Comparative Cytogenetic Analysis of Sex Chromosomes in Several *Canidae* Species Using Zoo-FISH*

Monika BUGNO-PONIEWIERSKA, Agnieszka SOJECKA, Klaudia PAWLINA, Andrzej JAKUBCZAK, and Grażyna JEŻEWSKA-WITKOWSKA

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Sex chromosome differentiation began early during mammalian evolution. The karyotype of almost all placental mammals living today includes a pair of heterosomes: XX in females and XY in males. The genomes of different species may contain homologous synteny blocks indicating that they share a common ancestry. One of the tools used for their identification is the Zoo-FISH technique. The aim of the study was to determine whether sex chromosomes of some members of the Canidae family (the domestic dog, the red fox, the arctic fox, an interspecific hybrid: arctic fox × red fox and the Chinese raccoon dog) are evolutionarily conservative. Comparative cytogenetic analysis by Zoo-FISH using painting probes specific to domestic dog heterosomes was performed. The results show the presence of homologous synteny covering the entire structures of the X and the Y chromosomes. This suggests that sex chromosomes are conserved in the Canidae family. The data obtained through Zoo-FISH karyotype analysis append information obtained using other comparative genomics methods, giving a more complete depiction of genome evolution.

Key words: Canidae, sex chromosomes, evolution of karyotypes, homologous synteny block, Zoo-FISH.

Monika BUGNO-PONIEWIERSKA, Klaudia PAWLINA, Laboratory of Genomics, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland.
E-mail: mbugno@izoo.krakow.pl
Agnieszka SOJECKA, Interdepartmental Biotechnology Studies Program, University of Agriculture, Kraków, Poland.
Andrzej JAKUBCZAK, Grażyna JEŻEWSKA-WITKOWSKA, Department of Biological Basis of Animal Production, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland.

The sex chromosomes of placental mammals evolved from a pair of homologous autosomes present in the genome of their common ancestor (BACHTROG 2006; GRAVES 2006; JANOUSEK & MRACKOVA 2010). This is also supported by the presence of the pseudoautosomal region and other homologous nucleotide sequences (CHARLESWORTH *et al*. 2005). Natural selection, by acting to preserve sex-specific regions on different chromosomes, contributed to suppression of recombination between them (CHARLESWORTH *et al*. 2005). Further limitation to the exchange of genetic material between the original X and Y chromosomes occurred in successive stages of their evolution (SKALETSKY *et al*. 2003). This was supplemented by intrachromosomal inversions and translocations of autosomal chromosomes fragments (BERGERO & CHARLESWORTH 2009; WILSON & MAKOVA 2009; CASTILLO *et al*. 2010; KIRKPATRICK 2010).

The inability to exchange genetic material during meiosis gave rise to the independent evolution of non-recombinant X and Y chromosome regions (GRAVES 2010). Crossing-over was limited to the pseudoautosomal region (PAR) (WATERS *et al*. 2007). Evolutionary processes occur independently between separate phylogenetic lines. This explains the considerable variation of DNA sequences and the different stages of Y chromosome degeneration in different, even closely related, species (GRAVES 2008). By contrast, the X chromosome shows a high degree of conservation in mammals (WATERS *et al*. 2007). The genome of most female placentals has two X chromosomes which may

pair and recombine during meiosis. This increases the efficiency of purifying selection and elimination of mutations. Except for some genes, the rate of change in DNA sequences of the X chromosome is lower compared to the Y chromosome and autosomes (Wilson & Makova 2009; Graves 2010). This helps to maintain a relatively low level of variation in this heterosome. Both the content of genes, their nucleotide sequence and often their localization are highly similar, also between phylogenetically distant individuals (Bachtrog 2006).

The Canidae family is represented by over 30 extant species. These include the gray wolf (*Canis lapus*) and its domesticated form, the domestic dog (*Canis familiaris*), the dhole (*Cuon alpinus*), the red fox (*Vulpes vulpes*), the arctic fox (*Alopex lagopus*), the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*), the Japanese raccoon dog (*Nyctereutes procyonoides viverrinus)* and the gray fox (*Urocyon cinereoargenteus*) (Graphodatsky *et al*. 2008). Analysis of the nucleotide sequence of nuclear DNA revealed that members of the Canidae family are closely related (Lindblad-Toh *et al*. 2005). However, the Canidae genome underwent many chromosomal rearrangements during its evolution. These were mainly fissions and centric and tandem fusions (Ferguson-Smith & Trifonov 2007). This is confirmed by a high degree of differentiation of karyotype structure between species in this family. The diploid chromosome number (A) ranges from 34 in the red fox to 78 in the domestic dog (Graphodatsky *et al*. 2000). There may be supernumerary chromosomes in the raccoon dog and the red fox genome (B) (Nie *et al*. 2003). The diploid chromosome number is also polymorphic in the arctic fox (2n = 48-50), which is due to the frequent incidence of Robertsonian translocations in this species (Graphodatsky *et al*. 2000). The evolutionarily most primitive members of the modern canids are the gray fox and the raccoon dog, evidenced by the presence of many chromosome segments with homology to the felid genome (Wayne & Ostrander 1999; Lindblad-Toh *et al*. 2005).

The aim of the study was to analyse sex chromosome conservation in selected Canidae species. Zoo-FISH with painting probes specific to X and Y chromosomes of the domestic dog was carried out to detect homologous synteny covering the complete heterosome structures of the red fox, the arctic fox, an interspecific hybrid (arctic fox × red fox) and the Chinese raccoon dog.

Material and Methods

Permission for this study was obtained from the II Local Ethics Committee Kraków – resolution number 771/2010 of 22 June 20101.

The experimental material used in this study came from several species of the Canidae family: the domestic dog, the red fox, the arctic fox, the interspecific hybrid (arctic fox × red fox) and the Chinese raccoon dog. Cytogenetic analysis was performed on microscope preparations of metaphase chromosomes obtained after a routine culture of peripheral blood lymphocytes.

Chromosome preparations were incubated in 10% pepsin solution at 37°C for 10 min. This was followed by washing in PBS buffer at room temperature (two times for 5 min) and in PBS containing $MgCl_2$ (once for 5 min). In the next stage, chromosomes were dehydrated in increasing ethanol concentrations of 70%, 80%, 95% (3 min each). Then, chromosomes were denatured by treatment with 70% formamide at 70°C for 2.5 min, and immediately placed in vessels with ethanol cooled to -20°C with serial concentrations of 70%, 80%, 90% and 100% (3 min each). The slides were dried at room temperature.

Commercial painting probes (WCP) specific to the X and Y chromosomes of the domestic dog were suspended in the hybridization mixture [Cambio, UK]. The probes were denatured at 70°C for 10 min, mounted on the preparations and the edges were sealed with fixogum. Hybridization was performed in an oven for 24-48 h at 37°C.

After hybridization, washing in 50% formamide at 42°C and then in Detergent Wash Solution was performed to remove the unbound probe. Signal amplification was performed using a commercial kit for detection of hybridization signals (Star*FISH) containing avidin-FITC and goat anti-avidin-FITC antibodies [Cambio, UK].

Microscopic observations were performed using a Zeiss Axiophot fluorescence microscope equipped with appropriate filters, a camera and LUCIA software (Laboratory Imaging LTD).

Results

The specificity of molecular probes used in the study was tested by control analysis of the domestic dog sex chromosomes (X and Y) using fluorescence *in situ* hybridization (FISH) (Fig. 1) which were later used for interspecific comparative analysis of karyotypes and chromosomes of selected Canidae species using Zoo-FISH. Clear hybridization signals were observed on the heterosomes of all animals studied (Fig. 2A-2D).

Discussion

Homologous synteny blocks are chromosome segments present in the genomes of two or more species which contain corresponding (homolo-
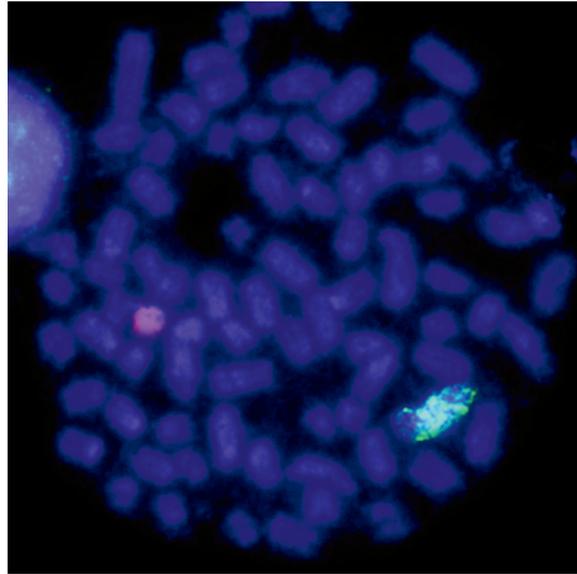
Fig. 1. Metaphase plate of the domestic dog (*Canis familiaris*). Fluorescence signals resulting from hybridization of molecular probes specific to the domestic dog sex chromosomes are visible (green – X chromosome, red – Y chromosome), 100x.
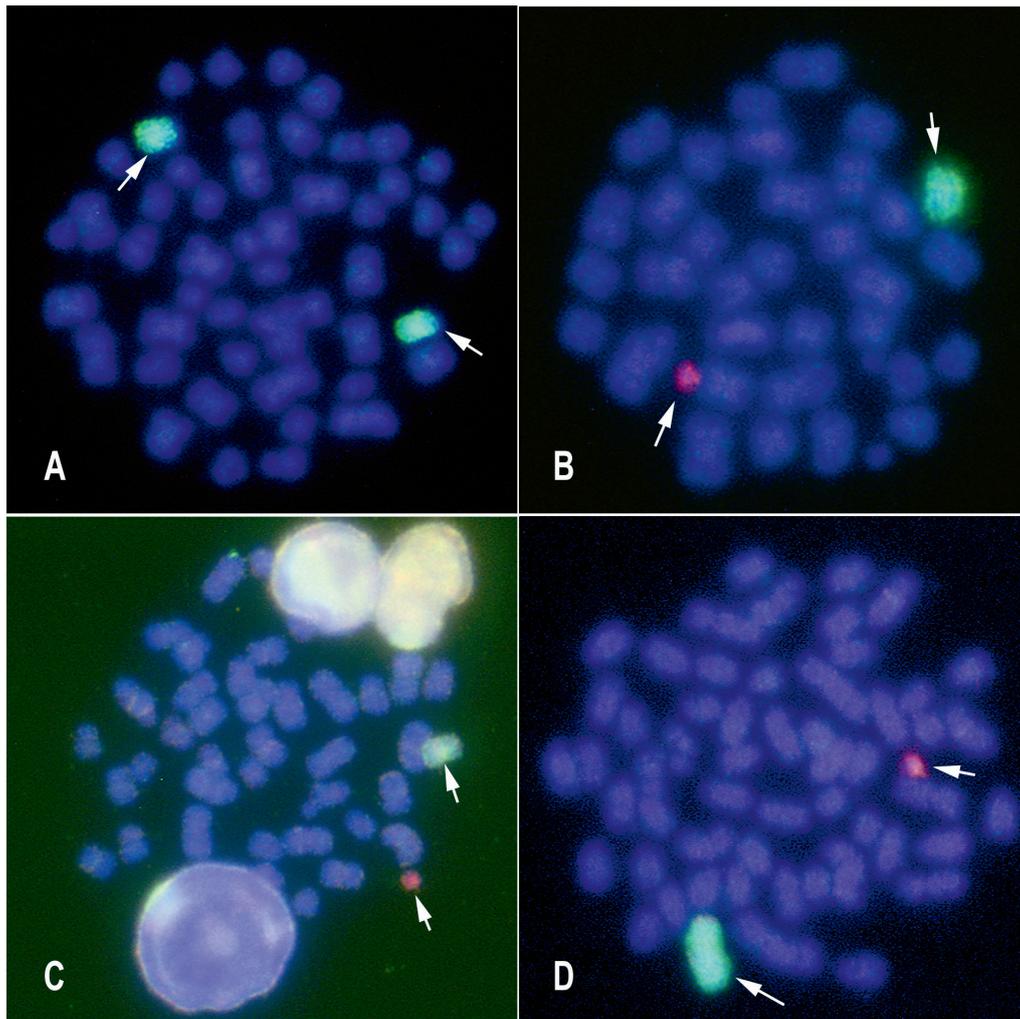
Fig. 2. A – Metaphase plate of the arctic fox (*Alopex lagopus*); B – Metaphase plate of the red fox (*Vulpes vulpes*); C – Metaphase plate of the interspecific hybrid (arctic fox × red fox); D – Metaphase plate of the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*). Arrows indicate fluorescence signals resulting from hybridization of probes specific to the domestic dog heterosomes (green – the X chromosome, red – the Y chromosome), x100.

gous) DNA sequences (HARDISON 2003; NG *et al*. 2009). Their presence is indicative of common ancestry (FERGUSON-SMITH & TRIFONOV 2007). The order in which certain sequences are located in a synteny block may be largely or completely preserved. It was observed, however, that small rearrangements leading to a new set of genes in a given synteny block are not a rare occurrence (PEVZNER & TESLER 2003; NG *et al*. 2009). Conservative genome segments may encompass smaller or greater chromosome areas as well as a chromosome arm or the whole chromosome structure (CHOWDHARY & RAUDSEPP 2001; MURPHY *et al*. 2004). One of the main tools used for their identification is the Zoo-FISH technique. In our study, we used whole chromosome painting probes specific to the sex chromosomes of the domestic dog which were hybridized with the genetic material from four other canids. This enabled the confirmation or exclusion of the presence of homologous synteny encompassing complete sex chromosome structures.

Our observations revealed that the X and the Y chromosomes of the domestic dog show a high degree of homology with the heterosomes of the red fox, the arctic fox, the interspecific hybrid (arctic fox × red fox) and the Chinese raccoon dog. This suggests that the sex chromosomes of the analysed species are conservative, meaning that their structure did not change much during the course of canid evolution. Earlier research showed that the ancestral carnivore karyotype (ACK) underwent many chromosome rearrangements during the evolution of Canidae species (GRAPHODATSKY *et al*. 2000). This is confirmed by marked differences in the karyotype structure of modern canids, paralleled by the presence of homologous syntenic regions in their genomes. GRAPHODATSKY *et al*. (2008) confirmed the presence of 42 conservative autosomal chromosomal regions characteristic of this family. They occur in different combinations in different species, which is evidence of intensive chromosomal rearrangements that have occurred during the speciation of modern Canidae. Research to date demonstrated, however, that unlike the high variation of autosomes, heterosomes have survived many years of independent evolution almost unchanged. This suggests that in this family, the structural changes in sex chromosomes were less frequent and covered smaller areas of the genome than in the autosomal chromosomes. The conservation of the sex chromosomes in Canidae manifests itself at several levels, from morphology to G-bands and localization of individual genes (YANG *et al*. 1999; NOWACKA-WOSZUK & SWITONSKI 2009).

Contemporary research on several canid species has revealed extensive homology of X and Y chromosomes between species. The homologous synteny covering the complete structures of both heterosomes has been retained, among others, between the Japanese raccoon dog and the arctic fox, between the Japanese raccoon dog and the crab-eating fox (*Cerdocyon thous*), between the domestic dog and the dhole, between the domestic dog and the gray fox and between the red fox and the corsac fox (*Vulpes corsac*) (NASH *et al*. 2001; GRAPHODATSKY *et al*. 2008).

The results of the present study confirm the earlier observations of sex chromosome conservation in Canidae. Complete homology of X chromosomes was found using Zoo-FISH in the domestic dog and the Chinese raccoon dog (NIE *et al*. 2003). The painting probe specific to the Y chromosome of *Canis familiaris*, used by the authors cited above, covered the entire Y chromosome and the distal fragment of the shorter arm of the X chromosome (Xpter) in the raccoon dog containing the pseudoautosomal region. Sex chromosome conservation in both species was also established at the level of GTG-banding patterns which turned out to be fully homologous. Analogous results were obtained when the domestic dog X chromosome was compared to the red fox and the arctic fox heterosomes (GRAPHODATSKY *et al*. 2000). In the work cited above, no information is provided on the homology of Y chromosomes in the species compared. Our observations prove, however, that also the domestic dog and the red fox Y chromosomes are evolutionarily conservative.

The X chromosome is the most conservative of all chromosomes that constitute the mammalian karyotype. The set of genes located on this chromosome and even their localization are identical or highly similar across species. Conservation was observed not only within the same family but also between less related species. Earlier cytogenetic studies showed that the X chromosome of the domestic dog is largely homologous to the X chromosome of the domestic cat (*Felis catus*), the African lion (*Panthera leo*), the clouded leopard (*Neofelis nebulosa*) and the Malayan sun bear (*Helarctos malayanus*), i.e. the species of one order, Carnivora (YANG *et al*. 2000; TIAN *et al*. 2004). X chromosome conservation also extends to more phylogenetically distant groups. Reciprocal chromosome painting revealed the presence of evolutionarily conservative segments on the X chromosome in the domestic dog and humans (BREEN *et al*. 1999; YANG *et al*. 1999).

The Y chromosome is usually the smallest or one of the smallest chromosomes in the karyotype. The relatively high heterochromatin content and the rapid rate of DNA changes, coupled with a significant effect of genetic drift have also contributed to the high morphological diversity and to the considerable differences in the content of coding and

non-coding sequences present on the Y chromosomes in distinct groups of mammals (STANYON *et al*. 2008; KIRSCH *et al*. 2009). Comparative analysis of the domestic dog and human genomes using chromosome painting showed a lack of homology between Y chromosomes of the investigated species (BREEN *et al*. 1999; YANG *et al*. 1999). This demonstrated that the Y chromosome is the least conservative of all chromosomes examined. The Y chromosome often varies also between species belonging to the same order or family. Interspecific chromosome painting analysis points to the loss of homologous synteny covering the Y chromosome between the domestic cat and the Japanese raccoon dog (NASH *et al*. 2001). However, the same authors detected intensive hybridization of the painting probes specific to the Y chromosome of the Japanese raccoon dog with the chromosomes of the arctic fox and the crab-eating fox, i.e. species of the same family. NIE *et al*. (2003) observed homologous synteny encompassing the whole Y chromosome of the domestic dog, the Chinese raccoon dog and the Japanese raccoon dog. Our findings also indicate that the Y chromosome of Canidae species is evolutionarily conservative. The high degree of Y chromosome homology that we observed speaks in favour of the high relatedness between the analysed species.

Our study revealed that the genomes of the arctic fox, the red fox, the interspecific hybrid (arctic fox × red fox) and the Chinese raccoon dog have synteny blocks covering the complete structures of the X and the Y chromosomes which are homologous to the domestic dog heterosomes. The results suggest that the structure of both sex chromosomes within the Canidae family is evolutionarily conservative. The X and Y conservatism is crucial for maintaining genes and their order intact during reproduction and inheritance, obviating unwanted changes which can be potentially harmful to an organism.

References

BACHTROG D. 2006. A dynamic view of sex chromosome evolution. Curr. Opin. Genet. Dev. **16**: 578-585.

BERGERO R., CHARLESWORTH D. 2009. The evolution of restricted recombination in sex chromosomes. Trends Ecol. Evol. **24**: 94-102.

BREEN M., THOMAS R., BEENS M. M., CARTER N. P., LANGFORD C. F. 1999. Reciprocal chromosome painting reveals detailed regions of conserved synteny between the karyotypes of the domestic dog (*Canis familiaris*) and human. Genomics **61**: 145-155.

CASTILLO R. D., BIDAU C. J., MARTI D. A. 2010. Neo-sex chromosome diversity in Neotropical melanopline grasshoppers (Melanoplinae, Acrididae). Genetica **138**: 775-786.

CHARLESWORTH D., CHARLESWORTH B., MARAIS G. 2005. Steps in the evolution of heteromorphic sex chromosomes. Heredity **95**: 118-128.

CHOWDHARY B. P., RAUDSEPP P. 2001. Chromosome painting in farm, pet and wild animals. Meth. Cell Sci. **23**: 37-55.

FERGUSON-SMITH M. A., TRIFONOV V. 2007. Mammalian karyotype evolution. Nature Rev. Genet. **8**: 950-962.

GRAPHODATSKY A. S., YANG F., O'BRIEN P. C. M., SERDUKOVA N., MILNE B. S., TRIFONOV V., FERGUSON-SMITH M. A. 2000. A comparative chromosome map of the Arctic fox, red fox and dog defined by chromosome painting and high resolution G banding. Chrom. Res. **8**: 253-263.

GRAPHODATSKY S. A., PERELMAN P. L., SOKOLOVSKAYA N. V., BEKLEMISHEVA V. R., SERDUKOVA N. A., DOBIGNY G., O'BRIEN S. J., FERGUSON-SMITH M. A., YANG F. 2008. Phylogenomics of the dog and fox family (Canidae, Carnivora) revealed by chromosome painting. Chrom. Res. **16**: 129-143.

GRAVES J. A. M. 2006. Sex chromosome specialization and degeneration in mammals. Cell **124**: 901-914.

GRAVES J. A. M. 2008. Weird animal genomes and the evolution of vertebrate sex and sex chromosomes. Ann. Rev. Genet. **42**: 565-586.

GRAVES J. A. M. 2010. Review: sex chromosome evolution and the expression of sex specific genes in the Placenta. Placenta **31**: S27-S32.

HARDISON R. C. 2003. Comparative genomics. PLoS Biol., 1:E58, doi:10.1371/journal.pbio.0000058.

JANOUSEK B., MRACKOVA M. 2010. Sex chromosomes and sex determination pathway dynamics in plant and animal models. Biological Journal of the Linnean Society **100**: 737-752.

KIRKPATRICK M. 2010. How and why chromosome inversions evolve. PLoS Biol., 8:e1000501, doi:10.1371/journal.pbio.1000501.

KIRSCH S., HODLER C., SHEMPP W. 2009. A non-human primate BAC resource to study interchromosomal segmental duplications. Cytogen. Gen.Res. **25**: 253-259.

LINDBLAD-TOH K., WADE C. M., MIKKELSEN T. S., KARLSSON E. K., JAFFE D. B., KAMAL M., CLAMP M., CHANG J. L., KULBOKAS III E. J., ZODY M. C., MAUCELI E., XIE X., BREEN M., WAYNE R. K., OSTRANDER E. A., POINTING C. P., GALIBERT F., SMITH D. R., deJONG P. J., KIRKNESS E. *et al*. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature **438**: 803-819.

MURPHY W. J., PEVZNER P. A., O'BRIEN S. J. 2004. Mammalian phylogenomics comes of age. Trends Genet. **20**: 631-639.

NASH W. G., Menninger J.C., Wienberg J., Padilla-NASH H. M., O'Brien S. J. 2001. The pattern of phylogenomic evolution of the Canidae. Cytogenet. Cell. Res., **95**: 210-224.

NIE W., WANG J., PERELMAN P., GRAPHODATSKY A. S., YANG F. 2003. Comparative chromosome painting de˘nes the karyotypic relationships among the domestic dog, Chinese raccoon dog and Japanese raccoon dog. Chromosome Res. **11**: 735-740.

NG M. P., VERGARA I. A., FRECH C., CHEN Q., ZENG X., PEI J., CHEN N. 2009. OrthoClusterDB: an online platform for synteny blocks. BMC Bioinform. **10**:192, doi:1

NOWACKA-WOSZUK J., SWITONSKI M. 2009. Comparative cytogenetic mapping of three genes involved in sex determination in four species of the family Canidae. J. Anim. Food Scien. **19**: 5-12.

PEVZNER P., TESLER G. 2003. Genome rearrangements in mammalian evolution: lessons from human and mouse genomes. Genome Res. **13**: 37-45.

SKALETSKY H., KURODA-KAWAGUCHI T., MINX P. J., CORDUM H. S., HILLIER L., BROWN L. G., REPPING S., PYNTIKOVA T., ALI J., BIERI T., CHINWALLA A., DELEHAUNTY A., DELEHAUNTY K., DU H., FEWELL G., FULTON L., FULTON R., GRAVES T., HOU S. F., LATRIELLE P., LEONARD S., MARDIS E., MAUPIN R., McPHERSON J., MINER T., NASH W., NGUYEN C., OZERSKY P., PEPIN K., ROCK S., ROHLFING T., SCOTT K., SCHULTZ B., STRONG C., TIN-WOLLAM A.,

Yang S. P., Waterston R. H., Wilson R. K., Rozen S., Page D. C. 2003. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature **423**: 825-837.

Stanyon R., Rocchi M. Capozzi O., Roberto R., Misceo D., Ventura M., Cardone M., Bigoni F., Archidiacono N. 2008. Primate chromosome evolution: Ancestral karyotypes, marker order and neocentromeres. Chromosome Res. **16**: 17-39.

Tian Y., Nie W., Wang J., Ferguson-Smith M. A., Yang F. 2004. Chromosome evolution in bears: reconstructing phylogenetic relationships by cross-species chromosome painting. Chromosome Res. **12**: 55-63.

Waters P. D., Wallis M. C., Graves J. A. M. 2007. Mammalian sex - origin and evolution of the Y chromosome and SRY. Semin. Cell Develop. Biol. **18**: 389-400.

Wayne R. K., Ostrander E. A. 1999. Origin, genetic diversity and genome structure of the domestic dog. BioEssays **21**: 247-57.

Wilson M. A., Makova K. D. 2009. Evolution and survival on Eutherian sex chromosomes. PLoS Genet., 5:e1000568, doi:10.1371/journal.pgen.1000568.

Yang F., O'Brien P. C., Milne B. S., Graphodatsky A. S., Solanky N., Trifonov V., Rens W., Sargan D., Ferguson-Smith M. A. 1999. A complete comparative chromosome map for the dog, red fox, and human and its integration with canine genetic maps. Genomics **62**: 189-202.

Yang F., Graphodatsky A. S., O'Brien P. C. M., Colabella A., Solanky N., Squire M., Sargan D. R., Ferguson-Smith M. A. 2000. Reciprocal chromosome painting illustrates the history of genome evolution of the domestic cat, dog and human. Chromosome Res. **8**: 393-404.