interindividual
2 differences; the Kruskal-Wallis and the Mann-Whitney tests with Bonferroni's corrections were
3 used for multiple comparisons and for class differences.

4 5 **3. Results**

6 Table 1 shows the number and frequency of the X-Y bearing sperm and rates of X-Y
7 aneuploidy in sperm of bulls of the Modicana and Agerolese breeds of cattle. The efficiency of the
8 FISH procedure was higher than 99% in both breeds.

9 10 *3.1. X-Y ratio*

11 In the Modicana breed, the fraction of X- and Y- bearing sperm was similar (49.87% vs
12 49.61%, respectively), being close the 1:1 ratio, whereas in the Agerolese breed the Y-fraction was
13 significantly higher ($P < 0.002$) compared to the X-counterpart (51.98% vs 47.55%, respectively).

14 Within each breed, the 'inter-individual' variations in the X/Y ratio were statistically
15 significant only in the Agerolese breed ($P < 0.004$).

16 17 *3.2. X-Y aneuploidy rates*

18 In the Modicana breed, the X-Y aneuploidy rates varied from 0.334% to 0.656%, with an
19 average value of 0.510%. Inter-individual differences were not statistically significant. In the
20 Agerolese breed, the X-Y aneuploidy rates varied from 0.270% to 0.792%, with an average value of
21 0.466%. Inter-individual differences were not statistically significant.

22 23 *3.3. Disomy versus diploidy*

24 In the Modicana breed, the incidence of total disomy varied from 0.220% to 0.636%, with
25 an average of 0.425%, while diploidy varied from 0% to 0.238%, with an average of 0.085%.
26 Similarly, in the Agerolese breed, the corresponding values for disomy varied from 0.213% to

1 0.698%, with an average of 0.380%, while diploidy varied from 0.038% to 0.176%, with an average
2 of 0.086%. In both breeds, no significant differences were found among the bulls investigated in the
3 mean rate of disomy and diploidy.

4

5 3.4. XY-XX-YY disomy

6 Table 2 shows the statistical significance of the comparisons within each breed in the
7 frequency of the different aneuploidy classes. In the Modicana breed, the incidence of the YY
8 disomic sperm (0.309%) was significantly higher compared with the XY (0.023%) and XX
9 (0.093%) counterparts ($P < 0.001$ and $P < 0.05$, respectively). Similarly, in the Agerolese breed, the
10 fraction of YY disomic sperm (0.293%) was significantly higher compared to the XY (0.022%) and
11 XX (0.065%) counterparts ($P < 0.001$ and $P < 0.01$, respectively). Totally, the Modicana and
12 Agerolese breeds showed similar levels of disomic sperm (0.425% and 0.380%, respectively).

13

14 3.5. XY-XX-YY diploidy

15 As shown in table 2, the frequencies of the XY, XX, and YY diploid sperm were quite
16 similar in the two breeds, being 0.039% vs 0.019% vs 0.027%, respectively in the Modicana breed,
17 and 0.034% vs 0.028% vs 0.024%, respectively, in the Agerolese breed. Totally, the two breeds
18 showed similar rates of diploid sperm (0.085% vs 0.086%, respectively).

19 To analyze possible differences in the occurrence of errors during meiosis I (XY
20 disomic/diploid sperm) or meiosis II (XX and YY disomic/diploid sperm) we applied the Mann-
21 Whitney test. In both breeds, meiotic errors giving rise to disomies were significantly more frequent
22 ($P < 0.01$) in M-II than in M-I (0.402% vs 0.023% in the Modicana, and 0.358% vs 0.022% in the
23 Agerolese).

24 Concerning the diploidy, the differences between M-I and M-II were not statistically
25 significant in both breeds.

26

1 3.6. *Interbreed comparison*

2 To investigate possible interbreed differences in the rate of X-Y aneuploidy, we compared
3 the present results with those previously reported in two 'indigenous' breeds [12] and in 'dairy'
4 breeds [6, 8] (Table 3). The significance level (P) of the comparisons is shown in table 4 on the
5 basis of the Kruskal-Wallis test with Bonferroni correction.

6 The mean rate of X-Y aneuploidy in sperm of 'minor' breeds (0.486%) was found to be
7 significantly higher ($P < 0.03$) compared to that previously reported on 'indigenous' (0.190%) as
8 well as on 'dairy' breeds (0.159%) ($P < 0.002$); this was mainly due to the incidence of disomy
9 (0.400%) which was significantly higher ($P < 0.01$) compared to diploidy (0.086%).

10

11 **4. Discussion**

12 The results of the present study indicate that sperm of the Modicana and Agerolese cattle
13 breeds show quite similar and high rates of X-Y aneuploidy (0.510% and 0.466%, respectively).
14 This high level of aneuploidy was mainly due to the higher incidence of disomy (0.425% and
15 0.380%, respectively) compared to diploidy (0.085% and 0.086%, respectively).

16 Within each breed, no significant inter-individual differences were found in the mean rates
17 of X-Y aneuploidy. This probably indicates that the tested animals belonged to quite uniform
18 samples. Our findings are in agreement with previous reports on Italian cattle breeds [8, 12].
19 Conversely, inter-individual variability in X-Y aneuploidy was recently observed in dairy bulls by
20 Rybar et al. [18]. In human, such a variability is often correlated to pollution factors (smoking,
21 drugs, caffeine and alcohol consumption or chemotherapy) and it is more frequent in subjects with
22 altered basic spermatological parameters like motility, morphology and concentration [19, 20].
23 However, such difference was not confirmed in other studies, where also the rate of total
24 aneuploidy was not influenced by such potential factors of risk [21, 22]. Therefore, the origin of
25 inter-individual differences is still controversial and further studies are necessary.

1 The X-Y ratio was substantially close to 1:1 in the Modicana breed, while in the Agerolese
2 breed the fraction of Y-bearing sperm was significantly higher ($P<0.002$) compared to the X-
3 counterpart. This finding is similar to that already observed in the ‘indigenous’ and ‘dairy’ breeds
4 analyzed so far [12, 8].

5 The overall incidence of disomy was similar in the two breeds (0.425% and 0.380% in the
6 Modicana and Agerolese, respectively), the same for diploidy (0.085% and 0.086%, respectively).
7 The higher incidence of disomy (0.400%) compared to diploidy (0.086%) was mainly due to the
8 YY disomic fraction, which was significantly higher compared to the XX and XY counterparts in
9 both breeds ($P<0.001$ and $P<0.05$, respectively). No significant differences were found in the XX-
10 XY and YY diploid fractions, in both breeds.

11 Errors in MII were significantly higher ($P<0.01$) than those in MI in both breeds, mainly due
12 to the YY fractions.

13

14 *‘Inter-breed’ comparison*

15 The results of the present study indicate that the two ‘minor’ breeds investigated (Modicana
16 and Agerolese) show significantly higher mean rates of X-Y aneuploidy (0.486%) compared to the
17 ‘indigenous’ (Podolian and Maremmana) (0.190%) and ‘dairy’ (Italian Friesian and Brown)
18 (0.159%) cattle breeds previously reported (Tables 3 and 4). The limited number of heads belonging
19 to the minor breeds and the likely high level of inbreeding might be a possible evidence for such
20 differences, as already observed for the rate of aneuploidy in somatic cells [23]. However, in cattle,
21 also the genetic selection seems to concur to the reduction of the baseline level of aneuploidy in
22 cattle. Such remark is also confirmed by a recent investigation on 49 selected young dairy bulls
23 candidates for artificial insemination [18]. Therefore, our finding can be explained, at least in part,
24 by the fact that the zootechnical selection in these minor breeds is hampered by the small number of
25 breeding animals.

1 In addition, the lack of suitable political actions specifically oriented to the rescue of the
2 breeds poses further limitations and constraints to the genetic improvement of these breeds, whose
3 actual situation is the exclusive result of the own action of the breeders.

4 Aneuploidy was mainly due to the higher incidence of disomy compared to diploidy, as
5 observed in the ‘minor’ (0.400% vs 0.086%), ‘indigenous’ (0.122% vs 0.068%), and ‘dairy’
6 (0.106% vs 0.053%) breeds, respectively. The higher incidence of disomy was mainly due to the
7 MII errors (YY-XX sperm) compared to MI (XY sperm) in all breeds investigated. These
8 observations are in agreement with previous studies [6, 18] and they confirm that chromosomal
9 non-disjunctions occur mainly in the second meiotic division.

10 In a previous study in the pig (*Sus scrofa domestica*), Rubes et al. [9] reported no breed
11 effects on disomy and diploidy rates. In the present study, we demonstrated that in cattle (*Bos*
12 *taurus*) variations might be detected ‘among’ different breeds, especially in the disomy rate. This
13 aspect, however, requires further investigations.

14 15 ‘Baseline’ level of X-Y aneuploidy in cattle

16 Assuming no significant inter-chromosomal effect (i.e. each chromosome has the same
17 likelihood to undergo non-disjunction), and according to the conservative law (Disomy x N. haploid
18 chromosomes), the baseline level of aneuploidy can be estimated as 12% in the ‘minor’, 3.65% in
19 the ‘indigenous’ and 3.18% in the ‘dairy’ breeds, respectively. By considering all the breeds
20 analyzed so far, the baseline frequency of aneuploidy in sperm of the species *Bos taurus* can be
21 estimated as 5.19%. However, since the X and Y chromosomes may have -as demonstrated in
22 humans- significantly higher rates of disomy compared to autosomes [24, 25, 26, 27], these baseline
23 levels might be overestimated. Unfortunately, in domestic animals, reports upon the inter-
24 chromosomal effect are quite scarce. Recently, Bonnet-Garnier et al. [28] investigated upon the
25 inter-chromosomal effect in 2 boars carriers of two different reciprocal translocations (12;14) and
26 (3;15) and reported no significant inter-chromosomal effect (ICE), except for chromosome 1 in the

1 t(3;15) in which, however, the significance level was very weak. Also this aspect, however, requires
2 further investigations.

3 Establishing the baseline level of aneuploidy in the sperm population of the various
4 species/breeds engaged in animal production could reveal useful for monitoring future trends of
5 their reproductive health, especially in relation to management errors (nutritional mistakes, diet
6 unbalancements, etc.), the particular animal production system for the populations with limited
7 distribution and/or environmental hazards (pollutants, mitotic poisons, etc.) which are known to
8 damage the mitotic/meiotic machinery of the cell.

9

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14

15 **6. References**

16 [1] Hecht F, Hecht BK. Environmental chromosome damage. *Am J Med Genet* 1987;27:399-400.

17 [2] Martin HR, Ko E, Rademaker A. Distribution of aneuploidy in human gametes: comparison
18 between human sperm and oocytes. *Am J Med Genet* 1991;39:321-331.

19 [3] Hassold TJ. Nondisjunction in the human male, in Handel MA (ed): *meiosis and gametogenesis*,
20 pp383-406 Academic Press, New York,1998.

21 [4] Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev*
22 *Genet* 2001;2:280-291.

23 [5] King WA. Chromosome abnormalities and pregnancy failure in domestic animals, in McFeely
24 RA (ed): *Domestic Animal Cytogenetics*, pp229-250, Academic Press, New York,1990.

- 1 [6] Hassanane M, Kovacs A, Laurent P, Lindblad K, Gustavsson I. Simultaneous detection of X-
2 and Y-bearing bull spermatozoa by double color fluorescence *in situ* hybridization. Mol Reprod
3 Dev 1999;53:407-412.
- 4 [7] Di Berardino D, Vozdova M, Kubickova S, Cernohoska H, Coppola G, Coppola GF, Enne G,
5 Rubes J. Sexing river buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*) and cattle
6 spermatozoa by double color FISH using bovine (*Bos taurus*) X-and Y-painting probes. Mol
7 Reprod Dev 2004;67:108-115.
- 8 [8] Nicodemo D, Pauciullo A, Castello A, Roldan E, Gomendio M, Cosenza G, Peretti V, Perucatti
9 A, Di Meo GP, Ramunno L, Iannuzzi L, Rubes J, Di Berardino D. Sperm aneuploidy in two cattle
10 (*Bos taurus*) breeds as determined by dual color fluorescent *in situ* hybridization (FISH).
11 Cytogenetics Genome Res 2009;126:217-225.
- 12 [9] Rubes J, Vozdova M, Kubickova S. Aneuploidy in pig sperm: multicolor fluorescence *in situ*
13 hybridization using probes for chromosomes 1-10 and Y. Cytogenet Cell Genet 1999;85:200-204.
- 14 [10] Bugno M, Jablonska Z, Tischner M, Klukowska-Rotzler J, Pienkowska-Schelling A, Schelling
15 C, Slota E. Detection of Sex chromosome aneuploidy in equine spermatozoa using fluorescence *in*
16 *situ* hybridization. Reprod Dom Anim 2010;45:1015-1019.
- 17 [11] Di Berardino D, Vozdova M, Nicodemo D, Kubickova S, Cernohoska H, Rubes J. Recent
18 developments on genetic information among river type buffaloes by chromosome microdissection
19 and cloning. Proc. 7th World Buffalo Congress, 20-23 october 2004;185-201.
- 20 [12] Pauciullo A, Cosenza G, Peretti V, Iannuzzi A, Di Meo GP, Ramunno L, Iannuzzi L, Rubes J,
21 Di Berardino D. Incidence of X-Y aneuploidy in sperm of two indigenous cattle breeds by using
22 dual color fluorescent *in situ* hybridization (FISH). Theriogenology 2011;76:328-333.
- 23 [13] Iannuzzi L, Di Berardino D. Tools of the trade: diagnostic and research applied to domestic
24 animal Cytogenetics. J.Applied Genetics 2008;49:357-366.

- 1 [14] Di Berardino D, Di Meo GP, Gallagher DS, Hayes H, Iannuzzi L. International System for
2 Chromosome Nomenclature of Domestic Bovids (ISCNDB). *Cytogenet Cell Genet* 2001;92:283-
3 299.
- 4 [15] Engelen JM, Albrechts J, Hamers G, Jeraedts J. A simple and efficient method for
5 microdissection and microFISH. *J Med Genet* 1998;35:265-268.
- 6 [16] Han TL, Webb GC, Flaherthy SP, Correll A, Matthews CD, Ford JH. Detection of
7 chromosome 17 and X-bearing human spermatozoa using fluorescence in situ hybridization. *Mol*
8 *Reprod Dev* 1992;33:189-194.
- 9 [17] Robbins WA, Baulch JE, Moore D, Weier HU, Blakey D, Wyrobek AJ. Three-probe
10 fluorescence in situ hybridization to assess chromosome X,Y and 8 aneuploidy in sperm of 14 men
11 from two healthy groups: evidence for a paternal age effect on sperm aneuploidy. *Reprod Fertil Dev*
12 1995;7:799-809.
- 13 [18] Rybar R, Kopecka V, Prinosilova P, Kubickova S, Veznik Z, Rubes J. Fertile bull sperm
14 aneuploidy and chromatin integrity in relationship to fertility. *Int J Androl* 2010;33:613-622.
- 15 [19] Shi Q, Martin RH. Aneuploidy in human sperm: a review of the frequency and distribution of
16 aneuploidy, effects of donor age and lifestyle factors. *Cytogenet Cell Genet* 2000;90:219–226.
- 17 [20] Collodel G, Capitani S, Baccetti B, Pammolli A, Moretti E. Sperm aneuploidies and low
18 progressive motility. *Human Reproduction* 2007;22:1893–1898.
- 19 [21] Sram RJ, Binkova B, Rössner P, Rubes J, Topinka J, Dejmek J. Adverse reproductive
20 outcomes from exposure to environmental mutagens. *Mutation Research* 1999;428:203–215
- 21 [22] Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, Perreault
22 SD. Episodic air pollution is associated with increased DNA fragmentation in human sperm without
23 other changes in semen quality. *Human Reproduction* 2005;20:2776–2783.
- 24 [23] Zartman DL, Fechheimer NS. Somatic aneuploidy and polyploidy in inbred and linecross
25 cattle. *J Anim Sci* 1967;26:678-682

- 1 [24] Bischoff FZ, Nguyen DD, Burt KJ, Shaffer LG. Estimates of aneuploidy using multicolor
2 fluorescence in situ hybridization on human sperm. *Cytogenet Cell Genet* 1994;66:237-243.
- 3 [25] Chevret E, Rousseaux S, Monteil M, Pellettier R, Cozzi J, Sele B. Meiotic segregation of the X
4 and Y chromosomes and chromosome 1 analyzed by three color FISH in human interphase
5 spermatozoa. *Cytogenet Genome Res* 1995;71:126-130.
- 6 [26] Martin HR, Spriggs E, Rademaker AW. The relationship between paternal age, sex ratios and
7 aneuploidy frequencies in human sperm, as assessed by multicolour FISH. *Am J hum. Genet*
8 1995;57:1395-1399.
- 9 [27] Spriggs EL, Rademaker AW, Martin RH. Aneuploidy in human sperm: the use of multicolour
10 FISH to test various theories of non disjunction. *Am J hum Genet* 1996;58:356-362.
- 11 [28] Bonnet-Garnier A, Guardia S, Pinton A, Ducos A, Yerle M. Analysis using sperm-FISH of a
12 putative interchromosomal effect in boars carrying reciprocal translocations. *Cytogene Genome Res*
13 2009;126:194-201.
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Table 1 - Number and frequency (%) of X- and Y- bearing sperm and rates of X-Y aneuploidy in sperm of bulls of the Modicana and Agerolese 'minor' breeds of cattle.

Bulls	Sperm													
	Analyzed	Without Signal (1)	With Signal (1)	Normal (2)		X-Y Aneuploid (2)							total	
				X	Y	disomic			diploid					
	(a)	(b)	XY			XX	YY	total	XY	XX	YY	total		
<i>Modicana breed</i>														
1	5,091	57 (1.120)	5,034 (98.880)	2,382 (47.318)	2,627 (52.185)	0 (0)	10 (0.199)	15 (0.298)	25 (0.497)	0 (0)	0 (0)	0 (0)	0 (0)	25 (0.497)
2	5,045	12 (0.238)	5,033 (99.762)	2,525 (50.169)	2,475 (49.175)	1 (0.020)	7 (0.139)	25 (0.497)	32 (0.636)	0 (0)	0 (0)	1 (0.020)	1 (0.020)	33 (0.656)
3	5,122	36 (0.703)	5,086 (99.297)	2,593 (50.983)	2,476 (48.683)	1 (0.020)	3 (0.059)	11 (0.216)	15 (0.295)	1 (0.020)	0 (0)	1 (0.020)	2 (0.039)	17 (0.334)
4	5,288	8 (0.151)	5,280 (99.849)	2,615 (49.526)	2,634 (49.886)	1 (0.019)	3 (0.057)	21 (0.398)	25 (0.474)	2 (0.038)	3 (0.057)	1 (0.019)	6 (0.114)	31 (0.588)
5	5,537	82 (1.480)	5,455 (98.520)	2,798 (51.292)	2,632 (48.250)	3 (0.054)	1 (0.018)	8 (0.148)	12 (0.220)	7 (0.129)	2 (0.036)	4 (0.073)	13 (0.238)	25 (0.458)
All	26,084	195 (0.748)	25,889 (99.252)	12,913 (49.878)	12,844 (49.612)	6 (0.023)	24 (0.093)	80 (0.309)	110 (0.425)	10 (0.039)	5 (0.019)	7 (0.027)	22 (0.085)	132 (0.510)
<i>Agerolese breed</i>														
1	5,348	43 (0.804)	5,305 (99.196)	2,543 (47.936)	2,745 (51.744)	3 (0.056)	0 (0)	12 (0.226)	15 (0.282)	1 (0.019)	1 (0.019)	0 (0)	2 (0.038)	17 (0.320)
2	5,173	82 (1.585)	5,091 (98.415)	2,436 (47.850)	2,625 (51.561)	0 (0)	2 (0.039)	19 (0.374)	21 (0.413)	4 (0.078)	3 (0.059)	2 (0.039)	9 (0.176)	30 (0.589)
3	5,224	56 (1.072)	5,168 (98.928)	2,401 (46.460)	2,753 (53.270)	1 (0.019)	2 (0.038)	8 (0.156)	11 (0.213)	1 (0.019)	2 (0.038)	0 (0)	3 (0.057)	14 (0.270)
4	5,316	15 (0.282)	5,301 (99.718)	2,533 (47.784)	2,726 (51.424)	1 (0.019)	6 (0.113)	30 (0.566)	37 (0.698)	3 (0.056)	1 (0.019)	1 (0.019)	5 (0.094)	42 (0.792)
5	5,505	22 (0.400)	5,483 (99.600)	2,596 (47.346)	2,858 (52.125)	1 (0.018)	5 (0.092)	19 (0.347)	25 (0.457)	1 (0.018)	1 (0.018)	2 (0.036)	4 (0.072)	29 (0.529)
6	6,119	68 (1.111)	6,051 (98.889)	2,898 (47.893)	3,134 (51.793)	1 (0.016)	6 (0.100)	7 (0.116)	14 (0.232)	1 (0.016)	1 (0.016)	3 (0.050)	5 (0.082)	19 (0.314)
All	32,685	286 (0.875)	32,399 (99.125)	15,407 (47.554)	16,841 (51.980)	7 (0.022)	21 (0.065)	95 (0.293)	123 (0.380)	11 (0.034)	9 (0.028)	8 (0.024)	28 (0.086)	151 (0.466)

(1) percentage values refer to column (a); (2) percentage values refer to column (b);

Table 2- Statistical significance of the comparisons 'within' each breed in the frequency of the different aneuploidy classes.

	Modicana			Agerolese		
	%	comparison	P	%	comparison	P
<i>Disomy</i>						
XY(1)	0.023	1-2	N.S.	0.022	1-2	N.S.
XX(2)	0.093	2-3	0.05	0.065	2-3	0.01
YY(3)	0.309	1-3	0.001	0.293	1-3	0.001
Total (4)	0.425	4-10	0.01	0.380	4-10	0.01
MI(5)	0.023	5-6	0.01	0.022	5-6	0.01
MII(6)	0.402	-	-	0.358	-	-
<i>Diploidy</i>						
XY(7)	0.039	7-8	N.S.	0.034	7-8	N.S.
XX(8)	0.019	8-9	N.S.	0.028	8-9	N.S.
YY(9)	0.027	7-9	N.S.	0.024	7-9	N.S.
Total (10)	0.085	-	-	0.086	-	-
MI(11)	0.039	11-12	N.S.	0.034	11-12	N.S.
MII(12)	0.046	-	-	0.052	-	-

Table 3 - Number and frequency (%) of X- and Y- bearing sperm and rates of XY- aneuploidy in sperm of bulls of ‘minor’, ‘indigenous’ and ‘dairy’ cattle breeds.

Breed	Bulls	Sperm With signals	Normal		X-Y aneuploid						<i>Total</i>
			X	Y	disomic			diploid			
					M-I	M-II	total	M-I	M-II	total	
<i>A. Minor breeds</i>											
Modicana ⁽¹⁾	5	25,889	12,913 (49.878)	12,844 (49.612)	6 (0.023)	104 (0.402)	110 (0.425)	10 (0.039)	12 (0.046)	22 (0.085)	132 (0.510)
Agerolese ⁽¹⁾	6	32,399	15,407 (47.554)	16,841 (51.980)	7 (0.022)	116 (0.358)	123 (0.380)	11 (0.034)	17 (0.052)	28 (0.086)	151 (0.466)
Total A	11	58,288	28,320 (48.586)	29,685 (50.928)	13 (0.022)	220 (0.378)	233 (0.400)	21 (0.036)	29 (0.050)	50 (0.086)	283 (0.486)
<i>B. Indigenous breeds</i>											
Podolian ⁽²⁾	5	25,512	12,441 (48.765)	13,025 (51.055)	6 (0.024)	32 (0.125)	38 (0.149)	4 (0.016)	4 (0.016)	8 (0.031)	46 (0.180)
Maremmana ⁽²⁾	5	28,514	13,952 (48.930)	14,505 (50.870)	5 (0.017)	23 (0.081)	28 (0.098)	14 (0.049)	15 (0.053)	29 (0.102)	57 (0.200)
Total B	10	54,026	26,393 (48.852)	27,530 (50.958)	11 (0.020)	55 (0.102)	66 (0.122)	18 (0.033)	19 (0.035)	37 (0.068)	103 (0.190)
<i>C. Dairy breeds</i>											
Italian Friesian ⁽³⁾	10	51,885	24,793 (47.784)	27,008 (52.053)	16 (0.031)	42 (0.081)	58 (0.112)	15 (0.025)	11 (0.021)	26 (0.050)	84 (0.162)
Italian Brown ⁽³⁾	10	50,835	24,313 (47.827)	26,450 (52.031)	6 (0.012)	34 (0.067)	40 (0.079)	21 (0.041)	11 (0.022)	32 (0.063)	72 (0.142)
Swedish Friesian ⁽⁴⁾	5	53,224	26,316 (49.044)	26,816 (49.976)	16 (0.029)	52 (0.096)	68 (0.125)	n.d. n.d.	24 (0.045)	24 (0.045)	92 (0.170)
Total C	25	155,944	75,422 (48.365)	80,274 (51.476)	38 (0.024)	128 (0.082)	166 (0.106)	36 (0.023)	46 (0.030)	82 (0.053)	248 (0.159)
<i>A + B + C</i>											
ALL	46	268,258	130,135 (48,512)	137,489 (51,252)	62 (0.023)	403 (0.150)	465 (0.173)	75 (0.028)	94 (0.035)	169 (0.063)	634 (0.236)

⁽¹⁾ Present study; ⁽²⁾ Pauciullo et al. [12]; ⁽³⁾ Nicodemo et al. [8]; ⁽⁴⁾ Hassanane et al. [6]; n.d.= not detected.

Table 4 – Significant p-value of XY- aneuploidy in sperm of ‘minor’, ‘indigenous’ and ‘dairy’ cattle breeds detected through Kruskal-Wallis test with Bonferroni correction.

P-value	Disomy			Diploidy			Disomy + Diploidy		
	<i>Minor</i> (1)	<i>Indigenous</i> (2)	<i>Dairy</i> (3, 4)	<i>Minor</i> (1)	<i>Indigenous</i> (2)	<i>Dairy</i> (3, 4)	<i>Minor</i> (1)	<i>Indigenous</i> (2)	<i>Dairy</i> (3, 4)
<i>Minor</i> ⁽¹⁾	-	<0.002	<0.0001	-	N.S.	N.S.	-	<0.004	<0.002
<i>Indigenous</i> ⁽²⁾		-	N.S.		-	N.S.		-	N.S.
<i>Dairy</i> ^(3, 4)			-			-			-

⁽¹⁾ Present study; ⁽²⁾ Pauciullo et al. [12]; ⁽³⁾ Nicodemo et al. [8]; ⁽⁴⁾ Hassanane et al. [6].