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Comparative cytogenetic analysis of selected chromosomes in Felidae family members using zoo-FISH

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Original article

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The karyotypes of felids are considered to be very conservative. Comparative chromosome painting is a technique used to determine the homology of regions between the chromosomes of different species. In order to supplement the existing knowledge and to obtain information on large-scale genome structure variations within the Felidae family, the fluorescence in situ hybridisation technique was performed in this study. Molecular probes specific to the cat chromosome A1, B1, C1, X and Y were used. The probes were hybridised with chromosomes belonging to the Amur tiger (♀), African lion (♂) and the leopard (♀). No significant differences were observed in the morphology or the banding pattern of both autosomes and heterosomes, and the homology of the tested DNA of fragments was demonstrated.

Key words: wild felids, molecular probes, fluorescence in situ hybridisation. *Barbara Kij-Mitka✉, Zuzanna Tarasek, Wiktoria Głowacz, Monika Bugno-Poniewierska, Department of Animal Reproduction, Anatomy and Genomics, University of Agriculture in Kraków, Poland.*
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The domestic cat (*Felis catus*, FCA, 2n=38), Amur tiger (*Panthera tigris altaica*, PTI, 2n=38), African lion (*Panthera leo*, PLE, 2n=38) and the leopard (*Panthera pardus*, PPA, 2n=38) are all representatives of the Felidae family. The karyotypes of felids are believed to be highly conservatised and to closely resemble the putative karyotype of the mammalian ancestors (Nash & O'Brien 1982; Rettenberger *et al.* 1995; Murphy *et al.* 2001; Davis *et al.* 2009). Most representatives of this family, apart from *Leopardus geoffroyi*, *Leopardus pajeros*, *Leopardus tigrinus* and *Leopardus wiedii* (2n=36; Eroğlu 2017), have the same number of chromosomes, 2n=38, characterised by a similar morphology.

Comparing karyotypes between different species is possible using various techniques. One of them is fluorescence *in situ* hybridisation (FISH), which uses comparative chromosome painting to determine the homology of regions between the chromosomes of different species (Yang *et al.* 2000; Tian *et al.* 2004). . This technique involves the hybridisation of molecular probes between individuals representing

the same or different families, or between phylogenetically more distant individuals. Zoo-FISH is also used to create gene maps, track evolutionary changes and to search for diagnostic possibilities (Kehler *et al.* 2007; Nie *et al.* 2011; Figueiró *et al.* 2017). This technique was used, among others, to create comparative chromosomal maps between domestic dogs (*Canis familiaris*, CFA) and other canids (Yang *et al.* 1999; Graphodatsky *et al.* 2000; Nie *et al.* 2003), as well as dogs and species from the Felidae family such as the African lion and clouded leopard (*Neofelis nebulosa*, NNE) (Tian *et al.* 2004). Moreover, data on the comparison of karyotypes between humans and domestic cats was described (Murphy *et al.* 2000; Pontius *et al.* 2007; Davis *et al.* 2009).

FISH-mapping studies are available that compare the genomes of wild cats with the domestic cat genome at the genetic level (Davis *et al.* 2009; Montague *et al.* 2014). Gene-specific probes offer high-resolution and localisation data, while the use of chromosomal probes allows for a large-scale view and a structural comparison of chromosomes at the macro level. This approach complements the existing knowledge and provides a more complete evolutionary picture.

Taking the above into account, in this study, the FISH technique was performed using molecular probes specific to the cat chromosomes A1, B1, C1, X and Y, in order to supplement the existing knowledge and to obtain information on large-scale genome structure variations within the family. The probes were hybridised with chromosomes belonging to the Amur tiger (♀), African lion (♂) and the leopard (♀).

Material and Methods

The research material was peripheral blood from the domestic cat (FCA – ♂), Amur tiger (PTI – ♀), African lion (PLE – ♂) and the leopard (PPA – ♀), collected in Vacutainer tubes lined with lithium heparin from zoological gardens in Poland. An aliquot of blood samples collected for the purpose of preventive examinations or other medical procedures was used for these cytogenetic studies , therefore no ethical approval was required.

Lymphocyte culture

The blood was subjected to a lymphocyte culture using the procedure described by Bugno-Poniewierska *et al.* (2020) for 72 h at 38°C, while introducing pokeweed mitogen (250 µl) as a division stimulator. In order to stop the cell division, 0.1% colchicine solution was added to the test tubes two hours before the end of the culture.

After 72 hours of culture, the tubes were centrifuged (900 rpm/10 min), the supernatant was collected and the pellet was suspended in a 0.05 M KCl solution. After 20 min of incubation in the hypotonic buffer, the tubes were centrifuged again, and after removing the supernatant, they were fixed using Carnoy's fixative in a 3:1 ratio (methanol: glacial acetic acid). The resulting suspensions were stored at -20°C until the further analyses.

G-banding

Twenty µl of chromosome suspension was placed on a cooled coverslip, fixed for 45 min at 45°C, and then left to age at room temperature for 5 days. The preparations were incubated for 4 seconds in Sorensen's buffer with the addition of 0.01g of trypsin. The digestion process was interrupted by immersing the slides in Sorensen's buffer, and the slides were then stained with 10% Giemsa solution for 8 min. Finally, the preparations were rinsed in distilled water and left to dry. The analysis of the preparations was performed using a Zeiss Axioimager (Carl Zeiss MicroImaging GmbH, Munich, Germany) microscope equipped with IKAROS 6.3 software (MetaSystems Hard & Software GmbH, Altlussheim, Germany).

Probe preparation

A1, B1, C1, X and Y chromosome-specific painting probes (200-800 bp) were prepared by a laser microdissection (PALM Microlaser system, Carl Zeiss MicroImaging GmbH, Munich, Germany) of the domestic cat chromosomes. To obtain chromosome-specific paints, we decided to destroy the telomeric ends of selected chromosomes prior to the dissection to avoid the amplification of repetitive DNA sequences, called FA-SATs (see Chaves *et al.* 2017), which are mainly located on the telomeres of different chromosomes in domestic cats. In the case of probes A1 and X, we obtained probes that painted the centromeric region in this way.

DNA amplification of the pooled DNA was performed using the GenomePlex SingleCell Whole Genome Amplification Kit (Sigma-Aldrich, USA). The probes were labelled by Green-dUTPs (Abbott, USA) using the Invitrogen™BioPrime™ Array CGH Genomic Labeling Module (Thermofisher Scientific, MA USA), according to the suppliers' instructions. Prior to use, the probes were denatured at 70°C for 10 min, then put on ice.

Fluorescence *in situ* hybridization (FISH)

The preparations were incubated in PBS buffer (2x5 min) and PBS with $MgCl_2$ (5 min) and then

passed through an alcohol series (70%, 80%, 90%). After drying the preparations (60°C/15min), they were denatured in 70% formamide for 2.5 min and immediately transferred through a frozen alcohol series. Probes were placed on dry slides and covered with a coverslip. The preparations secured with Fixogum glue (Marabu, Germany) were placed in a humid chamber and incubated overnight at 38°C.

After the hybridisation, the preparations were washed in 50% formamide (Sigma-Aldrich, St. Louis, MO, USA) (3x5 min) and 2x SSC (Sigma-Aldrich, St. Louis, MO, USA) (3x5 min). The preparations were transferred to a Wash Solution (400 ml of water, 250 µl of Tween 20 and 100 ml of 20xSSC – Sigma-Aldrich, USA) for 10 min. DAPI (Cambio, Great Britain) was applied to the wet slide, then the cover was slipped and it was analysed in a Zeiss Axioimager microscope equipped with Zeiss ZEN software (Carl Zeiss MicroImaging GmbH, Munich, Germany).

Results

G-banding

Figures 1-3 show the karyotypes of the Amur tiger, African lion and leopard. The comparison of A1, B1 and C1 chromosomes of the examined species is shown in Figures 4-6. Figure 7 shows the X chromosomes of the four studied species and Figure 8 shows the Y chromosomes of a domestic cat and an African lion.

The presence of an additional G-positive band below the centromere of the corresponding FCA A1 chromosome was observed in PTI, PLO and PPA. Additionally, no G-negative bands around the

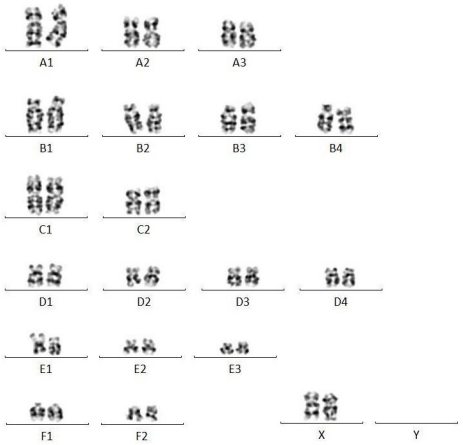

Fig. 1. G-banded karyotype of the female Amur tiger (*Panthera tigris altaica*), 2n=38 (magnification 100x).

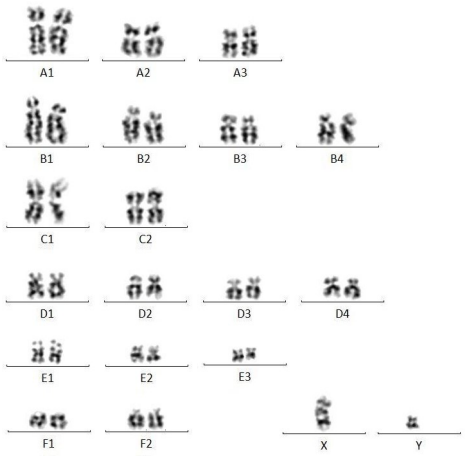

Fig. 2. G-banded karyotype of the male African lion (*Panthera leo*), 2n=38 (magnification 100x).

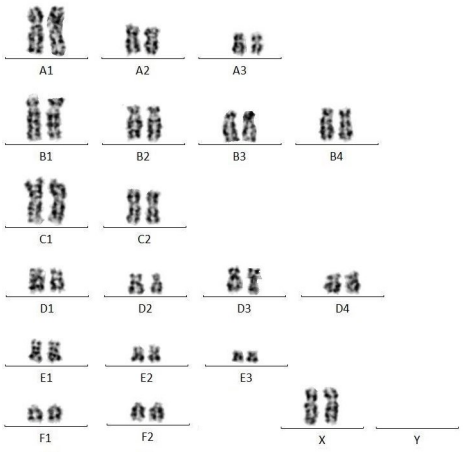

Fig. 3. G-banded karyotype of the female leopard (*Panthera pardus*), 2n=38 (magnification 100x).

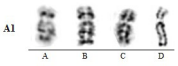

Fig. 4. G-banded A1 chromosomes in the domestic cat (A), Amur tiger (B), African lion (C) and leopard (D).

Fig. 5. G-banded B1 chromosomes of the domestic cat (A), Amur tiger (B), African lion (C) and leopard (D).

Fig. 6. G-banded C1 chromosomes of the domestic cat (A), Amur tiger (B), African lion (C) and leopard (D).

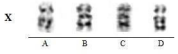

Fig. 7. G-banded X chromosomes of the domestic cat (A), Amur tiger (B), African lion (C) and leopard (D).

centromere were observed on the PLO A1 chromosome (Fig. 4). Moreover, the PLO B1 chromosome shows the presence of an additional G-positive band at the telomeric region of the p (PLO B1p ter), contrary to FCA B1, PTI B1 and PPA B1 (Fig. 5). Chromosomes C1 and X showed similarities in the G-banding patterns in all the species examined (Figs 6 and 7). The Y chromosome in the African lion appeared to be more intensely stained than FCA Y (Fig. 8).

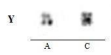

Fig. 8. G-banded Y chromosomes of the domestic cat (A) and African lion (C).

Comparative chromosome painting

After carrying out the Zoo-FISH technique, the specificity of the cat molecular probes for chromosomes FCA A1 (Fig. 9), FCA B1 (Fig. 10), FCA C1 (Fig. 11) and FCA X (Fig. 12) was observed in all the tested felids, showing full homology. In the leopard, it was observed that probes specific to the autosomal chromosomes FCA A1, FCA B1, FCA C1 also hybridised with the centromeric region of the X chromosome (Fig. 9D-12D). In the Amur tiger, additional fluorescent signals on the X chromosome were observed after the hybridisation with a chromosome-specific probe FCA B1.

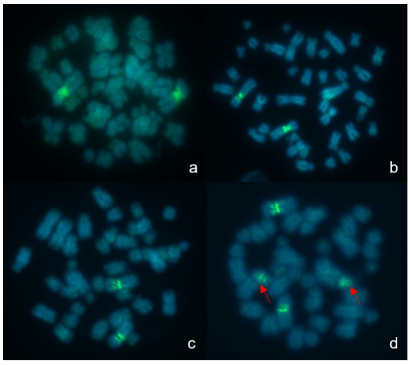

Fig. 9. Metaphase plate of the domestic cat (a), Amur tiger (b), African lion (c) and leopard (d), with fluorescent signals specific to the feline A1 chromosome. Additional fluorescent signals were observed on the X chromosome in the leopard (red arrows) (magnification 100x).

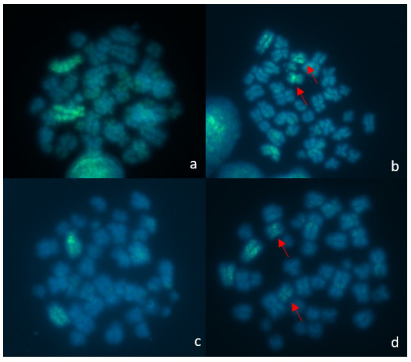

Fig. 10. Metaphase plate of the domestic cat (a), Amur tiger (b), African lion (c) and leopard (d), with fluorescent signals specific to the feline B1 chromosome. The presence of additional fluorescent signals on the X chromosome was observed in the Amur tiger (b) and leopard (d) (red arrows) (magnification 100x).

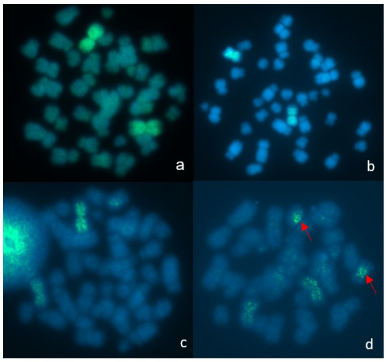

Fig 11. Metaphase plate of the domestic cat (a), Amur tiger (b), African lion (c) and leopard (d), with fluorescent signals specific to the feline C1 chromosome. Additional fluorescent signals were observed on the X chromosome in the leopard (red arrows) (magnification 100x).

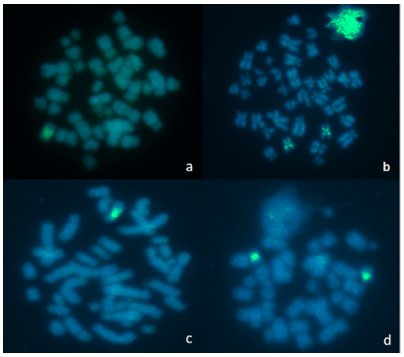

Fig 12. Metaphase plate of the domestic cat (a), Amur tiger (b), African lion (b) and leopard (d), with fluorescent signals specific to the feline X chromosome (magnification 100x).

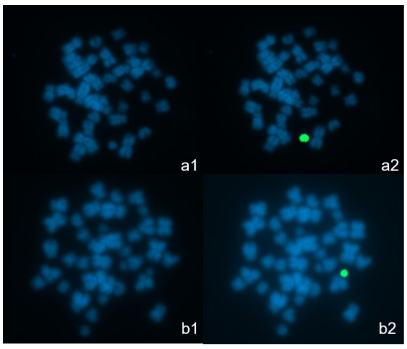

Fig 13. Metaphase plate of the domestic cat (a1) and an African lion (b1) after DAPI staining and with fluorescent signals specific to the FCAY chromosome (a2 and b2, respectively) (magnification 100x).

Table 1

Hybridisation results between probes specific for the domestic cat chromosomes (FCA) and chromosomes of the Amur tiger (PTI), African lion (PLE) and leopard (PPA)

Domestic cat chromosome (FCA)	Corresponding Amur tiger chromosome (PTI)	Notes	Corresponding African lion chromosome (PLE)	Notes	Corresponding leopard chromosome (PPA)	Notes
FCA A1	PTI A1	Conserved	PLE A1	Conserved	PPA A1	Conserved Secondary singal observed on X chromosome
FCA B1	PTI B1	Conserved Secondary singal observed on X chromosome	PLE B1	Conserved	PPA B1	Conserved Secondary singal observed on X chromosome
FCA C1	PTI C1	Conserved	PLE C1	Conserved	PPA C1	Conserved Secondary singal observed on X chromosome
FCA X	PTI X	Conserved	PLE X	Conserved	PPA X	Conserved
FCA Y	no data	no data	PLE Y	Conserved	no data	no data

The FISH with the probe prepared from FCA Y gave a strong fluorescent signal to the African lion Y chromosome (Fig. 13). The results of the hybridisation between the chromosomes of the species studied are presented in Table 1.

Discussion

Comparative chromosomes painting used between distant or closely related animal species is a technique that allows for the creation of comparative gene maps, the search for chromosomal rearrangements and the determination of evolutionary changes occurring in mammalian karyotypes (Bugno *et al.* 2007; Pieńkowska-Schelling *et al.* 2008; Bratuś *et al.* 2009; Figueiró *et al.* 2017). A visualisation of the chromosomes using fluorescently labelled molecular probes is an accurate and relatively quick diagnostic tool (Scherthan 2002).

The karyotypes of felids are considered to be very conservative. In almost all cats, except for *Leopardus geoffroyi*, *Leopardus pajeros*, *Leopardus tigrinus* and *Leopardus wiedii*, both from the Pantherinae and Felinae subfamily, 38 chromosomes with a similar morphology can be observed. There are scientific reports presenting very detailed gene maps of Felidae (Murphy *et al.* 2000; 2001; Davis *et al.* 2009; Montague *et al.* 2014; Yuan *et al.* 2024). The results indicate a high level of conservatism within the genomes of these animals; however, genetic separation between some cat species is also observed (Yuan *et al.* 2024).

In our study, the Zoo-FISH technique was used to determine the homology between the chromosomes of FCA (♂), PTA (♀), PLE (♂) and PPA (♀), in order to supplement the existing knowledge and to obtain information on large-scale genome structure variations within family.

As indicated by the studies presented by Wurster-Hill and Centerwall (1982), the chromosomes G-banding pattern of the Pantherinae subfamily members is identical (Wurster-Hill & Centerwall, 1982). In our study, a different pattern of G bands on the A1 and B1 chromosomes in the African lion was found compared to the other studied cats. Despite this difference, the homology of the tested DNA fragments of all the examined animals was demonstrated.

Additionally, secondary fluorescent signals were observed on the leopard X chromosome after using the FCA A1, FCA B1 and FCA C1 specific probes and on the African lion X chromosome after hybridisation with the FCA B1 specific probe. These observations may be related to the presence of repetitive sequences in the centromeric regions. The observation of homology between the X chromosomes of the leopard and the African lion was possible thanks to the use of molecular painting probes that allowed for viewing a large segment. As was noted by Chaves *et al* (2017), in species that have large blocks of repetitive sequences – for example, in centromeric regions – a large signal may appear after the FISH technique.

Conservatism of sex chromosomes is important in the context of gene maintenance. The X chromosome is characterised by the greatest conservatism among all mammalian chromosomes. This has to do with the genes, the location of which does not change. In turn, the Y chromosome contains a relatively large

amount of heterochromatin, and the DNA located on it undergoes rapid changes. For this reason, homology of this chromosome is not observed in many animal species (Yang *et al*. 2000; Tian *et al.* 2004; Stanyon *et al*. 2008; Kirsch *et al*. 2009).

Our observations showed homology between the X chromosomes in the examined species. It was also observed that the Y chromosome belonging to the PLE was characterised by the presence of a larger dark band compared to the domestic cat; however, it should be emphasised that hybridisation with a probe painting the Y chromosome (FCA Y) showed full homology between the individuals belonging to the species of FCA and PLE. Other authors would like to point out that full homology also occurs in the case of the sex chromosomes of the domestic dog and the heterosomes of the red fox, arctic fox, an interspecific hybrid (arctic fox × red fox) and the Chinese raccoon dog. As the authors noted, the observation of homology in the heterosomes of these animals may suggest that evolutionary changes occurring in the sex chromosomes of the studied canids included small fragments of them, compared to autosomes (Bugno-Poniewierska *et al*. 2012). In the present study, no significant differences were observed in the morphologies or the banding patterns of both autosomes and heterosomes. Unlike canids, autosomal chromosomes in felids have not undergone major evolutionary changes, resulting in a high degree of conservatism among these chromosomes.

Research conducted by Tian *et al*. (2004) showed chromosomal conservatism in the African lion and clouded leopard (*Neofelis nebulosa*, 2n=38). The authors used dog-chromosome specific paints to create comparative chromosome maps and no differences were shown between homologous regions (Tian *et al*. 2004). Similar results were obtained by Davis *et al* (2009) who compared the chromosomes of a domestic cat, a serval (*Profelis serval,* 2n=38) and a snow leopard (*Panthera uncia*, 2n=38). Hybridisation of the BAC probes to cat chromosomes was 100% successful, demonstrating the similarity of the tested genomes and determining the location of individual genes (Davis *et al*. 2009). Such a detailed analysis is not possible using probes responsible for painting whole chromosomes (WCPP – whole chromosome painting probes) or their parts (PCPP – partial chromosome painting probes); however, they are a supplement to the knowledge and provide a more complete evolutionary picture.

A comparison of chromosomes using *in situ* techniques is also possible between human and animal genomes. As studies have shown, probes specific to human chromosomes can be successfully used to visualise cat chromosomes (Rettenberger *et al*. 1995; Murphy *et al*. 2005). Therefore, the possibility of using human probes on wild felids seems highly probable, due to the lack of significant differences between the chromosomes of the domestic cat, Amur tiger, African lion and the leopard.

Conclusions

The use of molecular probes specific to cat chromosomes can be successfully used to perform a cytogenetic analysis of the chromosomes of wild felines, thereby expanding the current knowledge about the conservatism and chromosomal polymorphism of species belonging to this family.

Data availability. Data is available from the corresponding author on request.

Acknowledgments

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Author Contributions

Research concept and design B.K.-M., M.B.-P.: Collection and/or assembly of data: B.K.-M., H.C., S.K., M.P., Ł.R., M.Z., Z.T., W.G.; Data analysis and interpretation: B.K.-M., Z.T., W.G., M.B.-P.; Writing the article: B.K.-M., H.C., M.P., M.B.-P.; Critical revision of the article: H.C., S.K., M.B.-P.; Final approval of article: B.K.-M., H.C., S.K., M.P., M.B.-P.

Conflict of Interest

The authors declare no conflict of interest.

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