Short communication

Isolation and characterization of tandem repeat sequences in the growth hormone gene of the red seabream, Pagrus major (Temminck & Schlegel, 1843)

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Introduction

Growth hormone (GH) is one of the main regulators of postnatal somatic growth and stimulates anabolic processes such as cell division, skeletal growth and protein synthesis (Goodman, 1993). Several studies have reported the GH gene as a candidate for enhancing production traits such as growth (Tambasco et al., 2003), disease resistance, egg production (Kuhnlein et al., 1997), fat content (Knorr et al., 1997) and milk production (Høj et al., 1993; Dybus, 2002) in livestock. Polymorphisms of the GH gene are also known to be associated with some level of growth performance in several aquacultured fish species such as Atlantic salmon Salmo salar (Gross and Nilsson, 1999), Japanese flounder Paralichthys olivaceus (Kang et al., 2002) and gilthead seabream Sparus aurata (Almuly et al., 2005). Red seabream, Pagrus major is an important sparid fish species in the Japanese aquaculture industry (Murata, 2005). Several hatcheries successfully developed strains showing useful economic traits other than fast growth (Sawayama and Takagi, 2014), and efficient methods are needed to select for growth in these strains. Therefore, understanding the genetic architecture of the GH gene of red seabream (pmaGH) and polymorphisms of tandem repeats located in pmaGH is important for future breeding programs of this fish species.

In this study, we isolated the GH gene from red seabream (including introns and 566 bp of the 5'-flanking region) and characterized the variability in pmaGH based on polymorphisms in the satellite DNA using a wild population of this species. The genomic structure of pmaGH was also compared with some fish GH genes.

Materials and methods

In this study, we used one cultured specimen for isolation of tandem repeat sequences located in pmaGH and 39 wild-caught specimens for evaluation of polymorphisms of tandem repeat sequences of pmaGH. Total DNA was extracted from the caudal fin of a cultured red seabream using the standard SDS-phenol/chloroform procedure (Harris et al., 1991). The primer pair, GH-F and GH-R, was designed based on previously isolated complementary DNA (Acc. No. X00962; Momota et al., 1988) to amplify the entire pmaGH. The PCR protocol used was 50 ng of extracted DNA, 0.1 μm primer, 0.4 μm dNTPs, five units LA taq polymerase (Takara Bio, Shiga, Japan) with 10 × × buffers in total volumes to 10 μl. Thermocycling was done with one initial denaturing step for 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 40 s at 60°C, and 4 min at 72°C, and a final extension step for 5 min at 72°C. The PCR product was cloned into pGEM®-T Easy vector (Promega, Wisconsin, USA) and sequenced using pUC/M13 primers and three gene specific primers (GH Seq F1, GH Seq F2, and GH Seq R1). The primers used for cloning are shown in Table 1.

The 5'-flanking region of the gene was also isolated following the method of Siebert et al. (1995). Genomic DNA was digested separately with DraI, EcoRV, PvuII and SphI blunt-end restriction enzymes, and a blunt adaptor (A and B) was ligated to each digested genomic DNA sequence. Two gene specific primers, GSP1 and GSP2, were designed for the suppression PCR. The DNA library was then subjected to the primary PCR reaction using GSP1 and AP1 primers, and the secondary reaction using GSP2 and AP2 primers. An approx. 600 bp fragment was obtained using the PvuII restriction enzyme. The fragment was cloned into pGEM®-T Easy vector and sequenced using pUC/M13 primers. The primers used for isolating the 5'-flanking region are shown in Table I. Potential transcription binding domains in the promoter region were predicted using TRANSFAC database version 7.0 (http://www.gene-regulation.com).

The pmaGH sequence including the 5'-flanking region was searched for repeat regions using the program, Tandem Repeats Finder (Benson, 1999). We identified several tandem repeat sequences in introns of pmaGH and designed primer pairs using PRIMER3 (Untergasser et al., 2012).

Polymorphism of pmaGH using the newly developed markers was assessed using 39 red seabream individuals caught in the Seto Inland Sea. Total DNA was extracted by High Pure PCR Template Preparation Kit (Roch Diagnosis, Tokyo, Japan) from the caudal fin of wild specimens according to the manufacture’s protocol. The PCR protocol for
allele detection and amplification of short tandem repeats (2–4 bp) has been described previously (Sawayama and Takagi, 2014). Forward primers were labeled with fluorescent dyes and reverse primers were tailed (Life Technologies, California, USA). For larger repeat size (22 bp) analysis, the PCR protocol used was 25 ng of extracted DNA, 0.3 μM primer, 0.4 mM dNTPs, 0.2 unit KOD FX polymerase (TOYOBO, Osaka, Japan) with 2× buffers in a total volume of 10 μl. The PCR reactions were done as follows: one initial denaturing step for 2 min at 94°C, followed by 30 cycles at 98°C for 10 s, 63°C for 30 s, and 68°C for 60 s. The PCR products were separated by Fragment Analyzer (Advanced Analytical, Iowa, USA) on 8 kV for 85 min using a DNF-910 dsDNA Reagent Kit. Band sizes were calculated by PRO Size™ software (Advanced Analytical). Fragments scored at ≤20 bp were electrophoresed in the same capillary and their exact size determined. Polymorphisms among these markers were evaluated using GenAlEx 6.5 (Peakall and Smouse, 2012).

Results
The total length of pmaGH was found to be 3299 bp, comprising six exons (I–VI) of length 72, 134, 114, 144, 147, and 296 bp, respectively, and five introns (1–5) of length 563, 696, 969, 85, and 79 bp, respectively (Fig. 1).

We obtained a 566 bp long DNA sequence upstream of the 5′-untranslated region by means of chromosome walking of pmaGH. The 229 bp upstream from exon I showed high homology (87%) with the 5′-flanking region of gilthead seabream (Acc. No. AY138985). We also compared the promoter region (proximal 150 bp) among the Perciformes based on the data of Almuly et al. (2005) and cis-elements located in the promoter region were highly conserved (data not shown). A typical TATA box (TATAAA) was located at -107 to -112 bp upstream from the start codon. There were two more TATA boxes (also TATAAA) in the pmaGH 5′-flanking region located -450 bp to -455 bp and -500 bp to -505 bp, respectively, which are not seen in the gilthead seabream GH (Almuly et al., 2005). The pmaGH sequence including the 5′-flanking region was deposited in GenBank (Acc. No. AB905431).

Two large repeat sequences were found in introns 1 and 3. The core sequence in these repeats was 17 bp and 22 bp long, respectively. The 17 bp repeat motif contained two elements known to bind chromatin-disrupting factors: unliganded heterodimer of thyroid hormone receptor (TRE; Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH-F</td>
<td>ATCAGATCCAGTCACCAGAACTT</td>
<td>Whole GH sequence amplification</td>
</tr>
<tr>
<td>GH-R</td>
<td>CTGGAATGCAAACAGCACAGA</td>
<td>Whole GH sequence amplification</td>
</tr>
<tr>
<td>GH Seq F1</td>
<td>CTGTCAAGAGTTCACACACCT</td>
<td>Sequencing by primer walking</td>
</tr>
<tr>
<td>GH Seq F2</td>
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<tr>
<td>GH Seq R1</td>
<td>CAGGTGTCCAATGACACACACACT</td>
<td>Sequencing by primer walking</td>
</tr>
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<td>GSP1</td>
<td>TACCTCTTGCTAGCTGGGTCTGGTT</td>
<td>Chromosome walking</td>
</tr>
<tr>
<td>GSP2</td>
<td>CAGCTTCAGGTCAGGTTCTGGTACT</td>
<td>Chromosome walking</td>
</tr>
<tr>
<td>AP1</td>
<td>CCACTGAAATGACACTATAGGGCC</td>
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<td>AP2</td>
<td>CTATAGGACCCAGGCTG</td>
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<tr>
<td>Blunt-adaptor A</td>
<td>GATAATGACTGCTATATAGGGCCAGGCTG</td>
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</tr>
<tr>
<td>Blunt-adaptor B</td>
<td>ACCAGGCCC-NH2</td>
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<tr>
<td>pUC/M13 F</td>
<td>CGCCAGGTTTTTTCAGACTACG</td>
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<tr>
<td>pUC/M13 R</td>
<td>TCACACAGGAAACAGCTATGAC</td>
<td>Sequencing at 3′ end of the insert</td>
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</tbody>
</table>

Fig. 1. Structure of Pagrus major growth hormone gene. Open boxes = Exons, untranslated regions; dark boxes = translated regions. Lines between boxes = Introns. Dotted line = 5′-flanking region. Brackets below figure = size (in base pairs) of each intron and exon. Gray boxes = tandem repeat motifs detected. Brackets = sequence motifs of each tandem repeat, with marker name (upper part of figure). Bold = regulatory elements located in tandem repeats (TGTTCT as GRE; TGACCT as TRE; TCTCTC as GAGA factor).
TGACCT) and GAGA-binding factor (TCTCTC). Also, di- and tetra-nucleotide repeats were observed in introns 2 and 3, respectively. In addition, three repeat sequences (2, 4, and 10 bp) were found in the 5'-flanking region. The 10 bp repeat motif contains several glucocorticoid response elements (GRE; TGTTC). All repeat motifs are shown in Fig. 1.

A total of seven primer pairs were designed, of which two (17 bp of intron 1 and 10 bp of the flanking region) were rejected due to the presence of many non-specific bands (data not shown). The remaining five primer pairs were used for further analysis.

Polymorphisms of the newly developed tandem repeat markers located in pmaGH are shown in Table 2. Three loci were polymorphic (pmaGH4-P, pmaGH22 and pmaGH4), but two di-nucleotide repeats were monomorphic (pmaGH2-P and pmaGH2). The observed and expected heterozygosity values of these markers ranged from 0.077 to 0.821 and 0.074 to 0.864, respectively. One of these markers (pmaGH4) conformed to departure from Hardy–Weinberg equilibrium when tested with a chi-square test using GenAlEx 6.5 (Table 2).

Discussion

In the present study, we first determined the nucleotide sequence of the GH gene and its 5'-flanking region of red seabream. The red seabream GH gene is composed of six exons and five introns, the same as other fish species (Agellon et al., 1988; Male et al., 1992; Ber and Daniel, 1993; Devlin, 1993; Tanaka et al., 1995; Ohkubo et al., 1996; Venkatesh and Brenner, 1997; Tanaka et al., 1995; Ohkubo et al., 1996; Venkatesh and Brenner, 1997; Almuly et al., 2005; Quéré et al., 2010; Kocour and Kohlmann, 2011; Ma et al., 2012). The 5'-flanking region of pmaGH (−229 bp to -1 bp) was highly conserved between red seabream and gilthead seabream GH, and cis-elements located in the promoter (proximal 150 bp) were also conserved among the Perciformes when compared with the result of Almuly et al. (2005). Tandem repeat sequences located in the 5'-flanking region and introns are as reported in other fish species (Almuly et al., 2005; Quéré et al., 2010). Therefore, the genetic architecture of GH gene is highly conserved in fish species.

The microsatellite DNA located in the 5'-flanking region of GH is highly polymorphic in several aquacultured fish species (Almuly et al., 2005; Quéré et al., 2010), and its polymorphism is known to be associated with growth performance in S. aurata (Almuly et al., 2005). Unlike the fish GH gene, microsatellite DNA located in the 5'-flanking region (AC) of pmaGH was monomorphic in the wild red seabream population used in this study. Our result suggests the expression of GH in red seabream is not controlled by the microsatellite length polymorphism in the 5'-flanking region of pmaGH. Almuly et al. (2008) also reported the length of the first intron of the gilthead seabream GH gene regulates its expression. A similar minisatellite motif was observed in intron 1 of pmaGH, but unfortunately in this study this motif was difficult to amplify and we were not able to develop the marker for evaluation of the length polymorphism of intron 1. Further study is required to identify...
whether the length of intron 1 of the pmaGH regulates GH expression, and if so, an evaluation technique of the length of intron 1 of pmaGH needs to be developed.

The 22 bp of the tandem repeat sequence located in intron 3 (the pmaGH2 marker) has a large variant of polymorphisms and contains 23 alleles in the wild red seabream population. The number of alleles observed in this marker is close to that of the other neutral microsatellite markers analyzed in the same wild populations of red seabream (Sawayama and Takagi, 2014). Similar repeat motifs located in intron 3 have been reported in some fish GH genes (Quéré et al., 2010) and used for a population genetic study in European sea bass, Dicentrarchus labrax (Quéré et al., 2010). Our newly developed marker will also be useful for population genetic studies of red seabream as a gene-related marker.

In conclusion, we confirmed that the red seabream growth hormone gene contains polymorphic tandem repeat sequences in its promoter region and introns, and evaluated these polymorphisms using a wild population of red seabream. If these markers are related to growth trait in this species, the markers will accelerate growth selection of red seabream.

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References


Tandem repeat sequences in *Pagrus major GH*


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