Cytogenetic investigation in two endangered pig breeds raised in Southern-Italy: clinical and environmental aspects

Viviana Genualdo[1], Angela Perucatti[1], Donata Marletta[2], Bianca Castiglioni[3], Salvatore Bordonaro[2], Marco Iannaccone[4], Francesca Ciotola[5], Vincenzo Peretti[5], Alessandra Iannuzzi[1]

[1]National Research Council (CNR) of Italy, ISPAAM, Laboratory of Animal Cytogenetics and Genomics, Naples, Italy; [2]Di3A, University of Catania, Catania, Italy; [3]CNR-IBBA, Milan, Italy; [4]University of Naples "Federico II", Portici, Italy, [5]Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples, Italy.

Corresponding author: Dr. Alessandra Iannuzzi, National Research Council (CNR), Institute for Animal Production System in Mediterranean Environments (ISPAAM), Laboratory of Cytogenetics and Genomics, Via Argine 1085, 80147 Naples, Italy; Phone +39 0815966006; Fax +39 0815965291; E-mail: Alessandra.Iannuzzi@ispaam.cnr.it

Abstract

Representative groups of animals of two endangered local pig breeds (Casertana and Nero Siciliano, both with black skin and raised in Southern-Italy) were cytogenetically investigated and compared to check for the presence of chromosomal abnormalities and to test their chromosome stability by Sister Chromatid Exchange (SCE) test, in their normal breeding conditions. Forty-two Casertana pigs (22 males and 20 females, raised in 3 farms) and 39 Nero Siciliano pigs (19 males and 20 female, raised in 3 farms) were investigated. All animals showed normal CBA- and RBA-banded karyotypes (2n=38), except for two Nero Siciliano boars (both from the same farm) which showed 2n=37 being heterozygous carrier of rob(15;17). The translocation was confirmed by FISH-mapping with specific BAC-clones. Both animals were probably hybrids from crosses with the wild pig (2n=36) present in the Nebrodi mountains where Nero Siciliano is reared with extensive or semi-extensive systems. SCE-test applied on all studied animals revealed no statistical differences between the SCE-mean number of Casertana (7.13±3.20) and Nero Siciliano (6.87±3.12) breeds. Statistical differences were found between SCEs mean values of males (7.26±3.38) and females (6.59±2.90) of Nero Siciliano breed, as well as between females of Casertana (7.24±3.26) and Nero Siciliano (6.59±2.90) breeds, while no statistical differences were found between SCE mean values of males of Casertana (6.98±3.10) and Nero Siciliano (6.45±2.97) breeds, as well as between males and females of Casertana breed.

Key words: pig, breed, chromosome abnormality, environment, SCE-test, hybrid

1. Introduction

Casertana and Nero Siciliano pigs are two endangered breeds (both with black skin) raised in the Southern of Italy. The Casertana pig breed (Figure 1A) is raised in Campania region, mainly in

Caserta province. In 2017 the Birth Register maintained by the National Swine Breeders Association (ANAS) recorded a total of 706 pigs (137 sows, 31 boars and 538 young animals) in 21 farms located mainly in Caserta province. It's a very rustic breed, suitable for wild or half-wild farming with fattening trend. The colour of skin is black or slate-grey. Its meat is particularly suited for processing or direct consumption.

The Nero Siciliano pig breed (Figure 1B) is today farmed with extensive or semi-extensive systems in the Nebrodi mountains, Sicily (Guastella et al., 2010). Known locally as the "suino nero dei Nebrodi", it has a prominent ridge of spinal bristles running from its large head to about midway along its back and stands about 70 cm high. In 2017 the Birth Register maintained by ANAS recorded a total of 5.842 pigs (624 sows, 69 boars and 5.149 young animals). The swines are allowed to graze and forage over wide areas, including woods, and this diet influences the meat's flavor.

Crosses between domestic (2n=38) and wild (2n=36) pigs are possible and the hybrids (fertile) have 2n=37, being two acrocentric chromosomes of domestic pig (15 and 17) fused in the wild pig by a centric fusion translocation (Bosma, 1976; Sisa et al., 1984; Arroyo-Nombela et al., 1990). Chromosome stability ensures that genetic information is correctly transmitted during the DNA replication, cell proliferation and specie generations. Several studies report a variable rate of genome stability (or instability) by using Sister Chromatid Exchange (SCE) test according to the species analysed, like cattle (Di Berardino and Shofner, 1979; Iannuzzi et al., 1991; Iannuzzi et al., 1991; Ciotola et al., 2005; Di Meo et al., 2011; Perucatti et al., 2016), river buffalo (Iannuzzi et al., 1988; Genualdo et al., 2012; Iannuzzi et al., 2015), sheep (Di Meo et al., 2000; Iannuzzi et al., 2004; Perucatti et al., 2006; Genualdo et al., 2015), goat (Di Meo et al., 1993; Lopez and Arruga, 1992; Wojcik and Smalec, 2012) and pig (Rubes, 1987; Peretti et al., 2006; Ciotola et al., 2014), while only few data are available about the genome stability variation in different breeds of the same species. Iannuzzi et al. (1991) found statically different mean values of SCE/cell between

Podolian and Friesian cattle breeds reared under similar conditions including diet, sex (all males) and age. Indeed, SCE-test may be influenced by the by technical conditions of cellular growth, BrdU-doses, sex, age, dietary habits and genes (Carrano et al., 1980; Latt et al., 1981; Waksvik et al., 1981; Soper et al., 1984; Wulf et al., 1986). No direct cytogenetic comparisons between different pig breeds have been performed so far. SCE-test has been largely applied to test chromosome stability in cells of animals naturally or in vitro exposed to mutagens. The number of SCEs in the cells of single animal or of animal groups is higher than the normal, showing higher chromosome fragility with increasing probability to get genetic mutations and/or chromosome aberrations.

In this study, representative groups of animals of two endangered pig breeds were, cytogenetically analysed and compared by using both CBA- and RBA-banding techniques, as well as the SCE-test to check for the presence of chromosomal abnormalities and their chromosome stability under their normal breeding and environmental conditions, respectively.

2. Materials and methods

2.1 Animals

Forty-two pigs from Casertana breed (22 males and 20 females, from 3 different farms) and 39 pigs from Nero Siciliano breed (19 males and 20 females, from 3 different farms) randomly selected, were cytogenetically investigated by using both C- and R-banding techniques for karyotype analyses, as well as the SCE-test for chromosome stability testing.

2.2. Cell cultures, CBA and RBA-banding, SCE, FISH-mapping

Peripheral blood lymphocytes were cultured for 72 h at 38° C in RPMI-medium enriched by foetal calf serum (10%), Antibiotics and Antimicotic mixure (1%) and Lectin (1.5%) as mitogen.

Three different types of cell cultures were set up: (**a**) normal culture (without addition of any base-analogue) for CBA-banding technique; (**b**) cultures treated for late incorporation of both BrdU (15 µg/ml) and Hoescht 33258 (30 µg/ml) both added six h before harvesting for R-banding and FISH-mapping techniques; (**c**) cultures treated with BrdU (10 µg/ml) 26 h before harvesting for SCE-test. Colcemid (0.1 µg/10 ml) was added 1.5 h before harvesting. A hypotonic treatment and three fixations with acetic acid/methanol (1:3) solution followed. Three drops of cell suspension were spread on wet and warm slides, air dried and kept at room temperature for some days. FISH-mapping analyses were performed for animals carrying chromosome abnormalities to confirm the chromosomes involved. The following BAC-clones (CHORI) were used: CH242-379A2 (start 4.740.641; end 5.086.289) mapping in the proximal q-arm region of SSC15; CH242-501J6 (start 4.104.594; end 4.360.253) mapping in the q-arm proximal region of SSC17. BAC 379A2 and 501J6 were labelled with biotin and digoxigenin, respectively using nick-translation kit (Roche applied science Inc.). Slides were then treated for FISH analysis with BAC clones overnight, allocated in a moist chamber. After detection steps with FITC-avidin and anti-digoxigenin antibodies, chromosomes were counterstained with Vectashield H 1000 (Vector Lab) antifade solution.

For more detailed information about CBA- and RBA-banding, as well as for FISH-mapping and SCE-test protocols see in Iannuzzi and Di Berardino (2008).

Fifty metaphases for CBA-banding, twenty RBA-banded metaphases (with five RBA-banded karyotypes), and at least 35 metaphase plates for SCE-test were studied each animal. The images were captured by using a Leica CTR 5500 fluorescence microscope equipped with 100x oil immersion lens, DAPI, FITC, Texas Red specific filters and Photometrics Sensys camera. R-banded karyotypes were arranged according to the standard karyotype (Gustavsson, 1988).

2.3. Statistical analyses

Mean values and standard deviations of SCE-data were calculated for both single animal and animal groups. Statistical analyses were performed between animal groups by using a t-student test. Bonferroni correction was applied as default restriction and differences were considered significant if $P \leq 0.05$.

3. Results and Discussion

3.1. CBA- and RBA-banding, FISH-mapping.

All studied animals showed normal diploid number (2n=38) and CBA-banding (Fig. 2a) except for two males (boars) from Nero Siciliano breed from the same farm, which showed an abnormal karyotype (2n=37) for the presence of an extra biarmed chromosome showing two HC-blocks at the centromere (dicentric chromosome) (Fig. 2b), differently from the remaining biarmed pairs which showed single HC-blocks. Subsequent RBA-banding revealed that this abnormal biarmed chromosome was originated by centric fusion translocation of pig chromosomes (SSC) 15 and 17 (Fig. 3a-b), as also confirmed by FISH-mapping analyses with two specific markers of SSC15 and SSC17 (Fig. 4). Since the animals were raised with an extensive or semi-extensive system in the same area where are largely present wild pigs (2n=36), it is possible that they were originated from crosses between domestic and wild pigs. Being the hybrids between Nero Siciliano and wild boar similar (see Fig. 1b and supplementary Fig. 1 for comparison), it is difficult to distinguish them from the purebred Nero Siciliano. After these results the two boars were eliminated from reproduction and a specific project for cytogenetic selection of Nero Siciliano breeders, at least the boars, before their use for reproduction is ongoing. This study underlines the importance of karyotype analyses in the animals before their use as breeders, especially in boars of this endangered breed for the reasons previously reported.

3.2. Sister Chromatid Exchange (SCE)-test.

Figure 5 shows a pig metaphase plate treated for SCE-test, while table 1 reports the data achieved in all animal groups of Casertana and Nero Siciliano breeds, respectively. No statistical differences were found between the SCE-mean values of Casertana (7.13±3.20) and Nero Siciliano (6.87±3.12) breeds, while statistical differences ($P<0.01$) were found between SCE-mean values of males (7.26±3.38) and females (6.59±2.90) in Nero Siciliano, as well as between females of Casertana (7.24±3.26) and Nero Siciliano (6.59±2.90) breeds. No statistical differences were found between males of the two breeds, as well as between males and females of Casertana breed.

The SCE-mean value found in the present study in Casertana were very similar to those earlier achieved in the same breed (Peretti et al., 2006). Therefore, the genome of this breed seems to be stable after many years, indicating that the environmental conditions have not changed so far. The SCE-test, applied for the first time in the Nero Siciliano, shows a SCE-mean value very similar to that obtained in the Casertana. Generally, SCE-mean values in swine species appear lower than those achieved in other species, supporting the hypothesis that pig genome is more stable than that of other species, probably depending also for the younger age of animals, compared to other domestic species, in consideration that SCE-test may be influenced also by the age (Soper et al., 1984; Peretti et al., 2006).

SCE-test appears very useful not only to establish the normal genome stability baseline of the animal species and breeds raised in wild breeding, but also to test animals, which are naturally (*in vivo* exposure) or *in vitro* (cells) exposed to mutagens. Recent studies performed in animals naturally exposed to dioxins revealed a statistical higher number of SCEs in sheep and cattle, compared to control groups raised in dioxins free areas (Iannuzzi et al., 2004; Perucatti et al., 2006; Di Meo et al., 2011; Genualdo et al., 2012). Significant higher values of SCEs have been found in sheep raised in polluted areas of Sardinia (Italy), compared to sheep raised in unpolluted areas (Genualdo et al., 2015). These differences were also confirmed in the same study by analysing the

Redox-status of sheep, supporting a possible relationship between physiological stress and chromosome fragility. SCE-test has been also used to test lymphocytes of sheep exposed *in vitro* with furocumarin extracts from *Bituminosa bituminosa*, an interesting legume which is in full production during the summer (when the other legumes are not) (Iannuzzi et al., 2016).

In conclusion, the presence of hybrids between domestic and wild boar in the Nero Siciliano breed demonstrates that it is necessary and urgent to develop a specific breeding program to avoid contamination in this endangered breed with the wild boar present in the same area where this breed is raised in Sicily. In particular, cytogenetic analyses should be performed at least in all boars used in both natural and AI inseminations, for a radical eradication of hybrid animals. SCE-test revealed a good chromosome stability in both pig breeds studied supporting the thesis that breeding conditions in which these animals are managed is the best for the two endangered breeds.

Acknowledgements. This study has been supported by PSR Campania 2007-2013: Misura 214 azione e2, - RARECa and, Misura CISIA-Pig (National Research Council of Rome).

Ethical approval. The experimental project has been approved by the Ethical Commission of the National Research Council (CNR), ISPAAM of Naples, with registered number 643 of May 30, 2017

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

REFERENCES

Arroyo-Nombela, J.J., Rodriguez-Murcia, C., Abaigar, T., Vericad, J.R., 1990. Cytogenetic analysis (GTG, GBG and NOR bands) of wild boar population (*Sus scrofa scrofa*) with chromosomal polymorphism in the southeast of Spain. Genet. Sel. Evol. 22, 1-9.

Bosma, A.A., 1976. Chromosomal polymorphism and G-banding patterns in the wild boars (*Sus scrofa* L) from the Netherlands. Genetica 46, 391-399.

Carrano, A.V., Minkler, G.L., Setka, D.G., Moore, D.H., 1980. Variation in the baseline sister chromatid exchange frequency in human lymphocytes. Environm. Mutag. 2, 325-337.

Ciotola, F., Peretti, V., Di Meo, G.P., Perucatti, A., Iannuzzi, L., Barbieri, V., 2005. Sister chromatid exchanges (SCE) in the Agerolese cattle population. Vet. Res. Comm. 29(2), 359-61.

Ciotola F., Albarella S., Scopino G., Carpino S., Monaco F., Peretti V., 2014. Crossbreeding effect on genome stability in pig (Sus scrofa scrofa). Folia Biologica-Krakow 62(1):23-28.

Di Berardino, D., Shoffner, R.N., 1979. Sister chromatid exchange in chromosomes of cattle.

J. Dairy Sci. 62(4), 627-32.

Di Meo, G.P., Iannuzzi, L., Perucatti, A., Ferrara, L., Pizzillo, M., Rubino, R., 1993. Sister chromatid exchange in the goat (*Capra hircus*). Hereditas 118, 35–38.

Di Meo, G.P., Perucatti, A., Fornataro, D., Incarnato, D., Ferrara, L., Matassino, D., Iannuzzi, L., 2000. Sister chromatid exchange in chromosomes of sheep (*Ovis aries*). Cytobios 101(397), 71-8.

Di Meo, G.P., Perucatti, A., Genualdo, V., Caputi-Jambrenghi, A., Rasero, R., Nebbia, C., Iannuzzi, L., 2011. Chromosome fragility in dairy cows exposed to dioxins and dioxin-like PCBs. Mutagenesis 26(2), 269-72.

Genualdo, V., Perucatti, A., Iannuzzi, A., Di Meo, G.P., Spagnuolo, S., Caputi-Jambrenghi, A., Coletta, A., Vonghia, G., Iannuzzi, L., 2012. Chromosome fragility in river buffalo cows exposed to dioxins. J. Appl. Genet. 53, 221-226.

Genualdo, V., Perucatti, A., Pauciullo, A., Iannuzzi, A., Incarnato, D., Spagnuolo, M.S., Solinas, N., Bullitta, S., Iannuzzi, L., 2015. Analysis of chromosome damage by sister chromatid exchange (SCE) and redox homeostasis characterization on sheep flocks from Sardinian pasturelands. Sci. Total Env. 527–528, 393–400.

Guastella, A.M., Criscione, A., Marletta, D., Zuccaro, A., Chies, L., Bordonaro, S., 2010. Molecular characterization and genetic structure of the Nero Siciliano pig breed. Genetics and Molecular Biology, 33 (4), 650-656.

Gustavsson, I. (Co-ordinator), 1988. Standard karyotype of the domestic pig. Committee for the Standardized Karyotype of the Domestic Pig. Hereditas 109, 151-157.

Iannuzzi, A., Perucatti, A., Genualdo, V., Pauciullo, A., Melis, R., Porqueddu, C., Marchetti, M., Usai, M., Iannuzzi, L,, 2016. Sister chromatid exchange test in river buffalo lymphocytes treated in vitro with furocoumarin extracts. Mutagenesis 31(5), 547-51.

Iannuzzi, L., Di Berardino, D., 2008. Tools of the trade: diagnostics and research in domestic animal cytogenetics. J. Appl. Genet. 49(4), 357-66.

Iannuzzi, L., Perucatti, A., Di Meo, G.P., Ferrara, L., 1988. Sister chromatid exchange in chromosomes of river buffalo (*Bubalus bubalis* L.). Caryologia 41, 237-244.

Iannuzzi, L., Di Meo, G.P., Perucatti, A., Ferrara, L., 1990. Mitomycin C-induced sister chromatid exchange in X-chromosomes of Bovidae. J. Hered. 81(1), 78-80.

Iannuzzi, L., Di Meo, G.P., Perucatti, A., Ferrara, L., Gustavsson, I., 1991. Sister chromatid exchange in chromosomes of cattle from three different breeds reared under similar conditions. Hereditas 114(3), 201-5.

Iannuzzi, L., Di Meo, G.P., Perucatti , A., Ferrara, L., Gustavsson, I., 1991. Sister chromatid exchange in chromosomes of cattle from three different breeds reared under similar conditions. Hereditas 114, 201-205.

Iannuzzi, L., Perucatti, A., Di Meo, G.P., Polimeno, F., Ciotola, F., Incarnato, D., Peretti, V., Caputi-Jambrenghi, A., Pecoraro, A., Manniti, F., D'Alessandro, A., Vonghia, G., 2004. Chromosome fragility in two sheep flocks exposed to dioxins during pasturage. Mutagenesis 19(5), 355-359.

Latt, S.A., Allen, J., Bloom, S.E., Carrano, A., Falke, A., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitfield, B., Wolff, S., 1981. Sister chromatid exchanges: A report of the Gene-Tox program. – Mut. Res 87, 17-62.

Lopez, N.L., Arruga, M.V., 1992. Sister chromatid exchanges (SCEs) analysis in goats. Caryologia 45(2), 135-144.

Peretti, V., Ciotola, F., Cataldo, D., Albarella, S., Di Meo, G.P., Perucatti, A., Barbieri, V., Iannuzzi, L., 2006. Sister chromatid exchange (SCE) for the first time in Casertana pig. Hereditas 143, 113-116.

Perucatti, A., Di Meo, G.P., Albarella, S., Ciotola, F., Incarnato, D., Caputi-Jambrenghi, A., Peretti, V., Vonghia, G., Iannuzzi, L., 2006. Increased frequencies of both chromosome abnormalities and SCEs in two sheep flocks exposed to high dioxin levels during pasturage. Mutagenesis 21, 67-75.

Perucatti, A., Genualdo, V., Colonna, M.A., Giannico, F., Incarnato, D., Lubrano-Lavadera, G., Iorio, C., Vonghia, L., Caputi-Jambrenghi, A., Iannuzzi, L., Iannuzzi, A., 2016. Chromosome instability in lymphocytes of Friesian cows naturally exposed to dioxins being raised close to a metallurgic factory area in southern Italy. Caryologia 69(2), 1-8.

Rangel-Figueiredo, M.T., Di Meo, G.P., Iannuzzi, L., 1995. Sister chromatid exchange (SCE) in cattle: a comparison between normal and rob(1;29) carrying karyotypes. Hereditas 123, 25-29.

Rubes, J., 1987. Chromosomal aberrations and sister-chromatid exchanges in swine. Mut. Res. 191(2), 105-9.

Soper, K., Stalley, P.D., Galloway, S.M., Smith, J.G., Nichols, W.W., Wolman, S.R., 1984. Sister chromatid exchange (SCE) report (in control subject\ in a study of occupationally exposed workers. Mut. Res. 129, 77-88.

Sysa, P.S., Sławomirsk, i J., Gromadzka, J., 1984. Cytogenetic studies of hybridization in wild boars (*Sus scrofa ferus*) and domestic swine (*Sus scrofa dom.*). Arch. Vet. Pol. 24(1), 89-95.

Waksvik, H., Magnus, P., Berg, K., 1981. Effects of age, sex and genes on sister chromatid exchange. Clin. Genet. 20, 449–454.

Wójcik, E., Smalec, E., Danielewicz, A., 2011. Sister chromatid exchange in selected horse breeds (*Equus caballus*). Archiv Tierz. 54(2), 107-114.

Wulf, H.C., Kromann, N., Kausgaard, N., Hansen, J.C., Niebuhr, E., Alboge, K., 1986. Sister chromatid exchange (SCE) in Greenlandic Eskimoes. Dose-response relationship between SCE and seal diet, smoking, and blood cadmium and mercury concentrations. Sci. Total Envir. 48, 81-94.

Figure legend

Figure 1. Casertana (A) and Nero Siciliano (B) breeds.

Figure 2. CBA-banded metaphase plates in two boars with normal (2n=38) (A) and abnormal (2n=37) (B) diploid number, the latter in Nero Siciliano boar carrying a centric fusion translocation showing a pronounced HC-block involving both chromosome arms (large arrow). The Y-chromosome is also shown.

Figure 3. RBA-banded metaphase (A) and relative karyotype (B) of a pig boar 2n=37 carrying the rob(15;17) being probably hybrid between domestic and wild pigs.

Figure 4. A metaphase plate (2n=37) of a boar carrying rob(15;17) and treated for FISH-mapping technique with specific BAC-clones mapping SSC15 (green signals) and SSC17 (red signals) (arrows).

Figure 5. A male pig metaphase (2n=38) treated for SCE-test and showing three SCEs (arrows).

Supplementary figure 1. One of two boars found carrier of rob(15;17) being probably derived by crossing between domestic (Nero Siciliano, 2n=38) and wild (2n=36) boar. Note the similarity between the hybrid pig with the purebred Nero Siciliano (see figure 1B for comparison).

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

All Authors

Figure 1
Click here to download high resolution image

Figure 2

Click here to download high resolution image

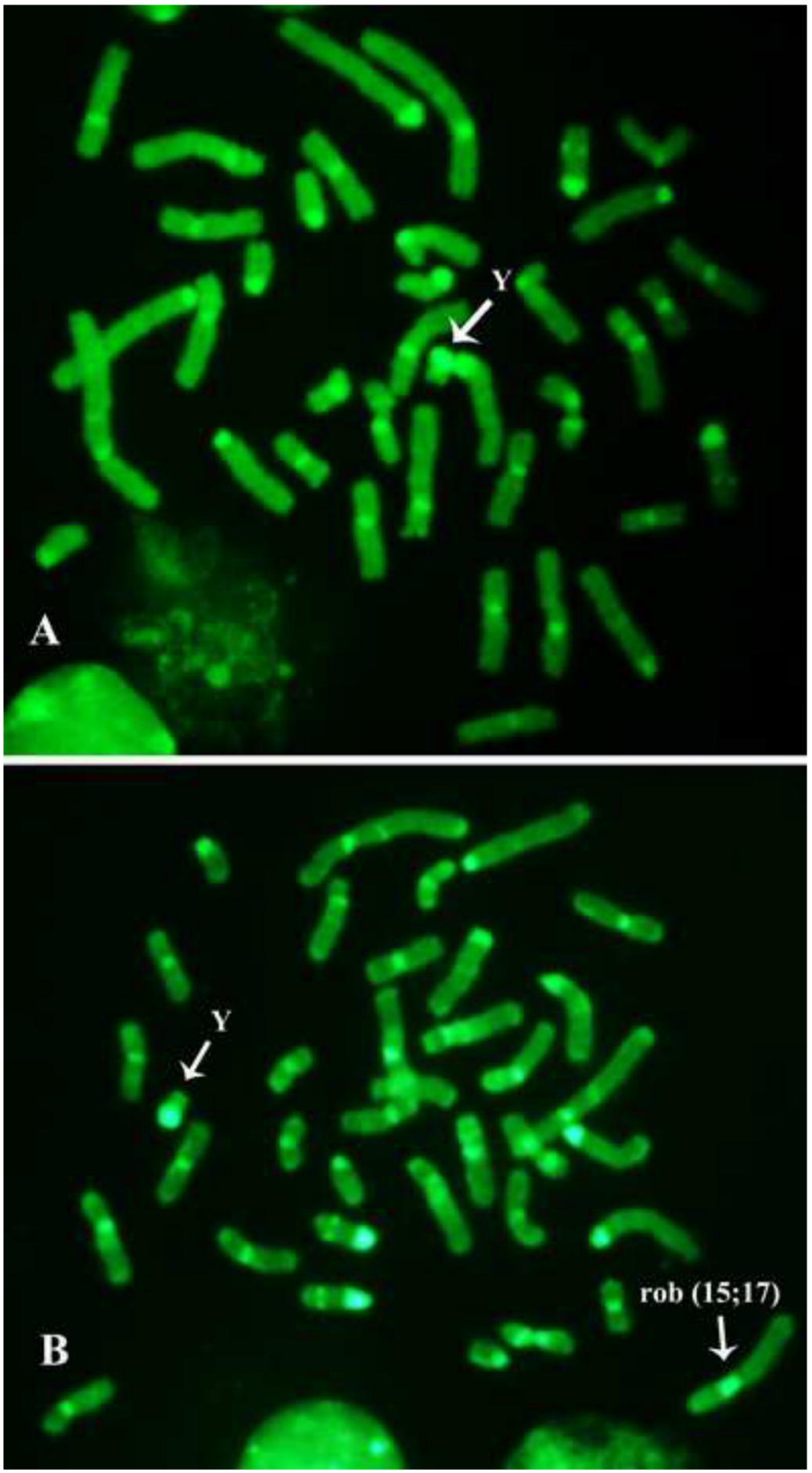
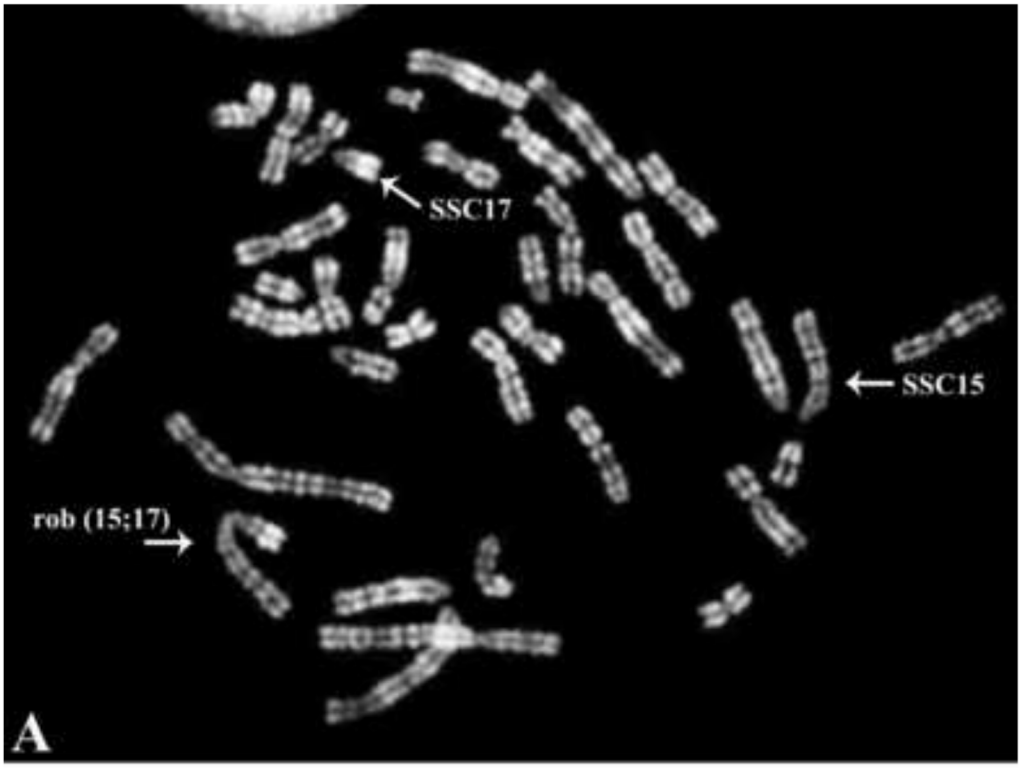


Figure 3

Click here to download high resolution image



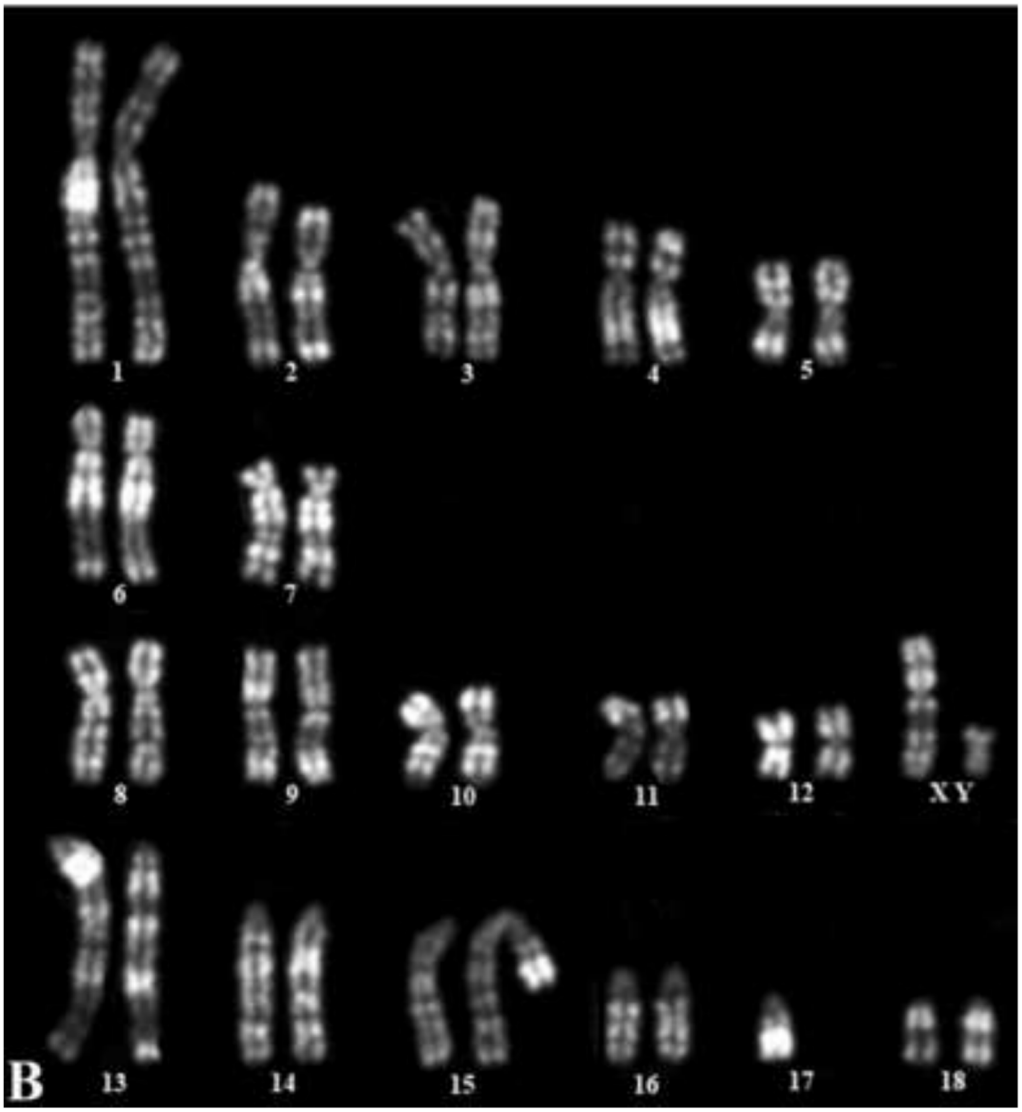


Figure 4

Click here to download high resolution image

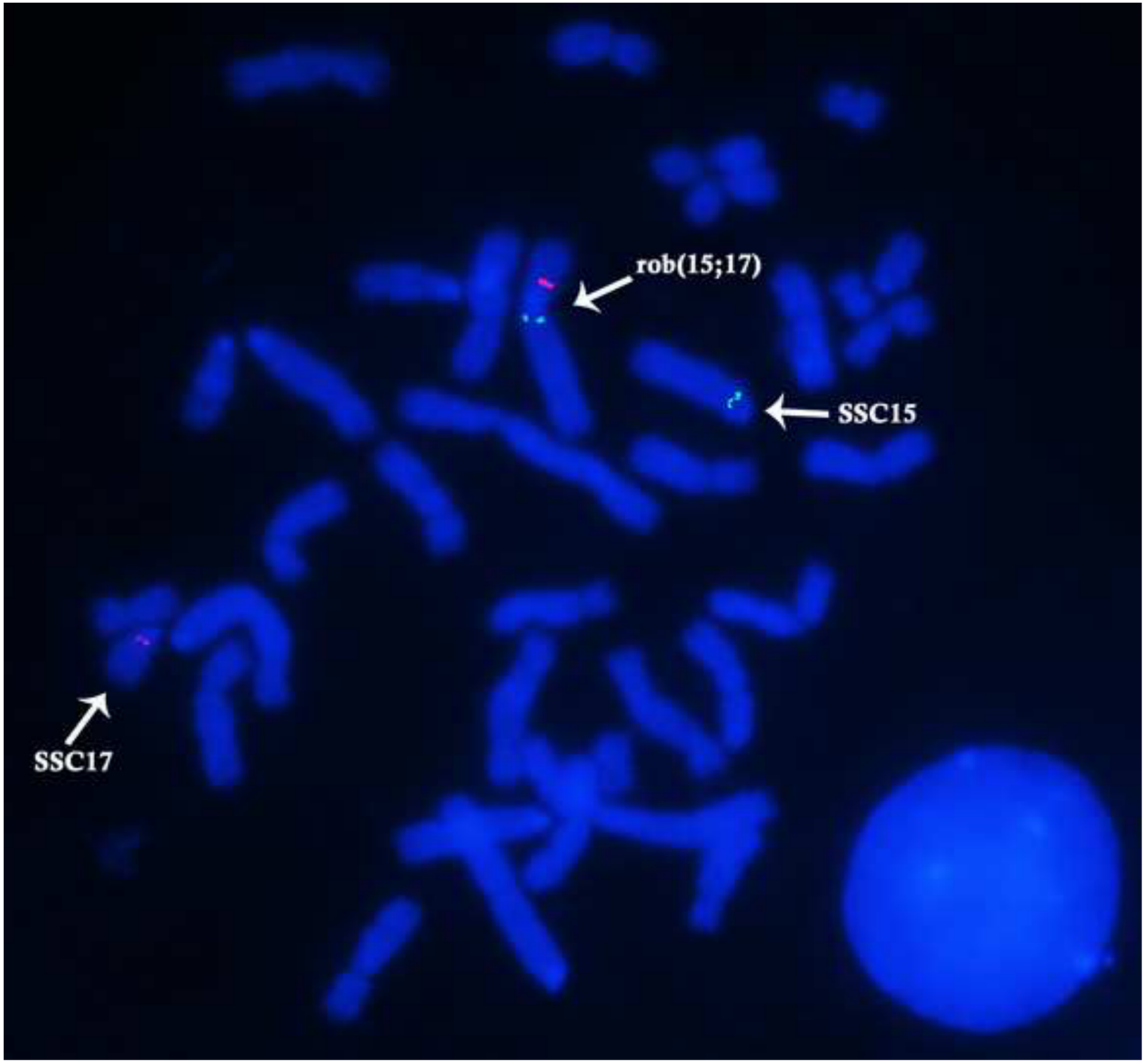


Figure 5

Click here to download high resolution image

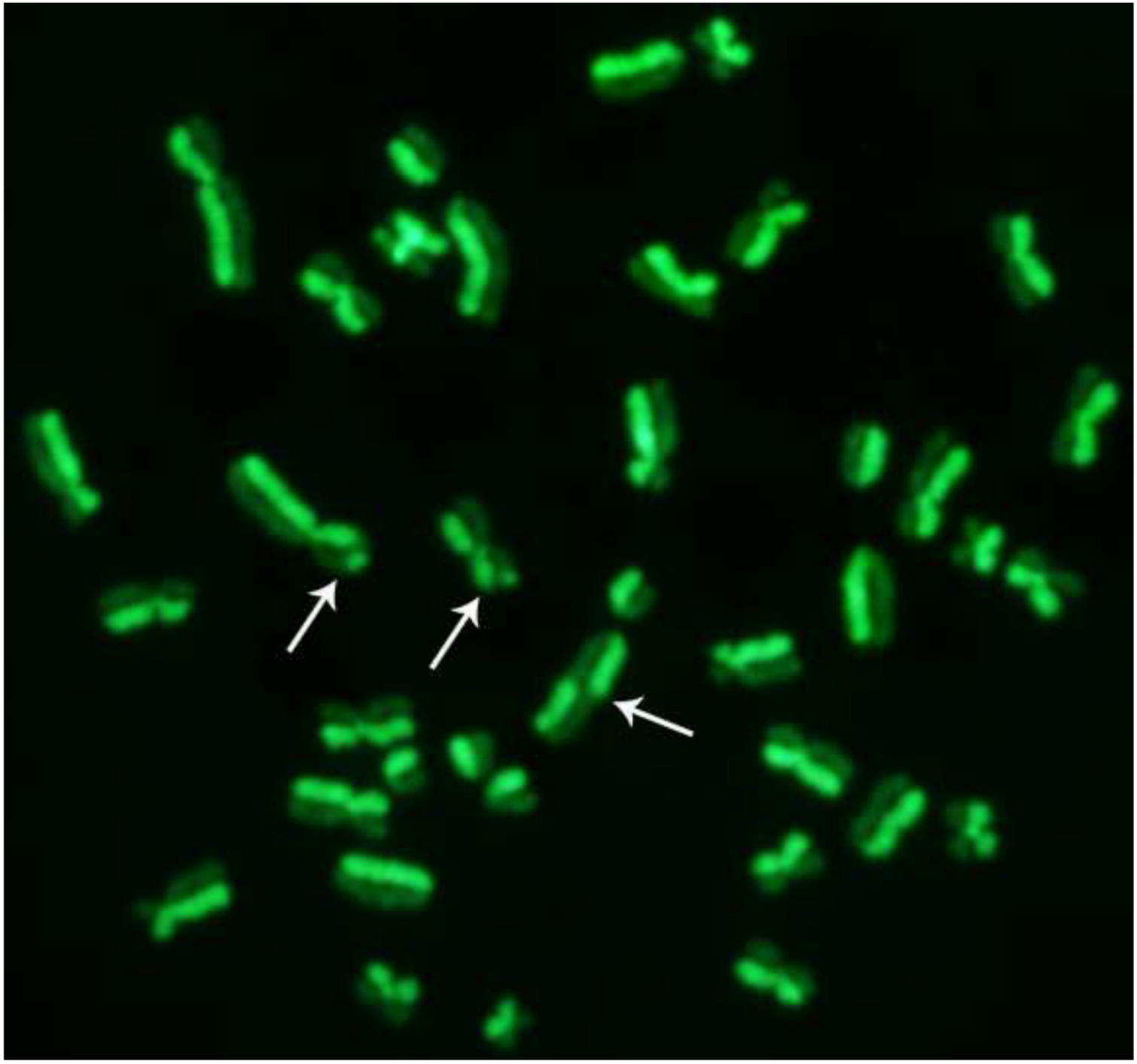


Table 1

Table 1. Sister chromatid Exchanges (SCEs) in Casertana and Sicily black pig breeds

Pig breed	Animals Males (M) Females (F) N	Examined Cells N	SCEs Mean/cell ± sd
Casertana Black (*Nero Casertano*)	M 22	770	6.98 ± 3.10
	F 20	700	7.24 ± 3.26
	M+F 42	**1470**	**7.13 ± 3.20**
Sicily Black (*Nero Siciliano*)	M 19	665	7.26 ± 3.38
	F 20	700	6.59 ± 2.90
	M+F 39	**1365**	**6.87 ± 3.12**

Optional e-only supplementary files

Click here to download Optional e-only supplementary files: Supplementary Figure 1.jpg