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RESEARCH ARTICLE

Growth Hormone Gene Polymorphisms in Mature Healthy Male Muscovy Ducks in Egypt

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Abstract

Blood samples were obtained from 28 mature healthy male Muscovy ducks for screening of a partial sequence of Growth hormone gene (GH gene) for single nucleotide polymorphisms using PCR amplification of the extracted DNA. The results of our research revealed one locus of exon 2 of growth hormone gene and its partial flanking intron. Polymorphisms in this locus were investigated by using direct sequencing and single strand conformational polymorphism (SSCP) technique. One unique pattern of SSCP was detected. Nucleotide sequencing was performed to endorse the current result which proved the absence of single nucleotides' polymorphisms (SNPs) or any other type of polymorphisms in the studied locus. The DNA sequencing and PCR-SSCP patterns confirmed that there was no polymorphism in all studied birds. In turn, it is spelled that PCR-SSCP failed to produce electrophoresis patterns capable of discriminating between the GH gene loci in Muscovy duck breed revealing no polymorphism in all studied individuals. Also, we compared our sequences with the published sequence on the gene bank of GH gene in Mallard ducks under the following accession number AB158762.1. Three SNPs were detected between Muscovy and Mallard breeds which are (94 A-G, 271 C-T and 298 G-A) these SNPs led to change of two amino acids proline and valine amino acids in GH protein in mallard ducks into serine and isoleucine in Muscovy ducks. In conclusion, these changes in the nucleotide sequence, amino acid and protein chain may be the main cause of differences of the phenotypic features, productive and reproductive traits between Muscovy and mallard duck breeds.

Keywords: Growth hormone gene, Polymorphism, Muscovy duck, PCR-SSCP.

Introduction

Ducks are a widespread poultry species found worldwide. A great interest has been paid for maximizing ducks' eggs and meat production in Egypt, because these birds are easily domesticated, disease resistant and also have highly feed conversion ratio [1]. In Egypt there are many of duck breeds including Native Sudanese, White Pekin, Mallard Muscovy and Campbell [2]. Not only large amounts of cheap raw materials and significant amounts of high quality palatable meat can be provided by ducks, but also, large number of eggs can be obtained from these birds in a short period of time [3].

Ducks were domesticated for many centuries and had provided worldwide commercial projects and food resource for individuals around the globe. Eggs and meat as well as down-feathers are provided by ducks for man. Ducks can be fed on simple moderate diets derived from food stuffs provided locally and grow to maturity within short time. Ducks' meat and eggs are considered a very good source for nutritional requirements including protein of high quality and energy, besides that, it provides a lot of vitamins and essential minerals [4].

Domestic ducks can be classified into the following; Mallard (*Anas platyrhynchos*) and

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Muscovy Ducks (*Cairina moschata*) ducks. Mallard ducks include the majority of known breeds; the most famous breeds of these groups are the Pekin, Albio, Tsaiya, Rouen, Call, Khaki Campbell, Indian Runner, Cayuga, Asylesbury, and Maya breeds. The Muscovy Ducks (*Cairina moschata*) has enormous genetic variations from mallard derived breeds. [2]

Muscovy breeds are known for more tasting meat than the usual domestic ducks, sometimes compared to roasted beef, not only for its leanness and tenderness like veal, but also, for their heavier carcass [5]. Unfortunately, Muscovy ducks are weak egg layers (about 75-80 egg per year), which results in high costs for rearing [6].

Chicken growth hormone which is a very interesting hormone secreted from the anterior pituitary gland [7], has a molecular weight approximately 4 kb, and consisted of five exons and four introns [8]. Unlike the mammalian GH gene which is extended over 2-3 kb [9], the duck GH gene is about 5.25 kb in size, but similar in structure to the GH genes of mammals and chickens (comprises five exons split by four introns) [10].

The growth hormone gene (GH) usually affects large number of physiological parameters such as control of feed intake, feed conversion, growth rate, ageing, body composition and reproduction [11, 12] beside the immunity itself [13]. Genetic variations in GH gene are responsible for abnormal changes like dwarfism and acromegaly [14], indicating the presence of great phenotypic mutations with critical effects.

Growth hormone (GH) is a peptide hormone which is secreted from the exocytosis of somatotrophs in the adenohypophysis via a series of physiological stimulations including the actions of GH releasing hormone (GHRH) and somatostatin, and fluctuations in the blood concentrations of glucagon, insulin, estrogen and thyrotrophin releasing [15-17]. Previous studies on the polymorphisms in the gene of the GH proved the relationship between GH and growth rate, fat deposition, and egg production [18-20].

Molecular markers are considered as the most powerful tools for the genomic analysis. It easily associates the heritable traits with the corresponding genomic variation. Candidate genes investigations and their effect on the phenotypic traits play very important role in marker-associated selection (MAS) for improved productive and reproductive performance of animals; one of these genes is the growth hormone gene (GH) [21].

SNPs are single nucleotide change in certain sequence of the genetic material of the individuals of a population. SNPs usually are recurrent in the non-coding region of the genetic material, however, in the coding regions, it becomes either non-synonymous, means the SNP which causes alteration in an amino acid sequence or synonymous that have not alter the amino acid sequences in the protein chain. However, change mRNA splicing causing phenotypic differences [22]. PCR- Single Strand Conformation Polymorphism (PCR-SSCP) can confirm mutations accurately as it has significant advantages over any other nucleic acid techniques due to its efficiency in the analysis of allelic and mutational sequence variation [23].

This research aimed to detect the polymorphism in GH gene in Muscovy ducks by direct sequencing and single strand conformation polymorphism (SSCP).

Materials and methods

This experiment was performed during the period from 2016 to 2017 at the research unit of molecular biology and biotechnology, Animal Wealth Development Department, Faculty of Veterinary Medicine, Zagazig University, Egypt. This study was approved by the committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University.

Animals:

A total number of 28 mature healthy male Muscovy ducks were raised under normal

management conditions, had free access to water and were fed on complete commercial diets *ad libitum*.

Sampling

Blood samples were collected under strict aseptic conditions by wing vein puncture into vacutainer tubes containing EDTA (Qingdao Hiprove Medical Technologies Co. Ltd., China) as an anticoagulant and then transmitted to the laboratory in ice box containing gel cool packs without delay. Blood samples were stored at -20°C till the extraction of DNA for further analysis.

Extraction of genomic DNA

The genomic DNA was extracted from the collected blood samples (n=28) according to Miller *et al.* [24] with few modifications, using Gene JET genomic DNA extraction kit according to the manufacturer's protocol (Fermentas, #K0721). Samples were digested with Proteinase K in either the supplied Digestion or Lysis Solution. RNA was cleared by treating the samples with RNase A. The lysate is then mixed with ethanol and transmitted to the purification column where the DNA binds to the silica membrane. Impurities were effectively removed by washing the column with the prepared wash buffers. The genomic DNA was eluted under low ionic strength circumstances with the Elution Buffer. Then the efficiency of DNA was examined by agarose gel electrophoresis, the DNA samples free from smearing were selected for further examination.

Polymerase chain reaction (PCR)

One pair of gene-specific primer was used in this experiment according to GH gene sequence (Gene Bank accession no.: AB158760) that had been used before by Chang *et al.* [25].

The primer sequence was as the following:

F: 5`** TTAGGGTTAGCACAGGCAGAAGA **3`

R: 5`** TCTGACAGGAGACACCAGTAGTTGA **3`

The annealing temperature of this primer is 56^{o}C and it produces 384 bp amplicon. To ensure primer sequence is unique for the template sequence, its similarity was compared to previously mentioned sequences with Blast (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Polymerase chain reaction mixtures were carried out in a volume of 50 µl which contained the following: 4 µl of DNA template + 25 µl of PCR Master Mix + 1.5 µl of Primer forward + 1.5 µl of Primer reverse + 18 µl of nuclease free water. The final reaction mixture was placed in T-professional thermal cycler (Biometra, Germany) under the following PCR conditions: initial denaturation at 94^{o}C for 5 min followed by 35 cycles of 94^{o}C for 1 min for DNA denaturation, annealing at 56^{o}C for 1 min, extension at 72^{o}C for 90 sec and final extension at 72^{o}C for 7 min. The samples were finally held at 4^{o}C.

Gel Electrophoresis Procedure

Gel electrophoresis was made following the procedure of Buitkamp *et al.* [26], in which 8 µl of PCR product samples were loaded into the gel and the products of the reaction were electrophoresed on 1.5% agarose gels using 1X TAE buffer containing 200 µg/ml ethidium bromide. The stained gel was then examined with UV light of gel documentation system. Fragment size was determined using Gene Ruler™ 50 bp Ladder (Fermentas).

Single stranded conformation polymorphism (SSCP)

SSCP was used to detect the different genotype patterns that appeared as a result of the existence or absence of the SNPs in the gene under study. Following PCR amplification of genomic DNA from each sample under study, the PCR products were subjected to SSCP analysis according to the procedure previously used by Orita *et al.* [27] in which aliquots of 5 µL of PCR products were added to 5 µL of formamide denaturing dye, heated for10 min at 98°C, and chilled on ice for 5 min. The samples were separated by electrophoresis on 8% neutral polyacrylamide gel at 120 V for 6–8 h at 4°C. The gels were stained with ethidium bromide 0.5 mg/mL.

DNA sequencing

The purified PCR products (n = 4) obtained by using silica – gel membrane adsorption kit (Jena Bioscience # pp-201×s) were sent to MacroGen Company (South Korea) to be sequenced in both directions using ABI 3730XL DNA automated sequencer (Applied Bio system, USA), using GH gene primers which were used for PCR amplification.

The sequences were analyzed using the Chromas Lite 2.1 program (**http://technelysium.com.au/?page_id=13)** and the identity of the sequenced PCR product was examined using Blast search against Gene bank database of duck growth hormone gene **(http://blast.ncbi.nlm.nih.gov/Blast.cgi).** The alignments, annotations and assembly of the sequences were performed using **CLC Main Workbench 7.**

Results

The amplified product of the GH gene (exon 2 and its partial flanking intron) was about 384 bp (Figure 1).

The PCR-SSCP showed only one pattern in all individuals under investigation which reveal no polymorphisms between them (Figure 2). Nucleotide sequencing confirmed the results and showed no SNPs in the GH gene locus. To detect polymorphic sites/SNPs, all sequenced amplicons (4 samples) of GH gene locus were aligned and compared using CLC Main Workbench7 software (Figure 3). The results revealed that there were no differences between all sequences of GH gene (exon 2 and its partial flanking intron) in all individuals under investigation.

Comparing the sequence of the GH gene locus under study with the existed database in the Gene bank for mallard ducks with the following accession numbers AB158760.2 showed 99% similarity with 3 SNPs (94 A-G, 271 C-T and 298 G-A) of which one is synonymous and two are non-synonymous at the positions illustrated in Figure (4). These two non-synonymous SNPs lead to change of proline and valine amino acids in GH protein in mallard ducks into serine and isoleucine in Muscovy ducks

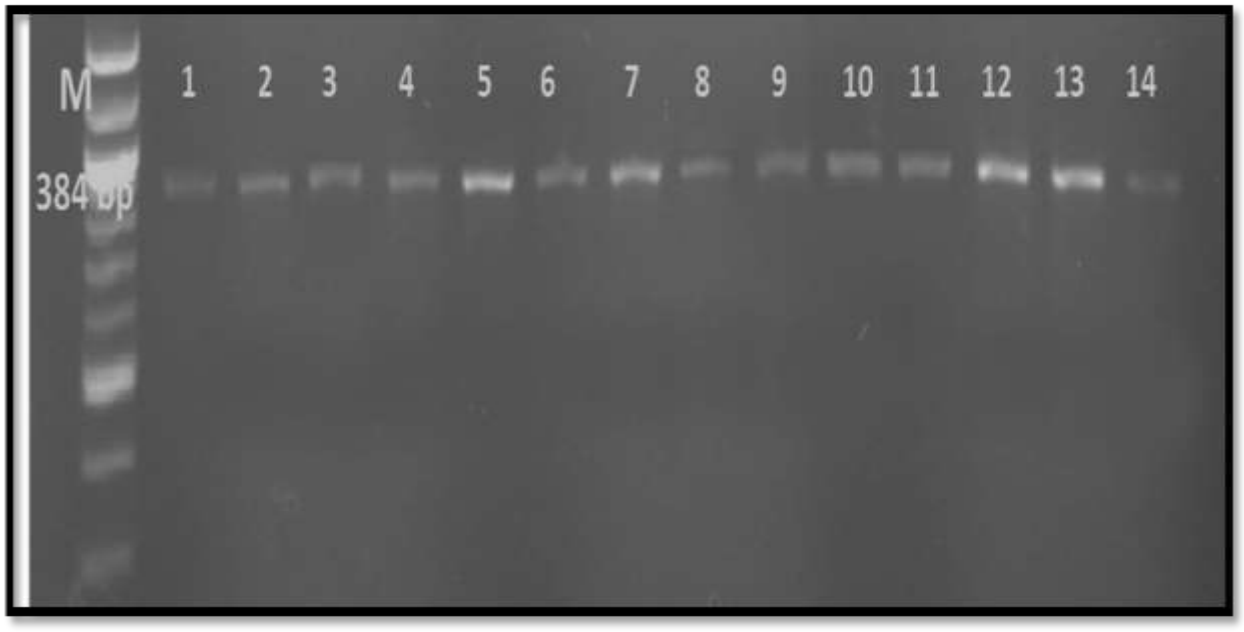

Fig. (1): 1.5% Ethidium bromide stained agarose gel showing 14 PCR products of growth hormone gene from different individuals from Muscovy ducks (1-14). M refers to 50 bp DNA ladder.

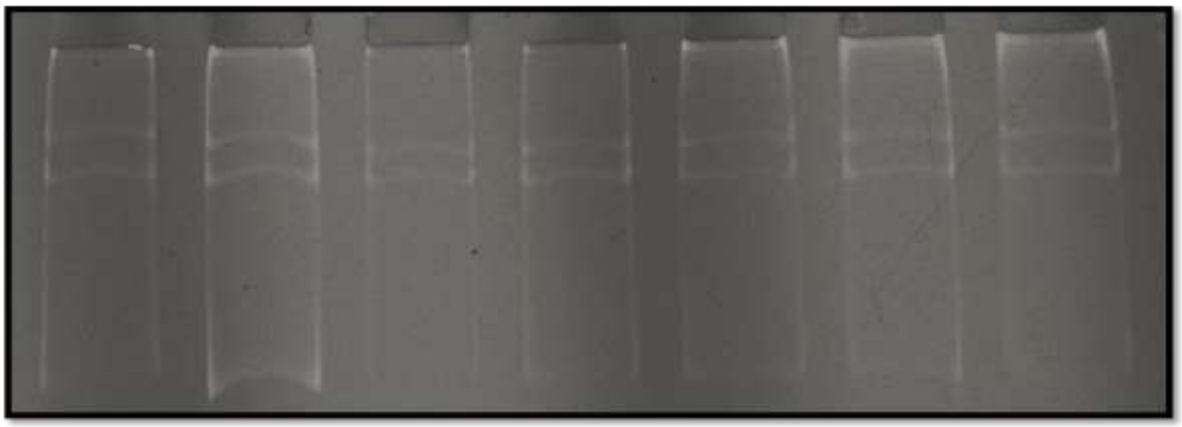

Fig. (2): 1.5% Ethidium bromide stained agarose gel showing one PCR-SSCP pattern of Growth hormone gene (exon 2) in Muscovy ducks, all tested individuals are dimorphic (lanes from 1 to 7).

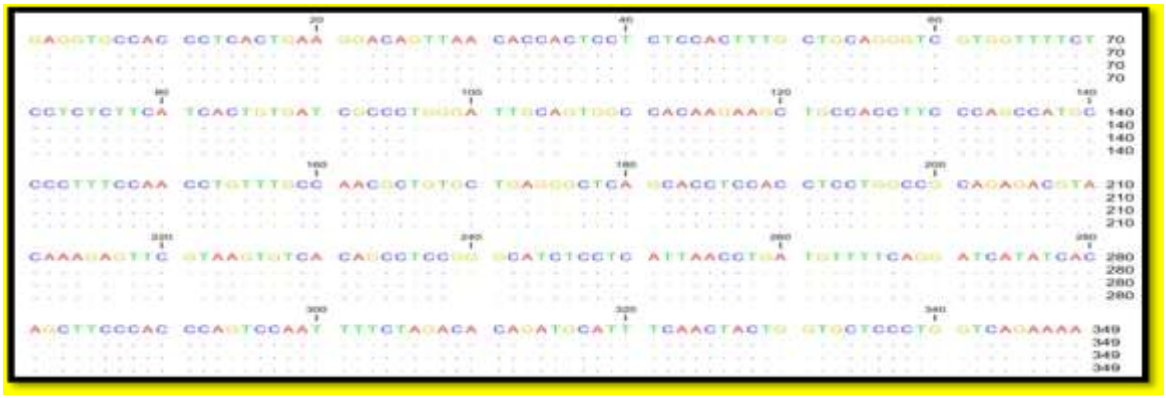

Fig. (3): Alignment of DNA sequence of GH gene locus (exon 2) in 4 sequenced samples using CLC main Workbench7 software, showing no SNPs between all nucleotide sequences of GH gene (exon 2).

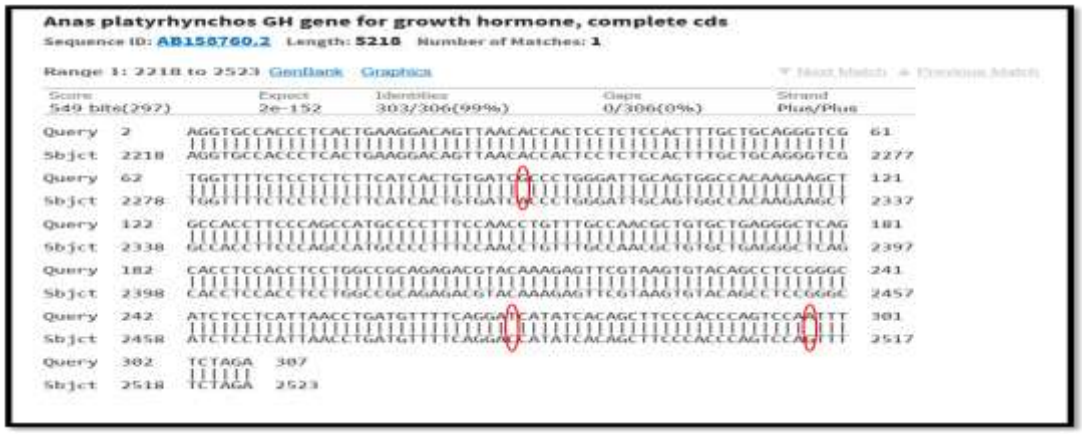

Fig. (4): Alignment analysis of Growth hormone nucleotide sequences (exon 2) of Muscovy ducks (query) and Mallard duck (subject) showing SNPs at positions (94 A-G, 271 C-T and 298 G-A).

Discussion

The advancement of molecular genetics and DNA molecular markers established new methods to study livestock genetics and animal performance. Depending on genotypic selections, a great improvement of productivity of farm animals, and the adaptation to environmental conditions can be reached while keeping the genetic diversity between different individuals [28]. Considering the molecular markers one of the most effective method for the analysis of the genetic diversity in the herd and achieving the association of heritable characteristics with underlying genomic variation, we can detect the variations of the candidate genes and their effect on the phenotypic traits. This resembles the basis for MAS (marker assisted selection) [21].

In this investigation a fragment which is about 384 bp in length was obtained for the GH gene (exon 2 and its partial flanking intron), this is in agreement with the results reported by Chang *et al.* [25]. However, Wu *et al.* [29] Who isolated a fragment of GH gene with total length of 348 bp.

PCR - SSCP showed no differences between sequenced amplicons of four individuals of Muscovy breed. This may be an evidence for that our sequence may be conservative for Muscovy ducks. These results are different from the results of the research conducted by Wu *et al.* [29], where, they detected one single nucleotide polymorphism (A 3270 G) by sequencing, and the frequencies of the A and G alleles in the population were 0.65 and 0.35, respectively. Also, Nie *et al.* [30] found a total of 46 SNPs of which, 5 in exons of GH gene of four divergent chicken breeds. In the research made by Chang *et al.* [25], they found 19 SNPs in a region of 2087 base pairs (bp) in the Tsaiya ducks GH gene of which three coding SNPs were recorded. However, Kansaku *et al.* [19] stated that five single nucleotide polymorphisms (SNPs) were found in the promoter region of the duck GH gene. This difference with other studies may be due to limited sample size tested in the present experiment.

Absence of SNPs in the tested GH gene loci among the studied individuals of Muscovy ducks does not constitute an evidence for the low significance of this locus in differential genomic studies. Therefore we aligned the coding sequences of this locus with the sequence of Mallard ducks and found three SNPs (94 A-G, 271 C-T and 298 G-A), one of these SNPs is synonymous and two are non-synonymous (lead to change in the amino acid sequence of the protein chain) between the coding sequence of GH gene locus in Muscovy ducks when aligned with the same locus of Mallard ducks. These two non-synonymous SNPs lead to change of proline and valine amino acids in GH protein in mallard ducks into serine and isoleucine in Muscovy ducks. These changes in the nucleotide sequence, amino acid and protein chain may be the main cause of the phenotypic features and productive and reproductive traits between Muscovy and mallard duck breeds.

These results disagreed with the results of Nie *et al.* [30] in which they detected one SNP in exon II (G 951A) which led to change of Threonine amino acid to Alanine amino acid (T13A) this SNP was significantly associated with body weight at all ages measured. Also, our results differ from the results of Wu *et* al. [29]who could not detect any SNPs in Exon 2 of Tsaiya ducks.

Evidence with several white goose breeds suggests that exon 2 of the GH gene is long, while the other four exons are short, and that all single-nucleotide polymorphisms (SNPs) are in exon 2 [31], making exon 2 the logical target for genetic studies.

Conclusion

PCR-SSCP failed to produce electrophoresis patterns capable of discriminating between the GH gene loci in Muscovy duck breed revealing no polymorphism in all studied individuals. Also, we compared our sequences with the published sequence on the gene bank of GH gene in

Mallard ducks under the following accession number AB158762.1. Three SNPs were detected between Muscovy and Mallard breeds which are (94 A-G, 271 C-T and 298 G-A) these SNPs led to change of two amino acids proline and valine amino acids in GH protein in mallard ducks into serine.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

تعدد الاشكال الوراثية لهرمون النمو فى ذكور البط المسكوفى البالغة والسليمة ظاهريا فى مصر

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تم تجمع عينات دم من ٢٨ طائر من ذكور البط المسكوفى البالغة و السليمة صحيا لفحص تسلسل تم تجمع عينات دم من ٢٨ طائر من ذكور البط المسكوفى البالغة و السليمة صحيا لفحص تسلسل جزئي لجين هرمون النمو لوجود تعدد لأشكال النيوكليوتيدات الأحادية والمترتب على انحرافات النيوكليوتيدات بواسطة تفاعل البلمرة المتسلسل فى الحمض النووى المستخلص. اظهرت نتائج البحث ان التتابع المعزول يمثل الاكسون الثانى لهرمون النمو مع جزء من الانترون المحيط به ويتكون من ٣٨٤ قاعدة نيتروجينية وتم تحديد تعدد الطرز المظهرى بواسطة تحليل البلمرة المتسلسل و وتقنية تعدد الأشكال المطابقة ذات الأحادية SSCP. تم تحديد طرز مظهرى واحد لجين هرمون النمو فى جميع الافراد محل الدراسة .اكدت نتائج فحص تسلسل القواعد النيتروجينية للجزء المعزول من الجين نتائج اختبار ا تعدد الأشكال المطابقة ذات الأحادية حيث لم يظهر اى اختلاف بالقواعد النيتروجينية للبط محل الإختبار. وبمقارنة التتابع المعزول بالتتابع المنشور على بنك الجينات لجين هرمون النمو للبط المالل تلاحظ وجود ثلاثة طفرات وهى (271 C-T and 298 G-A , 94 A-G) والتى ادت الى تغيير اثنان من الاحماض الامينية هى البرولين و الفالين فى جين هرمون النمو للبط المالل الى السيرين و الأيزوليوسين فى البط المسكوفى. لذا من الممكن ان تكون هذه الطفرات هى المسبب الرئيسى لاختلاف الطرز المظهرى والصفات الانتاجية بين السلالتينز وحيث ان هذه الدراسة قد خلصت الى عدم وجود عدم وجود اى فروقات جينية بين افراد البط المسكوفى محل الدراسة لذلك من الممكن ان يكون هذا التتابع المعزول من جين هرمون النمو هو جين محافظ لسلالة البط المسكوفى