

# Isolation of the pituitary gonadotrophic $\alpha$ -subunit hormone of the giant amazonian fish: pirarucu (*Arapaima gigas*)

M. T. Faria · R. F. Carvalho · T. C. A. Sevilhano ·  
N. A. J. Oliveira · C. F. P. Silva · J. E. Oliveira ·  
C. R. J. Soares · R. Garcez · P. R. E. Santo · P. Bartolini

Received: 2 June 2012 / Accepted: 26 September 2012 / Published online: 17 October 2012  
© Springer Science+Business Media Dordrecht 2012

**Abstract** The cDNAs of the  $\alpha$ -subunit of the pituitary gonadotrophic hormones (GTH $\alpha$ ) of fish of the order Osteoglossiformes or the superorder Osteoglossomorpha have never been sequenced. For a better understanding the phylogenetic diversity and evolution of PGH $\alpha$  in fish and for future biotechnological synthesis of the gonadotrophic hormones (ag-FSH and ag-LH), of *Arapaima gigas*, one of the largest freshwater fishes of the world, its GTH $\alpha$  cDNA was synthesized by reverse transcriptase and the polymerase chain reaction starting from total pituitary RNA. The ag-GTH $\alpha$ -subunit was found to be encoded by 348 bp, corresponding to a protein of 115 amino acids, with a putative signal peptide of 24 amino acids and a mature peptide of 91 amino acids. Ten cysteine residues, responsible for forming 5 disulfide linkages,

2 putative N-linked glycosylation sites and 3 proline residues, were found to be conserved on the basis of the known sequences of vertebrate gonadotrophic hormones. Phylogenetic analysis, based on the amino acid sequences of 38 GTH $\alpha$ -subunits, revealed the highest identity of *A. gigas* with members of the Acipenseriformes, Anguilliformes, Siluriformes and Cypriniformes (87.1–89.5 %) and the lowest with Gadiformes and Cyprinodontiformes (55.0 %). The obtained phylogenetic tree agrees with previous analysis of teleostei, since *A. gigas*, of the order of Osteoglossiformes, appears as the sister group of Clupeocephala, while Elopomorpha forms the most basal group of all other teleosts.

**Keywords** *Arapaima gigas* · Follicle-stimulating hormone · Luteinizing hormone · Reproduction

M. T. Faria (✉) · C. F. P. Silva  
Embrapa Amazônia Oriental, Trav. Dr. Enéas Pinheiro  
s/n°, Marco, Belém, PA 66095-100, Brazil  
e-mail: tucun\_br@yahoo.com.br

M. T. Faria · R. F. Carvalho · T. C. A. Sevilhano ·  
N. A. J. Oliveira · J. E. Oliveira · C. R. J. Soares ·  
P. R. E. Santo · P. Bartolini  
Biotechnology Department, IPEN-CNEN/SP, Av. Prof.  
Lineu Prestes 2242, Cidade Universitária, São Paulo,  
SP 05508-000, Brazil

R. Garcez  
Genetic Ichthyology Laboratory, Bioscience Institute,  
University of São Paulo, Rua Matão 277,  
São Paulo 05508-090, Brazil

## Introduction

The two pituitary gonadotrophic hormones (GTHs), that is, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), play important roles in the gonadal development and are the most likely candidates to elicit gonadal differentiation, being involved in regulating essential reproductive processes such as gametogenesis and follicular growth in vertebrates, including fish (Gen et al. 2000; Villeneuve et al. 2007; Ohta et al. 2008; Cao et al. 2009; Huang et al. 2009;

Schulz et al. 2010; Senthilkumaran 2011). These hormones can be employed as artificial spawning inductors to improve breeding and establish reproduction centers. This is particularly useful for endangered and overexploited species of fish used for human consumption, like *Arapaima gigas* (Zohar and Mylonas 2001; Yaron et al. 2009; Garcia et al. 2009; Chukoo et al. 2009; Mylonas et al. 2010; Castello et al. 2011). For these reasons, the production of recombinant FSH and LH by biotechnology is highly desirable (Kobayashi et al. 2003; Yu et al. 2010). For vertebrates, these hormones are non-covalently bound heterodimeric glycoproteins composed of a common  $\alpha$ -subunit, essential for signal transduction, protein-folding and heterodimer stabilization, and a hormone-specific  $\beta$ -subunit that is involved in determining the metabolic clearance rate being responsible for eliciting a particular biological response (Pierce and Parsons 1981; Bousfield et al. 2007; Carvalho et al. 2009). The  $\alpha$ -subunit amino acid sequence is highly conserved, with fishes of the same order showing 90–100 % identity among them and 55–70 % identity when compared to other teleosts (Gen et al. 1993; Kobayashi et al. 1997; Han and Yu 2002; Cerda et al. 2008). Since the first teleost GTH $\alpha$ -subunit sequencing (Chang et al. 1988; Suzuki et al. 1988; Kitahara et al. 1988), the GTH $\alpha$  cDNA of more than forty species of Actinopterygii has now been characterized, with the data either reported in the literature or deposited in the Genbank. However, none of these teleosts represent the superorder Osteoglossomorpha or the order Osteoglossiformes to which *A. gigas* belongs.

*Arapaima gigas* (pirarucu) is a giant Osteoglossidae native to the Amazon River basin that can reach 2 m in length and weigh up to 100 kg and is present in Ecuador, Colombia, Peru, Bolivia and Brasil. This species is in danger of disappearing due to exploitation by the fishing industry and increasing human presence. It is largely used for food and extractivism purposes, and commercial breeding is still incipient due to insufficient technological knowledge. Despite its importance, information about the current population is either lacking or missing (Stone 2007), and the physiology and reproduction of *A. gigas* have been the object of relatively few scientific investigations (Junk and Piedade 2004; Faria 2008; Silva and Faria 2011).

In order to better understand the phylogenetic diversity and evolution of the GTH $\alpha$ -subunit in fish, to

better situate the Osteoglossiformes within the teleosts and to provide the amino acid sequence for future synthesis of ag-FSH and ag-LH useful for fertility studies and related applications, we synthesized its GTH $\alpha$ -subunit cDNA starting from pituitary material. Its complete amino acid sequence was thus determined and used for phylogenetic analysis by comparison with 34 known sequences from other teleosts, using 3 Acipenseriformes as the outgroup.

## Materials and methods

### Animals

Sexually mature *A. gigas*, total length 150–200 cm, were obtained in fishing stations in districts of Santarém, Pará, Brazil. Pituitaries glands were removed immediately following decapitation, frozen in liquid nitrogen, stored at Embrapa Amazonia Oriental (Belém, Pará, Brazil) and transported to IPEN-CNEN/São Paulo for the experiments (Sample collection license: 16623-1\2008, Brazilian Institute of Environment (IBAMA) and Board of Genetic Heritage Management (CGEN), license: 02001.005724/2008-21). Seven fish were employed and each region of the gene was repeatedly analyzed using at least three different hypophyses per region.

### Total RNA extraction

Total RNA was extracted from individual pituitaries glands (70–180 mg) of *A. gigas* using the Purelink<sup>®</sup> Micro-to-Midi total RNA purification kit (Invitrogen, Carlsbad, CA, USA) and kept at the temperature of  $-70^{\circ}\text{C}$ .

### Design of primers and reverse-transcribed polymerase chain reaction (RT-PCR)

All oligonucleotides used as primers were designed using Bio Edit<sup>®</sup> softwares (Hall 1999). Initially, 13 primers (sense and antisense) were designed based on the conserved regions of 17 fish GTH $\alpha$  sequences deposited in the GenBank (Table 1), and these were used for different RT-PCR test reactions. One microgram of pituitary total RNA was reverse-transcribed to cDNA using a one-step kit (Invitrogen<sup>®</sup>). The reaction

**Table 1** Fish species used in sequence analysis of glycoprotein  $\alpha$ -subunits

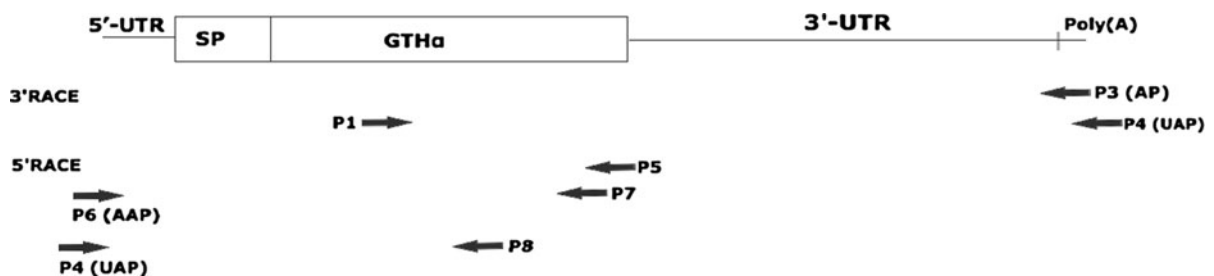
Order	Taxa name	Abbreviation	Accession No.	References
Synbranchiformes	<i>Monopterus albus</i>	Albus swamp eel	AF502395	Han and Yu (2002)
	<i>Ophisternon bengalense</i>	Bengalense swamp eel	AF502394	Han and Yu (2002)
Perciformes	<i>Sebastes schelegelii</i>	Rockfish	AY609078	Kim et al. (2005)
	<i>Thunnus obesus</i>	Tuna	P37204	Okada et al. (1994)
	<i>Pseudolabrus sieboldi</i>	–	BAF81899	Ohta et al. (2008)
	<i>Dicentrarchus labrax</i>	European seabass	AAK49431	Mateos et al. (unpublished)
	<i>Epinephelus coioides</i>	Orange-spotted grouper	AAN18038	Zhou and Gui (unpublished)
	<i>Acanthopagrus latus</i>	Yellowfin porgy	M94038	Tsai,H and Chen (unpublished)
	<i>Acanthopagrus schlegelii</i>	Black porgy	ABQ96863	An et al. (unpublished)
	<i>Pagrus major</i>	Red seabream	AB028211	Gen et al. (2000)
	<i>Sparus aurata</i>	Gilthead seabream	AF300425	Meiri et al. (unpublished)
	<i>Morone saxatilis</i>	Striped bass	L35071	Hassin et al. (1995)
	<i>Oreochromis mossambicus</i>	Tilapia	AF303087	Gur et al. (unpublished)
	Tetraodontiformes	<i>Takifugu rubripes</i>	Fugu rubripes	DAA06175
Pleuronectiformes	<i>Hippoglossus hippoglossus</i>	Atlantic halibut	AJ417770	Weltzien et al. (unpublished)
	<i>Solea senegalensis</i>	Senegalese sole	ABW81405	Cerda et al. (2008)
	<i>Paralichthys olivaceus</i>	Bastard halibut	AF268692	Lee and Kim (unpublished)
Cyprinodontiformes	<i>Fundulus heteroclitus</i>	Killifish	U12923	Lin et al. (unpublished)
Gadiformes	<i>G. morhua</i>	Cod fish	DQ402372	Mittelholzer et al. (2009)
Salmoniformes	<i>Oncorhynchus tshawytscha</i>	Chinook salmon	S77059	Suzuki et al. (1995)
	<i>Oncorhynchus masou</i>	Masu salmon	S69273	Gen et al. (1993)
	<i>Oncorhynchus keta</i>	Chum salmon	M27652	Kitahara et al. (1988)
	<i>Oncorhynchus mykiss</i>	Rainbow trout	AB050834	Morita et al. (unpublished)
Cypriniformes	<i>Ctenopharyngodon idella</i>	Grass carp	X61050	Chang et al. (unpublished)
	<i>Hypophthalmichthys molitrix</i>	Silver carp	P37037	Chang et al. (1990)
	<i>Cyprinus carpio</i>	Common carp	M37379	Chang et al. (1988)
	<i>Carassius auratus</i>	Goldfish	D86551	Kobayashi et al. (1997)
	<i>Rutilus rutilus</i>	Roach minnow	ABR67465	Trubiroha et al. (2009)
Siluriformes	<i>Clarias gariepinus</i>	African catfish	X97760	Rebers et al. (1997)
	<i>Ictalurus punctatus</i>	Channel catfish	AF112190	Liu et al. (1997)
Anguilliformes	<i>Anguilla anguilla</i>	European eel	X61038	Querat et al. (1990)
	<i>Muraenesox cinereus</i>	Pike eel	P12836	Liu et al. (1989)
	<i>Anguilla japonica</i>	Japanese eel	BAD14301	Nagae et al. (1996)
	<i>Anguilla marmorata</i>	Giant mottled eel	ACK87151	Huang et al. (2009)
Acipenseriformes	<i>Acipenser baerii</i>	Siberian sturgeon	AJ310342	Querat (unpublished)
	<i>Acipenser gueldenstaedtii</i>	Russian sturgeon	AY519658	Hurvitz et al. (2005)
	<i>Acipenser sinensis</i>	Chinese sturgeon	EU656137	Cao et al. (2009)

was performed at 37 °C for 30 min, using the moloney-monkey-leukemia-virus (MMLV) reverse transcriptase, then heating at 94 °C for 2 min to inactivate it. PCRs were performed in a thermocycler (Applied Biosystems®, Foster City, CA,USA) with 10 mM of sense and antisense primers and 2.5 U of Taq DNA polymerase (Life Technologies, Carlsbad,

CA, USA) followed by 35 cycles: denaturation at 94 °C for 15 s, annealing at 45 °C for 30 s and extension at 68 °C for 1 min. Final elongation was at 68 °C for 5 min before holding at 4 °C. Based on these tests, the two primers (primers #1 and #2; Table 2; Fig. 1) that provided the best reaction and a single band on 1.2 % agarose gel, corresponding to a putative

**Table 2** Primers used in cloning *A. gigas* GTH $\alpha$ 

Number	Direction	Name	Sequence
Primer 1	Sense	agGTH $\alpha$ 1	5' CAT GGG CTG CTG CTT CTC 3'
Primer 2	Antisense	agGTH $\alpha$ 2	5' CTC TTT GGT ATG TCT GAC G 3'
Primer 3	Antisense	Adapter primer	5' GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T 3'
Primer 4	Sense or Antisense	Universal amplification primer	5' CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3'
Primer 5	Antisense	agGTH $\alpha$ 3	5' TTT GCT GTT CTG CCT TA 3'
Primer 6	Sense	Abridged anchor primer	5' GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG 3'
Primer 7	Antisense	agGTH $\alpha$ 4	5' CTT GTG ATA GTA GCA GGT GTT G 3'
Primer 8	Antisense	agGTH $\alpha$ 5	5' CGT TGC CTC GGA TGT TAT GTT C 3'

**Fig. 1** Sequencing strategy and primer positions for the *A. gigas* GTH $\alpha$  cDNA identification. The primers correspond to oligonucleotides listed in Table 2. 5'-UTR, 5'-untranslated

region; 3'-UTR, 3'-untranslated region; SP, signal peptide sequence; poly (A), poly (A+) tail

partial sequence of GTH- $\alpha$  cDNA, were thus ready to be used for the following reactions.

#### Rapid amplification of cDNA ends (RACE)

Partial sequences of *A. gigas* GTH- $\alpha$  cDNAs were obtained using 3' and 5'-RACE System kits (Invitrogen<sup>®</sup>) according to manufacturer's instructions.

3'-RACE was carried out using 0.5–1.0  $\mu$ g of mRNA to synthesize the first-strand cDNA and 10 pmol of Adapter Primer (#3) and 200 U Superscript II reverse transcriptase (Invitrogen<sup>®</sup>), followed by PCR carried out using primers #1 and #4 (Universal Amplification Primer) in a reaction at 94 °C for 3 min and 35 cycles: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, according to kit instructions. PCR products were then sequenced at the Human Genome Research Center (University of São Paulo, Brazil) and analyzed by comparison with the literature data using the Bio Edit<sup>®</sup> software. Gene-specific primers (#5, #7 and #8) were then designed on the basis of the partial sequence obtained with the 3'-RACE and used together with the

primers supplied by the two kits (#3, #4 and #6) to obtain and repeatedly confirm the full sequence of GTH- $\alpha$  cDNA.

For 5'-RACE, 0.8–1.5  $\mu$ g of total RNA were reverse-transcribed with 200 U of Superscript II reverse transcriptase (Invitrogen<sup>®</sup>) using primer #5, according to kit instructions. Single-strand cDNA was column-purified, and tailed oligo-dC was added using terminal deoxynucleotidyl transferase. PCR was then performed using the Abridged Anchor Primer (#6) and gene-specific primer #7 in a reaction at 94 °C for 2 min and 35 cycles: 94 °C for 60 s, 55 °C for 60 s and 72 °C for 2 min, followed by a final extension at 72 °C for 5–7 min, according to kit instructions. Nested PCR was then carried out on the primary PCR products using the Universal Amplification Primer (#4) and gene-specific primer #8 under the same conditions described above. PCR products were sequenced and analyzed in comparison with the literature data.

The complete amino acid sequence of ag-GTH $\alpha$  was determined and a putative signal peptide

**Fig. 2** Nucleotide and deduced amino acid sequence of the cDNA encoding the GTH $\alpha$ -subunit of *A. gigas*. M start coding region, ◇ first amino acid of the mature peptide, N or C glycosylation sites; ○ cysteine residues; ATTAAA polyadenylation signal; AAAAAAAAAAAAAAAA poly (A<sup>+</sup>) tail

1	TGAACAGCTCACTACAAAAGTACAGAAACCTCCCGTCACTACGTTATTTGCAGGCACCATG	60
61	AGCTACACAGGAAAAGTACCATTGCATCTGTTCTGGCATTACTGGCCATCTTACATATT	120
121	GTAGACTCTAATTTCAATGTGGGTTGTGAAGAGTGCAAACCTAAGGAGAACAAGTACTTC	180
181	TCAAGGCTGGGAGCACCCATCTTTCAGTGCATGGGCTGTTGCTTCTCCAGAGCATACCC	240
241	ACACCTCTGAGGTCCAAGAAAACAATGCTGGTCCCAAGAACAATCCGAGGCAACG	300
301	TGCTGTGGTAAAGAAGTCAAACGGCTGATCACACTGAACAACGTGAGACTGGAAAAC	360
361	CACACGGACTGCTCACTGCAACACCTGCTACTATCACAAGTCATAAGGCAGAACAGCAAAA	420
421	CTTTATTTAACCTGATTTGAAGGTAGATTAATAAATGACATTGTTGGATTTAATTGTCAT	480
481	TGTTTTTCATTTATAGACGTAACATGCTGAAGACTCATTAGACTATACAAGGAAAAC	540
541	TGTGATGTGGTAATCTTTTGTGTTAGAAAAAGTTCTGAATAGTCATCATATTTCTTAA	600
601	TTTTTTGTTTACATAAAAACAGTACTCACAAGCAATTAAGCGGGAATCATTAGAG	660
661	GCAATCTTCNNNNNAAAATTGTATTGATTTTGCAGTACCAACAAGCTTTTCATTTA	720
721	CTT <span style="border: 1px solid black; padding: 0 2px;">ATTAAA</span> AGAGCCACATAAAACATG <span style="border: 1px solid black; padding: 0 2px;">AAAAAAAAAAAAAAAA</span>	767

identified using the “Signal peptide 4.1 software” (Petersen et al. 2011). The alignment of the mature peptide sequences was made using Clustal W (Thomson et al. 1994).

### Phylogenetic analysis

For the phylogenetic analysis, the amino acid sequence of the GTH $\alpha$ -subunit mature peptide of *A. gigas* obtained in this study was compared with that of 37 other homologous sequences retrieved from the GenBank/EMBL or from published papers. The phylogenetic tree and related analyses were conducted using the computer program MEGA 5 (Tamura et al. 2011) based on the Neighbor-Joining method, rooted with 3 Acipenseriformes species as the outgroup, with a bootstrap robustness derived from 1,000 replicates. Evolutionary molecular distances were corrected using the Poisson method (Kimura and Ohta 1979). Branches corresponding to partitions reproduced in less than 50 % of the bootstrap replicates automatically collapsed. All positions containing gaps and missing data were eliminated.

### Results

The sequences obtained via 3' and 5'-RACE by using material from 7 different pituitary glands were compared and aligned. A short overlapping enabled their proper joining into a single nucleotide sequence spanning the entire cDNA. The *A. gigas* GTH $\alpha$ -cDNA sequence was 767 bp in total length and had an open reading frame of 348 bp beginning with the first

ATG codon at position 58 bp (57 bp 5'-UTR) and with the stop codon at position 403 bp and a 342 bp 3'-UTR. A polyadenylation signal (ATTAAA) was recognized 18 bp upstream from a poly (A<sup>+</sup>) tail of 20 bp. The coding region translates into a peptide of 115 amino acids, while the cleavage site for the putative signal peptide was calculated to be between amino acid 24 and 25. This provides a mature peptide of 91 and a signal peptide of 24 amino acids. The proposed mature peptide of ag-GTH $\alpha$  thus contains 10 conserved cysteines and 3 conserved prolines. Putative N-linked glycosylation sites were identified at amino acid positions 51–53 (NIT) and 77–79 (NHT) of the mature peptide (Fig. 2). Figure 3 shows the alignment of the amino acid sequences of ag-GTH $\alpha$ -subunit with other 34 teleosts and 3 Acipenseriformes, while Table 3 presents the percentage identities of GTH $\alpha$  peptides for 12 fish orders. The phylogenetic tree was carried out using 35 GTH $\alpha$ -subunit peptide sequences from teleosts, with 3 Acipenseriformes species, forming the outgroup (Fig. 4).

### Discussion

The full-length cDNA that encodes *A. gigas* GTH $\alpha$ -subunit was synthesized and analyzed for the first time. When compared with the corresponding GTH $\alpha$  peptide sequences of fish that have already been reported, its sequence was found to have high amino acid identity with that of other teleosts (Anguilliformes and Ostariophysi) and even with Chondrostei (Acipenseriformes), with identity values varying from 87.1 to 89.5 %. This is similar to that found in monophyletic groups



**Table 3** Percentage identity of GTH $\alpha$  peptides among fish orders

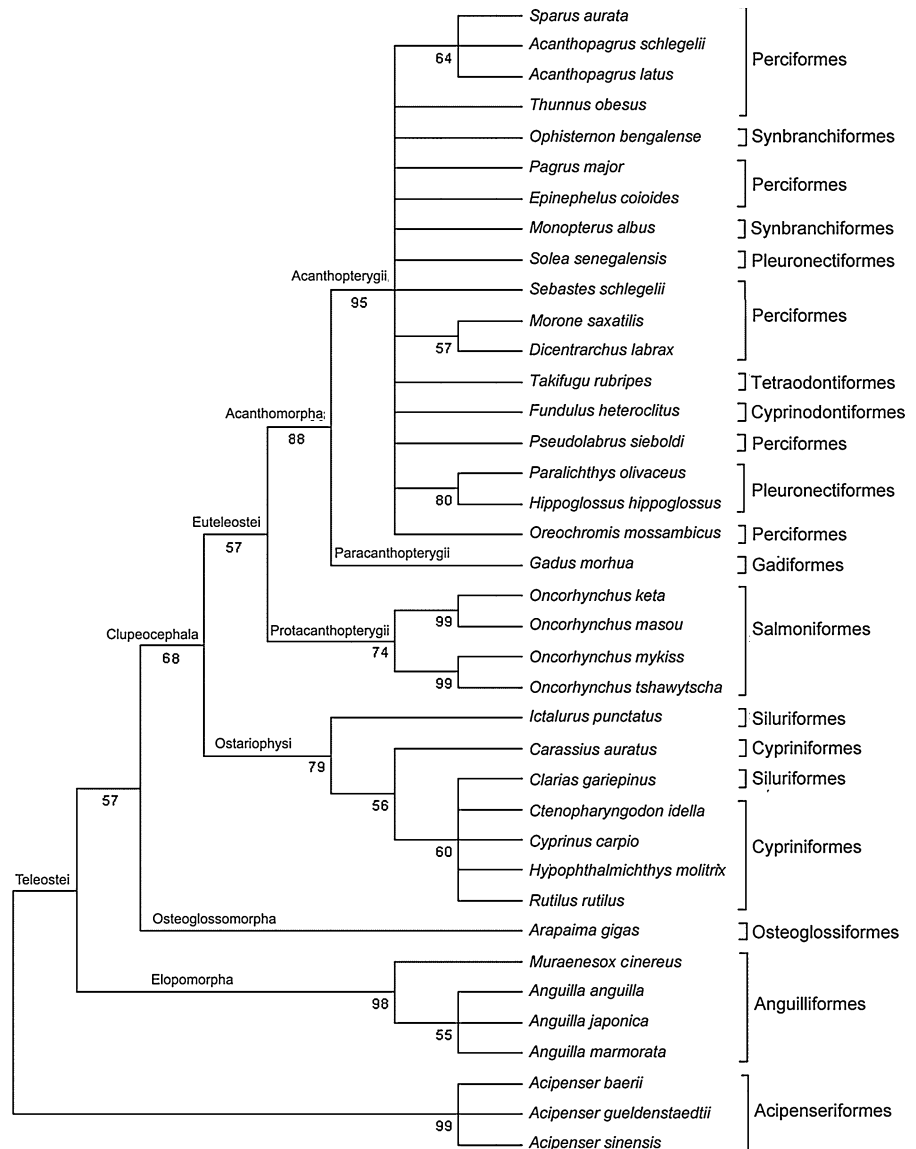
	1	2	3	4	5	6	7	8	9	10	11	12
1 Osteoglossiformes (1) <sup>a</sup>	100	87.1	88.1	89.5	88.1	75.7	67.4	55.0	55.0	70.0	63.6	62.8
2 Acipenseriformes (3) <sup>a</sup>		100	88.1	84.8	84.1	74.6	66.2	55.0	55.0	67.7	64.5	65.3
3 Anguilliformes (4) <sup>a</sup>			98.2	83.7	83.1	73.5	64.0	51.6	57.0	64.7	60.5	62.2
4 Cypriniformes (5) <sup>a</sup>				99.3	97.9	79.8	71.9	60.3	62.8	70.5	63.7	67.2
5 Siluriformes (2) <sup>a</sup>					96.5	79.0	71.7	60.3	61.6	70.0	63.6	67.7
6 Salmoniformes (4) <sup>a</sup>						87.4	63.0	58.9	56.4	62.2	61.1	65.3
7 Perciformes (11) <sup>a</sup>							93.3	81.6	58.4	94.4	83.6	88.2
8 Cyprinodontiformes (1) <sup>a</sup>								100	52.3	82.1	76.8	81.1
9 Gadiformes (1) <sup>a</sup>									100	57.7	54.0	57.7
10 Synbranchiformes (2) <sup>a</sup>										94.7	86.0	88.1
11 Pleuronectiformes (3) <sup>a</sup>											82.4	80.3
12 Tetraodontiformes (1) <sup>a</sup>												100

<sup>a</sup> The number in parenthesis represents species numbers of each order

such as Salmoniformes, Pleuronectiformes and Acanthopterygii (see Table 3). Salmoniformes and Pleuronectiformes present, although the lowest identity within orders, which could be related to the existence of two divergent forms of GTH $\alpha$ , as can be clearly seen in Fig. 3, in the variable regions of the salmoniform *Oncorhynchus*. Acanthomorpha, represented by Acanthopterygii and Gadiformes, present low identity not only with *A. gigas* but also with the other orders presented, with the lowest values for Gadiformes and Cyprinodontiformes. The identity level between *A. gigas* and Acanthomorpha, varying from 55 to 70 %, was similar to that found between *A. gigas* and terrestrial vertebrates: 66.1 % in comparison with rats (*Rattus norvegicus*), 67.8 % with mice (*Mus musculus*) and 60.0 % with humans (*Homo sapiens*). The phylogenetic relationships thus help for the interpretation of the amino acid variation found for GTH $\alpha$  in fishes, indicating that a relatively well-conserved subunit present in the basal orders of Teleostei and Acipenseriformes, gradually evolved to the GTH $\alpha$  that are present in Acanthopterygii and Gadiformes (Paracanthopterygii). Analysis of *A. gigas* GTH $\alpha$  in relation to that of 34 other species of Teleosts confirmed four conserved regions in their mature peptide. In particular, 10 cysteine residues responsible for forming 5 disulfide linkages, two N-linked glycosylation sites and 3 proline residues, possibly related to the backbone directions of the protein structure, appeared as preserved functional structures in *A. gigas* GTH $\alpha$ .

As is known, the sugar chains present at N-glycosylation sites regulate the process of subunit assembly and stimulate post-receptor binding functions upstream from G-protein activation and second messenger stimulation (Roch et al. 2009). The 12 orders and 38 species investigated here, including *A. gigas* (Osteoglossiformes), had in general NIT and NHT sequences providing two glycosylation sites in their GTH  $\alpha$ -subunit. The exception was *Muraenesox sinereus* (Anguilliformes), which had D instead of N at amino acid position 56, thus losing the first glycosylation site, while *Gadus morhua* (Gadiformes) had Q instead of H at amino acid position 86, which still maintains the glycosylation site (Fig. 3). The putative cleavage site for the signal peptide of GTH $\alpha$  of *A. gigas* appears between amino acid 24 (S) and 25 (N). Of the 38 species reported in Fig. 3, 33 presented tyrosine (Y), 4 presented other amino acids (D,F,G,H) and only one (*A. gigas*) presented asparagine (N) as a putative first amino acid of the mature  $\alpha$  peptide. The cleavage site position agrees with the majority of reported putative signal peptides, varying from 18 to 28 amino acids in Teleosts. The *A. gigas* GTH $\alpha$  polyadenylation signal ATTAAA is a non-consensus signal in comparison with the very highly conserved AATAAA present in all vertebrates, typically located 15–25 nucleotides upstream from the poly (A) addition site, but it agrees with signals from the Russian sturgeon, striped bass, coho salmon and tilapia (Hassin et al. 1995; Dickey and Swanson 2000; Gur et al. 2001;

**Fig. 4** Consensus phylogenetic tree of GTH $\alpha$  peptide subunits from 12 fish orders and 38 species on the basis of their amino acid identities. The tree was constructed with the Neighbor-Joining method, using the 3 Acipenseriformes as the outgroup. The numbers indicate the bootstrap robustness from 1,000 replicates



Hurvitz et al. 2005). The sequence ATTAAA has been demonstrated, in fact, to be the mildest mutation, corresponding to the most common natural variant, still providing a RNA that is 70–80 % as efficiently polyadenylated and cleaved as AATAAA-containing RNA (Sheets et al. 1990).

Few studies have reported phylogenetic hypotheses for the Osteoglossomorpha based on molecular data. O'Neill et al. (1998) suggested their non-monophyly with basis on a derived teleost form of GnRH. Employing sequences from mitochondrial cytochrome b and ND2 genes, Kumazawa and Nishida (2000)

suggested that Heterotidinae (*Heterotis niloticus* and *A. gigas*) form a sister group of the Osteoglossidae. Al-Mahrouki et al. (2001) isolated and sequenced preproinsulin cDNA in four members of the Osteoglossomorpha, showing them to be monophyletic and suggesting that they were not the most basal living teleosts. On the basis of 5 molecular markers, Lavoué and Sullivan (2004) strongly confirmed the Osteoglossomorpha group to be monophyletic. Mu et al. (2010) used the complete mitochondrial cytochrome b gene sequences to clarify the genetic structure and evolutionary relationship within four specific genera

of the Osteoglossidae family. On the other hand, Han and Yu (2002), in a study of the phylogenetic diversity and evolution of GTH $\alpha$  in fish, obtained the cDNA of swamps eels (Synbranchiformes) and compared it with that of 31 other GTH  $\alpha$ -subunit mature peptide sequences from 9 fish orders, none of which, however, represented the superorder Osteoglossomorpha.

The present study established a phylogenetic hypothesis by comparing the same conserved peptide (GTH $\alpha$ ) derived from *A. gigas* with that of 34 other teleosts. It represents 12 orders and 38 species of fish, including the 3 Acipenseriformes that were chosen as outgroup because they are the nearest relatives to the teleosts for which the GTH $\alpha$  sequences are known (Fig. 4). Unlike other studies that recently analyzed Osteoglossomorpha topology but excluded other teleosts or used them as the outgroup (Lavoué and Sullivan 2004; Mu et al. 2010), the present study considered a large fraction of teleosts, that is, 35 species. The resulting phylogenetic tree placed Osteoglossomorpha as the sister group of Clupeocephala, while Elopomorpha (Anguilliformes) appears as the most basal group of all other teleosts analyzed, confirming previous studies (Arratia 1991; Li and Wilson 1999; Hilton 2003). This diverges from traditional phylogenesis, which placed Osteoglossomorpha as the most basal branch of the living Teleosts (Patterson and Rosen 1977). This phylogenetic analysis thus contributes to the attempts to identify the extant sister group of all other living teleosts (Arratia 2001), although more taxonomic samples of Osteoglossomorpha should be investigated.

In conclusion, *A. gigas* GTH $\alpha$  cDNA was identified and sequenced in this study and its putative mature peptide sequence compared to those of other teleosts, providing additional data that confirmed the phylogenetic position of *A. gigas*. The characterization of *A. gigas* GTH $\alpha$  is the first step necessary for the biotechnological production of ag-FSH and ag-LH, extremely vital hormones for use in fertility studies and preservation of this important, potentially endangered species.

**Acknowledgments** This work was supported by EMBRAPA, project: 02.07.01.01.70.00 and CNPQ project: 556893/2009-3; 19/2009 CTAqua. The authors are grateful to Raimundo Nonato Guimarães Teixeira, Edilvar Santos Pimentel, Luiz Alberto Vasconcelos, for helping in collecting pituitaries, to the fishing communities for capturing *A. gigas* and to Andrew Macrae for helping in the design of primers.

## References

- Al-Mahrouki AA, Irwin DM, Graham LC, Youson JH (2001) Molecular cloning of preproinsulin cDNAs from several osteoglossomorphs and a cyprinid. *Mol Cell Endocrinol* 174:51–58
- Arratia G (1991) The caudal skeleton of Jurassic teleosts: a phylogenetic analysis. In: Chang MM, Liu YH, Zang GR (eds) Early vertebrates and related problems in evolutionary biology. Science Press, Beijing, pp 249–340
- Arratia G (2001) The sister-group of Teleostei: consensus and disagreements. *J Vertebr Paleontol* 21:767–773
- Bousfield GR, Butnev VY, Walton WJ, Nguyen VT, Huneidi J, Singh V, Kolli VSK, Harvey DJ, Rance NE (2007) All-or-none N-glycosylation in primate follicle-stimulating hormone  $\beta$  subunits. *Mol Cell Endocrinol* 260:40–48
- Cao H, Zhou L, Zhang YZ, Wei QW, Chen XH, Gui JF (2009) Molecular characterization of Chinese sturgeon gonadotropins and cellular distribution in pituitaries of mature and immature individuals. *Mol Cell Endocrinol* 303:34–42
- Carvalho CM, Oliveira JE, Almeida BE, Ueda EKM, Torjesen PA, Bartolini P, Ribela MTCP (2009) Efficient isolation of the subunits of recombinant and pituitary glycoprotein hormones. *J Chromatogr A* 1216:1431–1438
- Castello L, Stewart DJ, Arantes CC (2011) Modeling population dynamics and conservation of arapaima in the Amazon. *Rev Fish Biol Fish* 21:623–640
- Cerda J, Chauvigne F, Agulleiro MJ, Marin E, Halm S, Martinez-Rodriguez G, Prat F (2008) Molecular cloning of Senegalese sole (*Solea senegalensis*) follicle-stimulating hormone and luteinizing hormone subunits and expression pattern during spermatogenesis. *Gen Comp Endocrinol* 156:470–481
- Chang YS, Huang CJ, Huang FL, Lo TB (1988) Primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. *Int J Pept Protein Res* 32:556–564
- Chang YS, Huang FL, Lo TB (1990) Purification, characterization, and molecular cloning of gonadotropin subunits of silver carp (*Hypophthalmichthys molitrix*). *Gen Comp Endocrinol* 78:23–33
- Chu-Koo F, Dugue R, Aguilar MA, Daza AC, Bocanegra FA, Veintemilla CC, Duponchelle F, Renno JF, Tello S, Nunes J (2009) Gender determination in the Paiche or Pirarucu using plasma vitellogenin, 17 beta-estradiol and 11-ketotestosterone levels. *Fish Physiol Biochem* 35:125–136
- Dickey JT, Swanson P (2000) Effect of salmon gonadotropin-releasing hormone on follicle stimulating hormone secretion and subunit gene expression in Coho Salmon (*Oncorhynchus kisutch*). *Gen Comp Endocrinol* 118:436–449
- Faria MT (2008) Pirarucu: o gigante indomado. In: Gorayeb I (ed) Amazônia, 1st edn. RM Graph, Belém, pp 289–296
- García A, Tello S, Vargas G, Duponchelle F (2009) Patterns of commercial fish landings in the Loreto region (Peruvian Amazon) between 1984 and 2006. *Fish Physiol Biochem* 35:53–67
- Gen K, Maruyama O, Kato T, Tomizawa K, Wakabayashi K, Kato Y (1993) Molecular cloning of cDNAs encoding two types of gonadotrophin alpha subunit from the masu salmon, *Oncorhynchus masou*: construction of specific

- oligonucleotides for the alpha 1 and alpha 2 subunits. *J Mol Endocrinol* 11:265–273
- Gen K, Okuzawa K, Senthilkumaran B, Tanaka H, Moriyama S, Kagawa H (2000) Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. *Biol Reprod* 63:308–319
- Gur G, Rosenfeld H, Melamed P, Meiri I, Elizur A, Laron Z (2001) Tilapia glycoprotein hormone  $\alpha$  subunit: cDNA cloning and hypothalamic regulation. *Mol Cell Endocrinol* 182:49–60
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Han YS, Yu JYL (2002) Molecular cloning and sequence analysis of the cDNAs for pituitary glycoprotein hormone alpha subunits from two species of Synbranchiformes, *Monopterus albus* and *Ophisternon bengalense*. *Fish Physiol Biochem* 26:111–120
- Hassin S, Elizur A, Zohar Y (1995) Molecular cloning and sequence analysis of striped bass (*Morone saxatilis*) gonadotrophin-I and -II subunits. *J Mol Endocrinol* 15:23–35
- Hilton EJ (2003) Comparative osteology and phylogenetic systematics of fossil and living bony-tongue fishes (Actinopterygii, Teleostei, Osteoglossomorpha). *Zool J Linn Soc* 137:1–100
- Huang H, Zhang Y, Huang W, Li S, Zhu P, Liu Y, Yin S, Liu X, Lin H (2009) Molecular characterization of marbled eel (*Anguilla marmorata*) gonadotropin subunits and their mRNA expression profiles during artificially induced gonadal development. *Gen Comp Endocrinol* 162:192–202
- Hurvitz A, Degani G, Goldberg D, Din SY, Jackson K, Levav-Sivan B (2005) Cloning of FSHbeta, LHbeta, and glycoprotein alpha subunits from the Russian sturgeon (*Acipenser gueldenstaedtii*), expression, gonad development, and steroid levels in immature fish. *Gen Comp Endocrinol* 140:61–73
- Junk WJ, Piedade MTF (2004) Status of knowledge, ongoing research and research needs in Amazonian Wetlands. *Wetl Ecol Manag* 12:597–609
- Kim DJ, Cho YC, Sohn YC (2005) Molecular characterization of rockfish (*Sebastes schlegelii*) gonadotropin subunits and their mRNA expression profiles during oogenesis. *Gen Comp Endocrinol* 141:282–290
- Kimura M, Ohta T (1979) Population genetics of multigene family with special reference to decrease of genetic correlation with distance between gene members on a chromosome. *Proc Natl Acad Sci USA* 76:4001–4005
- Kitahara N, Nishizawa T, Gatanaga T, Okazaki H, Andoh T, Soma GI (1988) Primary structure of two mRNAs encoding putative salmon  $\alpha$ -subunits of pituitary glycoprotein hormone. *Comp Biochem Physiol* 91B:551–556
- Kobayashi M, Kato Y, Yoshiura Y, Aida K (1997) Molecular cloning of cDNA encoding two types of pituitary gonadotropin alpha subunit from the goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 105:372–378
- Kobayashi M, Morita T, Ikeguchi K, Yoshizaki G, Suzuki T, Watabe S (2003) Production of recombinant goldfish gonadotropins by baculovirus in silkworm larvae. *Fish Physiol Biochem* 28:469–471
- Kumazawa Y, Nishida M (2000) Molecular phylogeny of osteoglossoids: a new model for Gondwanian origin and plate tectonic transportation of the Asian arowana. *Mol Biol Evol* 17:1869–1878
- Lavoué S, Sullivan JP (2004) Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei). *Mol Phylogenet Evol* 32:171–185
- Li GQ, Wilson MVH (1999) Early divergence of Hiodontiformes sensu stricto in East Asia and phylogeny of some late mesozoic teleosts from China. In: Arratia G, Schultze HP (eds) *Mesozoic Fishes 2—systematic and fossil records*. Verlag Dr Friedrich Pfeil, München, pp 369–384
- Liu CS, Huang FL, Chang YS, Lo TB (1989) Pike eel (*Muraenesox cinereus*) gonadotropin. Amino acid sequences of both alpha and beta subunits. *Eur J Biochem* 186:105–114
- Liu Z, Li P, Argue BJ, Dunham RA (1997) Gonadotropin alpha-subunit glycoprotein from channel catfish (*Ictalurus punctatus*) and its expression during hormone-induced ovulation. *Mol Marine Biol Biotechnol* 6:217–227
- Mittelholzer C, Andersson E, Taranger GL, Karlson O, Norberg B (2009) Quantification of gonadotropin subunits GPalpha, FSHbeta, and LHbeta mRNA expression from Atlantic cod (*Gadus morhua*) throughout a reproductive cycle. *Comp Biochem Physiol B* 153:288–295
- Mu X, Wang P, Hu Y, Wang X, Song H, Li X, Luo J (2010) Molecular phylogenetic studies of Osteoglossidae based on the sequences of complete mitochondrial cytochrome b gene. *J South China Agricult Univ* 31:100–103
- Mylonas CC, Fostier A, Zanuy S (2010) Broodstock management and hormonal manipulations of fish reproduction. *Gen Comp Endocrinol* 165:516–534
- Nagae M, Todo T, Gen K, Kato Y, Young G, Adachi S, Yamauchi K (1996) Molecular cloning of the cDNAs encoding pituitary glycoprotein hormone alpha- and gonadotropin II beta-subunits of the Japanese eel, *Anguilla japonica*, and increase in their mRNAs during ovarian development induced by injection of chum salmon pituitary homogenate. *J Mol Endocrinol* 16:171–181
- O'Neill DF, Powell JFF, Standen EM, Youson JH, Warby CM, Sherwood NM (1998) Gonadotropin-releasing hormone (Gn-RH) in ancient teleosts, the bonytongue fishes: putative origin of salmon GnRH. *Gen Comp Endocrinol* 112:415–425
- Ohta K, Mine T, Yamaguchi A, Matsuyama M (2008) Sexually dimorphic expression of pituitary glycoprotein hormones in a sex-changing fish (*Pseudolabrus sieboldi*). *J Exp Zool Part A* 309:534–541
- Okada T, Kawazoe I, Kimura S, Sasamoto Y, Aida K, Kawauchi H (1994) Purification and characterization of gonadotropin I and II from pituitary glands of tuna (*Thunnus obesus*). *Int J Pept Protein Res* 43:69–80
- Patterson C, Rosen DE (1977) Review of the ichthyodectiform and other mesozoic fishes and the theory and practice of identifying fossils. *B Am Mus Nat His* 158:81–172
- Petersen TN, Brunak S, Heijne GV, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* 8:785–786
- Pierce JC, Parsons F (1981) Glycoprotein hormones: structure and function. *Ann Rev Biochem* 50:465–495

- Querat B, Jutisz M, Fontaine YA, Counis R (1990) Cloning and sequence analysis of the cDNA for the pituitary glycoprotein hormone alpha-subunit of the European eel. *Mol Cell Endocrinol* 71:253–259
- Rebers FEM, Tensen CP, Schulz RW, Goos HJT, Bogerd J (1997) Modulation of glycoprotein hormone alpha- and gonadotropin II beta-subunit mRNA levels in the pituitary gland of mature male African catfish, *Clarias gariepinus*. *Fish Physiol Biochem* 17:99–108
- Roch GJ, Wu S, Sherwood NM (2009) Hormones and receptors in fish: do duplicates matter? *Gen Comp Endocrinol* 161:3–12
- Schulz RW, de França LR, Lareyre JJ, Le Gac F, Chiarini-García H, Nóbrega RH, Miura T (2010) Spermatogenesis in fish. *Gen Comp Endocrinol* 65:390–411
- Senthilkumaran B (2011) Recent advances in meiotic maturation and ovulation: comparing mammals and pisces. *Front Biosci* 16:1898–1914
- Sheets MD, Ogg SC, Wickens MP (1990) Point mutations in AAUAAA and in the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Res* 18:5799–5805
- Silva CFP, Faria MT (2011) Ilha de São Miguel. A pesca Sustentável. In: Gorayeb I (ed) *Amazônia sustentável*, 1st edn. RM Graph, Brazil, pp 141–144
- Stone R (2007) Aquatic ecology. The last of leviathans. *Science* 316:1684–1688
- Suzuki K, Kawauchi H, Nagahama Y (1988) Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen Comp Endocrinol* 71:292–301
- Suzuki K, Liu D, Hew CL (1995) A gene encoding chinook salmon (*Oncorhynchus tshawytscha*) gonadotropin alpha subunit: gene structure and promoter analysis in primary pituitary cells. *Mol Marine Biol Biotechnol* 4:10–19
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood evolutionary distance and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Thomson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Trubiroha A, Wuertz S, Frank SN, Sures B, Kloas W (2009) Expression of gonadotropin subunits in roach (*Rutilus rutilus*, Cyprinidae) infected with plerocercoids of the tapeworm *Ligula intestinalis* (Cestoda). *Int J Parasitol* 39:1465–1473
- Villeneuve DL, Miracle AL, Jensen KM, Degitz SJ, Kahl MD, Korte JJ, Greene KJ, Blake LS, Linnum AL, Ankley GT (2007) Development of quantitative real time PCR assays for fathead minnow (*Pimephales promelas*) gonadotropin beta subunit mRNAs to support endocrine disruptor research. *Comp Biochem Phys C* 145:171–183
- Wong AC, Van Eenennaam AL (2004) Gonadotropin hormone and receptor sequences from model teleost species. *Zebrafish* 1:203–221
- Yaron Z, Bogomolnaya A, Drori S, Biton I, Aizen J, Kulikovskiy Z, Levavi-Sivan B (2009) Spawning induction in the carp: past experience and future prospects—a review. *Isr J Aquac-Bamid* 61:5–26
- Yu X, Lin S, Kobayashi M, Ge W (2010) Expression of recombinant zebrafish follicle-stimulating hormone (FSH) in methylotropic yeast *Pichia pastoris*. *Fish Physiol Biochem* 36:273–281
- Zohar Y, Mylonas CC (2001) Endocrine manipulations of spawning in cultured fish: from hormones to genes. *Aquaculture* 197:99–136