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Single Nucleotide Polymorphism of Melanocortin-4 Receptor Gene in American Mink (*Neovison vison*)

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ABSTRACT

Melanocortins are an important group of proteins controlling food intake and body weight. The most important hormone involved in the regulation of appetite, body weight and systemic energy expenditure is α-melanocyte-stimulating hormone (α-MSH). These effects are caused by α-MSH interacting with its specific membrane receptors: MC1R, MC2R, MC3R, MC4R, and MC5R. In fur animals, growth-related features are particularly important as body weight is associated with the quantity of harvested skin/fur, which directly translates into its value. The objective of the study was to determine the nucleotide sequence of the *MC4R* gene in American mink (*Neovison vison* Schreb., 1777), as well as to detect the genetic polymorphisms within the coding sequence, as well as in 5'-and 3'-UTR fragments of that gene in different color ariations of minks bred at a mink farm. Gene sequencing facilitated the determination of the complete sequence and genomic variations of the *MC4R* gene in American mink, as well as the identification of two synonymic mutations at positions 291 and 330 within the coding sequence of the gene. The *MC4R* gene polymorphism was detected by means of the ACRS-PCR-RFLP technique. The obtained PCR products were digested by the following enzymes: *Nco*I (291), and *Bam*HI (330). Statistically significant differences were observed in the frequency of genotype CC between specimens of different color variations with regard to the single-nucleotide polymorphisms at positions 291 and 330. Also demonstrated was a disturbance of genetic equilibrium estimated according to the Hardy-Weinberg formula.

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MK conceived the project, worked out, created and deposited MC4R gene sequence in GenBank and wrote the article. AMM executed the experimental work.

Key words:
American mink, synonymous, mutation/SNP, melanocortin-4, receptor, MC4R polymorphism

INTRODUCTION

Melanocortins are an important group of proteins controlling food intake and body weight. The most important hormone in that group, involved in the regulation of appetite, body weight and systemic energy expenditure is α-melanocyte-stimulating hormone (α-MSH) (Hwa *et al*., 2001; Kim *et al*., 2000). These effects are caused by α-MSH interacting with its specific membrane receptors. Five types of membrane receptors were identified for α-MSH, namely MC1R, MC2R, MC3R, MC4R, and MC5R. Of these, MC3R and MC4R are located in hypothalamic nuclei and are involved in the control of food intake and systemic energy homeostasis (Mounien *et al*., 2005). The melanocortin 4 receptor protein regulates the food intake process and body weight in cooperation with leptin and agouti-related protein (AGRP), thus playing an important role in the maintenance of proper body weight (Dhillo *et al*., 2002; Świtoński, 2013). Polymorphism of the *MC4R* gene has been described in numerous farm animals, such as pigs and cattle, as well as in fur animals. The effects of that polymorphism on growth, food intake, adiposity and conformation-related features have been demonstrated (Kim *et al*., 2004, 2006; Meidtner *et al*., 2006; Houston *et al*., 2004; Jokubka *et al*., 2006). In the breeding of fur animals, including mink, animal weight is an important breeding target as it is related to animal size. Therefore, *MC4R* may be considered a candidate gene (Nowacka-Woszuk *et al*., 2011).

The objective of this study was to determine the nucleotide sequence of the *MC4R* gene in American mink (*Neovison vison*, Schreb, 1777), as well as to detect the genetic polymorphisms within the coding sequence, as well as in 5'- and 3'-UTR fragments of that gene in different color variations of minks bred at a mink farm.

MATERIALS AND METHODS

The study material consisted of 139 non-cognate adult male minks bred at a farm in Zachodniopomorskie province. After slaughter, muscle tissue was collected from carcasses and DNA was isolated from that tissue using a High Pure PCR Template Preparation Kit with High Pure Spin Filter Tubes from Roche® (Germany), according to the isolation protocol provided by the manufacturer.

Two pairs of primers were designed for

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determination of the nucleotide sequence of the Americanmink *MC4R* gene:
(5'-CACGGGACAGGTACTCAACA-3'/59.6°C;
5'-GCATGGTGAAGAACATGGTG-3'/60.0°C and
5'-TCTCAATTGCAGTGGACAGG-3'/59.8°C;
5'-TAACAATGCACAGCCCAAAA-3'/60.1°C)
on the basis of the canine *MC4R* gene sequence (GenBank NW_003726046.1), including the 5'-UTR, the entire exon and the 3'-UTR (1360 bp), overlapping over a sequence of 192 bp within the central part of the exon. Next, PCR was carried out to obtain amplicons for further sequencing. PCR was carried out in a total volume of 15µL according to the following temperature profile: initial denaturation of the DNA matrix for 5 minutes at 95°C followed by 33 cycles of denaturation for 1 minute at 95°C, annealing of primers for 1 min at 60°C, polynucleotide chain elongation for 2 min at 72°C, and final elongation for 10 min at 72°C. The quality of the obtained amplicons (756 bp and 796 bp in size, respectively) was verified by means of agarose gel electrophoresis, and then subjected to direct sequencing using Sanger's enzymatic method (Sanger *et al*., 1977). Gene sequencing facilitated determination of the complete sequence and genomic variations of the *MC4R* gene in American mink, as well as the detection of two synonymic mutations at positions 291 and 330 within the coding sequence of the gene.

The ACRS-PCR technique was used for the detection of mutations at positions 291 and 330 of the encoding sequence of the American mink *MC4R* gene. Specific primer sequences were designed for that purpose using Insilico® software (Bikandi *et al.,* 2010). The sequences specific for SNP at position 291 within the nucleotide sequence of the American mink *MC4R* gene included the forward primer
(291F): 5'-GTTGGTGAGCGTCTCCCA-3'
and the reverse primer
(291R): 5'-GGAAAGCAGGCTGCAAATC-3'.
The primers allowed for the generation of a product with a length of 154 bp. The introduction of an unpaired base (C→A) at position 17 of the forward primer, located near the mutation site, allowed for the establishment of an endonuclease *Nco*I restriction site. The sequences specific for SNP at position 330 within the nucleotide sequence of the American mink *MC4R* gene included the forward primer
(330F): 5'-GGGGTCATAAGCTTGTTGGA-3'
and the reverse primer
(330R): 5'-AACTCTGTGCGTCCGGATC-3'.
The primers allowed for the generation of a product with a length of 187 bp. The introduction of an unpaired base (A→C) at position 4 of the forward primer, located near the mutation site, allowed for establishment of an endonuclease *Bam*HI restriction site. PCR was carried out in a total volume of 15µL according to the following temperature profile: initial denaturation of the DNA matrix for 5 min at 95°C followed by 33 cycles of denaturation for 1 min at 95°C, annealing of primers for 1 min at 60°C, polynucleotide chain elongation for 2 min at 72°C, and final elongation for 10 min at 72°C.

The obtained product, with a size of 154 bp, was subjected to digestion using the *Nco*I restriction enzyme, which recognized and digested the C↓CATGG sequence to form two fragments sized 138 and 16 bp, respectively, when T was present at position 291 of the *MC4R* nucleotide sequence. No digestion occurred when position 291 of the *MC4R* nucleotide sequence was occupied by nucleotide C. The obtained product, with a size of 187 bp, was subjected to digestion using the *Bam*HI, restriction enzyme, which recognized and digested the G↓GATCC sequence to form two fragments sized 168 and 19 bp, respectively, when T was present at position 330 of the *MC4R* nucleotide sequence. No digestion occurred when position 291of the *MC4R* nucleotide sequence was occupied by nucleotide C.

Fragments obtained after endonuclease digestion were separated by electrophoresis on 3% agarose gel (Agarose MS, Roche®, Germany) containing ethidium bromide in TBE buffer for about 90 min at 90V in the presence of the pUC19/*Msp*I fragment size marker so as to determine the genotypes of individual specimens.

Visualization and archiving of PCR products as well as of the restriction fragments was carried out using a Vilber Lourmat® electrophoresis gel visualization, documentation and analysis kit (France).

RESULTS AND DISCUSSION

The obtained PCR products allowed for determination of the complete sequence of the American mink *MC4R* gene. Its total length was determined at 1206 nt. The sequence encompasses a fragment of 5'-UTR (119 bp), the entire exon (999 bp), and a fragment of 3'-UTR (88 bp). The sequence was deposited in GenBank (www.ncbi.nlm.nih.gov/genbank), access number 130 KC155630.1.

The total content of adenine and thymine (A+T) in the nucleotide sequence of the melanocortin 4 receptor gene was 50.17%, while the total content of guanine and cytosine (G+C) was 49.83%. Purines constituted 46.02% of the sequence, while pyrimidines accounted for the remaining 53.98%. The most prevalent nucleotide was deoxycytidine monophosphate, accounting for nearly 27% of all nucleotides within the studied *MC4R* gene

sequence. The content of individual nucleotides, as well as individual two- and three-nucleotide sequences, are presented in Tables I and II.

The comparison of the nucleotide sequence of the *MC4R* gene in American mink revealed 99% similarity with the sequence of the same gene in domestic dog (*Canis familiaris)*, red fox (*Vulpes vulpes)*, arctic fox *(Vulpes lagopus)*, raccoon dog (*Nyctereutes procyonoides*), as well as 92% similarity with the sequence of the same gene in European polecat (*Mustela putorius).*

Sequencing analyses performed in the study revealed two single gene polymorphisms within the encoding sequence of the gene at positions 291(*Nco*I) and 330 (*Bam*HI). Three genotypes, CC, CT, and CC, were detected within the former nucleotide. Their frequencies were 0.259, 0.683, and 0.058, respectively (Table I). Among the examined groups of different color variations, the highest frequency of the CC genotype was observed in the specimens of the Sapphire cross variation, while the lowest frequency of that genotype was observed in the specimens of the Brown variation. The observed differences between variations were statistically confirmed at the significance level of P<0.05. In contrast, the frequency of the CT genotype was highest in the specimens of the Brown variation, while the lowest frequency of that genotype was observed in the specimens of the Sapphire cross variation. The observed differences were statistically confirmed at significance levels of P<0.05 and P<0.01. Among all the examined color variation groups, the TT genotype was the least common. Within the TT genotype group, the highest frequency of that genotype was observed in specimens of Sapphire and Sapphire cross. No TT genotype was observed in the specimens of other color variations. The observed differences were not confirmed statistically. Analysis of the polymorphism within nucleotide 330 (*Bam*HI) revealed the presence of two genotypes: CC and CG. The frequency of both genotypes was as follows: CC: 0.889, CG: 0.101. No GG genotype was found among any of the examined color variants. The frequency of the CC genotype was highest in the Sapphire cross, Pearl, and Brown variations. In the remaining variations it was lower. The observed differences within the frequency of the CC genotype between individual variations were confirmed statistically (P<0.05 and P<0.01). Among the studied color variations, genotype CG was nearly nine times less common than genotype CC. The highest frequency of genotype CG was observed in the Black variation, while the lowest frequency was observed in the Sapphire variation. No CG genotype was observed in the remaining color variations. The observed differences

Table I.- Frequencies of genotypes and alleles *MC4R/Nco*I and *MC4R/Bam*HI.

Mink types	N	291 nucleotide polymorphism (NcoI)						PIC	330 nucleotide polymorphism (BamHI)						PIC
		Genotypes			Alleles				Genotypes			Alleles			
		CC	CT	TT	C	T			CC	CG	GG	C	G		
Sapphire	25	4/0.160a	18/ 0.720a	3/0.120	0.520	0.480	0.375		20/0.800ab	5/0.200	-	0.900ab	0.100	0.164	
Sapphire cross	31	13/0.419abc	13/0.419aBC	5/0.162	0.629	0.371	0.358		31/1.000aC	-	-	1.000aC	-	0.000	
Pearl	25	4/0.160b	21/0.840Bc	0/0.000	0.580	0.420	0.369		25/1.000D	-	-	1.000D	-	0.000	
Brown	28	4/0.143cd	24/0.857d	0/0.000	0.571	0.429	0.370		28/ 1.00bE	-	-	1.000bE	-	0.000	
Black	30	11/0.367d	19/0.633d	0/0.000	0.683	0.317	0.339		21/0.700CDE	9/0.300	-	0.850CDE	0.150	0.222	
Total	139	36/0.259	95/0.683	8/0.058	0.601	0.399	0.365		125/0.899	14/0.101	-	0.950	0.050	0.090	

* Differences between frequencies in columns marked with the same letters are statistically significant. Lower case letters indicate differences at the significance level of P < 0.05, while upper case letters indicate differences at the significance level of $p < 0.01$

Table II.- Observed (obs.) and expected (exp.) numbers for genotypes *MC4R/NcoI* and *MC4R/Bam*HI.

Mink types	Total obs./exp.	Genotypes *MC4R/NcoI*			Significance of differences	Total obs./exp.	Genotypes *MC4R/Bam*HI			Significance of differences
		CC	CT	TT			CC	CG	GG	
Sapphire	25/25.0	4/6.8	18/12.5	3/5.8	0.027*	25/25.0	20/20.3	5/4.5	0/0.3	0.899 NS
Sapphire cross	31/31.0	13/12.3	13/14.5	5/4.3	0.597 NS	31/31.0	31/31.0	-	-	1.000 NS
Pearl	25/25.0	4/8.4	21/12.2	0/4.4	0.000**	25/25.0	25/25.0	-	-	1.000 NS
Brown	28/28.0	4/9.1	24/13.7	0/5.1	0.000**	28/28.0	28/28.0	-	-	1.000 NS
Black	30/30.0	11/14	19/13.0	0/3.0	0.027*	30/30.0	21/21.7	9/7.7	0/0.7	0.783 NS

*$p \leq 0.05$; ** $p \leq 00.1$; NS – statistically insignificant differences .

within the frequency of genotype CG were not confirmed statistically.

The analyses of differences between the observed and the expected values *for MC4R/NcoI* genotype groups (Table II), as based on the Hardy-Weinberg law, showed that the differences were statistically significant for all color variations with the exception of the Sapphire cross. This is evidence of disturbed genetic equilibrium in relation to *MC4R/Ncol* genotypes within the study population. A dissimilar case is observed within the *MC4R/Bam*HI genotype groups. Calculations revealed genetic equilibrium of the study population in relation to these genotypes. This is indicative of a lack of statistically significant differences between the observed and expected numbers of specimens within individual *MC4R*/*Bam*HI genotype groups for all color variations.

The study allowed for identification of the nucleotide structure and the variability of the *MC4R* gene in American mink involving a fragment of 5'-UTR, the entire exon and a fragment of 3'-UTR. They also allowed for the detection of polymorphism within the *MC4R* gene at positions 291 and 330 of the coding nucleotide sequence of the gene. Statistically significant differences were also observed in relation to the frequency of the CC genotype between specimens of different color variations with regard to the single-nucleotide polymorphisms at positions 291 91 (S-0,16; SC-0,42; 181 P-0,16; BR-0,14; BL-0,37) and 330 (S-0,80; SC-1,00; P-1,00; BR-1,00; BL-0,7). Also demonstrated was a disturbance of genetic equilibrium in the animal groups, estimated according to the Hardy-Weinberg formula on the basis of the polymorphism within the *MC4R*/*Nco*I genotype groups.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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