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Growth Parameters Evaluation and Identification of Growth Hormone Receptor Gene Polymorphisms in Various Strains of Rainbow Trout *Oncorhynchus mykiss*

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Abstract

In the present study, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and restriction fragment length polymorphism (RFLP) methods were compared to analyze the polymorphism of growth hormone receptor (GHR) gene in French, Iranian and Danish strains of *Oncorhynchus mykiss*. A monomorphic SSCP pattern of AA genotype in the French and Iranian strains and a dimorphic AA and AB genotype in the Danish strain were observed in 3' non-coding regions of GHR gene. In the Danish strain, the AB genotype polymorphism of the GHR gene with its very low frequency (5%) has no effect on the production trait. While, RFLP-Dde1 showed no polymorphism with genetic variation in the location of GHR. Moreover, comparing the condition factors (K) of three different rainbow trout strains showed a significant correlation between the French (1.312 ± 0.13), Iranian (1.245 ± 0.17) and Danish strains (0.763 ± 0.1), respectively ($p<0.05$). In particular, the French strain obtained a higher K compared to the two others. The length-weight relationship is shown by the following equa'ions $W= 0.013 \times L^{2.921}$, $W= 0.012 \times L^{3.023}$ and $W= 0.007 \times L^{3.176}$ for French, Iranian and Danish strains, respectively. With the mean (b= 2.921), the studied French strain exhibited a negative allometric growth b<3 while the Iranian (b=3.023) and the Danish (b=3.176) showed positive allometric growths. Regardless of the underlying mechanism(s) responsible for the different relationships, the results of this study suggested that the AA and AB genotypes polymorphism of the GHR gene are not associated with condition factor and environmental variables can influence condition factor and length-weight relationship of rainbow trout strains in this study.

Keywords: Growth hormone receptor; Enzyme digestion; Single strand conformation polymorphism; Rainbow trout; Length-weight

Introduction

Hormones, growth factors, and other regulatory proteins associated with the so-called "somatotropic axis" are candidate markers for quantitative trait in farm animals [1]. The biological effect of growth hormone (GH) plays numerous important and central physiological roles in the growth, metabolism, reproduction, immune function, osmoregulation, and other physiological functions of fish and other vertebrates [2,3]. Growth hormone receptor (GHR) is the cell surface receptor for GH and is required for GH to carry out its effects on target tissues [4]. As a receptor, GHR mediates the biological actions of GH on target cells by transducing the GH stimulating signal across the cell membrane and subsequently inducing the transcription of many genes, including IGF-I [5]. Although there are few studies on GHR in fish, in Atlantic salmon GHR appears to play a similar role within the somatotropic axis as in humans and its differential expression is what mediates the cellular response to GH plasma levels, and in particular the production of IGF [6].

The fish GHR gene has been successfully cloned and characterized in several fish species such as gilthead sea bream (*Sparus aurata*) [7], Atlantic (*Salmo salar*) and masu salmon (*Oncorhynchus masou*) [8], and rainbow trout (*Onchorhynchus mykiss*) [9]. Significant associations have been observed between GHR gene polymorphism and growth rates by PCR restriction fragment polymorphism (RFLP) marker (using four restriction enzymes) in 353 individual *Cyprinus carpio* fish [10].

Accordingly, cost and time can be significantly reduced based on restriction analysis particularly when a large number of restriction enzyme needed. Additional gene segments can then be included in the analysis favored of population genetic studies and identification of species based on Single-Stranded Conformation Polymorphism (SSCP) analysis. The disadvantage of RFLP method is that mutations are recognized only when they occur in the sequence recognized by the restriction endonuclease, and that PCR products may need to be tested with many different restriction enzymes [11,12]. Because of this limitation, the SSCP technique is more appropriate for detecting point mutations and is more useful than PCR-RFLP to detect polymorphism [12].

In so far as our knowledge is concerned, no prevalent polymorphic site at this locus has been identified. However, given the significant associations between polymorphisms and growth modulation in other vertebrates, this gene should be further examined as a possible candidate for the enhancement of production in finfish. Therefore, the goal of this study is to compare morphometric parameters determine the employment of PCR-RFLP and SSCP methods, and find out whether or not the polymorphism in the 3'-UTR of the GHR gene can affect the condition factor (K) in the aforementioned strains (French, Iranian, and Danish) of rainbow trout.

Materials and Methods

DNA extraction

One hundred twenty rainbow trout of French, Iranian and Danish strains were collected from different rainbow trout breeding farms in

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Mazandaran province (40 samples per strain). In order to isolate the DNA and assay RFLP and SSCP, fin clips were cut from the fish and placed in phenol chloroform and 1 ml ethanol based on Sambrook et al. [13]. The fin clip tissue samples were transferred to the laboratory in dry ice and stored at a temperature -20°C until used for the assay. The DNA was extracted using phenol-chloroform method and the quality and concentration of DNA were assessed through the use of 1% agarose gel electrophoreses. The extracted DNA samples were then stored at -20°C until used for RFLP and SSCP assay.

Primer design and PCR amplification

The ethanol-precipitated DNA sample extracted from each individual fish was used as a template in SSCP and RFLP procedures. The oligonucleotide primers were designed with Primer 5 plus software [14] using information from a Genbank accession number (EU084720.1) on genomic GHR gene sequences of rainbow trout.

Forward and reverse primer sequences were: 5'TAAATGCCAT-CACAAGGA-3' and 5'-AAAGCCACAGTCAATCAG-3', respectively. This pair of primer is expected to amplify a 188 bp segment of the GHR gene, encompassing the sequence from 3'UTR. The optimized PCR reaction mix was performed in a 25 ml containing 10 mM of Tris-HCl with a 8.3 pH, 50 mM of KCl, 2.3 mM of $MgCl_2$, 0.01% gelatin, 100 mM of dATP, dCTP, dGTP and dTTP, 0.2 mM of each primer, 5-50 ng of genomic DNA template, and 0.625 unit of Taq DNA polymerase. Amplification of the GHR gene by PCR was done on a thermocycler (BIORAD) with an initial denaturation of 5 min at 95°C where it was processed through 35 cycles consisting of 30 s at 95°C, 30 s at 60°C and 20 s at 72°C with the last elongation step lengthened to 10 min at 72°C.

SSCP and RFLP

As in described below the DNA of each sample was amplified by PCR and analyzed by SSCP. Aliquots of 5µl of the above mentioned PCR products were mixed with 5 µl of the denaturing (95% formamide, 25 mm of EDTA, 0.025% xylenecyanole and 0.025% bromophenolblue), heated for 10 min at 95°C and chilled immediately on ice. Denatured DNA ran in a 1x TBE buffer (containing 89 mM of Tris-Borate, and 2 mM of EDTA, with a 8.3 PH) for 20 h at 4°C under a constant voltage (150 V) with 14% polyacrylamide gel. The gel was stained with 0.1% silver nitrate and was visualized with 2% NaOH solution (containing 0.1% formaldehyde) according to Zhang et al. [15,16].

For RFLP analysis, 7 µl of the PCR products were digested by 4 units of Dde1 restriction enzyme and were incubated at 37°C for 4-12 h. Digested DNA fragments were separated using electrophoresis employing 2% agarose gel in 1x TBE (containing 89 mM of Tris-Borate, and 2 mM of EDTA). The gel was stained with ethedium bromide and was visualized under UV light.

Length-weight relationship and condition factor

The exact relationship between length and weight differs among species of fish according to the inherited body shape for some species, and the condition (robustness) of the individual fish for others [17]. The relationship between the length (L) and weight (W) of fish is expressed by the following equation [18]:

$W = aL^b$

Where (a) and (b) are the parameters of the above nonlinear model with the latter being a numeral between 2 and 4. Taking logarithmic transformation on both sides of the above equation and the linearized model can be obtained:

$\text{Log } W = b \log L + \log a$

The correlation coefficient r^2, i.e., the degree of association between the length and weight was computed through the linear regression analysis:

$R = r^2$

Confidence intervals were calculated for b slops to see if these were statistically different from 3 (allometric ranges more than 3 show positive allometry while b<3 indicates negative allometry) or were equal with 3 (b=3 shown an isometric range). Growth pattern was estimated using Pauly's modified t-test (1983).

$$t = SdLnL / SdLnW \times |b-3| / \sqrt{1-r^2} \times \sqrt{n-2}$$

The condition factor (K) of the experimental fish was estimated from the relationship [18]:

$$K = W \times 100 / L^b$$

The study of condition, a standard practice in fisheries ecology, is based on the analysis of length-weight data and assumes that heavier fish of a given length are in better conditions [19]. The K of fish is influenced by the reproductive organs stage of development [20] therefore, when comparing K, it is important to sample the populations at the same time of the year when the populations are at the same stage of the reproductive cycle [21].

Statistical analyses

Genotype frequencies were determined for each strain by direct counting. For the association studies, the trait of interest was analyzed using the General Linear Model (GLM) procedure of the SAS program (SAS Institute Inc, Version 9.1 edition). Moreover regression and Correlation analysis (RECA) for linear regression of length and weight of fish were done with Microsoft Excel (2010), One-way ANOVA was used to determine the effects of strains on K using SPSS (version 17). Duncan's multiple range tests were then used to compare differences between the means at 5% probability.

Results

Based on the sequences of the rainbow trout GHR gene available in the GenBank, a pair of PCR primers amplifying the 3'UTR region was designed. Using them, 188 bp of GHR was amplified from nucleotide 285 to nucleotide 473 through the use of PCR-SSCP method (Figure 1).

Monomorphic AA genotype was observed in French and Iranian strains while AB pattern was observed in the Danish strain and the AA genotype was almost fixed in the all strains. On the other hand, RFLP-Dde1 showed no polymorphism with genetic variation in the location of GHR (Table 1).

Through SSCP method, the AB genotype of the Danish strain was presented at 5%. The mean values ± SD of the investigated trait and the effects of GHR genotypes are shown in (Table 2).

The length-weight (L-W) relationship equation 1-3, growth pattern and K (condition factor) model were fitted to the dataset. There was a strong variation in the K among French, Iranian and Danish rainbow trout strains. The K varied between 1.079 and 1.629 of the French, 0.680 and 1.584 for the Iranian and 0.514 and 0.954 for the Danish trains. K value in all three strains were presented in Table 3 and demonstrated that K in the three strains were significantly different ($p<0.05$).

The following equations were detected for each strain (Figure 2):

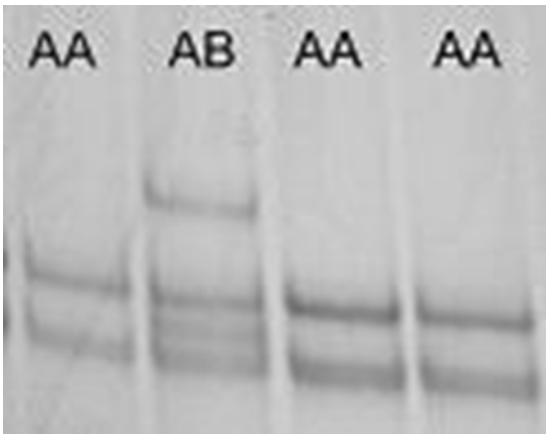

Figure 1: PCR-SSCP banding pattern of GHR in a 14% non-denaturing polyacryl-amide gel.

Methods	Strains	Genotype		Allele	
		AA	AB	A	B
SSCP					
	French	100	-	100	-
	Iranian	100	-	100	-
	Danish	95	5	97.5	2.5
RFLP				(-)	(+)
	French	100	-	100	-
	Iranian	100	-	100	-
	Danish	100	-	100	-

Table 1: Frequency of alleles and genotypes of GHR gene (%) with SSCP and RFLP analysis.

Genotype	K
AA	1.3501 ± 0.02
AB	1.2907 ± 0.12

Table 2: Means ± SD for K trait based on genotypes of Danish strain in rainbow trout.

(1) *Frenchstrains:* $W=0.013 \times L^{2.921}$ $R^2=0.856$

(2) *Iranian strains:* $W=0.012 \times L^{3.023}$ $R^2=0.920$

(3) *Dunish strains:* $W=0.007 \times L^{3.176}$ $R^2=0.877$

Discussion

In this study, the effect of GHR gene polymorphism and its association with production trait in the various strains of rainbow trout were analyzed. Monomorphic SSCP patterns of AA genotype in two French and Iranian strains and a dimorphic AA and AB genotype in the Danish strain were observed. Our data showed that in the latter strain, the AB genotype polymorphism of the GHR gene was of a very low frequency (5%) and not associated with K. Furthermore, the RFLP and SSCP markers were compared in order to detect and identify the polymorphism of the GHR gene. According to the data gathered through SSCP marker, RFLP could detect any polymorphism in the GHR 3'-UTR. Clearly, when dealing with short PCR fragments (188 bp in our case), cutting small PCR fragment with restriction enzymes decline, and long PCR fragments can increase the chance to detect polymorphisms as was demonstrated for GHR gene by RFLP. in this study and analyzed by SSCP is higher than the limit up that reported previously and considered to be exhaustive which is about 150-200 bp [22]. Advantages of SSCP analysis over RFLP analysis have been emphasized previously [23]. SSCP analysis can be done through the use of low level of PCR mixture and a single SSCP gel, which in the long run turned out to be a much more economic method [24]. Another advantage of SSCP analysis is that multiple polymorphism scan be easily identified by re-amplifying and directly sequencing each distinctive band on the gel without the need for long cloning procedures.

K is often associated with fitness, a bad condition for the individual fish. Reproductive success can be reduced through low fecundity, reduction in egg quality or low sperm quality [25] and [26]. Inappropriate conditions may also lower the chances of survival [27]. However, the results of this study showed the diversity of GHR gene in the Danish strain. Shaki et al. [28] reported the genetic distance in three different rainbow strains at 0.0008, 0.1976 and 0.1988 among Iranian-French, Iranian- Danish and French-Danish strains, respectively. According to this result, when K is an appropriate estimate of fitness in rainbow trout strains, our results are in agreement with population genetics theory, which predicts that higher genetic variability increases mean fitness of strains [29]. It should be noted that K in the French strain was higher than in the other two strains. The differences in weight for all strains maybe due to the individual K as it is related to the well-being and degree of fatness [18]. Our data showed that the farm condition of the Danish strain maybe almost unfavorable in comparison to the other strains. However, compared with the current study, the K values of the French and Iranian rainbow trout are relatively higher than those reported previously. Shah et al. [30] reported that the value of K ranged from 0.95 to 1.44 (average K = 1.15 ± 0.111) for *Oncorhynchus mykiss* Walbaum which is close to unity, indicating that the fish are in excellent conditions. Moreover, Rabe et al. [31] found the value of K to be between 0.859 and 1.104 for rainbow trout in Alpine lakes and Cada et al. [32] reported condition factors for rainbow trout collected from southern Appalachian streams that ranged from 0.82 to 1.17.

The results L-W regressions are useful for [17]:

- Calculating the total weight of fish taken from the length-frequency data.

Table 3: Mean ± S.D of L-W relationship and K in rainbow trout.

Strains	K
French	1.312 ± 0.13^{a}
Iranian	1.245 ± 0.17^{b}
Danish	0.763 ± 0.1^{c}

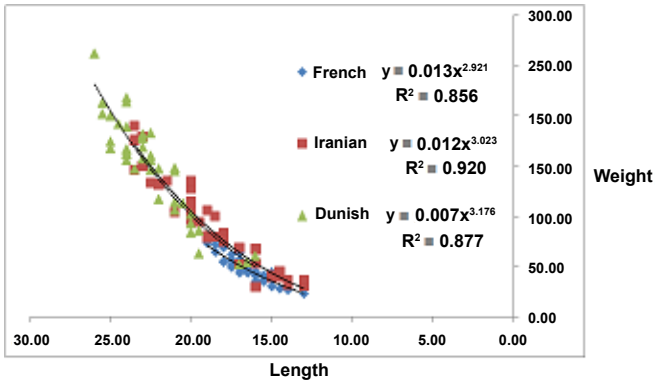

Figure 2: Length-weight relationships of French, Iranian and Danish strains.

- Measuring changes in robustness and health of this population.
- Pacifying the relative condition of small fish compared to large fish.
- Comparing the condition of this population to the state-wide standards discussed below.

The length exponent of 2.921, 3.023 and 3.176 for French, Iranian and Danish strains, respectively, showed isometric growth and our results are in agreement with the Shah et al. [30] and Ekeng [33]. However, the b association was the 0.714, 0.381 and 0.537 for French-Iranian, French-Danish and the Iranian-Danish, respectively, and showed no statistically significant relationship ($p>0.05$). The b value in the L-W relationship of fish can be used as an indicator of food intake and growth pattern, and may differ according to biotic and abiotic factors such as water temperature, food availability and habitat type [34].

In the beginning of this study, only one rainbow trout GHR gene sequence (accession no. EU084720.1) was available in the GenBank. To our knowledge, this is the first study to investigate, compare and determine whether the polymorphism in the 3'-UTR of the GHR gene is related to K using PCR-RFLP and SSCP methods. In the course of this study, we were not able to sufficiently investigate the relationship between genotype and K on the individual level. It may be due to the limited number of loci and individuals per strains in any cases, between strains, no correlation was found between the genotypes (using SSCP marker) and the K in the Danish strain.

Conclusion

In summary, K is an appropriate estimate of fitness in rainbow trout strains. Our results suggest that in strain Danish, the AB genotype polymorphism of the GHR gene were not associated with the K of rainbow trout. Furthermore, environmental conditions are evidently important in determining the K of rainbow trout strains. Emphasizing the importance of performing similar studies in different parts of the distribution range of a species, to get an impartial idea of the generality and importance of the relationship of interest. More studies are needed, and attention to positive as well as neutral results will allow for the explanation of possible patterns and processes.

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