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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)

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Abstract

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

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

1. Introduction

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

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

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

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

2. Material and methods

2.1. The Modicana and Agerolese breeds

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

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

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

2.2. Semen samples

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

2.3. Chromosome micro-dissection and probes preparations

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

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 min.

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

2.4. Sperm decondensation

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

2.5. In Situ Hybridization

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

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

2.6. Fluorescence Analysis and Scoring

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

2.7. Statistical analysis

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

3. Results

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

3.1. X-Y ratio

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

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

3.2. X-Y aneuploidy rates

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

3.3. Disomy versus diploidy

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

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

3.4. XY-XX-YY disomy

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

3.5. XY-XX-YY diploidy

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

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

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

3.6. Interbreed comparison

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

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

4. Discussion

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

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

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

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

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

'Inter-breed' comparison

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

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

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

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

'Baseline' level of X-Y aneuploidy in cattle

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

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

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

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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)								
						disomic				diploid				total
				X	Y									
	(a)		(b)			XY	XX	YY	total	XY	XX	YY	total	
Modicana breed														
1	5,091	57	5,034	2,382	2,627	0	10	15	**25**	0	0	0	**0**	**25**
		(1.120)	(98.880)	(47.318)	(52.185)	(0)	(0.199)	(0.298)	**(0.497)**	(0)	(0)	(0)	**(0)**	**(0.497)**
2	5,045	12	5,033	2,525	2,475	1	7	25	**32**	0	0	1	**1**	**33**
		(0.238)	(99.762)	(50.169)	(49.175)	(0.020)	(0.139)	(0.497)	**(0.636)**	(0)	(0)	(0.020)	**(0.020)**	**(0.656)**
3	5,122	36	5,086	2,593	2,476	1	3	11	**15**	1	0	1	**2**	**17**
		(0.703)	(99.297)	(50.983)	(48.683)	(0.020)	(0.059)	(0.216)	**(0.295)**	(0.020)	(0)	(0.020)	**(0.039)**	**(0.334)**
4	5,288	8	5,280	2,615	2,634	1	3	21	**25**	2	3	1	**6**	**31**
		(0.151)	(99.849)	(49.526)	(49.886)	(0.019)	(0.057)	(0.398)	**(0.474)**	(0.038)	(0.057)	(0.019)	**(0.114)**	**(0.588)**
5	5,537	82	5,455	2,798	2,632	3	1	8	**12**	7	2	4	**13**	**25**
		(1.480)	(98.520)	(51.292)	(48.250)	(0.054)	(0.018)	(0.148)	**(0.220)**	(0.129)	(0.036)	(0.073)	**(0.238)**	**(0.458)**
All	***26,084***	***195***	***25,889***	***12,913***	***12,844***	***6***	***24***	***80***	***110***	***10***	***5***	***7***	***22***	**132**
		(0.748)	***(99.252)***	***(49.878)***	***(49.612)***	***(0.023)***	***(0.093)***	***(0.309)***	***(0.425)***	***(0.039)***	***(0.019)***	***(0.027)***	***(0.085)***	***(0.510)***
Agerolese breed														
1	5,348	43	5,305	2,543	2,745	3	0	12	**15**	1	1	0	**2**	**17**
		(0.804)	(99.196)	(47.936)	(51.744)	(0.056)	(0)	(0.226)	**(0.282)**	(0.019)	(0.019)	(0)	**(0.038)**	**(0.320)**
2	5,173	82	5,091	2,436	2,625	0	2	19	**21**	4	3	2	**9**	**30**
		(1.585)	(98.415)	(47.850)	(51.561)	(0)	(0.039)	(0.374)	**(0.413)**	(0.078)	(0.059)	(0.039)	**(0.176)**	**(0.589)**
3	5,224	56	5,168	2,401	2,753	1	2	8	**11**	1	2	0	**3**	**14**
		(1.072)	(98.928)	(46.460)	(53.270)	(0.019)	(0.038)	(0.156)	**(0.213)**	(0.019)	(0.038)	(0)	**(0.057)**	**(0.270)**
4	5,316	15	5,301	2,533	2,726	1	6	30	**37**	3	1	1	**5**	**42**
		(0.282)	(99.718)	(47.784)	(51.424)	(0.019)	(0.113)	(0.566)	**(0.698)**	(0.056)	(0.019)	(0.019)	**(0.094)**	**(0.792)**
5	5,505	22	5,483	2,596	2,858	1	5	19	**25**	1	1	2	**4**	**29**
		(0.400)	(99.600)	(47.346)	(52.125)	(0.018)	(0.092)	(0.347)	**(0.457)**	(0.018)	(0.018)	(0.036)	**(0.072)**	**(0.529)**
6	6,119	68	6,051	2,898	3,134	1	6	7	**14**	1	1	3	**5**	**19**
		(1.111)	(98.889)	(47.893)	(51.793)	(0.016)	(0.100)	(0.116)	**(0.232)**	(0.016)	(0.016)	(0.050)	**(0.082)**	**(0.314)**
All	***32,685***	***286***	***32,399***	***15,407***	***16,841***	***7***	***21***	***95***	***123***	***11***	***9***	***8***	***28***	***151***
		(0.875)	***(99.125)***	***(47.554)***	***(51.980)***	***(0.022)***	***(0.065)***	***(0.293)***	***(0.380)***	***(0.034)***	***(0.028)***	***(0.024)***	***(0.086)***	***(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
Disomy						
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	-	-
Diploidy						
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						***Total***
			X	Y	disomic			diploid			
					M-I	M-II	total	M-I	M-II	total	
A. Minor breeds											
Modicana[(1)]	5	25,889	12,913	12,844	6	104	110	10	12	22	132
			(49.878)	(49.612)	(0.023)	(0.402)	(0.425)	(0.039)	(0.046)	(0.085)	(0.510)
Agerolese[(1)]	6	32,399	15,407	16,841	7	116	123	11	17	28	151
			(47.554)	(51.980)	(0.022)	(0.358)	(0.380)	(0.034)	(0.052)	(0.086)	(0.466)
Total A	**11**	**58,288**	**28,320**	**29,685**	**13**	**220**	**233**	**21**	**29**	**50**	**283**
			(48.586)	**(50.928)**	**(0.022)**	**(0.378)**	**(0.400)**	**(0.036)**	**(0.050)**	**(0.086)**	**(0.486)**
B. Indigenous breeds											
Podolian[(2)]	5	25,512	12,441	13,025	6	32	38	4	4	8	46
			(48.765)	(51.055)	(0.024)	(0.125)	(0.149)	(0.016)	(0.016)	(0.031)	(0.180)
Maremmana[(2)]	5	28,514	13,952	14,505	5	23	28	14	15	29	57
			(48.930)	(50.870)	(0.017)	(0.081)	(0.098)	(0.049)	(0.053)	(0.102)	(0.200)
Total B	***10***	***54,026***	***26,393***	***27,530***	***11***	***55***	***66***	***18***	***19***	***37***	***103***
			(48.852)	***(50.958)***	***(0.020)***	***(0.102)***	***(0.122)***	***(0.033)***	***(0.035)***	***(0.068)***	***(0.190)***
C. Dairy breeds											
Italian Friesian[(3)]	10	51,885	24,793	27,008	16	42	58	15	11	26	84
			(47.784)	(52.053)	(0.031)	(0.081)	(0.112)	(0.025)	(0.021)	(0.050)	(0.162)
Italian Brown[(3)]	10	50,835	24,313	26,450	6	34	40	21	11	32	72
			(47.827)	(52.031)	(0.012)	(0.067)	(0.079)	(0.041)	(0.022)	(0.063)	(0.142)
Swedish Friesian[(4)]	5	53,224	26,316	26,816	16	52	68	n.d.	24	24	92
			(49.044)	(49.976)	(0.029)	(0.096)	(0.125)	n.d.	(0.045)	(0.045)	(0.170)
Total C	***25***	***155,944***	***75,422***	***80,274***	***38***	***128***	***166***	***36***	***46***	***82***	***248***
			(48.365)	***(51.476)***	***(0.024)***	***(0.082)***	***(0.106)***	***(0.023)***	***(0.030)***	***(0.053)***	***(0.159)***
A + B+ C											
ALL	***46***	***268,258***	***130,135***	***137,489***	***62***	***403***	***465***	***75***	***94***	***169***	***634***
			(48,512)	***(51,252)***	***(0.023)***	***(0.150)***	***(0.173)***	***(0.028)***	***(0.035)***	***(0.063)***	***(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 bulls of ‘minor’, ‘indigenous’ and ‘dairy’ cattle breeds detected through Kruskal-Wallis test with Bonferroni correction.

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

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