THE POLYMORPHISM OF CYTOGENETIC MARKERS IN THE FARM AND WILD-LIVING RACCOON DOG (*NYCTEREUTES PROCYONOIDES*)*

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Abstract

The raccoon dog (*Nyctereutes procyonoides*) is a mammalian species that belongs to Canidae family, order Carnivora. This species represents both animals living in the wild and farm animals used in the fur industry. Raccoon dogs have the most 'primitive' karyotype among Canidae family. The Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*) is characterised by a variable number of chromosomes (2n = 54 + 0–4 B). B chromosomes are supernumerary chromosomes occurring in addition to the basic set of A chromosomes in the cells of many organisms. The function and origin of these additional chromosomes is not clear. The aim of this work was to determine possible karyotypic differences between wild-living and farm populations, using methods of classical and molecular cytogenetics. The most useful cytogenetic markers to analyse karyotype polymorphism of canine are the number of B chromosomes and nucleolar organizer regions. A variation was identified in the number of B chromosomes and nucleolar organizer regions (NORs) in cells between wild-living and breeding populations.

Key words: wild-living raccoon dog, farm raccoon dog, cytogenetic markers, polymorphism

The raccoon dog is considered the most ancestral member of Canidae family and constitutes a separate branch in the phylogenetic tree of this family. As a result its karyotype is considered as the basic, "primitive" karyotype. Six subspecies of the raccoon dog (*Nyctereutes procyonoides*), naturally inhabiting areas of Asia have been identified: *N. p. albus in* Japan, *N. p. koreensis* in Korea, *N. p. orestes* in South-

-West China, *N. p. procyonoides* in China and Northern Indochina, *N. p. viverrinus* in Japan, and *N. p. ussuriensis* in Eastern China and Russia (introduced in Europe) (Kauhala and Saeki, 2004).

Nyctereutes procyonoides naturally inhabits areas of the Far East: China, Korea, Siberia, Mongolia, and Japan (Ward and Wurster-Hill, 1990). In Europe, this species has been introduced in the twentieth century. Following the introduction of the species (100,000 individuals) in Russia in the years 1920–1950, raccoon dogs readily adapted to new habitats, and quickly spread to Eastern, Central and Northern Europe, displacing in some areas smaller carnivorous species (Mäkinen et al., 1986; Kowalczyk, 2006).

Currently, two subspecies are recognized in the *Nyctereutes procyonoides* species, which differ in the number and morphology of their chromosomes. These are the Japanese raccoon dog (*Nyctereutes procyonoides viverrinus*) and the Eurasian raccoon dog, including *Nyctereutes procyonoides procyonoides* originating from China and *Nyctereutes procyonoides ussuriensis* living in the areas of Finland (Mäkinen et al., 1986; Pieńkowska et al., 2002 b; Szczerbal et al., 2003; Nie et al., 2003).

This species has long been used in Asia as a valuable fur animal. The quality of the fur contributed significantly to the introduction of this species in Russia, and as a result, spreading of the raccoon dog also in Europe. When used in clothing, the fur of the raccoon dog is called "murmansky" fur. Many years of research on raccoon dog karyotype from the beginning of the last century did not answer the question whether the preference for certain features of the fur and the selection for these traits had a significant impact on the polymorphism of genetic markers at the molecular (Ślaska et al., 2010; Ślaska and Grzybowska-Szatkowska, 2011) and chromosome level between wild and farmed animals. The species of *Nyctereutes procyonoides* can be divided into two subspecies carrying different karyotypes. The Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*) subspecies is characterised by 2n = 54+B number of chromosomes, and the number of B chromosomes varies from 0 to 4, whereas the Japanese subspecies of the raccoon dog (*Nyctereutes procyonoides viverrinus*) possesses a diploid set of chromosomes (2n = 38+B) with B chromosomes number ranging from 1 to 7 (Mäkinen et al., 1986; Pieńkowska et al., 2002 b; Trifonov et al., 2002; Szczerbal et al., 2003, 2005; Nie et al., 2003; Sosnowski et al., 2011).

In the karyotype of the Chinese raccoon dog 5 pairs of metacentric or submetacentric chromosomes and 21 acrocentric chromosomes can be distinguished. The X chromosome is a medium-sized metacentric chromosome and the Y chromosome is the smallest acrocentric chromosome (Mäkinen et al., 1986; Pieńkowska et al., 2002 b). Silver nitrate staining allows specifying the location of active NORs in the three pairs of autosomes. They are localized on the short arm of chromosome 1, the long arm of chromosome 4, and on the acrocentric chromosome 11. Ag-NOR band can be also detected on the Y chromosome (Mäkinen et al., 1986; Pieńkowska et al., 2002 a).

B chromosomes in mammals have been described in only 1% of all cytogenetically examined mammals, representing 55 species, including 37 species of rodents and 3 species belonging to the Canidae family: the raccoon dog (*Nyctereutes procyo-*

noides), the red fox (*Vulpes vulpes*) and the short-eared dog (*Atelocynus microtis*) (Vujošević and Blagojević, 2004).

B chromosomes are acrocentric, and have similar size to autosomes. Three groups of chromosomes can be distinguished: type BI are large acrocentric chromosomes similar in size to chromosome 6, and type BII and BIII are smaller, similar in size to the medium-sized acrocentrics (Pieńkowska et al., 2002 b). Variation in the types of B chromosomes results in an overlap of two different kinds of mosaicism: the one pertaining to the number of B chromosomes in the cell and the second one that concerns particular types of these chromosomes (Szczerbal et al., 2005). In 2002 Trifonov and colleagues used FISH technique with bicolour probe complementary to a fragment of B chromosome and they did not find any homology between extra chromosomes and the basic set of A chromosomes (Vujošević and Blagojević, 2004).

While characterizing a karyotype it is also worth taking into consideration the analysis of chromosome conjugation, which is mainly based on the observation of synaptonemal complexes; especially in the case of bivalent sex chromosomes, whose specific morphology at different stages of conjugation helps to distinguish several basic substages of the prophase I pachytene. Deviations from the typical course of sex chromosome conjugation (early dissociation, association with autosome bivalents) may cause fertility problems in animals.

The aim of this work was to determine differences that may exist in the karyotype between wild-living and farm populations, using methods of classical and molecular cytogenetics.

Material and methods

Cytogenetic studies were carried out on wild-living and breeding Chinese raccoon dogs. Farmed animals came from a fur farm in Chorzelów. Samples from wild-living animals were obtained during planned culls in the hunting districts of the Warmia and Mazury voivodeship. Six wild-living (male and female) and 15 breeding (male and female) animals were analysed. Every animal was examined with the use of each technique.

Primed *in situ* DNA synthesis (PRINS) using an oligonucleotide complementary to the telomeric sequences

A reaction mixture, per slide, consisted of: 25 µl of premix (Roche Applied Science, Germany), 2U of Taq I polymerase and 1 nM of Cy3-dUTP. Slides were covered with cover slips, edges were sealed with rubber gum, and subsequently inserted into a thermal cycler (Eppendorf Mastercycler Gradient) with *in situ* adapter. Denaturation was carried out at 94°C for 5 minutes, followed by 30-minute annealing step at 58°C. After completion of the reaction, slides were immersed in a "stop" buffer with pH = 8.0 at 58°C for 5 minutes, and then transferred to the same buffer at room temperature for another 5 minutes. After this step, slides were washed in alcohol series, 3 minutes in each concentration. Next, slides were dried and mounted with DAPI antifade solution.

Analysis of synaptonemal complexes by immunofluorescence

In order to observe the chromosomes in the pachytene stage of prophase I, fixed preparations of synaptonemal complexes were immunofluorescently stained using antibodies conjugated to fluorescent FITC dye (fluorescein isothiocyanate) against SCP1 and SCP3 proteins that build the structure of the synaptonemal complex. Working solutions: – primary polyclonal antibody against rabbit SCP1 protein (Abcam, UK); primary rabbit polyclonal antibody against SCP3 protein (1 mg/ml) (Thermo Scientific, USA); secondary sheep antibody against rabbit, FITC-labelled 1 mg/ml (Abcam, UK); 10xPBS (Lab Empire, Poland); detergent, TritonX100 (Sigma, USA), phosphate buffer with detergent (PBST); 1x PBS + 0.25% Triton X100; preincubation solution: 1% BSA in PBST – DAPI fluorescent dye (0.4 μg/ml) in antifade (Cambio, UK). Fixed preparations were washed for 3 minutes in PBST solution, and then 250 μl of the 1% BSA in PBST solution was applied to the slides to block non-specific signals, and incubated for 30 minutes at room temperature. After the incubation, slides were rinsed once with PBST for 3 minutes. Then 150–200 μl of the primary antibody diluted 1:200 in preincubation solution was applied to the slide, covered with coverslips and placed in a humid chamber overnight at 4°C. Next day, slides were washed three times (each wash – 5 minutes) in PBST to remove unbound antibodies. Secondary antibody diluted 1:200 in preincubation solution was applied to slightly damp slides, covered with coverslips and incubated in a humid chamber for 1 h. Next, slides were washed three times (each wash – 3 minutes) in PBST and mounted with DAPI with antifade.

Results

Using CBG technique, it was shown that the autosomes of the Chinese raccoon dog present a distinct centromeric band. Among the autosomes, metacentric chromosomes of the third pair do not present a heterochromatin block. The characteristic pattern of C bands was not observed on B chromosomes when the aforementioned method was applied. However, dark staining was observed along the whole length of the arms of these chromosomes (Fig. 1).

The analysis of B chromosomes during the mitotic divisions in somatic cells of the Chinese raccoon dog

The analysis of mitotic metaphases in somatic cells of the Chinese raccoon dog revealed the presence of B chromosomes in the range from 0 to 4 in the farm animals and from 0 to 3 in the wild-living animals (Fig. 2).

The number of B chromosomes varied at both the interindividual and intracellular level. During the analysis of metaphase chromosomes in the breeding and wild-living raccoon dogs, most frequently cells with two B chromosomes were observed. However, in the wild-living raccoon dog metaphases there were no cells with four B chromosomes.
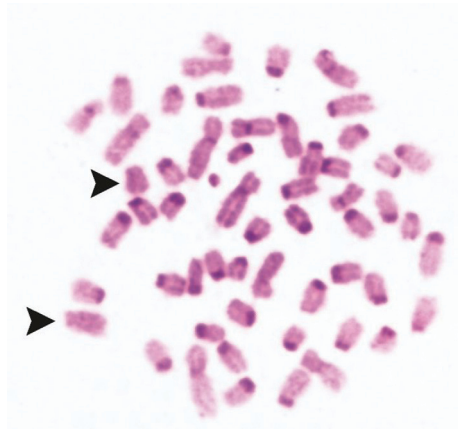
Figure 1. Metaphase spread of male Chinese raccoon dog (2n = 54, XY+2B), stained with CBG technique. Arrows indicate B chromosomes
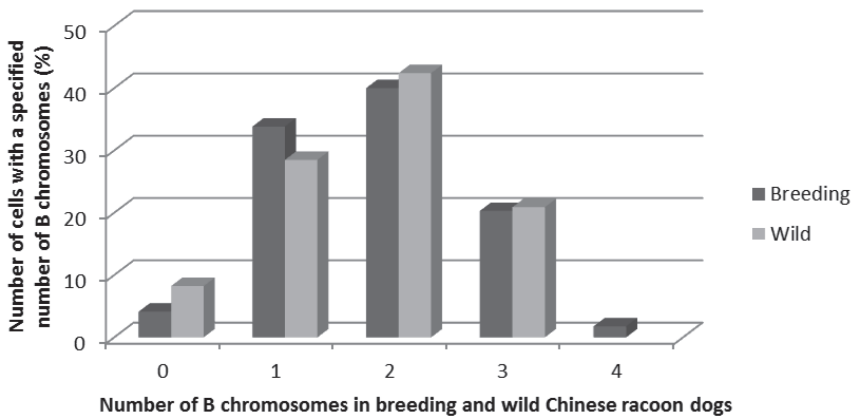

Figure 2. Percentage of cells with a specific number of B chromosomes in farm and wild-living animals

The average number of B chromosomes for all farm individuals was 1.82 and ranged from 1.54 to 2.05 for particular animals. The average for females was 1.83 and that for males 1.81. The average number of B chromosomes for all wild-living animals, based on the analysis of 158 metaphase spreads was 1.76, whereas for particular animals it ranged from 1.45 to 2.03. Females' average was 1.69 and males' average was 1.81.

Ag-NOR banding technique
Application of Ag-NOR banding technique allowed determining the number of active nucleolar organizer regions (NORs). The number of such regions ranged from

1 to 6 and was variable at the interindividual and intercellular level. In the case of farmed raccoon dogs the most frequent were cells with six active NORs, as opposed to animals living in the wild where four active NORs were the most common (Fig. 3). In addition, in one wild-living male (number 109), a metaphase spread with seven NORs was observed (Fig. 4).
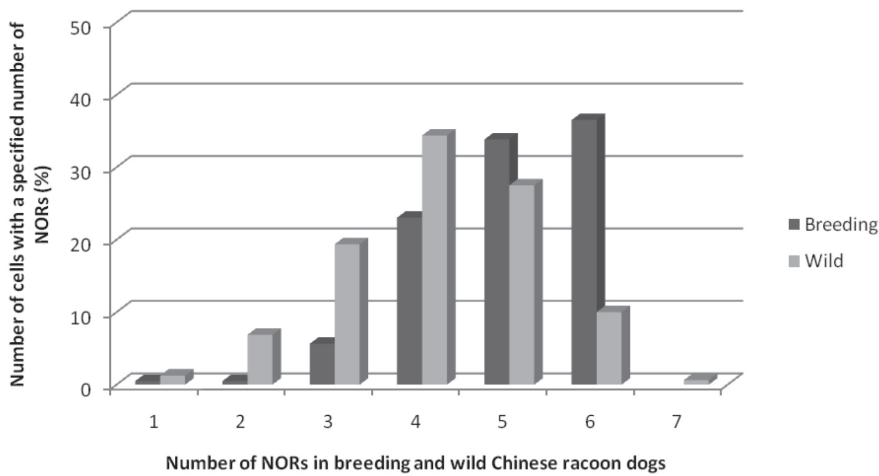

Figure 3. Percentage of cells with a specific number of active nucleolar organizer regions (NORs) in farm and wild-living animals

Figure 4. Metaphase spread of a wild-living male, number 109, with seven visible active nucleolar organizer regions (indicated with arrows). Ag-NOR staining

The average number of NORs for all farm animals was 4.99, whereas for individual animals it ranged from 4.21 to 5.44. Females' average was 5.08 and males' average was 4.9. The average number of NORs for all wild-living animals was 4.13,

whereas for particular animals it ranged from 3.62 to 4.63. The average of females was 3.81 and that of males 4.28.

FISH technique

The use of FISH painting probes specific to dog heterosomes, helped to determine the sex of all tested animals, both wild and farmed. On X chromosomes strong signals were additionally observed in the pseudoautosomal region (Fig. 5 A, B)

The applied painting probes specific to fox B chromosomes did not hybridize with B chromosomes of the raccoon dog.
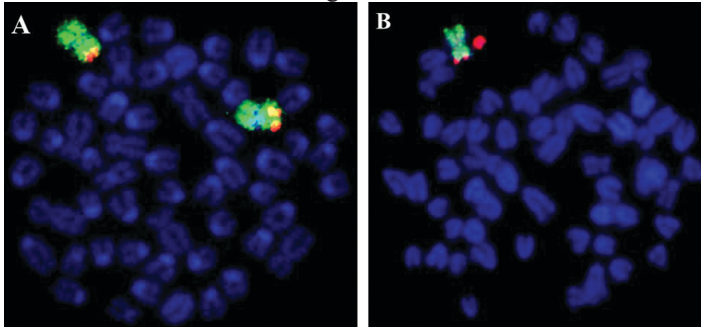

Figure 5. FISH with probes specific to X chromosome (FITC-green) and Y chromosome (Cy3-red):
A) Metaphase spread of female Chinese raccoon dog with two X chromosomes (green);
B) Metaphase plate of Chinese raccoon dog male. X chromosomes showed clear signals
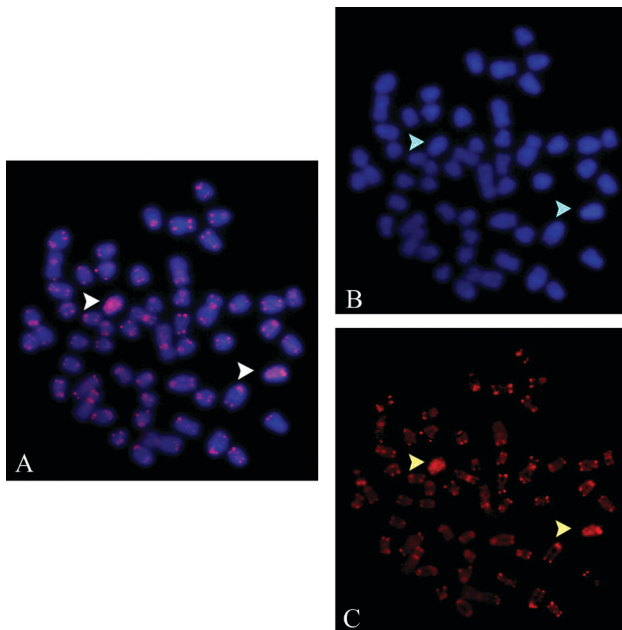in the pseudoautosomal region after hybridization of Y chromosome-specific probe

Figure 6. Metaphase spread of a raccoon dog (female, 2n = 54,XX+2B) after PRINS with primers specific to telomeres: A – merged image of fluorescent signals; B – metaphase chromosomes stained with fluorescent dye DAPI; C – localization of telomeric sequences. Arrows indicate B chromosomes

PRINS technique

PRINS with a telomeric probe showed evenly distributed signals along the whole length of B chromosomes as opposed to autosomes and sex chromosomes where signals were localized to the ends of chromosomes and pericentromeric regions (Fig. 6). Size and distribution of hybridization signals on B chromosomes suggest that they contain a large number of repetitive telomeric sequences.

Analysis of synaptonemal complexes by immunofluorescence

Immunofluorescence with antibody against SCP1 protein, building central part of the synaptonemal complex and against SCP3 protein that forms lateral part of the synaptonemal complexes, allowed for the identification and observation of the morphology of the XY chromosomes bivalent during the meiotic division of cells (Fig. 7 A, B).
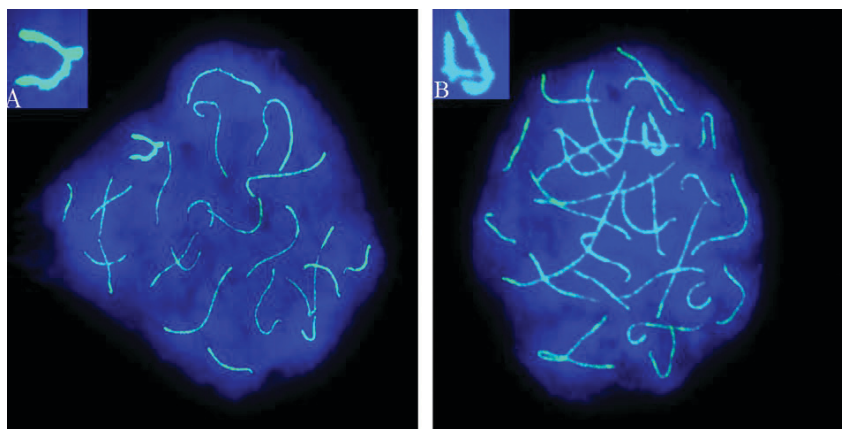

Figure 7. Immunofluorescence with antibodies against SCP1 protein (FITC). Synaptonemal complexes of raccoon dog: A) – conjugation of sex chromosomes (XY bivalent); B) – lack of sex chromosomes conjugation
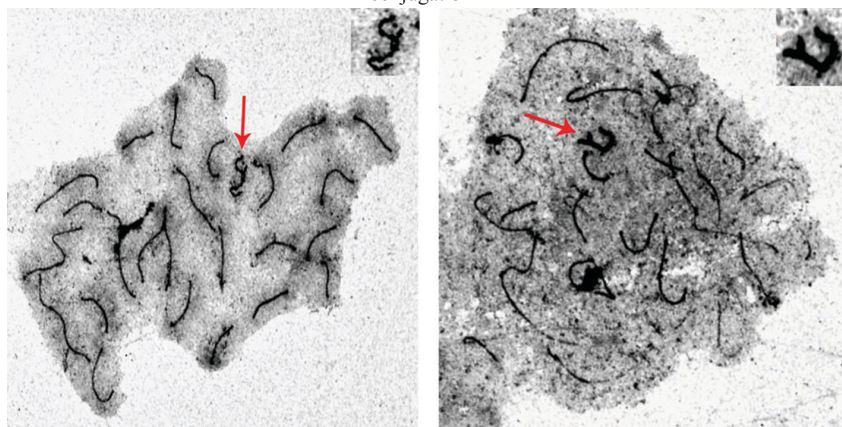

Figure 8. Synaptonemal complexes after silver nitrate staining. Arrows indicate the bivalents of sex chromosomes

Silver nitrate staining of synaptonemal complexes

The results of staining of synaptonemal complexes with silver nitrate are shown in Figure 8.

Discussion

In the Canidae family intraspecific polymorphism of the diploid number of chromosomes can be observed in three species: the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*) – 2n = 54 + 0–4 B, the fox (*Vulpes vulpes*) – 2n = 34 + 0–7 B, and the short-eared dog (*Atelocynus microtis*) – 2n = 74 + 0–2 B (Wurster and Benirschke, 1968; Mäkinen et al., 1986; Pieńkowska et al., 2002 b). Intraspecific variation results from the presence of a variable number of B chromosomes in the cells of these three species. It has been shown that B chromosomes are not subject to Mendelian inheritance and their variable number can occur both at the intraindividual and intracellular level. Available cytogenetic studies indicate that they probably arose during the evolution of one of the basic sets of chromosomes as a result of various processes taking place between the chromosomes in the cell, such as fusions and fissions (Trifonov et al., 2002; Vujošević and Blagojević, 2004; Kauhala and Saeki, 2004).

Cytogenetic studies of B chromosomes in raccoon dogs have been initiated in the 1970s. In order to learn more about their morphology and structure, various techniques of classical cytogenetics have been used, including CBG or GTG banding techniques (Wang and Fedoroff, 1974; Mäkinen, 1974; Mäkinen and Fredga, 1980; Mäkinen et al., 1986; Pieńkowska et al., 2002 b). Only the development of molecular cytogenetic techniques such as *in situ* hybridization with the use of molecular probes has enabled more detailed examination of the nature of these chromosomes. However, no definite answer has been found so far to the question whether they have an essential influence on the functioning of the organism. Until 2005, it had not been confirmed that the B chromosomes actually contained genes. Only Graphodatsky et al. (2005) located for the first time the proto-oncogene C-KIT, encoding a transmembrane tyrosine kinase on the B chromosomes of the red fox and raccoon dog, while in other mammals this gene is located on the autosomes.

In this work, CBG technique allowed for the identification of B chromosomes and their acrocentric structure. They show dark staining along the whole length without clear distinction of the centromeric region as it is the case in autosomes and sex chromosomes, indicating the heterochromatic character of B chromosomes.

Studies carried out by Pieńkowska and colleagues in 2002 with GTG, QFQ and CBG techniques revealed that supernumerary chromosomes can differ in size and morphology. These studies have also demonstrated that the B chromosomes are acrocentric and show a C banding pattern in the form of a darker colour along the entire length of the chromosome, without explicit distinction of the centromeric region. As a result of R bands staining (RBA technique) B chromosomes exhibit only a slight coloration which might indicate late replication in the S phase.

During the analysis of B chromosomes in the cells, it was observed that their number is variable both at the interindividual and intercellular level. In breeding raccoon dogs as well as in the wild-living animals the most frequently observed cells had two B chromosomes. Among the wild-living raccoon dogs there were no cells with four B chromosomes, and there were differences in the number of B chromosomes between males and females. Average number of B chromosomes was 1.69 in females and 1.81 in males.

Pieńkowska and Zagalska (2001) showed the presence of inactive NOR-like sequences in the telomeric fragments and the proximal part of the Chinese raccoon dog B chromosomes. They obtained these results using FISH with a human 28S rDNA probe. However, these sequences were not detected by Ag-NOR staining, which shows only active NORs. In the B chromosomes of the raccoon dog, nucleotide sequences specific to 18S rDNA have been found previously, using PRINS with primers specific to the porcine 18S rDNA (Wnuk et al., 2010). These studies suggest that during evolution of raccoon dog B chromosomes the accumulation and inactivation of NOR-like sequences took place. But this phenomenon is not unique to this species only. In 2000 Stitou and colleagues reported a similar phenomenon in some rodent species.

Molecular probes obtained from the microdissection of B chromosomes of the red fox did not hybridize with B chromosomes of the raccoon dog, which suggests differential evolution of these chromosomes in both species and their distinct nucleotide composition.

A probe specific to B chromosomes enables obtaining hybridization signals in these chromosomes but also weak signals on autosomes (Szczerbal and Świtoński, 2003; Graphodatsky et al., 2005). Trifonov and colleagues in 2002 and Graphodatsky and colleagues in 2005 used FISH technique with a probe specific to B chromosomes of the raccoon dog. They obtained evenly distributed signals on B chromosomes in both Eurasian and Japanese raccoon dogs. Moreover, they also detected weak signals on autosomes and allosomes. These studies suggest that sex chromosomes but also autosomes contain short sequences characteristic of supernumerary chromosomes.

Application of FISH technique with painting probes specific to heterochromosomes of the dog, allowed for the identification of sex chromosomes in all tested individuals. Additionally, on X chromosomes strong signals were observed in the pseudoautosomal region. Graphodatsky et al. (2001) obtained even signals on heterochromosomes of Chinese and Japanese raccoon dogs using FISH with a probe specific to dog sex chromosomes. Similar results were found for heterochromosomes of the red fox (Yang et al., 1999). Using Zoo-FISH, Bugno-Poniewierska et al. (2012) found that X and Y chromosomes of the domestic dog show a high level of homology with heterochromosomes of the raccoon dog, red fox, arctic fox and a hybrid of the latter two species. These results confirm the rule that heterochromosomes contain highly conserved DNA sequences that barely changed during the evolution of canids. For the molecular analysis of raccoon dog chromosomes PRINS technique was used with primers specific to human telomeric DNA sequences. Evenly distributed hybridization signals were obtained at the terminal ends of chromosomal arms, peri-

centromeric regions, and along the whole length of B chromosomes of the Chinese raccoon dog.

Wurster-Hill et al. (1988) used FISH with telomeric probes to test B chromosomes of two subspecies of the raccoon dog. The result of the experiment was a hybridization signal obtained at the telomeric regions of chromosomes from the basic A set, and additionally along the entire length of the arms of B chromosomes. This indicates clearly that the B chromosomes contain telomeric repetitive sequences along the entire length of the chromatid (Silva and Yonenaga-Yassuda, 1998).

Immunofluorescent staining carried out in this work, using antibodies against SCP1 and SCP3 proteins that form central and lateral structures of synaptonemal complexes enabled identification of the bivalent of XY chromosomes during meiosis. In some of the cells early dissociation of this bivalent was observed.

Sosnowski et al. (2011) conducted experiments with spermatocytes of the red fox and Chinese raccoon dog, analysing the frequency of cases with the lack of conjugation of sex chromosomes during meiotic divisions. They used antibodies against SCP3 protein, structural protein of the lateral parts of synaptonemal complexes and antibodies against γH2A.X histone. They also carried out observations of autosomes and B chromosome conjugation. The results of the study demonstrated a significant percentage of cases without conjugation of sex chromosomes in spermatocytes of the tested individuals. It was also observed that B chromosomes conjugating together formed diverse structures, such as bivalents, trivalents and tetravalents. The results of experiments carried out by Sosnowski's group (2011) indicate that with the increase in the number of B chromosomes in spermatocytes of the Chinese raccoon dog, the instances of lack of conjugation also become more frequent.

In summary, in our study we identified a variation in the number of B chromosomes and nucleolar organizer regions (NORs) in cells between wild-living and breeding populations.

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Polimorfizm markerów cytogenetycznych u jenota (*Nyctereutes procyonoides*) w populacjach hodowlanych i dziko żyjących

STRESZCZENIE

Jenot (*Nyctereutes procyonoides*) jest gatunkiem ssaka zaliczanym do rodziny psowatych (Canidae), rzędu drapieżne (Carnivora). Gatunek ten reprezentuje zarówno zwierzęta wolno żyjące, jak i hodowlane, wykorzystywane w przemyśle futrzarskim. Posiada najbardziej prymitywny kariotyp wśród psowatych. Jenot chiński (*Nyctereutes procyonoides procyonoides*) charakteryzuje się zmienną liczbą chromosomów 2n = 54+ 0–4 B. Chromosomy B są chromosomami nadliczbowymi, występującymi oprócz podstawowego zestawu chromosomów A w komórkach wielu organizmów. Nie są znane ich funkcja ani pochodzenie. Badania miały na celu określenie różnic, jakie mogą występować na poziomie chromosomowym między populacjami dziko żyjącymi i hodowlanymi, z wykorzystaniem metod cytogenetyki klasycznej i molekularnej. Zaobserwowano polimorfizm w liczbie chromosomów B i regionów jąderkotwórczych (NOR) w komórkach pomiędzy populacjami dziko żyjącymi i hodowlanymi.