cDNA CLONING AND PHYLOGENETIC ANALYSIS OF GROWTH HORMONE GENES FROM THE MULLET *MUGIL PLATANUS* (MUGILOMORPHA, MUGILIDAE) AND THE HALFBEAK *HEMIRAMPHUS BRASILIENSIS* (ATHERINOMORPHA, HEMIRAMPHIDAE)

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RESUMO

Os peixes aterinídeos e mugilídeos são normalmente considerados grupos irmãos, porém existem muitas controvérsias sobre esta classificação. Para investigar a relação filogenética entre aterinídeos, mugilídeos e percomorfos, as seqüências de cDNA do hormônio do crescimento da tainha *Mugil platanus* (Mugilomorpha) e do agulha-negra *Hemiramphus brasiliensis* (Atherinomorpha) foram obtidas utilizando o protocolo RACE (Rapid Amplification of cDNA Ends). O cDNA do agulha-negra apresentou uma seqüência de 615 nucleotídeos, codificando um polipeptídeo de 204 aminoácidos, enquanto o cDNA da tainha apresentou 597 nucleotídeos, codificando um polipeptídeo de 199 aminoácidos. Ambos os hormônios exibem aspectos típicos de GH quando comparados com a seqüência matura de GHs de outros peixes da superordem Acanthopterygii. As seqüências deduzidas de aminoácidos do GH foram utilizadas para a construção de uma árvore filogenética utilizando o método da máxima parcimônia. A topologia encontrada classificou Atherinomorpha e Mugilomorpha dentro de Percomorpha, indicando que os mugilídeos são provavelmente mais relacionados aos perciformes do que os aterinídeos.

PALAVRAS-CHAVE: Atherinomorpha; hormônio do crescimento; Mugilomorpha; filogenia

ABSTRACT

Clonagem do cDNA e análise filogenética dos genes do hormônio de crescimento da tainha *Mugil platanus* (Mugilomorpha, Mugilidae) e do agulha-negra *Hemiramphus brasiliensis* (Atherinomorpha, Hemiramphidae)

Atherinid and mugilid fishes are currently considered sister groups. However, there are many controversies about this hypothesis. In order to evaluate the phylogenetic relationship between aterinids, mugilids and percomorphs, GH (growth hormone) cDNA sequences of the mullet *Mugil platanus* (Mugilomorpha) and the halfbeak *Hemiramphus brasiliensis* (Atherinomorpha) were obtained using the RACE protocol (Rapid Amplification of cDNA Ends). The *H. brasiliensis* GH cDNA contains an open reading frame of 615 nucleotides encoding a preprotein of 204 amino acid residues, while the partial *M. platanus* GH cDNA contains an open reading frame of 597 nucleotides encoding a preprotein of 199 amino acid residues. Both hormones exhibit typical GH features when compared to those of mature GHs from other Acanthopterygian fishes. The deduced GH amino acid sequences were used to infer a phylogenetic tree using the maximum parsimony method. The topology found has placed Atherinomorpha and Mugilomorpha within Percomorpha, indicating that mugilids are probably more related to perciforms than atherinids.

KEY WORDS: Atherinomorpha; growth hormone; Mugilomorpha; phylogeny

1 – INTRODUCTION

Growth hormone (GH) is a polypeptide of fundamental importance for growth regulation in vertebrates and, together with prolactin and somatolactin, constitutes a family of pituitary hormones with similar structure and function which appear to have originated from a common ancestral gene before the evolution of fishes (Kawauchi & Yasuda 1989). Molecular data from nuclear genes such as the GH gene have been recently used as a source of information in order to evaluate evolutionary relationships of fishes at a variety of taxonomic levels, producing phylogenies with substantial statistical confidence (Bernardi et al. 1993; Rubin & Dores 1994, 1995; Venkatesh & Brenner 1997; Clements et al. 2004).

Within the superorder Acanthopterygii one of the most recurrent taxonomic problem is the relationship among the series Atherinomorpha, Mugilimorpha and Percomorpha. Since the classification of atherinomorphs was created by Rosen (1964), several hypotheses about sister-group and family relationship were suggested based only on morphological characters. Gosline (1971), pointed out the affinity of this group with members of the former perciform suborder Mugiloidei, which is currently in the series Mugilomorpha, order Mugiliformes (Stiassny 1990). This author proposed that atherinomorphs and mugilids are sister taxa based in four atherinomorph/mugilid synapomorphies. However, some reservations were done regarding to mugilids have the percomorph type pelvic girdle which represents a character conflict, suggesting the need for new approaches to solve this question (Stiassny 1993).

Parenti (1993) suggested that atherinomorphs are the sister group of some or all paracanthopterygian fishes. The same author states that the relationship between atherinomorphs, percomorphs and paracanthopterygians is not resolved because data are incomplete, arguing that atherinomorphs/percomorphs sister group relationship is rarely supported. However, atheriniform fishes were considered to be composed of two monophyletic groups: Atherinopsidae, the New World silversides, and Atherinidae, the Old World silversides both of them having percoids and mugilids as sister-groups (Saeed et al. 1994; Dyer & Chernoff 1996). Nelson (1994) accepted the alignment of mugilids with atherinomorphs and regarded the series Mugilomorpha and Atherinomorpha as sister groups.

Recently, Wiley et al. (2000) in a comprehensive study about the interrelationships of acanthomorph fishes using molecular and morphological data, have also shown evidence corroborating Stiassny's (1993) hypothesis that mullets are related to atherinomorphs.

In this paper, we report the isolation and characterisation of GH cDNAs using the RACE protocol (Rapid Amplification of cDNA Ends, Frohman et al. 1988), from the mullet (*Mugil platanus*) representing the series Mugilomorpha, and the halfbeak (*Hemiramphus brasiliensis*) representing the series Atherinomorpha. The deduced amino acid sequences for both GH cDNAs were used to perform a phylogenetic analysis in order to investigate the relationship among the series Mugilomorpha, Atherinomorpha and Percomorpha.

2 – MATERIAL AND METHODS

Total RNA was isolated from pituitaries of *M. platanus* and *H. brasiliensis* using Trizol Reagent (Invitrogen, Brazil) according to manufacturer's protocol. Fish collected at the Cassino beach (Southern Brazil) were anesthetized in ice-cold water before pituitaries dissection. About 5μg of total RNA from 3 individuals of each species were used as a template for Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) using the AP primer (5'- GGC CAC GCG TCG ACT AGT AC (T)17 - 3', Invitrogen, Brazil). First strand cDNA was then used as a template in a Polymerase Chain Reaction (PCR) with the gene specific primer (EXO1) and the AUAP primer (5'- GGC CAC GCG TCG ACT AGT AC - 3', Invitrogen, Brazil). The sequence of EXO1 (5'- CCC AGA CCA GCC ATG GAC AGA - 3') was designed from alignment of several known acanthopterygian GH nucleotide sequences and matches with the first exon region (data not shown). The PCR product obtained for *H. brasiliensis* was cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen, Brazil), while the PCR product obtained for *M. platanus* was sequenced directly. Before sequencing each amplified PCR product was purified with Shrimp Alkaline Phosphatase and Exonuclease I (Amersham Biociences). Recombinant plasmids and purified PCR products were sequenced through the chain terminators method (Amersham Biosciences ET terminator kit) in a thermocycler, purified through ethanol precipitation and finally were read on an automatic sequencer MegaBACE 1000. Both amino acid sequences were inferred using the standard eukaryotic genetic code.

Several GH amino acid sequences from GenBank (http://www.ncbi.nlm.nih.gov) were aligned using CLUSTAL X (Thompson et al. 1997) and phylogenetic analysis was performed using the Phylogeny Inference Package PHYLYP 3.6 (Felsenstein 1993). The aligned amino acid sequences were used to perform a phylogenetic analysis using the maximum parsimony method (PROTPARS, for details see PHYLYP 3.6 manual). The eel *Anguilla japonica* representing the subdivision Elopomorpha was used as outgroup. A bootstrapping analysis using 1000 iterations was performed using SEQBOOT.

3 – RESULTS AND DISCUSSION

3.1. *GH cDNA sequences of H. brasiliensis and M. platanus*

The *H. brasiliensis* GH cDNA (hbGH; GenBank no. AY775149) contains an open reading frame of 615 nucleotides encoding a preprotein of 204 amino acids residues as shown in the Fig. 1. The hbGH mRNA 3' untranslated region is 224 nucleotides long and it contains one polyadenylation consensus sequence AATAAA (Fig. 1).

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   1 atggacagagttatccttctcctgtccgtcatctgtctgagagtttcctctcagccaatc
     M  D  R  V  I  L  L  L  S  V  I  C  L  R  V  S  S ↑Q  P  I   20
  61 acagacagccagcgtctgttctccattgccgttagcagagtccaacatctgcatctgctg
     T  D  S  Q  R  L  F  S  I  A  V  S  R  V  Q  H  L  H  L  L   40
 121 gctcagaggctcttctcagacttcgagagctctctgcagacggaggagcagaggcagctc
     A  Q  R  L  F  S  D  F  E  S  S  L  Q  T  E  E  Q  R  Q  L   60
 181 aacaaaatcttcctgcaagatttctgcaactctgattacatcatcagccccatcgacaag
     N  K  I  F  L  Q  D  F (C) N  S  D  Y  I  I  S  P  I  D  K   80
 241 catgagacgcaacgcagctctgtcctgaagctattgtccatctcctaccgtctggtggag
     H  E  T  Q  R  S  S  V  L  K  L  L  S  I  S  Y  R  L  V  E  100
 301 tcctgggagttccccagtcgttccctgtctggaggctcttctcccaggaaccagatctcc
     S  W  E  F  P  S  R  S  L  S  G  G  S  S  P  R  N  Q  I  S  120
 361 ccgaaactttctgagctgaagacaggaatcctgctgctgatcaaggccaatcaggaccca
     P  K  L  S  E  L  K  T  G  I  L  L  L  I  K  A  N  Q  D  P  140
 421 gcagagatgttcaccgacacctcgaccctccagctggcaccgtacgggaactactaccag
     A  E  M  F  T  D  T  S  T  L  Q  L  A  P  Y  G  N  Y  Y  Q  160
 481 agtctgggagctgacgagtcactgagacgaacctacgagttgctggcatgtttcaagaag
     S  L  G  A  D  E  S  L  R  R  T  Y  E  L  L  A (C) F  K  K  180
 541 gatatgcacaaggtggagacctacctgaccgtggccaaatgtcgactctctccagaggct
     D  M  H  K  V  E  T  Y  L  T  V  A  K (C) R  L  S  P  E  A  200
 601 aactgcactctgtagcctcgcgcttccagcgggaagccctgtccctttagcagacaattc
    [N (C) T] L  *                                               204
 661 attagctttagtgtttgattagcattagtgtaggcagttgctgagggttttctgagtcac
 741 aggtgtagtccagaggggatgtcactgatgaatgttgaagacaggaactgatgtcacact
 801 tgcagacagtaaataaagtgagatgcattaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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FIGURA 1 – Nucleotide sequence of the halfbeak (*H. brasiliensis*) GH cDNA (GenBank no. AY775149) and the deduced amino acid sequence of the hormone. Nucleotides and amino acids are numbered on the left- and right-hand sides, respectively. The arrow indicates the possible site for cleavage of signal peptide. The cysteine residues in the mature hormone are circled. The asterisk indicates the termination codon. A potential N-glycosylation site is indicated by a box, and the polyadenylation signal is underlined.

Since the first 17 amino acid residues from the N terminus are highly hydrophobic and also have a high degree of homology to the signal peptide of other fish GHs, it is assumed that in the hbGH this region probably represents the signal peptide of the pre-GH which is cleaved upon hormone secretion. In the case of *M. platanus* GH cDNA (mpGH; GenBank no. AY775148), it was not possible access the nucleotide sequence encoding for the initial amino acid residues since the PCR product was sequenced directly. However, it was possible to identify an open reading frame of 597 nucleotides encoding a preprotein of 199 amino acid residues as shown in the Fig. 2. The signal peptide is not complete but it is assumed from the alignment that the first 11 amino acid residues represent part of the mpGH signal peptide. The mpGH mRNA 3' untranslated region is 181 nucleotides long and it contains one polyadenylation consensus sequence AATAAA (Fig. 2).

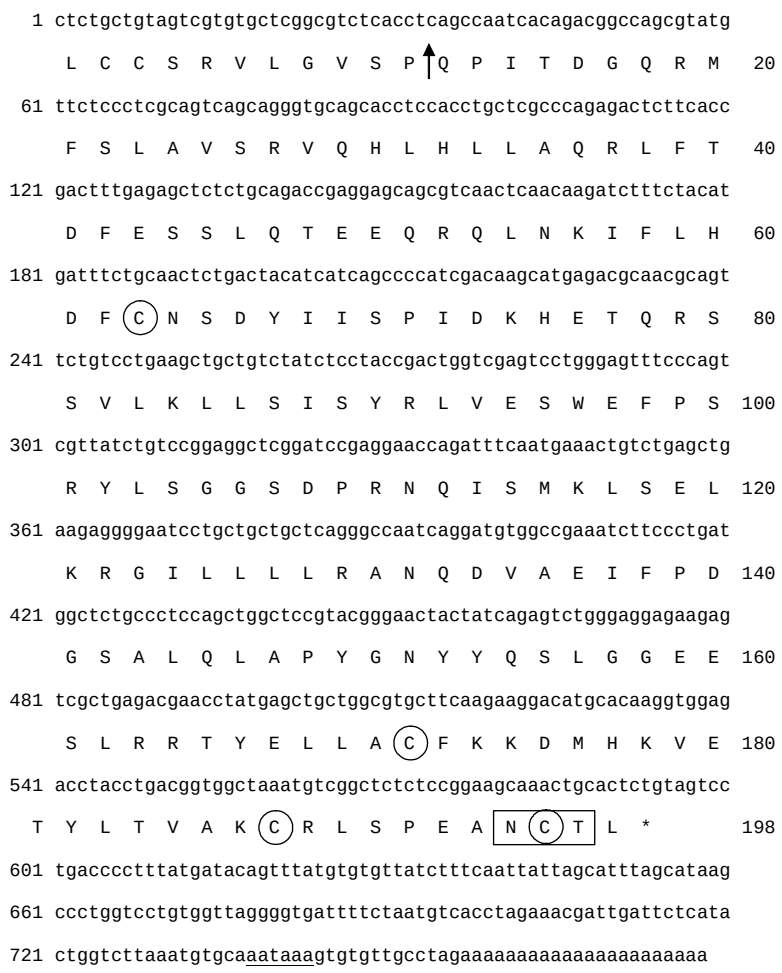

FIGURA 2 – Nucleotide sequence of the mullet (*M. platanus*) GH cDNA (GenBank no. AY775148) and the deduced amino acid sequence of the hormone. Nucleotides and amino acids are numbered on the left- and right-hand sides, respectively. The arrow indicates the possible site for cleavage of signal peptide. The cysteine residues in the mature hormone are circled. The asterisk indicates the termination codon. A potential N-glycosylation site is in the box, and the polyadenylation signal is underlined.

Both hormones exhibit typical GH features, such as four cysteine residues, capable of forming two disulphide bonds which are assumed to contribute to the tertiary structure of the hormone molecule, a single tryptophan residue and stretches of amino acids highly conserved in all known GHs. There is only one Asn-Xaa-Thr motif in both GH amino acid sequences at the C terminus region which is a potential site for N-linked glycosylation. The mature form of both GHs contains 187 amino acids, starting with a glutamine. Both mature amino acid sequences, when compared to those of mature GHs from other Acanthopterygian fishes, showed higher levels of homology to fish GHs representing the order Perciformes, with a similarity up to 91% for *M. platanus* and 89% for *H. brasiliensis*.

3.2. *Phylogenetic analysis*

The consensus phylogenetic tree obtained (Fig. 3) showed that members of superorders Ostariophysi, Protacanthopterygii and Paracanthopterygii grouped according to the current fish classification (Nelson 1994). Within Acanthopterygii, Tetraodontiformes and Pleuronectiformes have grouped as a monophyletic lineage. However, the main result coming from our analysis is the clade supported by a bootstrap value of 65% comprising perciforms (*Sparus aurata* and *Thunnus thynnus*), mugiliform (*M. platanus*), atherinomorphs (*H. brasiliensis* (Beloniformes), *Odontesthes bonariensis* and *Odontesthes argentinensis* (Atheriniformes)) and scorpaeniforms (*Cottus kazika* and *Sebastes schlegeli*). Similarly, Miya et al. (2003) analysing the complete mitochondrial genome of several fish species found a strongly supported clade (jackknife value of 87%) comprising perciforms,

mugiliforms, and atherinomorphs within the Percomorpha. The authors pointed out that although the latter two (mugiliforms and atherinomorphs) have often been treated as relatively primitive groups of higher teleosts (Nelson 1994; Parenti 1993; Stiassny 1993), their results have confidently placed them within the Percomorpha. Our results corroborate this statement.

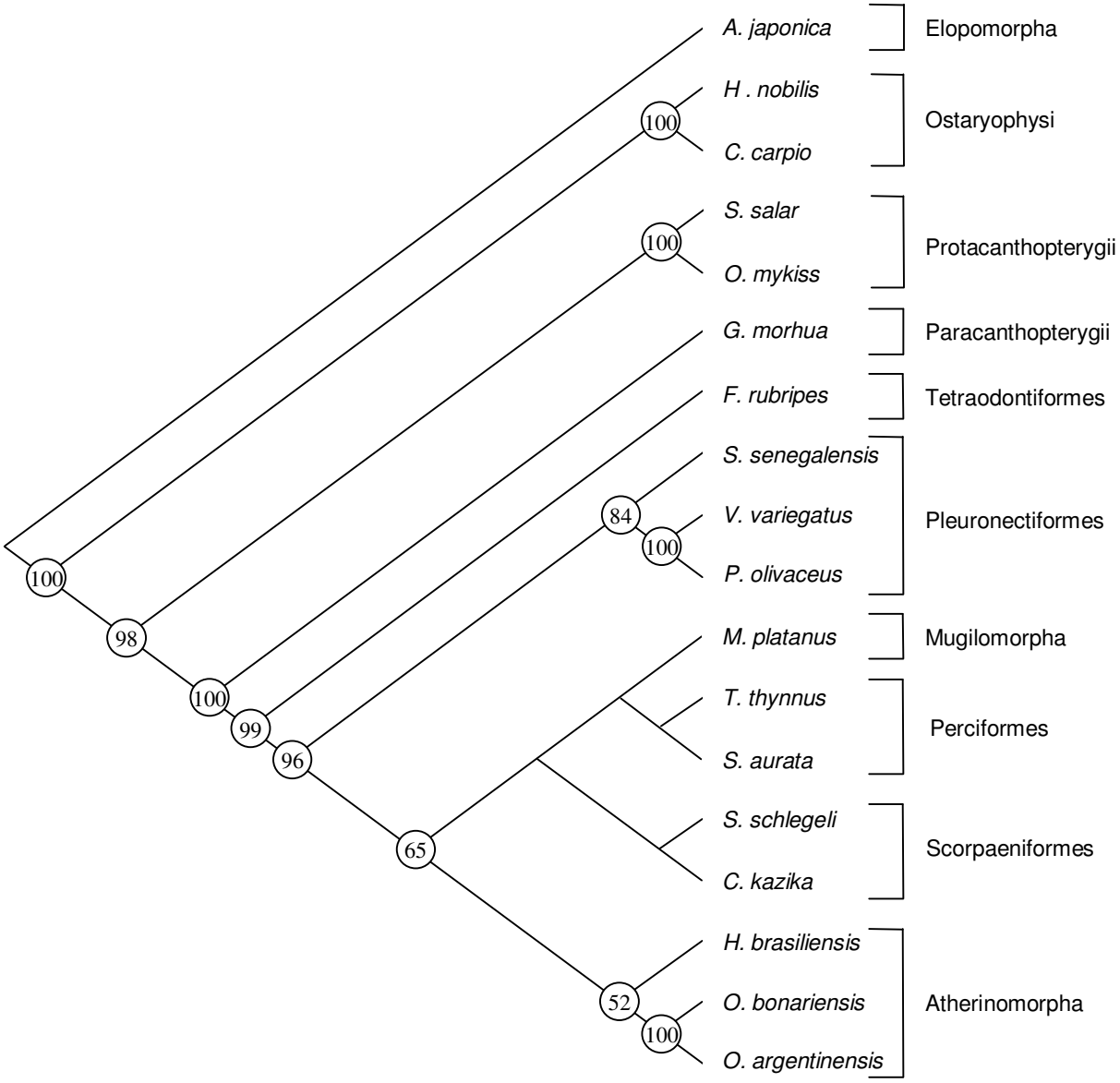

FIGURA 3 – Consensus phylogenetic tree of fish GH amino acid sequences obtained using the maximum parsimony method. Values at the branch point are the percentage of bootstrap replicates that supported that branch out of 1000 replicates. Only bootstrap values >50 are shown. The GH sequences included in this analysis other than *M. platanus* and *H. brasiliensis* were obtained from GenBank: A*nguilla japonica* (AAA48535), *Hypophthalmichthys nobilis* (CAA43006), *Cyprinus carpio* (CAA36228), *Salmo salar* (CAA32481), *Oncorhynchus mykiss* (AAA49555), *Gadus morhua* (B61128), *Fugu rubripes* (AAC60105), *Solea senegalensis* (AAA60372), *Verasper variegatus* (AAC36716), *Paralichthys olivaceus* (BAA06159), *Thunnus thynnus* (CAA29914), *Sparus aurata* (AAB19750), *Sebastes schlegeli* (AAB49492), *Cottus kazika* (BAC07248), *Odontesthes bonariensis* (AAO31982), *Odontesthes argentinensis* (AAF36995).

As expected, *H. brasiliensis* grouped with two other atherinomorphs (*O. bonariensis* and *O. argentinensis*), but the Atherinomorpha branch did not include the species representing Mugilomorpha (*M. platanus*). Even though there are many hypotheses for relationship between atherinomorphs and mugilomorphs, our results indicate that mugilids are more related to perciforms than atherinomorphs. Indeed, according to Nelson (1994) Perciformes is

not a monophyletic group, since most families in many suborders are not currently definable in terms of shared derived characters. In addition to its likely polyphyletic origin, Perciformes is the most diversified of all fish orders which is a problem when few species are used to perform phylogenetic analysis. However, there was a clear agreement of the molecular data obtained in the present work with the major fish groups and the currently accepted phylogenies. More recent approaches using molecular and morphological data indicate that it is important to increase the effort in finding nuclear genes with significant group-specific synapomorphies, which can be useful to solve the questions about sister group relationships in fish.

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