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

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/151321> since

*Published version:*

DOI:10.1016/j.theriogenology.2012.03.017

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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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)**

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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  $\chi^2$  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

## 10 **5. Acknowledgements**

11 This work was financially supported by the Ministry of Agriculture and Forestry Politics  
12 (MiPAAF) of Rome (SpermovoFISH project n. 291/7303/06) and by the MZE project CR n.  
13 002716202 of the Czech Republic.

14

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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 (a)	Without Signal (1)	With Signal (1) (b)	Normal (2)		X-Y Aneuploid (2)							total	
				X	Y	disomic			diploid					
						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)	<b>25</b> <b>(0.497)</b>	0 (0)	0 (0)	0 (0)	<b>0</b> <b>(0)</b>	<b>25</b> <b>(0.497)</b>
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)	<b>32</b> <b>(0.636)</b>	0 (0)	0 (0)	1 (0.020)	<b>1</b> <b>(0.020)</b>	<b>33</b> <b>(0.656)</b>
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)	<b>15</b> <b>(0.295)</b>	1 (0.020)	0 (0)	1 (0.020)	<b>2</b> <b>(0.039)</b>	<b>17</b> <b>(0.334)</b>
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)	<b>25</b> <b>(0.474)</b>	2 (0.038)	3 (0.057)	1 (0.019)	<b>6</b> <b>(0.114)</b>	<b>31</b> <b>(0.588)</b>
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)	<b>12</b> <b>(0.220)</b>	7 (0.129)	2 (0.036)	4 (0.073)	<b>13</b> <b>(0.238)</b>	<b>25</b> <b>(0.458)</b>
<b>All</b>	<b>26,084</b>	<b>195</b> <b>(0.748)</b>	<b>25,889</b> <b>(99.252)</b>	<b>12,913</b> <b>(49.878)</b>	<b>12,844</b> <b>(49.612)</b>	<b>6</b> <b>(0.023)</b>	<b>24</b> <b>(0.093)</b>	<b>80</b> <b>(0.309)</b>	<b>110</b> <b>(0.425)</b>	<b>10</b> <b>(0.039)</b>	<b>5</b> <b>(0.019)</b>	<b>7</b> <b>(0.027)</b>	<b>22</b> <b>(0.085)</b>	<b>132</b> <b>(0.510)</b>
<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)	<b>15</b> <b>(0.282)</b>	1 (0.019)	1 (0.019)	0 (0)	<b>2</b> <b>(0.038)</b>	<b>17</b> <b>(0.320)</b>
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)	<b>21</b> <b>(0.413)</b>	4 (0.078)	3 (0.059)	2 (0.039)	<b>9</b> <b>(0.176)</b>	<b>30</b> <b>(0.589)</b>
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)	<b>11</b> <b>(0.213)</b>	1 (0.019)	2 (0.038)	0 (0)	<b>3</b> <b>(0.057)</b>	<b>14</b> <b>(0.270)</b>
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)	<b>37</b> <b>(0.698)</b>	3 (0.056)	1 (0.019)	1 (0.019)	<b>5</b> <b>(0.094)</b>	<b>42</b> <b>(0.792)</b>
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)	<b>25</b> <b>(0.457)</b>	1 (0.018)	1 (0.018)	2 (0.036)	<b>4</b> <b>(0.072)</b>	<b>29</b> <b>(0.529)</b>
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)	<b>14</b> <b>(0.232)</b>	1 (0.016)	1 (0.016)	3 (0.050)	<b>5</b> <b>(0.082)</b>	<b>19</b> <b>(0.314)</b>
<b>All</b>	<b>32,685</b>	<b>286</b> <b>(0.875)</b>	<b>32,399</b> <b>(99.125)</b>	<b>15,407</b> <b>(47.554)</b>	<b>16,841</b> <b>(51.980)</b>	<b>7</b> <b>(0.022)</b>	<b>21</b> <b>(0.065)</b>	<b>95</b> <b>(0.293)</b>	<b>123</b> <b>(0.380)</b>	<b>11</b> <b>(0.034)</b>	<b>9</b> <b>(0.028)</b>	<b>8</b> <b>(0.024)</b>	<b>28</b> <b>(0.086)</b>	<b>151</b> <b>(0.466)</b>

(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.

	<b>Modicana</b>			<b>Agerolese</b>		
	%	comparison	P	%	comparison	P
<b><i>Disomy</i></b>						
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)	<b>0.425</b>	4-10	0.01	<b>0.380</b>	4-10	0.01
MI(5)	0.023	5-6	0.01	0.022	5-6	0.01
MII(6)	0.402	-	-	0.358	-	-
<b><i>Diploidy</i></b>						
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)	<b>0.085</b>	-	-	<b>0.086</b>	-	-
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		total	diploid		total	
					M-I	M-II		M-I	M-II		
<i>A. Minor breeds</i>											
Modicana <sup>(1)</sup>	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 <sup>(1)</sup>	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)
<b>Total A</b>	<b>11</b>	<b>58,288</b>	<b>28,320</b> <b>(48.586)</b>	<b>29,685</b> <b>(50.928)</b>	<b>13</b> <b>(0.022)</b>	<b>220</b> <b>(0.378)</b>	<b>233</b> <b>(0.400)</b>	<b>21</b> <b>(0.036)</b>	<b>29</b> <b>(0.050)</b>	<b>50</b> <b>(0.086)</b>	<b>283</b> <b>(0.486)</b>
<i>B. Indigenous breeds</i>											
Podolian <sup>(2)</sup>	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 <sup>(2)</sup>	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)
<b>Total B</b>	<b>10</b>	<b>54,026</b>	<b>26,393</b> <b>(48.852)</b>	<b>27,530</b> <b>(50.958)</b>	<b>11</b> <b>(0.020)</b>	<b>55</b> <b>(0.102)</b>	<b>66</b> <b>(0.122)</b>	<b>18</b> <b>(0.033)</b>	<b>19</b> <b>(0.035)</b>	<b>37</b> <b>(0.068)</b>	<b>103</b> <b>(0.190)</b>
<i>C. Dairy breeds</i>											
Italian Friesian <sup>(3)</sup>	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 <sup>(3)</sup>	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 <sup>(4)</sup>	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)
<b>Total C</b>	<b>25</b>	<b>155,944</b>	<b>75,422</b> <b>(48.365)</b>	<b>80,274</b> <b>(51.476)</b>	<b>38</b> <b>(0.024)</b>	<b>128</b> <b>(0.082)</b>	<b>166</b> <b>(0.106)</b>	<b>36</b> <b>(0.023)</b>	<b>46</b> <b>(0.030)</b>	<b>82</b> <b>(0.053)</b>	<b>248</b> <b>(0.159)</b>
<i>A + B + C</i>											
<b>ALL</b>	<b>46</b>	<b>268,258</b>	<b>130,135</b> <b>(48,512)</b>	<b>137,489</b> <b>(51,252)</b>	<b>62</b> <b>(0.023)</b>	<b>403</b> <b>(0.150)</b>	<b>465</b> <b>(0.173)</b>	<b>75</b> <b>(0.028)</b>	<b>94</b> <b>(0.035)</b>	<b>169</b> <b>(0.063)</b>	<b>634</b> <b>(0.236)</b>

<sup>(1)</sup> Present study; <sup>(2)</sup> Pauciullo et al. [12]; <sup>(3)</sup> Nicodemo et al. [8]; <sup>(4)</sup> 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> <sup>(1)</sup>	-	<0.002	<0.0001	-	N.S.	N.S.	-	<0.004	<0.002
<i>Indigenous</i> <sup>(2)</sup>		-	N.S.		-	N.S.		-	N.S.
<i>Dairy</i> <sup>(3, 4)</sup>			-			-			-

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