DOI: 10.2478/v10083-012-0030-0

ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA

VOL. XXX (4) SECTIO EE 2012

University of Life Sciences in Lublin, Department of Biological Basis of Animal Production,
Akademicka 13, 20-950 Lublin,
e-mail: andrzej.jakubczak@up.lublin.pl

ANDRZEJ JAKUBCZAK, GRAŻYNA JEŻEWSKA-WITKOWSKA,
BEATA HORECKA, MONIKA ŻELAZOWSKA,
ELEONORA KOPEĆ, ZOFIA RUPEĆ

Preliminary studies of polymorphism of selected microsatellite sequences in different colour types of raccoon dog (Nyctereutes *Procyonoides*)

Wstępne badania polimorfizmu wybranych sekwencji mikrosatelitarnych różnie umaszczonych jenotów *(Nyctereutes* procyonoides)

Summary. Primary sequences designed for microsatellite loci in the domestic dog genome (FH2054, CPH3, FH2168, FH2164, FH2097), which were used in this study, also allowed for the amplification of the corresponding loci in white and standard Raccoon dogs. In these groups the frequency of alleles, observed and expected heterozygosity, the polymorphic information content and the probability of parentage exclusion were analyzed. The studies allowed to define the expected degree of polymorphism of five microsatellite loci in the two color varieties of Raccoon dogs. All of the five selected microsatellite sequences may be used for checking the origin of Raccoon dogs and the construction of genetic maps within the canids. The obtained values of genetic similarity and genetic distance shows that there is a high genetic similarity between the groups of white and standard Raccoon dog genetic, and thus the small genetic distance. This suggests only small genetic differences between white and standard Raccoon dogs.

Key words: Raccoon dog, microsatellite, PCR-multiplex.

INTRODUCTION

In recent years, genetic markers have become a valuable material for both genetic researches, as well as a point of interest for breeders. In the modern animal husbandry, the most useful are studies of restriction fragment polymorphism of specific genes and microsatellite and minisatellite sequences. Development of knowledge about the polymorphism and the location of microsatellite sequences in the genome of the dog, is also very useful for other species from the canine family (*Canidae*), for example, Raccoon dogs (*Nyctereutes procyonoides*) [Jakubczak and Jeżewska 2008]. Evolutionary relations

studies carried out using RAPD technique showed that the Chinese Raccoon dog is a species highly genetically distinct from the other analyzed canine species (domestic dog, Red fox, Arctic fox) (Stępniak *et al.* 2002). However, short tandem repeated (STR) from the dog genome were also used in checking the origin of Raccoon dogs. For the first time such a research was conducted by Ślaska *et al.* [2007]. Using a set of highly polymorphic microsatellite loci, multiplex PCR amplification to their analysis, and automatic DNA genotyping in sequencers giving almost a hundred percent probability of parentage exclusion and ensuring the reproducibility of the obtained results [Radko 2008].

The basic colour variant of Raccoon dog is standard. Among these animals we can distinguish the following types of color: silver-gray, golden-brown and hybrids of both types. Colored mutant varieties (white and colorful) appeared among Raccoon dogs in the initial phase of their breeding. Multicolored animals, with lighter spots on the shoulder, appeared among standard Raccoon dogs. There are reports of individuals with small lighter spots, as well as that whose body was white, and head dark. However, the inheritance of this type of coloration remained unexplained. White Raccoon dogs started to be used on a large scale on Polish farms in 1979. Fur of these animals is completely white. However, black nose and colored eyes indicates that it is not albino variety. Dominant mutations in many cases are lethal and do not occur in homozygous form. In the case of white Raccoon dogs, it was not clearly proved that the white coloration gene was lethal in the homozygous system. White Raccoon dogs' genotype is labeled as *Ww*.

The objective of this study was a comparative analysis of polymorphism of selected microsatellite sequences in different colour types of Raccoon dogs.

MATERIALS AND METHODS

The studied material consisted of 30 unrelated Raccoon dogs from two breeding farms, including 10 white Raccoon dogs and 20 standard coloured animals. DNA was isolated from whole peripheral blood using QIAcube station with QIAamp DNA Blood

Table 1. Primer sequences used for amplification of analyzed loci
Tabela 1. Sekwencje starterowe wykorzystane do amplifikacji analizowanych loci

Locus	Motif Motyw	Primer sequences Sekwencja starterowa	Source Źródło
CPH3	$(GA)_2TA(GA)_{17}$	F: CAGGTTCAAATGATGTTTTCAG R:TTGACTGAAGGAGATGTGGTAA	Fredholm and Winterø, 1995
FH2054	$(GATA)_{16}$	F: GCCTTATTCATTGCAGTTAGGG R: ATGCTGAGTTTTGAACTTTCCC	Fransisco *et al*. 1996
FH2097	$(GAAA)_{16}$	F: CAATGTCGAATTCCATGGTG R: ATGGAGCAAGATGTGTTTGTG	Fransisco *et al*. 1996
FH2164	$(GAAA)_{43}$	F: GATTATGACTCGAACCAAAGGC R:TGGAGGAAGTTCATTAAGCAGC	Fransisco *et al*. 1996
FH2168	$(GAAA)_{20}$	F:GCAAATTACTTACTTCACTATGCC R: TTGCAAGACTTCAACATGGC	Fransisco *et al.* 1996

Mini Kit (QIAGEN) according to the manufacturer's procedures. The purity and concentration of the isolated DNA was determined spectrophometrically (BioPhotometer, Eppendorf). Quality was assessed by electrophoretic separation in 1% agarose gel containing ethidium bromide in 1x TBE buffer at a voltage 6V/cm. The samples were visualized under UV light and saved using Scion Image software. We examined five microsatellite sequences in the group of Raccoon dogs: FH2054, CPH3, FH2168, FH2097, FH2164. Multiplex PCR was performed in a MJ Research PTC - 225 Tetrad thermocycler. Primers for domestic dog microsatellite sequences were used including the Raccoon dog or the domestic dog belong to one family (Tab. 1).

To prepare the reaction mixture, the AmpliTaq Gold set was used (Applied Biosystems). Its composition is given in Table 2. The volume of one sample was 10 µl (9 µl of reaction mix + 1 µl of DNA).

Table 2. Composition of PCR mixture for PCR multiplex
Tabela 2. Skład mieszaniny reakcyjnej do PCR multipleks

Compoment Składnik	Initial concentration Stężenie wyjściowe	Amount Ilość	Volume per 1 sample (µl) Objętość na 1 próbę (µl)
Deionised water Woda dejonizowana	-	-	6,39
AmpliTaq Gold 360, 10X buffer AmpliTaq Gold 360, Bufor 10 X	10X	1X	1,00
360 GC Enhancer 360 GC Bufor	-	-	0,17
25 mM Magnesium Chloride 25 mM Chlorek Magnezu	25 mM (25 nmoli/µl)	17,25 nmoli	0,69
dNTP mix (2,5 mM of each: dATP, dCTP, dGTP, dTTP) – 10-mM solution mieszanina dNTP (2,5 mM każdego: dATP, dCTP, dGTP, dTTP) – 10-mM roztwór	10 mM (10 nmol/ µl)	4,9 nmol	0,49
Primer F Starter F Primer R Starter R } for each locus	100 pmoli/µl	2,0 pmoli	0,02
AmpliTaq Gold 360 DNA Polymerase Polimeraza DNA AmpliTaq Gold 360	5 units/µl	0,3 unit	0,06
Total – Łącznie			9,00

Temperature-time profile of the PCR has been chosen experimentally:

- nitial denaturation 95°C - 10 min
- denaturation 95°C - 30 s
- annealing 58°C - 45 s
- DNA extension 72°C - 60 s
- final extension 72°C - 20 min

} × 36

- cooling 4°C - ∞

Capillary electrophoresis results were analyzed by Gene Mapper v.3.5. Using Cervus v. 3.0.3 and PopGene v. 1.31 expected and observed heterozygosity, polymorphic information content and probability of exclusion when the data are available for one and for both parents, were calculated. There was also genetic similarity and genetic distance by Nei'a (1978) determined between studied groups of different coloured Raccoon dogs.

RESULTS

In the examined five microsatellite loci total of 28 alleles were identified. The number of alleles for individual loci ranged from 4 to 8 The average number of alleles in each of the analyzed loci was 6.40. Comparison of the two color varieties including number and length of alleles and frequency of their occurrence in different loci are shown in Table 3.

Table 3. Number (n) and length of alleles and frequency of their occurrence in analyzed loci in group of white and standard Raccoon dogs
Tabela 3. Liczba (n) i długość alleli oraz frekwencji ich występowania w analizowanych loci w grupie jenotów białych i standard

Locus	White Raccoon dogs Jenoty białe			Standard Raccoon dogs Jenoty standard		
	allele – allele		frequency (%) frekwencja (%)	allele – allele		frequency (%) frekwencja (%)
	n	length (bp) długość (pz)		n	length (bp) długość (pz)	
FH2054	4	141	5.00	4	141	2.94
		145	20.00		145	47.06
		149	60.00		149	47.06
		153	15.00		153	2.94
CPH_3	6	152	10.00	5		
		156	45.00		156	50.00
		160	10.00		160	2.94
		164	5.00		164	17.65
		170	25.00		170	17.65
		174	5.00		174	11.76
FH2168	7	249	5.56	5	249	37.50
					253	3.13
		257	11.11			
		261	33.33		261	15.63
		269	33.33		269	21.88
		273	5.56			
		297	5.56			
		301	5.56		301	21.88
FH2164	6	275	11.11	4		
		279	22.22		279	31.25
		283	5.56			
		287	33.34		287	12.50
		291	5.56		291	3.13
		295	22.22		295	53.13
FH2097	4	278	25.00	5	278	37.50
		282	10.00		282	6.25
		286	35.00		286	15.63
		290	30.00		290	37.50
					294	3.13

Table 4. Heterozygosity values for microsatellite sequences in group of white and standard coloured Raccoon dogs
Tabela 4. Wartości współczynników heterozygotyczności dla 5 sekwencji mikrosatelitarnych w grupie jenotów białych i standard

Locus	Heterozygosity (HET) Heterozygotyczność (HET)		Heterozygosity (HET) total Heterozygotyczność (HET) łącznie	
	white Raccoon dogs jenoty białe	standard Raccoon dogs jenoty standard	observed (Ho) obserwowana (Ho)	expected (He) oczekiwana (He)
FH2054	0.6053	0.5989	0.704	0.617
CPH3	0.7474	0.6934	0.630	0.710
FH2168	0.7974	0.7621	0.720	0.804
FH2164	0.8497	0.6230	0.760	0.724
FH2097	0.7526	0.7117	0.654	0.728
Average Średnia	0.7505	0.6778	0.6936	0.7166

Table 5. PIC values for 5 microsatellite sequences in group of white and standard coloured Raccoon dogs
Tabela 5. Wartości współczynników PIC dla 5 sekwencji mikrosatelitarnych w grupie jenotów białych i jenotów o umaszczeniu typu standard

Locus	PIC value Wartość PIC		Polymorphic information content (PIC) total Indeks stopnia polimorfizmu (PIC) łącznie
	white Raccoon dogs jenoty białe	standard Raccoon dogs jenoty standard	
FH2054	0.5261	0.4929	0.531
CPH_3	0.6710	0.6307	0.661
FH2168	0.7170	0.6947	0.755
FH2164	0.7745	0.5357	0.664
FH2097	0.6609	0.6332	0.660
Average Średnia	0.6699	0.5975	0.6543

In the group of white Raccoon dogs 27 alleles were found, 23 alleles in the group of standard Raccoon dogs. 21 alleles were common for both groups. The highest number of common alleles was observed at locus FH2054 (4 of 4 identified), CPH3 (5 to 6 identified), FH2097 (4 out of 5 identified). While the least common alleles were observed in the locus FH2168 (4 of 8 identified) and FH2164 (4 to 6 identified). HET-values in the group of white Raccoon dogs ranged from 0.6053 (locus FH2054) to 0.8497 (locus FH2164) and in standard Raccoon dogs from 0.5989 (locus FH2054) to 0.7621 (locus FH2168).

The average heterozygosity was slightly higher among white Raccoon dogs (0.7505) than in the standard Raccoon dogs (0.6778). The values of the expected heterozygosity ranged from 0.617 to 0.804. The lowest value of 0.617 was recorded for FH2054 sequence, while the highest value of 0.804 for the sequence FH2168. Average value of expected heterozygosity was 0.7166. Average observed heterozygosity was lower than

expected and was 0.6936. The highest observed heterozygosity value was observed in locus FH2164 (0.760). The lowest value occurred in the locus CPH3 and reached 0.630. The highest PIC value found was 0.7745 and it was recorded in white Raccoon dogs group for the FH2164 locus. The lowest PIC value in the same group of individuals was 0.5261 for locus FH2054. A similar situation occurred in the group of standard coloured Raccoon dogs, where the lowest PIC value was observed for the FH2054 locus (0.4929), while the highest PIC value of 0.6947 was found for FH2168 locus.

Table 6. Probability of exclusion in white coloured Raccoon dogs group for 5 microsatellite loci
Tabela 6. Prawdopodobieństwo prawidłowego wskazania rodziców u jenotów o umaszczeniu typu białego dla 5 loci mikrosatelitarnych

White Raccoon dogs Jenoty białe		
Locus	probability of exclusion (PE) prawdopodobieństwo prawidłowego wskazania rodziców (PE)	
	PE_1	PE_2
FH2054	0.8239	0.6647
CPH_3	0.6912	0.5125
FH2168	0.6358	0.4582
FH2164	0.5641	0.3873
FH2097	0.7129	0.5438

PE_1 – probability of exclusion for single locus, when one parent data are available.
PE_1 – prawdopodobieństwo prawidłowego wskazania rodziców dla pojedynczego locus, kiedy dostępne są dane jednego z rodziców.
PE_2 – probability of exclusion for single locus, when both parents data are available.
PE_2 – prawdopodobieństwo prawidłowego wskazania rodziców dla pojedynczego locus, kiedy dostępne są dane obojga rodziców.

Table 7. Probability of exclusion in standard coloured Raccoon dogs group for 5 microsatellite loci
Tabela 7. Prawdopodobieństwo prawidłowego wskazania rodziców u jenotów o umaszczeniu typu standard dla 5 loci mikrosatelitarnych

Standard Raccoon dogs Jenoty standard		
Locus	probability of exclusion (PE) prawdopodobieństwo prawidłowego wskazania rodziców (PE)	
	PE_1	PE_2
FH2054	0.8243	0.7045
CPH_3	0.7374	0.5607
FH2168	0.6747	0.4977
FH2164	0.8101	0.6650
FH2097	0.7316	0.5668

PE_1 – probability of exclusion for single locus, when one parent data are available.
PE_1 – prawdopodobieństwo prawidłowego wskazania rodziców dla pojedynczego locus, kiedy dostępne są dane jednego z rodziców.
PE_2 – probability of exclusion for single locus, when both parents data are available.
PE_2 – prawdopodobieństwo prawidłowego wskazania rodziców dla pojedynczego locus, kiedy dostępne są dane obojga rodziców.

For both groups of differently coloured Raccoon dogs the least informative turned out to be locus FH2054, where the PIC was the lowest (<0.6). The most informative was considered FH2164 locus in the group of white Raccoon dogs, where the PIC reached a value of 0.7745, and the FH2168 locus for standard Raccoon dogs where the PIC value was 0.6947. The probability of exclusion in the case when data is available for only one parent ranged from 0.5641 (locus FH2164) to 0.8239 (locus FH2054) in the white Raccoon dogs group, and from 0.6747 (locus FH2168) to 0.8243 (locus FH2054) for the standard Raccoon dogs. In cases when data were available for both parents, the probability of exclusion ranged from 0.3873 (locus FH2164) to 0.6647 (locus FH2054) in the group of white Raccoon dogs, and from 0.4977 (locus FH2168) to 0.7045 (locus FH2054) for the standard Raccoon dogs group.

High genetic similarity (0.8465), and thus a small genetic distance (0.1666) were found between white and standard Raccoon dogs groups.

DISCUSSION

The data obtained from the analyses demonstrate the usefulness of the loci studied to assess the genetic diversity within the different colour varieties of Raccoon dogs and to determine the probability of identifying the right parents. For a group of white Raccoon dogs' locus FH2164 turned out to be the most informative, with the highest PIC value (0.7745) and heterozygosity (0.8497). In standard coloured Raccoon dogs, the highest PIC occurred in the locus FH2168 and was 0.6947. At the same locus heterozygosity for these animals reached the highest value (0.7621).

In parentage testing, in both color varieties, locus FH2054 proved to be the most useful, with the highest values of probability of exclusion. Higher values of this coefficient were observed in both groups if one parent data were available: 0.8239 for white and 0.8243 for standard Raccoon dogs. In the situation when data for both parents was available, the values of a probability of exclusion were much lower (white Raccoon dogs – 0.6647, standard Raccoon dogs – 0.7045).

Genetic similarity value between white and standard Raccoon dogs was high and reached 0.8465. Therefore, the genetic distance between the two color varieties appeared to be small (0.1666). Even lower values of genetic distance between the different colored varieties were recorded in the case of Arctic foxes. The genetic distance between blue and shadow Arctic Foxes was 0.0956 [Jakubczak *et al.* 2011]. Likewise, the genetic distance occurring between groups of Raccoon dogs from different breeding farms in Poland and wild individuals was small and ranged from 0.067 to 0.211 [Ślaska *et al.* 2010].

The differences in both the frequency of alleles, as well as the values of the polymorphic information content (PIC) and heterozygosity (HET) confirm that both Raccoon dogs color varieties: white and standard, are genetically distinct. Further research should be continued.

REFERENCES

Francisco L. V., Langston A. A., Mellersh C. S., Neal C. L., Ostrander E. A., 1996. A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. Mamm. Genome, 7, 359–362.

Fredholm M., Winterø A. K., 1995. Variation of short tandem repeats within and between species belonging to the Canidae family. Mamm. Genome, 6, 11–18.

Jakubczak A., Jeżewska G., 2008. Validation of StockMarks® Set for identifying origin of species from the canine family. Med. Wet., 64 (6), 832–835.

Jakubczak A., Bugno-Poniewierska M., Jeżewska-Witkowska G., Horecka B., Buś-Kicman M., 2011. Genetic differentiation of intergeneric hybrids of Blue Frost foxes and their original forms based on microsatellite polymorphism. Ann. Anim. Sci., 11, 2, 217–225.

Nei M., 1978. Estimation of average heterozygosity and genetic distance from a small numbers of individual.Genetics, 89, 583–590.

Radko A., 2008. Ocena polimorfizmu markerów mikrosatelitarnych DNA wykorzystywanych w kontroli rodowodów bydła w Polsce. Wiad. Zoot., 46, 4, 3–8.

Stępniak E., Zagalska M. M., Świtoński M., 2002. Use of RAPD technique in evolution studies of four species in the family Canidae. J. App. Genet., 43 (4), 489–499.

Ślaska B., Zięba G., Jeżewska G., 2007. Raccoon dog (Nyctereutesprocyonoides gray) parentage testing based on STR markers. EJPAU, 10 (3), 16.

Ślaska B., Zięba G., Rozempolska-Rucińska I., Jeżewska-Witkowska G., Jakubczak A., 2010. Evaluation of genetic biodiversity in farm-bred and wild Raccoon dogs in Poland. Folia Biol. (Kraków), 58, 3–4, 195–199.

Streszczenie. Sekwencje starterowe zaprojektowane dla loci mikrosatelitarnych w genomie psa (FH2054, CPH3, FH2168, FH2164, FH2097), które zostały wykorzystane w badaniach do niniejszej pracy, pozwoliły również na amplifikację analogicznych loci u jenotów odmiany standard i białej. W wymienionych grupach analizowano frekwencję alleli, wartości heterozygotyczności oczekiwanej i obserwowanej, indeks stopnia polimorfizmu oraz prawdopodobieństwo wykluczenia. W wyniku przeprowadzonych badań określono oczekiwany stopień polimorfizmu pięciu loci mikrosatelitarnych u obu odmian barwnych jenotów. Wszystkie z 5 wybranych sekwencji mikrosatelitarnych mogą być wykorzystywane w kontroli pochodzenia jenotów i konstruowaniu map genetycznych w obrębie rodziny psowatych. Otrzymane wyniki podobieństwa i dystansu genetycznego dowodzą, że między grupą jenotów białych i grupą jenotów standard występuje duże podobieństwo genetyczne, a co za tym idzie mały dystans genetyczny. Sugeruje to istnienie tylko niewielkich różnic genetycznych między jenotami białymi i jenotami typu standard.

Słowa kluczowe: jenoty, sekwencje mikrosatelitarne, PCR.