SaMSTNb23 and SaMSTNb33: Emerging Markers for Growth Traits in Huchen (*Hucho hucho*, Linnaeus, 1758)

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Abstract
Huchen or Danube salmon (*Hucho hucho,* Linnaeus, 1758) is an economically and ecologically valuable salmonid species, among the most endangered fish species inhabiting the Danube basin. Consequently, appropriate conservation measures are required to prevent the species extinction. Although *H. hucho* is a poorly studied species, there are several studies on other salmonid species regarding fast growth markers. Following these studies, the main purpose of the current study was to determine growth-related SNPs in myostatin gene in *H. hucho*. Therefore, we analysed 20 aquaculture individuals of same age separated in two groups according length and weight, by sequencing the amplification product of SaMSTNb23 and SaMSTNb33 primers pairs. We also statistically analysed the differences in the development of individuals over time regarding total length, weight and head length. Significantly variations were observed ($p < 0.001$) for the measured traits in both groups. Regarding the genetic analysis, we observed nine SNPs in the *mstn* gene structure. Considering these results, it appears that the analysed markers are not appropriate for genetic analysis of growth traits in *H. hucho*. To extend gene expression pattern analysis, other markers are recommended for further evaluation.

Keywords: aquaculture, growth traits, huchen, myostatin, single nucleotide polymorphisms.

1. Introduction

Among the salmonid fish species, the huchen (*Hucho hucho*, Linnaeus, 1758), is a poorly studied species worldwide [1,2]. The so-called Danube salmon is one of the largest member of the *Salmonidae* fish family [3] and it currently has an endemic distribution in the Danube drainage [4]. In 1873 it was caught caught in the Danube River the largest individual of this species, with a weight of 60 kg and [5,6]. *H. hucho* is an economically and ecologically valuable species due to its size and to its living conditions. Therefore, it is popular among anglers, but, most importantly, it is a flagship species being an indicator of an ecosystem's health [4].

Unfortunately, nowadays, the population status of the Danube salmon is critical, mainly because of various anthropogenic activities (overfishing, deforestation, pollution, dam construction) or climate change [7]. All these factors lead to habitat and spawning sites degradation [1]. It is estimated that the native distribution range decreased by 33% in the last decades [3,8]. There are reports that state the huchen can be sporadically found in Romania in isolated areas, such as the Vișeu, Tisa and Bistrița Rivers and their tributaries [2]. The conservation status of *H. hucho* is assigned as endangered (EN) by IUCN and it is also assessed as a protected species in Bern Convention Appendix III and Annexes II and V of the EU Habitat Directive [9]. Due to its

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special status, this species requires *in-situ* conservation of its habitat and *ex-situ* conservation by hatchery stocking [4].

Morphological aspects (length, weight, age, food) and habitat aspects (temperature, oxygen level, water current, substrate composition) have been studied in order to recommend appropriate conservation measures, especially to manage aquaculture production of this species [10,11,12]. A new approach in such studies is the genetic approach of molecular markers for growth traits analysis. Although no such study has been published so far on *H. hucho*, there are several potential growth trait markers analysed in other fish species. In this regard, SNPs related to growth traits in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) were observed in myostatin (*mstn*), insulin-like growth factor 1 (*igf1*) and growth hormone (*gh*) genes [13,14,15,16,17,18]. Furthermore, MSTN acts as a negative regulator in the growth process [19] and SNPs in *mstn* gene can lead to protein inactivation by altering the gene expression or affecting the splicing process in farmed fish [18].

The aim of this study was to determine whether the *mstn* gene is a potential marker for growth traits in *H. hucho*, improving the huchen aquaculture production.

2. Materials and methods

a. Sampling

Same age hatchlings were raised in the same aquaculture conditions until they were separated in two groups by their total length. Afterwards, each individual was measured monthly, for total length, head length and weight for a period of 4 months, from October 2017 until January 2018. For possible SNPs identification in *mstn* gene, fin clips were collected from 10 individuals of group 1 and 10 of group 2. The samples were preserved in 96% ethanol until DNA extraction.

b. DNA extraction, amplification and sequencing

For DNA extraction the phenol/chloroform protocol was used. The nucleotide sequences for SaMSTNb23 and SaMSTNb33 primers [16] used for partial amplification of the *mstn* gene are listed in Table 1. The PCR reactions were performed in 25 µl reaction volumes, with 50 ng DNA, 1X PCR Buffer, 1.5 mM of $MgCl_2$, 0.64 mM of dNTPs (Promega), 0.32 pmol/µl of each primer, 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems), and nuclease free water. Verity Thermal Cycling PCR System (Applied Biosystems) was programmed with the following amplification program: 94°C for 2 minutes, followed by 35 cycles of denaturation 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The PCR products were processed using Wizard SVGel and PCR Clean-Up System (Promega), BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and BigDye XTerminator Purification Kit (Applied Biosystems). The amplicons were then loaded on ABI 3130 Genetic Analyzer (Applied Biosystems).

c. Data analysis

The initial separation criterion between the two groups was tested using Student's t test. To identify if there are any statistically significant differences among the monthly observations the nonparametric Kruskal-Wallis with Dunn's post hoc test was implemented in GraphPad Prism 6.01. To test whether the slopes and the intercepts are statistically different, the two groups were compared through linear regression analysis in GraphPad Prism 6.01. We used the BLAST algorithm on GenBank to confirm that the primers amplified the *mstn* gene. The resulted sequences were visualized and analyzed with BioEdit [20]. Two sets of sequence alignments were carried out, one for each pair of primers, using the ClustalW option [21].

3. Results and discussion

The development of the individuals under normal culture conditions was monitored and although they are of the same age, the growth process has taken place differently.

The measured traits were total length, weight and head length (Figures 1-2) and the evaluation of these traits was done mainly to determine if there are major phenotypical differences in the development of the individuals over time. The difference between the two groups in regard to total length was statistically significant, with $p < 0.001$ for the initial separation condition. Among the investigated months, a clear growth for total length, head length and weight were observed (Figure 3). For the group 1 individuals, the total length varied significantly between October 2017 and December 2017 ($p < 0.001$), October 2017 and January 2018 ($p < 0.001$), and November 2017 and January 2018 ($p < 0.001$). The same significant differences were observed for head length and weight. Group 2 presented the same significant variation pattern as group 1 with respect to the investigated measurements. As for the linear regression analysis, the slopes differed significantly for weight ($p < 0.001$), while for total length and head length, the corresponding slopes were not statistically different ($p > 0.2$) (Figure 3). The difference between the elevations are extremely significant ($p < 0.001$) for total length and head length, therefore for any given month group 2 will have a significantly longer total length as well as head length compared to group 1. In case of weight evolution, the slopes differ so much; therefore, it is not possible to test whether the intercepts differ significantly.

Table 1. Primer pairs used for molecular analysis of growth markers in *H. hucho*

Primer Name	Primer Sequence
SaMSTNb23 F	5’AAACTGGGCATTCAATGTTCCACCATACCA3’
SaMSTNb23 R	5’CTGCAGTGGCTATTGGGAAAGCTCGCTAAT3’
SaMSTNb33 F	5’ACTTGACGTACGAGCCGAGTTCC3’
SaMSTNb33 R	5’TTGCCGCAGCCACACCGACAAC3’

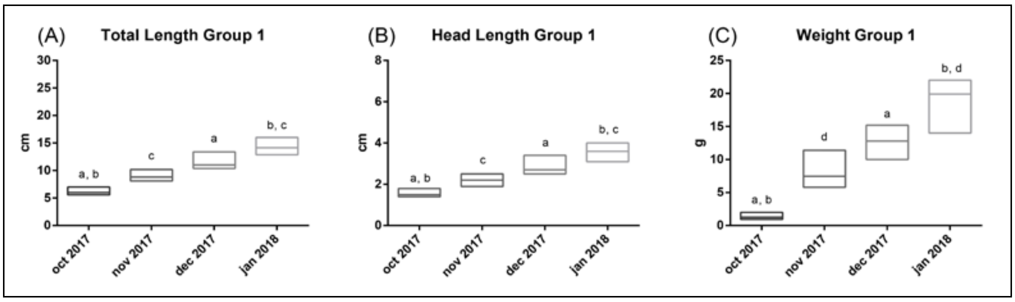

Figure 1. Total length (A), head length (B) and weight (C) measurements for individuals from group 1. The measurements were taken at 1-month interval for 4-months. The nonparametric Kruskal-Wallis with Dunn’s post hoc test compares each two statistically significant sets. a, b, c – $p < 0.001$, .

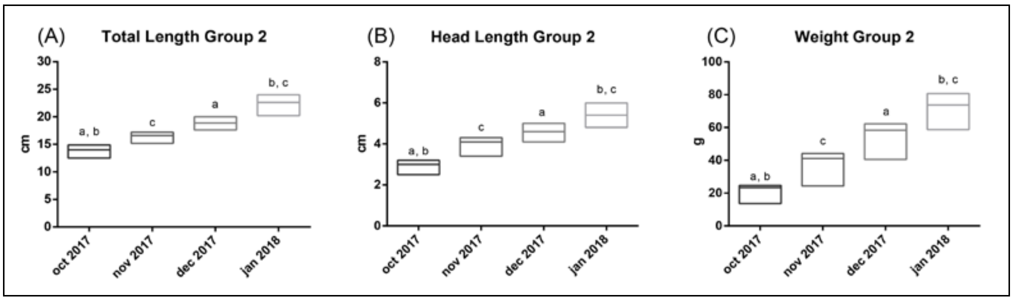

Figure 2. Total length (A), head length (B) and weight (C) measurements for individuals from group 2. The measurements were taken at 1-month interval for 4 months. The nonparametric Kruskal-Wallis with Dunn’s post hoc test compares each two statistically significant sets. a, b, c – $p < 0.001$, .

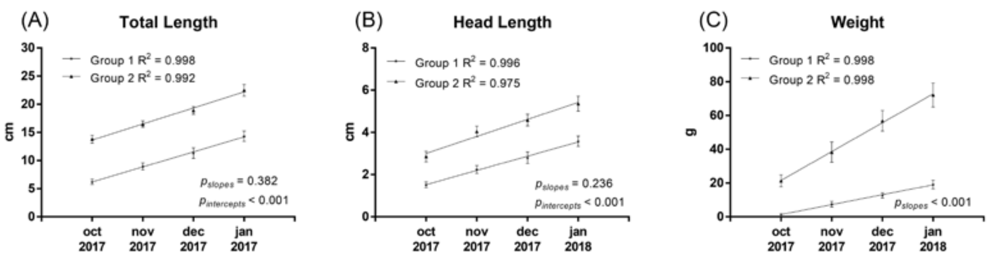

Figure 3. Total length (A), head length (B) and weight (C) linear regression comparison between groups.

R^2 – coefficient of determination, p_{slope} – p value for testing if the slopes are equal, $p_{intercepts}$ – p value for testing if the intercepts are equal.

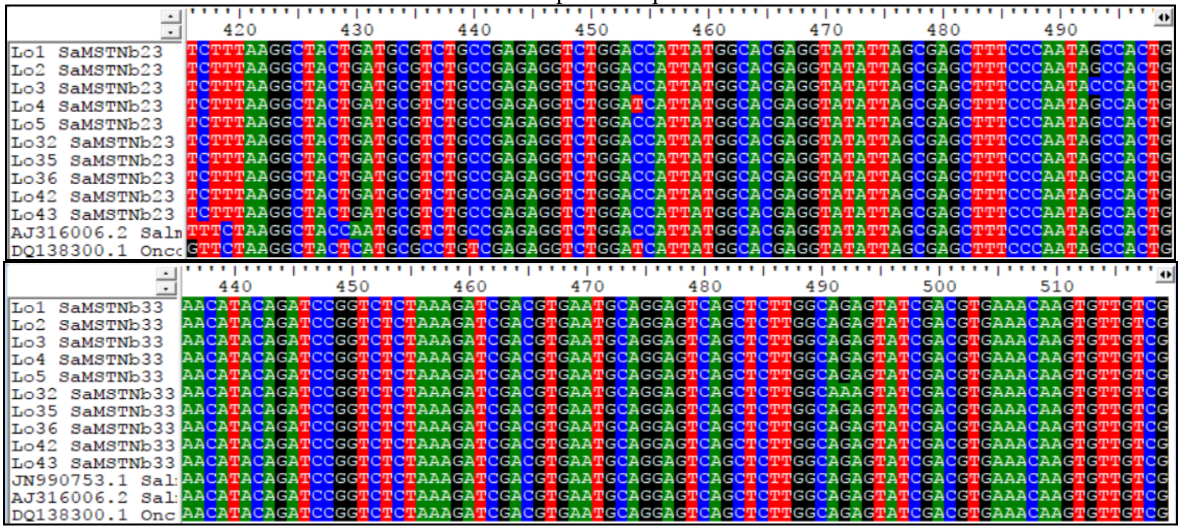

Figure 4. Partial alignments for gene fragments amplified with SaMSTNb23 and SaMSTNb33 primers and reference sequences. Group 1 - individuals Lo1-Lo5, group 2 - individuals Lo32, Lo35, Lo36, Lo42, Lo43.

Considering that the *mstn* gene was not sequenced before in *H. hucho*, the BLAST verification of the sequences obtained in the laboratory was crucial in order to confirm the specificity of the primers. A sequence similarity with the myostatin 1b gene from *S. salar* JN990753.1, AJ316006.2 sequences and *O. mykiss* DQ138300.1 sequence was observed.

For further analysis, the sequences were cut and aligned with reference sequences, the final dataset being comprised of sequences of 503 bp for SaMSTNb23 and 519 bp for SaMSTNb33. The partial alignment of 5 sequences per group is shown in Figure 4. The BLAST analysis for SaMSTNb23 showed similarity to the promoter region of myostatin 1b in both *S. salar* and *O. mykiss*. In the case of SaMSTNb33, the sequence similarity was observed in the regulatory region of *S. salar* JN990753.1, 3` flanking region of the intron 1 and 5` flanking region of exon 2 of *S. salar* AJ316006.2 and exon 2 of *O. mykiss* DQ138300.1. After aligning all the sequences obtained for the 20 analyzed individuals, the following SNPs were identified: 3 T > A, 65 A > T, 101 G > T, 104 C > T, 114 G > A, 453 T > C, and 492 C > G, in the sequences amplified with the SaMSTNb23 primer pair, and 491 A > G, in the sequences amplified with the SaMSTNb33 primer pair. Considering these results, the amplification with the SaMSTNb23 and SaMSTNb33 primers in huchen does not lead to the same results as the ones presented in other studies which were focused on various other salmonid fish species. [16] used the same pairs of primers on *S. salar* and observed that a SNP 1086 C > T in the regulatory region is associated with changes in the growth process.

Furthermore, [14] performed a complex study on *O. mykiss* and the team tried to discover growth associated SNPs markers by sequencing the transcriptome of muscle tissue coming from both fast growing and slow growing individuals. Most of the discovered growth-related SNPs were located in regulatory regions as well. Therefore, the localization of the SNPs plays an important role in the development of the individuals. Despite being part of the same family with *S. salar* and *O. mykiss*, it appears that in huchen the growth signaling is differently controlled. Thus, it appears that the proposed and the analyzed markers are not appropriate for the genetic analysis of growth traits in *H. hucho* and no specific growth correlated SNPs were observed.

4. Conclusions

Fast growth is a trait that presents high interest in aquaculture-oriented studies, thus, genetic understanding of growth associated markers could increase the farmers' profit. By analyzing the myostatin 1b gene as a growth marker in *H. hucho* no SNPs were observed in the gene structure. However, detailed genetic analysis and expression patterns of the individuals are needed in order to explain the size differences. Further studies are recommended in order to evaluate other possible growth markers that should be examined in larger populations as well. Moreover, since the genetic databases lack information about huchen studies, the data gathered in this study could represent a start for various detailed gene expression pattern analysis.

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