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Characteristics of Structure and Nucleotide Polymorphism of the American Mink (*Neovison vison*) Growth Hormone Gene

Jakub Jan Skorupski

Department of Ecology and Environmental Protection, University of Szczecin, Wąska 13 St., 71-415 Szczecin, Poland
Department of Genetics and Animal Breeding, West Pomerania University of Technology, Piastów 45 Ave., 70-311 Szczecin, Poland

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ABSTRACT

The nucleotide sequence, variation and gene-structure of the American mink (*Neovison vison*) growth hormone gene was analyzed. For this purpose set of 3 amplicons were sequenced, including 5'-UTR and all exons and introns. The study involved 389 animals. The sequencing results revealed the presence of 5 exons (10 bp, 161 bp, 117 bp, 162 bp, 201 bp) and 4 introns (245 bp, 171 bp, 176 bp, 290 bp), with a total length of 1745 bp. Fourteen polymorphic variable sites were identified: 12 SNP substitutions, one single nucleotide deletion and one ins/del polymorphism. The assessment of informativeness and conclusiveness of the identified variable sites shows that the highest ability to differentiate the American mink growth hormone gene genotypes has tri-allelic polymorphism g.1219C>G (PIC = 0.3039), and three di-allelic polymorphisms - g.616G>C, g.846A>G and g.931C>T (PIC>0.2400). The recognized SNPs enrich the database of single nucleotide polymorphisms for American mink.

INTRODUCTION

American mink (*Neovison vison* Schreb. 1777) is a semiaquatic, mesocarnivore mustelid, native to North America (Ray, 2000). Despite the increasing economic importance of the American mink and its wide geographical spread, the genome of this species remains one of the least studied among livestock species (Benkel *et al*., 2012). Genetic studies of this species are needed in relation to both, its economic use and wide geographical spread as harmful, invasive alien species (Skorupski, 2015, 2016).

This study focuses on the growth hormone gene whose structure and nucleotide variation have been thoroughly described for a number of other mammalian species, *e.g.* house mouse, rat, guinea pig, European rabbit (Barta *et al*., 1981; Das *et al*., 1996), as well as for human (Farooq and Sami, 2013). There are also many studies on the relationship between its genetic variability and traits of economic importance in livestock (Barta *et al*., 1981; Lioupis *et al*., 1997; Panicz *et al*., 2012).

cDNA of the American mink growth hormone gene's (*mGH*) was reported for the first time by Shoji *et al*. (1990). The open reading frame includes 648 bp and product of its translation consist of 216 amino acids with app. 24.5 Da (Shoji *et al*., 1990). The complete nucleotide sequence, structure and genetic variation of the *mGH* was not previously described. Thus, the main objective of this study was to determine the complete nucleotide sequence and structure of the growth hormone gene in the American mink and to analyze genetic diversity within this gene.

MATERIALS AND METHODS

DNA sampling and isolation

The biological material came from 389 animals - wild, ranch and feral minks from Canada, Poland and Iceland. Samples came from slaughter-waste and from carcasses of animals killed on roads (no animal has been killed in order to carry out the experiment). Samples were kept frozen at -20°C until analysis.

Genomic DNA was isolated from muscle tissue, using the High Pure PCR Template Preparation Kit from Roche (Germany). Extraction was performed in accordance with the manufacturer's protocol and followed by assessment of the extracted DNA quality, by agarose gel electrophoresis on 1.0% w/v agarose.

DNA amplification

Standard PCR and nested-PCR were used to amplify the growth hormone gene. In order to obtain amplicons

* Corresponding author: jakub@gajanet.pl
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with an optimal length for sequencing, two sets of internal (Set I, For.: 5'- GGCTGCAATGGCTGCTG-3', Rev.: 5'-CCAGGAGCATGGAGTT-3'; Set II, For.: 5'- CCCTTGTCCAGCCTGTTTG-3', Rev.: 5'-CAGGAGAGCAGCCCATAGTT-3') and external primers(SetI, For.:5'-GTCCTGTGGACAGCTCACCT-3', Rev.: 5'-GTTGGCAAACAGGCTGGAC-3'; Set II, For.: 5'-ACTCCATGCTCCTGGTGTTC-3', Rev.: 5'-AGATACGTCTCGGCCTTGTG -3') were designed for two separate nested PCRs, and one set of primers for standard PCR (For.: 5'-GGGAAGGGACCAGGGTATAA-3', Rev.: 5'-CTCTTCTCCCCTCCAGCAG-3').

All primers were designed based on the recognized sequences of CDS and 5'-UTR in the *GH* of the American mink (GenBank: X56120.1, GenBank: E04303.1) and domestic dog (GenBank: AF166119.1), so that the primer sequences were in the GenBank database and at the same time were flanking unknown sequences.

DNA amplification was performed in a mixture with a volume of 15 ml. A ready-to-use 2xPCR mixture from A&A Biotechnology (Poland) was used, comprised of a recombinant Taq DNA polymerase (0.1 U/µl), PCR buffer optimized by the manufacturer, magnesium chloride (4.0 mM), a mixture of deoxyribonucleotide triphosphates (0.5 mM of each dNTP), a red dye, gel loading buffer (enabling direct application of the reaction mixture on an agarose gel) and deionized water. The following composition of PCR reaction mixtures were used:

1) standard PCR reaction - 6,3 µl of deionized water, 6,0 µl of 2xPCR mix, 0,9 µl of forward primer, 0,9 µl of reverse primer, 0,9 µl of extracted DNA,
2) nested-PCR reaction with internal primers-6,6 µl of deionized water, 6,0 µl of 2xPCR mix, 0,8 µl of forward primer, 0,8 µl of reverse primer, 0,8 µl of extracted DNA,
3) nested-PCR reaction with external primers - 8,3 µl of deionized water, 5,0 µl of 2xPCR mix, 0,7 µl of forward primer, 0,7 µl of reverse primer, 0,3 µl of products of a nested-PCR reaction with internal primers.

All PCRs consisted of initial denaturation at 94°C for 5 min., 35 cycles of denaturation at 94°C for 40 s, annealing at 52°C (for the nested-PCR) or 55°C (standard PCR) for 40 s, and polynucleotide chain elongation at 72°C for 40s, and final extension at 72°C for 5 min.

Products of standard PCR and nested-PCR with internal primers were separated by 2.0% agarose gel electrophoresis. In order to assess the size of the isolated DNA fragments, pUC19/*Msp*I DNA marker (A&A Biotechnology, Poland) and GeneRulerTM1kb DNA Ladder (Fermentas, Lithuania) were used.

DNA sequencing

In order to determine the nucleotide sequence, the resulting amplicons were subjected to Sanger sequencing. Besides determination of the full nucleotide sequence of the American mink growth hormone gene, sequencing aimed at detecting genetic polymorphisms within the gene and direct genotyping. The templates for sequencing reactions were the products of standard PCR and nested-PCR using internal primers. Sequencing involved amplicons obtained from all 389 tested subjects; in each case, sequencing was carried out both from the 5' and 3' ends.

Sequential PCR amplification was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), comprising DNA polymerase, a mixture of deoxyribonucleotide triphosphates, and fluorochrome labeled dideoxynucleotide triphosphate. The sequential PCR amplification consisted of initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50-52°C for 5 s, and polynucleotide chain synthesis at 60°C for 4 min. The next step was to clean the sequential PCR products using a ready-to-use ExTerminator kit (A&A Biotechnology, Poland). The purified sequencing reaction products were separated and read in a capillary sequencer, a 3730xl DNA Analyzer (Applied Biosystems, USA).

Bioinformatic analysis

In assessing the variation of segregating sites, the incidence of variable nucleotides within the established *mGH* gene sequence was determined. The simple ratio of variable nucleotides to all the nucleotides in the sequence was calculated (entire gene, coding and non-coding regions) as well as the percentage of variable nucleotides in the examined sequence (variable nucleotides/total number of nucleotides in the length x 100%). Using DnaSP v5.10.01 software, nucleotide diversity (π) per segregating site, the average number of nucleotide differences (k), and parameter θ - population mutation rate, reflecting the expected resources of neutral variation in the population, were calculated (Librado and Rozas, 2009).

In order to evaluate the genetic variation of the identified segregating sites, analyses of observed number of alleles, effective number of alleles, Shannon's information index, as well as the average heterozygosity for each variable site was performed using POPGENE v1.32 software (Yeh and Boyle, 1997; Riaz *et al*., 2011).

In order to assess the informativeness and conclusiveness of a given segregating sites as a potential genetic marker, polymorphic information content (PIC) was determined, taking into account the number of alleles per given variable site and their frequency (Botstein *et al*., 1980). Identification of specific sequence motifs was made

based on the direct analysis of the determined nucleotide sequence of the *mGH* gene.

RESULTS

The obtained sequences of PCR products (lengths of 232 bp, 286 bp and 1113 bp), after alignment with known sequences (GenBank: X59786.1, GenBank: X56120.1, GenBank: E04303.1, GenBank: E04303.1), allowed determination of the complete nucleotide sequence of the mink growth hormone gene - the total length of the established gDNA sequence is 1745 bp (submitted to the GenBank under accession number JX489617.2 and KR025486- KR025490). The guanine and cytosine content (G+C) is 65.6%. The contents of purines and pyrimidines in the established nucleotide sequence of mink somatotropin are 51.0% and 49.0%, respectively. The most numerous type of nucleotide was deoxyguanosine monophosphate, at a little more than 34% of all nucleotides.

Comparison of the complete sequence of the *mGH* gene with the previously described cDNA sequence of this gene enabled determination of its exon-intron structure. It consists of five exons (10 bp, 161 bp, 117 bp, 162 bp, 201 bp) and four introns (245 bp, 171 bp, 175-176 bp, 272-290 bp), wherein the ratio of the length (in bp) of exons to introns was 0.74 (Fig. 1).

Detection of specific sequence motifs concerned the following signal sequences: the Kozak sequence - at position g.105-111 (5'-AXXATGG-3'), the promoter region - at position g.8-57, the transcription start site (TSS) - at position g.48, the transcription initiator region - at position g.95-100 (5'-TCACCT-3'), and the polyadenylation signal - at position g.1724-1729. Also location of the TATA box, a *cis*-regulatory element, has been detected at position g.16-21.

Direct analysis of the *mGH* gene nucleotide sequence showed typical 5' exon/intron junction's sequence - 5'-GT-3', as well as 3' intron/exon junction's - 5'-AG-3'.

Within the nucleotide sequence of the mink growth hormone gene, 30 segregating sites (S) were detected, comprising fourteen different types of variation in nucleotide structure of the gene (polymorphisms and mutations). In 12 cases this variation was a single nucleotide substitution - 9 cases corresponded typologically to transitions (g.703G>A, g.742G>A, g.748T>C, g.775G>A, g.778G>A, g.846A>G, g.931C>T, g.1156A>G, g.1329T>C), and three to transversion (g.616G>C, g.837G>C, g.1219C>G). Other cases included deletion (del), which applied alternately to a single nucleotide (g.885delC) and 18-nucleotide motif (g.1219_1236delCTCTTGCAGGGGCAGGGG).

The incidence of variable nucleotides within the identified *mGH* gene sequence was 1/58.2 bp (1.72%), while the coding sequences for this parameter assumed a value of 1/130.2 bp (0.77%) and for non-coding sequences 1/43.8 bp (2.29%).

Taking into account only single nucleotide polymorphisms (total number of mutations, η=13), nucleotide diversity was 0.1581. Given this, the average number of nucleotide differences was 1.7390, while parameter θ was equal to 0.1634. When taking deletions into account, and assuming a three-allele model (η=2), parameter π was 0.0057, k=0.1720, and θ (per all nucleotides subject to deletion) was 0.2760.

For variable sites g.616G>C, g.703G>A, g.748T>C, g.778G>A, g.837G>C, g.846A>G, g.931C>T, g.1156A>G and g.1329T>C 2 alleles and 3 genotypes were identified; for variable sites g.742G>A g.775G>A, g.885delC and g.1219_1236delCTCTTGCAGGGGCAGGGG-2 alleles and 2 genotypes, whereas for the variable site g.1219C>G - 3 alleles and 4 genotypes. The average value of the effective number of alleles in all analyzed variable sites was 1.2143. The greatest value of the average heterozygosity was found for variable site g.748T>C (0.0889) and the lowest for variable sites g.742G>A (0.0020) and g.775G>A (0.0041). Average heterozygosity for all variable sites was 0.1489.

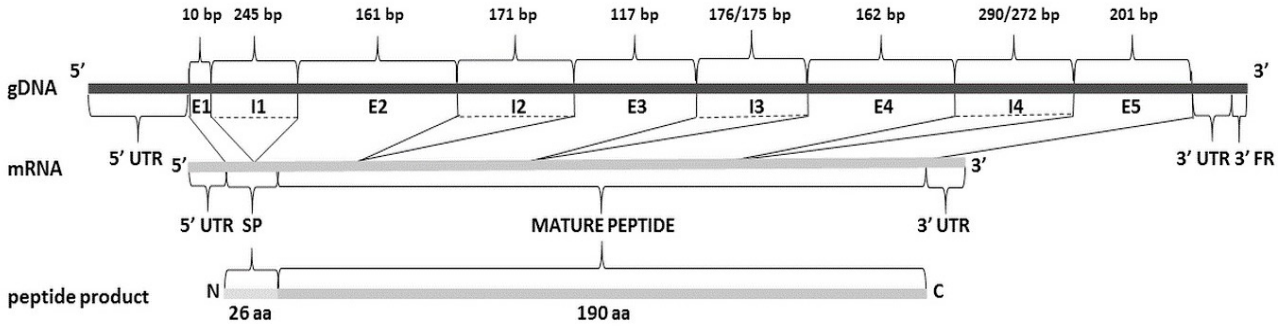

Fig. 1. The structure of the *mGH* gene (FR, flanking region; E, exson; I, intron; UTR, untranslated region; SP, signal peptide).

Among the two-allele variable sites, the highest PIC were observed for g.931C>T (0.2850), g.846A>G (0.2638) and g.616G>C (0.2452), while the lowest PIC for g.742G>A (0.0020) and g.775G>A (0.0060). For a three-allele variable site, g.1219C>G, PIC=0.3039. No significant deviation from HWE were found for g.616G>C, g.742G>A, g.748T>C, g.837G>C, g.846A>G and g.1329T>C. Detailed information on all described genetic diversity parameters are listed in Table I.

Table I.- Genetic diversity parameters of the *mGH* variable sites.

Variable site	Parameter				
	n_a	n_e	I	H_{ave}	PIC
g.616G>C	2	1.4022	0.4614	0.2725	0.2452
g.703G>A	2	1.1571	0.2620	0.1179	0.1262
g.742G>A	2	1.0026	0.0098	0.0020	0.0020
g.748T>C	2	1.0940	0.1835	0.8890	0.0823
g.775G>A	2	1.0052	0.0179	0.0041	0.0060
g.778G>A	2	1.1778	0.2843	0.1339	0.1392
g.837G>C	2	1.1053	0.1989	0.1101	0.0905
g.846A>G	2	1.4553	0.4921	0.2943	0.2638
g.885delC	2	1.0912	0.1795	0.0641	0.0806
g.931C>T	2	1.5253	0.5283	0.3265	0.2850
g.1156A>G	2	1.1689	0.2749	0.1369	0.1335
g.1219C>G	3	1.5110	0.6136	0.2901	0.3039
g.1219_ 1236del18bp	2	1.2079	0.3142	0.0625	0.0839
g.1329T>C	2	1.0968	0.1874	0.1804	0.1572

n_a, observed number of alleles; n_e, effective number of alleles; I, Shannon's information index; H_{ave}, average heterozygosity; PIC - polymorphic information content.

DISCUSSION

A characteristic feature of the *mGH* gene is the relatively high (reaching 65.6%) content of G+C nucleotides. For comparison, the growth hormone gene of the domestic dog contains about 63.3% G+C nucleotides (GenBank: U92533.1), in pig it is ca 61% (Rothschild and Ruvinsky, 2011), llama about 59.4% (GenBank: HM921333.1), and for carp and tench about 39.8% (GenBank: JX392402.1, GenBank: HM114351.1).

The high content of guanine and cytosine nucleotides is characteristic of mammalian genes, especially with relatively short introns. The high G + C content is also associated with high levels of recombination (Galtier *et al*., 2001). The biological sense of maintaining high-energy-consuming genome regions rich in G+C nucleotides is explained in the reduced risk of nonsense mutation. This is particularly important in the case of genes determining the condition of the entire body, such as the *GH* gene (Schmid and Flegel, 2011).

Interestingly, although a high G+C content is found in areas rich in cDNA, a higher C+G content in introns (average about 67.0%) than in exons (on average around 63.3%) was observed. A similar situation was observed in the porcine growth hormone gene, in which cDNA contains approximately 59.0% G+C nucleotides (Rothschild and Ruvinsky, 2011). In the case of the mink growth hormone gene there was no variation in the percentage of G+C nucleotides in individual introns, unlike the rat growth hormone where it is a characteristic feature (Barta *et al*., 1981).

The identified exon-intron structure of the mink growth hormone did not differ from the general scheme found for mammals - 5 exons separated by 4 introns of different lengths (Lioupis *et al*., 1997). The *mGH* gene exon lengths are similar to the lengths of exons identified in the mouse (10, 161, 117, 162, 299 bp) (Breier, 1999), deer (13, 161, 117, 162, 198 bp) (Lioupis *et al*., 1997), and even tench (9, 141, 117, 162, 201 bp) (Panicz *et al*., 2012).

The specific sequence motifs, located within the nucleotide sequence of the American mink growth hormone gene, do not differ from the general structure scheme of eukaryotic genes (Lynch and Conery, 2003). The structure and location of sequence motifs associated with the initiation of transcription and polyadenylation signal for the *mGH* gene are not significantly different from the findings in other mammals (Barta *et al*., 1981; Das *et al*., 1996; Lioupis *et al*., 1997). Significant differences concern only the TSS site which, in case of different species, is different nucleotide-guanine nucleotide in the American mink, cytosine nucleotide in mouse and hamster, adenine nucleotide in rat (Barta *et al*., 1981; Southard *et al*., 1991; Das *et al*., 1996).

The most characteristic *cis*-regulatory element, detected in the *mGH*, is the TATA box (Goldberg-Hogness box), a binding site of the TATA-Binding Protein (TBP) that is part of the transcription preinitiation complex (Schluesche *et al*., 2008). A similar location of the TATA box was found in the growth hormone gene sequence of domestic dog (GenBank: U92533.1), rat (Barta *et al*., 1981), mouse (Das *et al*., 1996) and red deer (Lioupis *et al*., 1997).

Intron boundaries of the *mGH* are designated by typical splicing signals. Hence, the 5'-GC-3' sequence at the beginning of the intron 1, found in the pig and other animals (Vize and Wells, 1987), is not a case in the

American mink.

The number of identified cases of single nucleotide variation (mutations and SNPs) and deletion variation within the exons and introns of the *mGH* gene were higher than in cattle, the domestic pig or Atlantic salmon, with about 7, 4, and 7 SNPs or indels, respectively (Theilmann *et al*., 1989; Lucy *et al*., 1991; Høj *et al*., 1993; Yao *et al*., 1996; Li *et al*., 2006; Ryynänen, 2006; Mullen *et al*., 2010).

The incidence of variable nucleotides within the *mGH* gene is different for coding and non-coding sequences- 0.77% and 2.29%, respectively. Therefore, there is a characteristic uneven distribution of the identified genetic variation- 83.3% of variable sites in the *mGH* gene were located within non-coding sequences, and only 16.7% within coding sequences; in the central region were 30.0% of the variable nucleotides, which constituted as many as 64.3% of the identified types of *mGH* gene structural variation (polymorphic/mutation sites). This situation is similar in the case of the growth hormone gene in cattle in which ca 60.0% of identified genetic variation is located within non-coding sequences (Theilmann *et al*., 1989; Lucy *et al*., 1991; Høj *et al*., 1993; Yao *et al*., 1996).

Importantly, all mutations and polymorphisms identified for the coding sequences in the mGH and located in exon 3 were synonymous. It is worth noting that for some other species, the total number of nucleotide variations detected for the cDNA in the growth hormone gene was greater and often included non-synonymous SNPs and mutations that change the primary structure of the gene protein product. This has also been described, inter alia, for cattle (Lucy *et al*., 1991; Yao *et al*., 1996; Mullen *et al*., 2010).

Nucleotide diversity in the *mGH* gene was very high at 0.1581 (or 0.1592, depending on the applied calculation algorithm) for single nucleotide variation and 0.0057 for deletion variation, and greatly exceeded that for the growth hormone gene in Atlantic salmon (0.0015) (Barta *et al*., 1981). The calculated value of nucleotide diversity was also high in genomic terms; average nucleotide diversity for mammals is 0.0036 (Lynch and Conery, 2003). The reason for this is probably the very high genetic heterogeneity of the studied minks, and the existence of a complex internal structure in the study population. The choice of nucleotide sequences for which π is determined is likely also of great importance. This value, calculated for 49 selected segments of DNA in humans, gorillas, bonobo and the common chimpanzee is 0.087, 0.158, 0.076, and 0.134, respectively (Yu *et al*., 2004).

The informativeness and conclusiveness of individual segregating sites as evaluated by polymorphism information content, shows that the ability of the identified segregating sites to distinguish genotypes for the *mGH* gene was relatively high. The highest suitability for the assessment of genetic differentiation between the study groups was found for the three-allele polymorphism g.1219C>G (PIC=0.3039), and the two-allele polymorphisms g.616G>C, g.846A>G and g.931C>T (PIC>0.2400). These most informative variable sites can be used to determine from which parent a given allele originates with a probability of 24-30%. This highlights the need for simultaneous examination of several SNPs or indels, or by resorting to microsatellite markers.

The assessment of genetic variation of the segregating sites in the *mGH* gene was also based on the observed number of alleles, the effective number of alleles at a variable site (determining how many alleles are responsible for the observed level of heterozygosity in the population) and the Shannon information index. The highest values of these indicators, and thus the greatest genetic variation (genetic polymorphism), was found for the three-allele variable site g.1219C>G, indicating a particularly high informativeness and usefulness in determining the resources of genetic diversity in an individual, and also in inter-individual and inter-population comparisons. A high usefulness in this respect is also shown by variable sites g.931C>T, g.846A>G and g.616G>C. The lowest informativeness and conclusiveness for analysis of genetic variation was found for the mutation variable sites g.742G>A and g.775G>A, and also polymorphic site g.748T>C, g.885delC>C and g.1329T. These results are entirely consistent with the results of analysis based on PIC, and, apart from variable site g.748T>C, also those based on the average heterozygosity. All this proves that an efficient evaluation of genetic diversity in the *mGH* gene, both with respect to an individual and to inter-individual analyses as well as population-based studies, may be limited to the aforementioned most informative variable sites.

CONCLUSIONS

The growth hormone gene of the American mink has a structure and size typical for *GH* genes in other mammals. Furthermore, the overall scheme of the *mGH* gene structure fits perfectly into the overall *GH* gene structure in the subphylum Vertebrata, demonstrating a relatively high level of conservatism (Kawauchi *et al.*, 2002). However, analysis of available literature data shows the unique character of the 18-nucleotide deletion g.1219_1236delCTCTTGCAGGGGCAGGGG in the American mink. SNPs reported in this paper enrich single nucleotide polymorphisms data-base for the American mink, which can be used in the construction of physical

maps of this species, identification of genes responsible for traits of economic importance, in comparative and functional genomics research, or the implementation of genetic marker-assisted breeding programs (MAS).

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Statement of conflict of interest

Authors have declared no conflict of interest.

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