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A NOVEL POLYMORPHISM WITHIN INTRON B OF GROWTH HORMONE GENE (*GH2*) OF THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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K e y w o r d s: rainbow trout, *Oncorhynchus mykiss,* growth hormone gene, PCR-RFLP.

A b s t r a c t

Nucleotide composition of both growth hormone variants of rainbow trout (*Oncorhynchus mykiss*) has been strongly preserved evolutionally what might suggest that any change within these sequences can have an influence on the functioning of the somatotropic axis. A 121 bp fragment that contained nearly the entire B intron was amplified by the polymerase chain reaction. PCR products were bidirectionally sequenced. PCR products were digested by TaiI according to manufacturer's instructions and resulting DNA was subjected to electrophoresis. An analysis of the gene fragment for growth hormone 2 showed the presence of SNP, easily identifiable by means of digestion with *Tai*I restriction enzyme. Statistical analysis confirmed that homozygous GH^{BB} fish were the longest (31.77 cm) and the heaviest (404.70 g) and were statistically significantly different ($P \leq 0.05$) from heterozygous GH^{AB} fish. Mean length of GH^{AA} homozygous fish was insignificantly lower (30.06 cm) with mean body weight of 339.12 g than homozygotes GH^{BB}.

CHARAKTERYSTKA POLIMORFIZMU W SEKWENCJI INTRONU B GENU HORMONU WZROSTU (*GH2*) PSTRĄGA TĘCZOWEGO, *ONCORHYNCHUS MYKISS*

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S ł o w a k l u c z o w e: pstrąg tęczowy, *Oncorhynchus mykiss,* gen hormonu wzrostu, PCR-RFLP.

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Abstrakt

Sekwencja nukleotydowa obu wariantów genu hormonu wzrostu pstrąga tęczowego (*Oncorhynchus mykiss*) jest silnie zakonserwowana ewolucyjnie. Każde nowo powstałe miejsce polimorficzne może mieć więc wpływ na funkcjonowanie osi somatotropowej. Badano niepełną sekwencję intronu B genu hormonu wzrostu typu 2 o długości 121 pz, uzyskaną po przeprowadzeniu dwukierunkowej reakcji sekwencjonowania. Otrzymane produkty PCR poddano analizie z wykorzystaniem endonukleazy *Tai*I, a wynik tej reakcji zobrazowano poprzez rozdział otrzymanych fragmentów restrykcyjnych w żelu agarozowym. Podczas analiz wykazano obecność mutacji punktowej, którą można łatwo zidentyfikować poprzez trawienie sekwencji nukleotydowej intronu B enzymem restrykcyjnym *Tai*I. W analizie statystycznej otrzymanych danych potwierdzono, że homozygotyczne GH^{BB} pstrągi o średniej długości 31,77 cm oraz masie 404,70 g różniły się statystycznie istotnie ($P \leq 0,05$) od osobników heterozygotycznych GH^{AB}. Średnia długość (30,06 cm) oraz masa (339,12 g) homozygotycznych GH^{AA} ryb były nieistotnie niższe od analogicznych parametrów osobników z genotypem GH^{BB}. Scharakteryzowany polimorfizm ma istotny statystycznie wpływ na tempo wzrostu badanych osobników pstrąga tęczowego.

Introduction

The rainbow trout (*Oncorhynchus mykiss*) plays an important role in Polish and world aquaculture. In 2010, Polish aquaculture production of this species amounted to approximately 13 000 tonnes and came second after the carp (*Cyprinus carpio*) with 15 400 tonnes (FAO 2010). In order to speed up process of trout growth a complex mechanism of growth regulation was analysed, especially taking into account the somatotropic axis. A major element of the axis is the growth hormone, whose synthesis and secretion take place in somatotropic cells of the pituitary gland under control of *Pit-1* factor (LEFEVRE et al. 1987, BOLLIET et al. 2001). An analysis conducted on *O. mykiss* lead to a discovery of the presence of two *GH* genes: *GH1* and *GH2*, resulting from genome-doubling event that occurred 25-100 million years ago (ALLENDORF and THORGAARD 1984). *GH1* and *GH2* mRNA consist of 630 nucleotides and encode 210 amino acid residues. Both forms differ by 22 nucleotides in the protein-coding region and their synthesis depend on sex, age and density of the fish. Level of mRNA *GH2* was reported lower than *GH1*, specifically in pituitary glands of 10-day-old fry and 2-year-old females (AGELLON et al. 1988, YANG et al. 1997). Moreover, YANG with co-authors (1997) revealed within 5' flanking regions, exons and introns of *GH1* and *GH2* sequences relating to the cAMP-response elements, thyroid hormone-response elements, retinoic acid-response elements, estrogen-response element (only in *GH1*), and glucocorticoid-response elements. Nucleotide sequences of both growth hormone variants have been strongly preserved in the course of evolution which might suggest that any change within these sequences can have an influence on the functioning of both the somatotropic axis and growth performance (RENTIER-DELRUE et al. 1989).

Selection of fish based on various genetic markers has resulted in a faster growth rate of the trout (O'MALLEY et al. 2003, DREW et al. 2007). One from the interesting markers are SNP's (single nucleotide polymorphism) that may occur in both coding and non-coding regions (BLACK 2003, DE-SANTIS, JERRY 2007, HE et al. 2012). Scientific literature provide examples in which point mutations located within *GH* sequence influence some productive traits of fowls, goats, cows or less frequently fish (LAGZIEL et al. 1999, MARQUES et al. 2003, LEI et al. 2007, AMINAFSHAR and REZA 2012, NI et al. 2013). Despite the fact that most SNP's occur in introns, these non-coding regions of gene may influence processes of transcription, translation or expression, which in turn might affect growth performance (NI et al. 2013). None of the point mutations found in the less abundant *GH2* (comparing to *GH1*) were associated with growth performance of rainbow trout so far. Therefore, the purpose of this study was to analyse the non-coding part of *GH2* sequence for polymorphism and to assess if the found polymorphism might be associated with a higher growth rate.

Materials and Methods

A total of 97 trout individuals were randomly caught alive during spring time from concrete tank of the fish farm Mołstowo which is constantly supplied with water form River Mołstowa. Fish had the same culture conditions (feed, temperature) and were at the same age. Total length was measured using callipers with 0.01 mm accuracy and weight was assessed using weighting scale with 1g accuracy. Before the fish was released a small piece of caudal fin from each of the trout was dissected and placed in 1.5 ml Safe-Lock micro test tubes (Eppendorf Inc.). DNA extraction was carried out according to standard phenol chloroform extraction method. Purity and concentration of DNA extracts were analysed on a 1% agarose gel and Nanodrop ND-1000 (Thermo Fisher Scientific Inc.) spectrophotometer, then stored in -20°C until PCR (polymerase chain reaction) assays.

According to the sequence of the rainbow trout *GH*2 gene (GenBank acc. code DQ294400) a pair of specific primer sequences was designed using Primer3 program (Table 1). Sequence submitted into GenBank was obtained during our earlier (unpublished) studies. Primers that enabled amplification and sequencing of 381 bp sequence had to be redesigned and as a next step in the presented study authors designed and used *GH*2F and *GH*2R. Hence, length differences between these sequences. A 121-base pair (bp) fragment that contained nearly the entire B intron was amplified by the PCR. *GH* intron sequences had often been used to infer sub-familial phylogenetic relationships

amongst salmonids (OAKLEY and PHILLIPS 1999). Additionally, KIRKPATRICK (1992) described that point mutation in pig intron B of *GH* gene is correlated with important performance traits. The PCR reaction for each sample contained 90 ng of genomic DNA, 10 pmol of each primer, 2 μl 10x PCR Buffer with $(NH_4)_2SO_4$ (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 1.2 μl 25 mM $MgCl_2$, 2 μl dNTP mix and 0.5 units of Taq-polymerase (MBI Fermentas), amounting to total volume of 20 μl. The PCR reactions were performed in a thermal cycler (Perkin Elmer) programmed for initial denaturation in 5 mins at 94°C followed by 35 cycles of 45 secs at 94°C, 1 min at 59°C and 1 min at 72°C, and a final extension over 5 mins at 72°C. After the amplification, PCR products were subjected to electrophoresis on 2% argarose gel. Ten PCR products of each variant were bidirectionally sequenced (IBB PAN, Warsaw, Poland) and analysed with the aid of Chromas (Technely-sium Pty Ltd, Tewantin, Australia) and BioEdit software (HALL 1999). Additionally, all obtained sequences were analysed using the on-line Webcutter 2.0 program to select the restriction enzyme. PCR products were digested by *Tai*I (Fermentas) according to manufacturer's instructions and resulting DNA was subjected to electrophoresis on 2% argarose gel. Significance of the observed differences was analysed based on Duncan's multiple range test.

Table 1

Primers used to amplify the analysed region of *GH*2 gene of the rainbow trout

Name	Sequence	Position of the amplified fragment
*GH*2F	5' TTGAATCTTCTTTTGACACAGCA 3'	110–230*
*GH*2R	5' CAAAATCACAAAGACGGGAGA 3'	

* according to GenBank access code DQ294400

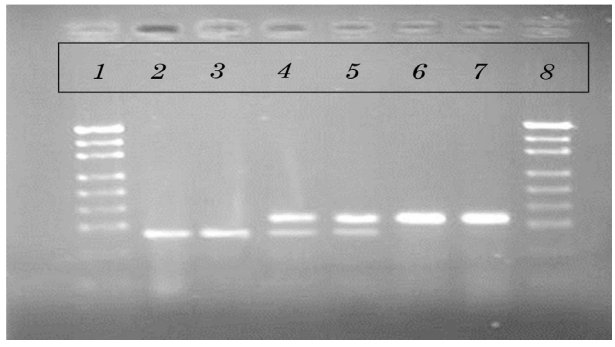
Results

Restriction site predicted using Webcutter 2.0 and verified by digestion proved that *Tai*I recognized the mutation site T → C (recognition sequence acgt↓). The following DNA restriction fragments were obtained for the *rtGH2*/*Tai*I polymorphism (Figure 1):

– 90 and 31 bp for the AA genotype (C at the position 138 (GenBank access code DQ294400) – 2 and 3 lanes;

– 121, 90 and 31 bp for the AB genotype (heterozygote) – 4 and 5 lanes;

– 121 bp for the BB genotype (T at the position 138 (GenBank access code DQ294400); no digestion) – 6 and 7 lanes.



1 and *8* lanes – DNA ladder pUC 19/*Tai* I (9 fragments in bp – 501, 404, 331, 242, 190, 147, 110, 67, 34)
Fig. 1. Electrophoretic pattern of polymorphic GH2 gene of the rainbow trout

Sequence analysis revealed in the analysed amplicons only one site where base pair was T or C, depending on the genotype of the trout. A total of 97 individuals were genotyped (Table 2).

Table 2

Frequencies of genotypes and alleles of the rainbow trout *GH*2 gene

Rainbow trout GH2 gene	rtGH2/TaiI genotype			All	Frequencies of alleles	
	AA	AB	BB		A	B
n	25	48	24	97	0.5052	0.4948
Frequencies of genotypes	0.2577	0.4948	0.2475	1.000		
Length [cm]	30.06 ± 3.91	29.27^a ± 3.92	31.77^a ± 4.13	–	–	–
Weight [g]	399.12 ± 105.41	322.25^a ± 133.36	404.70^a ± 145.08	–	–	–

a – $P \leq 0.05$

Statistical analysis confirmed that homozygous GH^{BB} fish were the longest (31.77 cm) and the heaviest (404.70 g) and were statistically significantly different ($P \leq 0.05$) from heterozygous GH^{AB} fish (322,25 g and 29,27 cm). Mean length (30.06 cm) and body weight (339.12 g) of GH^{AA} homozygous fish was insignificantly lower comparing to homozygous GH^{BB} trout. Mean length and weight of the analysed individuals are given in the Table 2.

Discussion

On the basis of the above data it is obvious that the genotype GH^{BB} had the biggest influence on growth rate of fish in the analysed stock. In spite of that, this kind of mutation occurring in the intron sequence might have a consider-

able influence on the growth and development of the rainbow trout. It is supposed that the location of this mutation might have an influence on mRNA *GH2* splicing and proper functions of growth hormone protein. Literature of the subject has provided examples of intron point mutations that had influence on fish weight gain, e.g. point mutation found within the third intron of growth hormone gene of the Atlantic salmon (*Salmo salar*), which turned out to have substantial influence on the growth rate (GROSS 1999). In their study, TAO and BOULDING (2003) examined the pituitary adenylate cyclase activating polypeptide gene (PACAP) and growth hormone-releasing hormone (GHRH) gene, which had a common mRNA promoter. The researchers concluded that point mutation within the fourth intron conditioned an alternative splicing and determined the presence of two mRNA forms: the shorter PACAP and the longer GHRH, in brains and intestines of the arctic charr (*Salvelinus alpinus*). A practical aspect of the study was the description of a genetic marker which, depending on whether it was G or C, affected growth rate of the species in a manner which was statistically significant. There are also point mutations which do not refer directly to GH structure but have a significant influence on fish growth. A mutation within the intron of laminin-$\alpha 2$ (LAMA 2) gene disturbs the process of splicing and thus non-functional protein is produced. Such a mutation results in the detachment of myofibers, damaged myosepta and growth defects in the brain and eye of the mutant fish, which adds up to congenital muscular dystrophy (GUPTA et al. 2012). In another analysis, SÁNCHEZ-RAMOS et al. (2012) showed a significant connection between MSTN-1 gene polymorphism and growth traits for the gilthead seabream (*Sparus aurata*).

The type of mutation presented in this paper has usually been regarded as unimportant and unlikely to have any possible influence on fish growth rate. However, the novel mutation found in this study in the intron sequence of growth hormone gene 2 has a substantial influence on the length and weight of the rainbow trout. Statistical analysis has confirmed that homozygous GH^{BB} fish were the longest and the heaviest among all fish in the tested group. The analysis discussed here will be continued to find additional polymorphic sites which will be tested in more numerous groups of individuals. A prepared group of genetic markers supported by statistical analysis might be applied to marker assisted selection (MAS) programs aimed at growth rate improvement.

Conclusions

1. Based obtained results it is highly probable that the genotype have influence on lengths and weights of rainbow trout in the analysed tank.

2. Mutation occurring in the intron B sequence of *GH* have a considerable influence on the growth rate of the rainbow trout.

3. Statistical analysis confirmed that fish with GH^{BB} genotype had the best predisposition for growth among all fish in the analysed group.

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