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# Molecular characterization of a cDNA from the gilthead sea bream (*Sparus aurata*) encoding a fish prion protein

Laurence Favre-Krey [a], Maria Theodoridou [a], Evridiki Boukouvala [a], Cynthia H. Panagiotidis [b,c], Athanassios I. Papadopoulos [d], Theodoros Sklaviadis [b,c], Grigorios Krey [a,*]

[a] *National Agricultural Research Foundation-Fisheries Research Institute, Nea Peramos, 64007, Kavala, Greece*
[b] *Institute of Agrobiotechnology-Center of Research and Technology Hellas, P.O. 361, GR-57001 Thermi, Thessaloniki, Greece*
[c] *Department of Pharmacy, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece*
[d] *Department of Biology, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece*

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**Abstract**

We have identified and characterized a cDNA from the brain tissue of the highly commercial marine fish species, the gilthead sea bream (*Sparus aurata*), which encodes a 496 amino acid residue protein sharing the organizational and structural features of the mammalian prion proteins. Tissue mRNA expression analyses revealed the presence of this transcript in various tissues of the gilthead sea bream including the brain, the spleen, and the heart. Sequence comparison and phylogenetic analysis showed the gilthead sea bream protein to be the homologue of one of the long form prion proteins identified from the model fish species *Takifugu rubripes* and *Danio rerio*. Unique to this fish prion protein is an extended Gly–Tyr–Pro-rich region, a structural feature that apparently resulted from multiple duplications of a core motif. The presence of this feature in other seemingly unrelated proteins suggests the involvement of common mechanism(s) in its formation and infers possible evolutionary trends related to its function.


*Keywords:* Gilthead sea bream; Phylogenetics; Prion protein; Tissue mRNA expression

## 1. Introduction

Transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative diseases in which the causative agent is thought to be the glycoprotein, $PrP^{Sc}$, an aberrant form of the normal cellular prion protein, $PrP^{C}$ (Prusiner, 1998). In comparison to $PrP^{C}$, the abnormal $PrP^{Sc}$ is partially protease resistant (Butler et al., 1988), a characteristic often used to distinguish the two PrP isoforms. $PrP^{Sc}$ is not present in healthy uninfected individuals, but the normal cellular isoform, $PrP^{C}$, is present in most tissues of mammals, with the nervous tissues showing the highest expression levels (Horiuchi et al., 1995; Ford et al., 2002). Although $PrP^{C}$ and $PrP^{Sc}$ share the same amino acid sequence they differ in tertiary structure and aggregation state. Thus, while protease sensitive $PrP^{C}$ is rich in α-helices, the pathogenic $PrP^{Sc}$ isoform has a higher β-sheet content that contributes to both its increased protease resistance and its decreased solubility (Pan et al., 1993). Studies with PrP knockout mice have shown that endogenous $PrP^{C}$ expression is required for infectious prion propagation (Sailer et al., 1994) but the precise mechanism by which $PrP^{Sc}$ converts normal $PrP^{C}$ to the pathogenic isoform is not completely understood (Silveira et al., 2005).

The TSE disease group includes Creutzfeldt–Jacob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and scrapie in sheep and goats. While TSEs have been studied extensively in higher organisms, including primates and rodents, little is known about TSE pathogenesis in fish and lower vertebrates (Ingrosso et al., 2006, and http://europa.eu.int/comm/food/fs/sc/ssc/out320_en.pdf).

Current knowledge of TSEs dictates that prerequisite for propagation of prion infectivity in other species is the presence

* Corresponding author. Tel.: +30 25940 29035; fax: +30 25940 22222.
*E-mail address:* krey@otenet.gr (G. Krey).

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of an endogenous cellular prion protein. Efforts in identifying prion encoding genes in fish have largely taken advantage of developments in fish genomics. Thus, database mining has been used to identify cDNAs encoding for similar to prion proteins from the puffer fish *Takifugu rubripes* (Suzuki et al., 2002, Rivera-Milla et al., 2003, Oidtmann et al., 2003) and *Tetraodon nigroviridis* (Premzl et al., 2004), and the zebrafish, *Danio rerio* (Cotto et al., 2005). Subsequently, PrP encoding cDNAs have been identified from the Atlantic salmon, *Salmo salar*, (Oidtmann et al., 2003), the Japanese sea bass, *Lateolabrax japonicus*, the Japanese flounder, *Paralichthys olivaceus* (Liao et al., 2005) and, more recently, from the common carp, *Cyprinus carpio*, rainbow trout, *Oncorhynchus mykiss*, and stickleback, *Gasterosteus aculeatus* (Rivera-Milla et al., 2006).

We report herein the identification and molecular characterization of a PrP cDNA from the highly commercial marine fish species, the gilthead sea bream (*Sparus aurata*), as the first step in assessing the risk of transmission and development of TSE in this species.

## 2. Methods

### 2.1. Animals

Gilthead sea bream (Acanthopterygii, Percomorpha, Perciformes, Percoidei; Sparidae; *S. aurata*,) were obtained from a commercial farm (INTERFISH, Larimna, Greece) and maintained in recirculating seawater. At sampling time fish aged 14 months and weighed, on average, 170 g. Gilthead sea bream is a protandrous species (Fischer et al., 1987) and at sampling time all fish were male approaching sexual maturity. Before sampling, fish were anaesthetized and killed by decapitation. National and institutional regulations, in accordance with the European Union's relevant legislation, have been followed regarding animal experimentation.

### 2.2. RNA isolation and synthesis of cDNA

Total RNA from sea bream tissues, i.e. brain, liver, intestinal (proximal to cecum), gills, spleen, heart, red muscle, white muscle, was extracted with the single-step method of Chomczynski and Mackey (1995). One μg of total brain RNA was used for reverse transcription and rapid amplification of cDNA ends (RACE) PCR with the SMART RACE cDNA amplification kit (BD Clonetech, Basingstoke, UK) according to the manufacturer's instructions. The sequence of the gene-specific primer used for 3'-RACE was 5'-ATG GGG AGG TTG TGT GAA GTG G (ATG being the initiation codon) and was derived from the long form of the pufferfish *T. rubripes* PrP1 (GenBank accession no. AY141106). Following first round amplification, a nested PCR was performed with the primer 5'-ACA TGG GCT AAA AAA AGT GG (corresponding to positions 250–269 of the *T. rubripes* PrP1 cDNA) and the nested universal primer A (NUP) provided by the kit. The major product of this reaction was gel purified and inserted into the pCR4-TOPO vector (Invitrogen, Groningen, The Netherlands) for further analyses. For 5'-RACE, the gene-specific primer was 5'-GGG ATG CAG ATA TAA TGG AGG AAG TAG and was derived from the sequence of the 3'-RACE product (complementary to nucleotides 1608–1634 of cDNA). The major product of the reaction was gel purified and inserted into the pCR-Script vector (Stratagene, La Jolla, CA, USA) for further analyses.

### 2.3. Sequence analysis

The 5'- and 3'-RACE products were sequenced on both strands, using plasmid- and gene-specific primers. All sequencing was performed by a commercial service (Macrogen, Seoul, South Korea). Motif identification on the nucleotide and the deduced protein sequence was performed with the OMIGA 2.0 software package (Accelerys, Cambridge, UK).

### 2.4. Phylogenetic analysis

The deduced amino acid sequences of known fish PrP proteins along with the human, mouse, bovine, dog, avian, chick, and turtle PrPs were obtained from the EMBL/GenBank and Swiss-Prot databases and were aligned using the OMIGA 2.0 software package. The alignment file was used to construct neighboring–joining trees using the MEGA 2 (Kumar et al., 2001) and/or the PAUP 4b (Sinauer Associates, Inc., Sunderland, MA, USA) software packages. Trees were constructed using the entire amino acid sequence of the proteins. Additional trees were constructed using either the N-terminal repeat region or the globular C-terminal region of the proteins (Rivera-Milla et al., 2003). Trees were tested for robustness by "bootstrapping" through 1000 iterations.

### 2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (2 μg) from each gilthead sea bream tissue was reverse-transcribed, in the presence of oligo$(dT)_{20}$ primer, with Expand reverse transcriptase (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. PCR was performed on 2 μl of the thus synthesized cDNA using the gilthead sea bream PrP-specific primers, 5'-CTG GAA CCC AAA TAA TAA GAT CCT CAG TC (forward primer, binds at positions 874–902 of the cDNA) and 5'-GGG ATG CAG ATA TAA TGG AGG AAG TAG (reverse primer, binds at positions 1634–1608 of the cDNA) and Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). The reaction products were loaded on 1% agarose gel and, following electrophoresis, the 760 bp amplified fragment was visualized by ethidium bromide staining.

RT-PCR was also performed for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, which was used as an internal control of expression. The specific primers for the gilthead sea bream GAPDH cDNA (GenBank accession no. DQ641630) were 5'-CCT TCA TCG ACC TGG AGT ACA TGG (forward primer, binds at positions 101–124 of cDNA) and 5'-CCA GCA TCA AAG ATG GAG GAG TGG C (reverse primer, binds at positions 887–863 of cDNA).