Albanian j. agric. sci. 2014;13 (2): 72-79 *Agricultural University of Tirana*

RESEARCH ARTICLE **(Open Access)**

Inducible heat shock protein 70 KDA phylogenetic analyses in the suborder Percoidei

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Abstract

Members of heat shock protein 70 kDa family have been shown to be responsive to a wide variety of physiological and environmental insults, including thermal shock, heavy metals, free radicals and microbial infection. Following stress stimuli and under normal physiological conditions, a major function of them is that of an intracellular molecular chaperone supporting protein folding and transport; they are required for growth, survival and apoptosis of cells. Hsp70 may play dual role as chaperone and cytokine in mammalian immunity. It could be used as a sensitive biomarker for different classes of environmental assault. Although Hsp70 is among the most studied genes at the molecular and cellular level, its molecular evolution is not well understood in fish. We studied the Hsp70 molecular evolution by performing phylogenetic analyses with coding and amino acid sequences of different aquaculture and fishery species. Our results suggested that the analyzed organisms Hsp70 molecular evolution didn't respect organism phylogeny.

Keywords: Sensitive biomarker; Phylogeny; Molecular clock.

1. Introduction

Heat shock proteins consist of several families of highly conserved proteins that play an essential role in a number of cellular processes. Among the 70 kDa family of heat shock proteins, heat shock cognate protein 70 kDa (Hsc70) and inducible heat shock protein 70 kDa (Hsp70) have been most extensively studied in mammals [1, 2]. As other members of heat shock protein family, Hsp70 and Hsc70 have been shown to be responsive to a wide variety of physiological and environmental insults, including thermal shock, heavy metals, free radicals and microbial infection. Following stress stimuli and under normal physiological conditions, a major function of them is that of an intracellular molecular chaperone supporting protein folding and transport [3], and they are required for growth, survival and apoptosis of cells [4, 5]. In addition to serving as molecular chaperones, evidence is accumulating that mammalian Hsp70 and Hsc70 have been implicated in both innate and adaptive immunity [6, 7] (Beside the function as molecular chaperones, mammalian Hsp70 and Hsc70 have also been implicated in both innate and adaptive immunity). According to different authors, both Hsp70 and Hsc70 play important roles in antigen presentation, T-cell receptor complex formation [8], autoimmunity and tumor immunity [9, 10, 11]. In particular, immunomodulatory functions of extracellularly located or membrane-bound Hsp70 have been established [12, 13] since the extracellular Hsp70 can be recognized by immune cells including macrophages, dendritic cells and natural killer cells [14, 15]. Actually, it has been suggested that Hsp70 may play dual role as chaperone and cytokine in mammalian immunity [16]. There is of great concern the monitoring of the impact of aquatic environments on fish. In view of their responsiveness to diverse forms of stress, Hsp70 or Hsc70 could be used as a sensitive biomarker for different classes of environmental assault [17]. Based on the considerations mentioned above, the identification and characterization of teleost Hsp70 and Hsc70 have received increasing attention in recent years. To date, the cDNA sequences of both Hsp70 and Hsc70 have been isolated from several fish species including sea bream [18], rainbow trout [19] and Wuchang bream [20]. More efforts are being made to examine the expression of Hsp70/Hsc70 under different stimuli stress, including thermal effect [21, 22], environmental pollution [23], heavy metals [24, 25], insecticides [26] and crowding stress [20]. As in mammals, both Hsp70 and Hsc70 are induced in the pattern of gene expression under stressful conditions, (it is suggested) that they may be used as indicators of cellular stress response, when fish is exposed to environmental contaminants. Recently, a few studies reported that mRNA levels of Hsp70 were increased in bacterially infected fish [25, 27], providing valuable knowledge on the involvement of Hsp70 in fish immunity. Although Hsp70 is among the most

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(Accepted for publication 20 May 2014)
***ISSN**: 2218-2020, © Agricultural University of Tirana*

studied genes at the molecular and cellular level, its molecular evolution is not well understood in fish. We decided to pay particular attention to the Hsp70 molecular evolution of percoid fish species, which are known to be very important in aquaculture and recreational fishery. The results of our phylogenetic analyses suggested that the analyzed organisms Hsp70 molecular evolution didn't respect organism phylogeny.

2. Material and Methods

Hsp70 amino acid and full coding cDNA sequences of the species reported in Table 1 were found in EMBL-EBI (www.ebi.ac.uk).

Table 1. All analyzed Perciformes Hsp70 sequences, which were found in EMBL-EBI database.

Species	Accession no.
Acanthopagrus schlegelii (**japanese black porgy**)	AY762970
Miichthys miiuy	JN969072
Siniperca chuatsi **(mandarin fish)**	AHK25483
	AHK25484
Rhabdosargus sarba **(goldlined seabream)**	AY436787
Dicentrarchus labrax **(european seabass)**	AAR01102
Sciaenops ocellatus **(red drum)**	GU244375
Oreochromis niloticus **(Nile tilapia)**	ACI25099

All respective sequences were aligned using T-Coffee multiple sequence alignment software package [28]. jModelTest [29] was used to carry out statistical selection of best-fit models of nucleotide substitution. Analyses were performed using 88 candidate models and three types of information criterion (Akaike Information Criterion-AIC, Corrected Akaike Information Criterion-cAIC and Bayesian Information Criterion-BIC). For selection of the best-fit model of analyzed protein evolution was used ProtTest3 [30]. 122 candidate models and three types of criterion (Akaike Information Criterion-AIC, Corrected Akaike Information Criterion-cAIC and Bayesian Information Criterion-BIC) were used in these statistical analyses. The Hsp70 coding region and amino acid sequences phylogenetic trees were build using the Bayesian inference (BI) method implemented in Mr. Bayes 3.2 [31]. Four independent runs, each one with four simultaneous Markov Chain Monte Carlo (MCMC) chains, were performed for 1,000,000 generations sampled every 1000 generations. FigTree v1.3.1 software was used to display the annotated phylogenetic trees.

3. Results and Discussion

3.1. Molecular Clock Tests

The molecular clock has become an indispensable tool within evolutionary biology, enabling independent timescales to be placed on evolutionary events. Despite these valuable contributions, date estimates derived from molecular data have not been without controversy. In particular, when molecular clocks have been employed to estimate the timing of recent events already tentatively dated on the basis of paleontological, archaeological or biogeographic sources, conflicting dates are frequently obtained. In its most extreme form, the molecular clock hypothesis postulates that homologous stretches of DNA evolve at essentially the same rate along all evolutionary lineages for as long as they maintain their original function [32]. Since the assumption of rate constancy is violated even within Mammalians, a truly universal molecular clock that applies to all organisms cannot be assumed to exist. In order to know which was the best-fit model to analyzed Hsp70 protein sequence evolution a Bayes factor comparison (Mr. Bayes 3.2) was performed to test the strict clock model against the non-clock model using Hsp70 full coding sequences. We used an accurate assessment of the marginal model likelihoods using the stepping-stone method. It estimates the model likelihood by sampling a series of distributions that represent different mixtures of the posterior distribution and the prior distribution [33]. The stepping-stone method was applied to the Hsp70 dataset using 510000 generations with a diagnostic frequency of 2500 in 2 independent runs for each of the tested models. The marginal likelihood values are shown in Table 2.

Table 2. The marginal likelihood values in each of the 2 independent runs and the resulting mean values for each of the tested models using the stepping-stone method.

Run	Unconstrained	Strict Clock
1	-7122,17	-7205,17
2	-7122,05	-7207,24
Mean of Marginal Likelihood	-7122,11	-7206,23

The non-clock model (-7122.11) was almost 84 log likelihood units better than the strict-clock model (-7206.23). A difference exceeding 5 log likelihood units is usually considered very strong evidence in favor of the better model [34]. Thus, the analyzed Hsp70 molecular evolution was not based on the clock model.

3.2. Phylogenetic Tree Construction

In order to build rooted trees we decided to use as outgroup, the coding and amino acid sequence of *Oreochromis niloticus*, which is member of a different order: *Cichliformes*. All the other sequences belong to members of the order *Perciformes*. The Hsp70 full coding sequences were aligned using T-Coffee in combined libraries of local and multiple alignments, which are known to induce high accuracy and performance in sequence alignments [28]. The residue consistency mean score of the all sequence alignment reported by T-Coffee aligner was very high (SCORE=86) demonstrating that Hsp70 coding sequences alignment is a high quality alignment. jModelTest 2 software [29] determined the TIM2+I model as being the best-fit model of coding cDNA sequence evolution, using three statistical criterion, Akaike Information Criterion (AIC), Corrected Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC) (-lnL= 7038.015). Phylogenetic relationships of all these different fish Hsp70 sequences were determined using one of the most powerful method (BI). The best phylogeny generated by the BI method is depicted in Figure 1.

T-Coffee was also used for Hsp70 amino acid sequences alignment and the alignment was characterized by a high score value (99). This amino acid sequences alignment (Figure 2) was better than the coding cDNA sequences alignment, because its mean score was higher than the mean score of cDNA sequences alignment.

ProtTest3 was used for determination of amino acid sequences evolution best-fit model. The best model resulted to be the JTT + G model (-lnL= 3194.91) with a gamma shape value (four rate categories) of 0.25. The best phylogeny (Figure 3) was obtained by using the BI method.

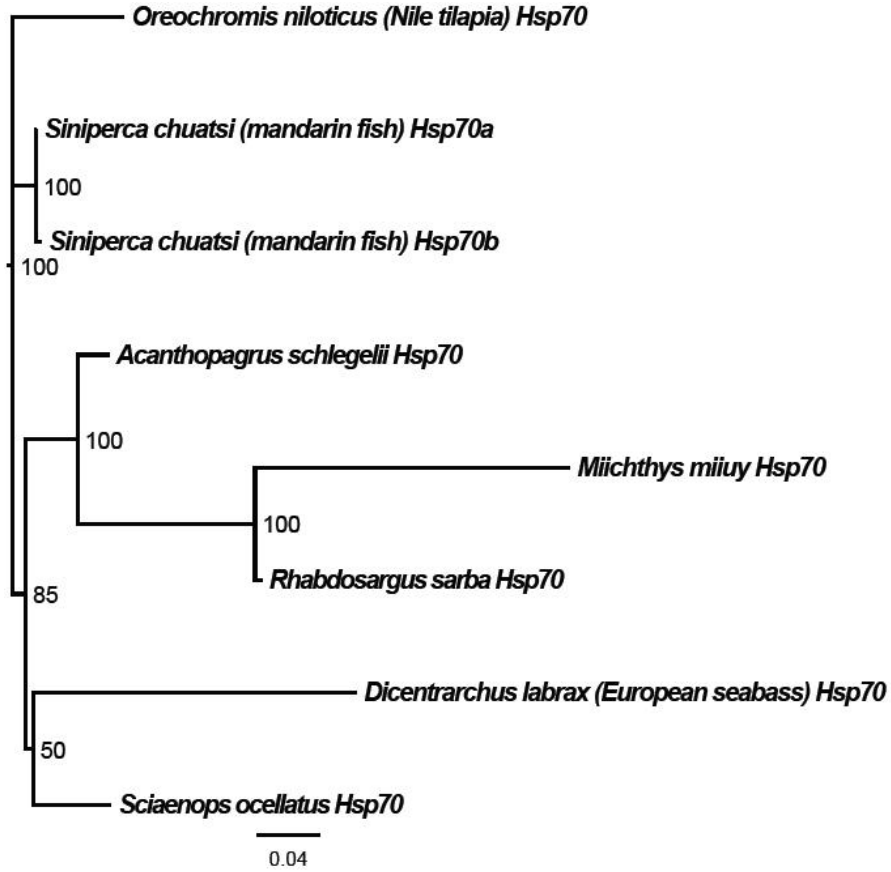

Figure 1. Phylogenetic relationships among Hsp70 coding cDNA sequences using BI method (arithmetic mean = -7066.756; harmonic mean = -7.076.779). Posterior probability values higher than 50% are indicated on each node. The scale for branch length (0.04 substitution/site) is shown below the tree.

```
AschHSP70         1 MAPAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNHLALNPSNTV  69
MmiiHsp70         1 MS-AKGISIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPVNTI  68
SchuHsp70a        1 MSAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVALNPSNTV  69
SchuHsp70b        1 MSAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVALNPSNTV  69
Rsarhsp70         1 MAPAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVALNPSNTV  69
DlabHSP70         1 MSAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVALNPSNTV  69
SoceHsp70         1 MPAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVSLNPSNTV  69
OnilHsp70         1 MSAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVALNPSNTV  69

cons              1 *. ***:::**********************************************:**********::::** **:  69


AschHSP70        70 FDAKRLIGRKMDDQVVQADMKHWPFKVVGDGGKPKIQVEYKGEDKTFYPEEISSMVLVKMKEIAEAYLG  138
MmiiHsp70        69 FDAKRLIGRKFTDPVVQSDMKLWPFKVINDSGKPKVQVEYKGETKAFYPEEISSMVLVKMKEIAEAYLG  137
SchuHsp70a       70 FDAKRLIGRKLDDPVVQADMKHWPFKVVGDGGKPKIQVEYKGEDKAFYPEEISSMVLVKMKEIAEAYLG  138
SchuHsp70b       70 FDAKRLIGRKLDDPVVQADMKHWPFKVVGDGGKPKIQVEYKGEDKAFYPEEISSMVLVKMKEIAEAYLG  138
Rsarhsp70        70 FDAKRLIGRKMDDQVVQADMKHWPFKVVGDGGKPKIQVEYKGEDKTFYPEEISSMVLVKMNEIAEAYLG  138
DlabHSP70        70 FDAKRLIGRKLDDPVVQADMKHWPFKVVGDGGKPKIQVEYKGEDKTFYPEEISSMVLLKMKEIAEAYLG  138
SoceHsp70        70 FDAKRLIGRKLDDSVVQADMKHWPFEVVGDAGKPKIQVQYKGEDKTFSPEEISSMVLVKMKEIAEAYLG  138
OnilHsp70        70 FDAKRLIGRKFDEPVVQADMKHWPFKVISDGGKPKIRVEYKGEDKAFYPEEISSMVLVKMKEIAEAYLG  138

cons             70 **********: : ***:*** ***:*:.*.****::*:**** *:* *********:**:******** 138


AschHSP70       139 QQVSNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKGKTGERNVLIFDLGGGTF  207
MmiiHsp70       138 QKVSNAVITVPAYFNDSQRQATKDAGVISGLNVLRIINEPTAAAIAYGLDKGKRGERNVLIFDLGGGTF  206
SchuHsp70a      139 HKVSSAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKGRTGERNVLIFDLGGGTF  207
SchuHsp70b      139 HKVSSAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKGRTGERNVLIFDLGGGTF  207
Rsarhsp70       139 QQVSNAVITVPAYFNDSQRQATKDAGVIAGLNVLKIINEPTAAAIAYGLDKNKTGERNVLIFDLGGGTF  207
DlabHSP70       139 HQVSNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKGKSRERNVLIFDLGGGTF  207
SoceHsp70       139 HQVSQAVITVPAYFNDSQRQATKDAGVIAGLNVMRIINEPTAAAIAYGLDKAKTGERNVLIFDLGGGTF  207
OnilHsp70       139 QKVSNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKGKSGERNVLIFDLGGGTF  207

cons            139 ::**.**:********************:****::**************** :  ************** 207


AschHSP70       208 DVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDISHNKRALRRLRTACERAKRTL  276
MmiiHsp70       207 DVSILTIEDGIFEVKSTAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTL  275
SchuHsp70a      208 DVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDIGQNKRALRRLRTACERAKRTL  276
SchuHsp70b      208 DVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDIGQNKRALRRLRTACERAKRTL  276
Rsarhsp70       208 DVSILTIEDGIFEVKATAGDTHLGGEKFDNRMVNHFVEEFKRKHKKDISHNKRPLRRLRTACERAKRTL  276
DlabHSP70       208 DVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTL  276
SoceHsp70       208 DVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTL  276
OnilHsp70       208 DVSILTIEDGIFEVKSTAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTL  276

cons            208 ***************:**********.**********************.:***.:************** 276


AschHSP70       277 SSSSQASIEIDSLFEGVDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDIVLVGGSTRI  345
MmiiHsp70       276 SSSTQASLEIDSLFEGIDFYTSITRARFEELNSELFRGTLDPVEKSLQDAKLDKSKIHEIVLVGGSTRI  344
SchuHsp70a      277 SSSSQASVEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDVVLVGGSTRI  345
SchuHsp70b      277 SSSSQASVEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDVVLVGGSTRI  345
Rsarhsp70       277 SSSTQASIEIDSLFEGIDFYTSITRARFEELNSELFRGTLDPVEKALQDAKLDKSKIHEVVLVGGSTRI  345
DlabHSP70       277 SSSSQASIEIDSLFEGIDFYTSITRARFEELNADLFRGTLDPVEKSLRDAKMDKGQIHDIVLVGGSTRI  345
SoceHsp70       277 SSSSQASIEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDIVLVGGSTRI  345
OnilHsp70       277 SSSSQASIEIDSLFEGVDFYASITRARFEELCSDLFRGTLEPVEKSLRDAKLDKGQIHDVVLVGGSTRI  345

cons            277 ***:***:*********:***:********** ::******:****:*:***:**.:**::********* 345


AschHSP70       346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILSGDTSGNVQDLLLLDVAPLSLGIETAGGVMTS  414
MmiiHsp70       345 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILMGDTSENVQDLLLLDVAPLSLGIETAGGVMTP  413
SchuHsp70a      346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILTGDTSGNVQDLLLLDVAPLSLGIETAGGVMTS  414
SchuHsp70b      346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILTGDTSGNVQDLLLLDVAPLSLGIETAGGVMTS  414
Rsarhsp70       346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILMGDTSENVQDLLLLDVAPLSLGIETAGGVMTP  414
DlabHSP70       346 PKIQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTV  414
SoceHsp70       346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILTGDTSANVQDLLLLDVAPLSLGIETAGGVMTS  414
OnilHsp70       346 PKIQKLLQDFFNGRELNKSINPDEAVAYGAAVQAAILSGDTSGNVQDLLLLDVAPLPLGIETAGGVMTA  414

cons            346 **************:********************** **.* **********:**.*********** 414
```

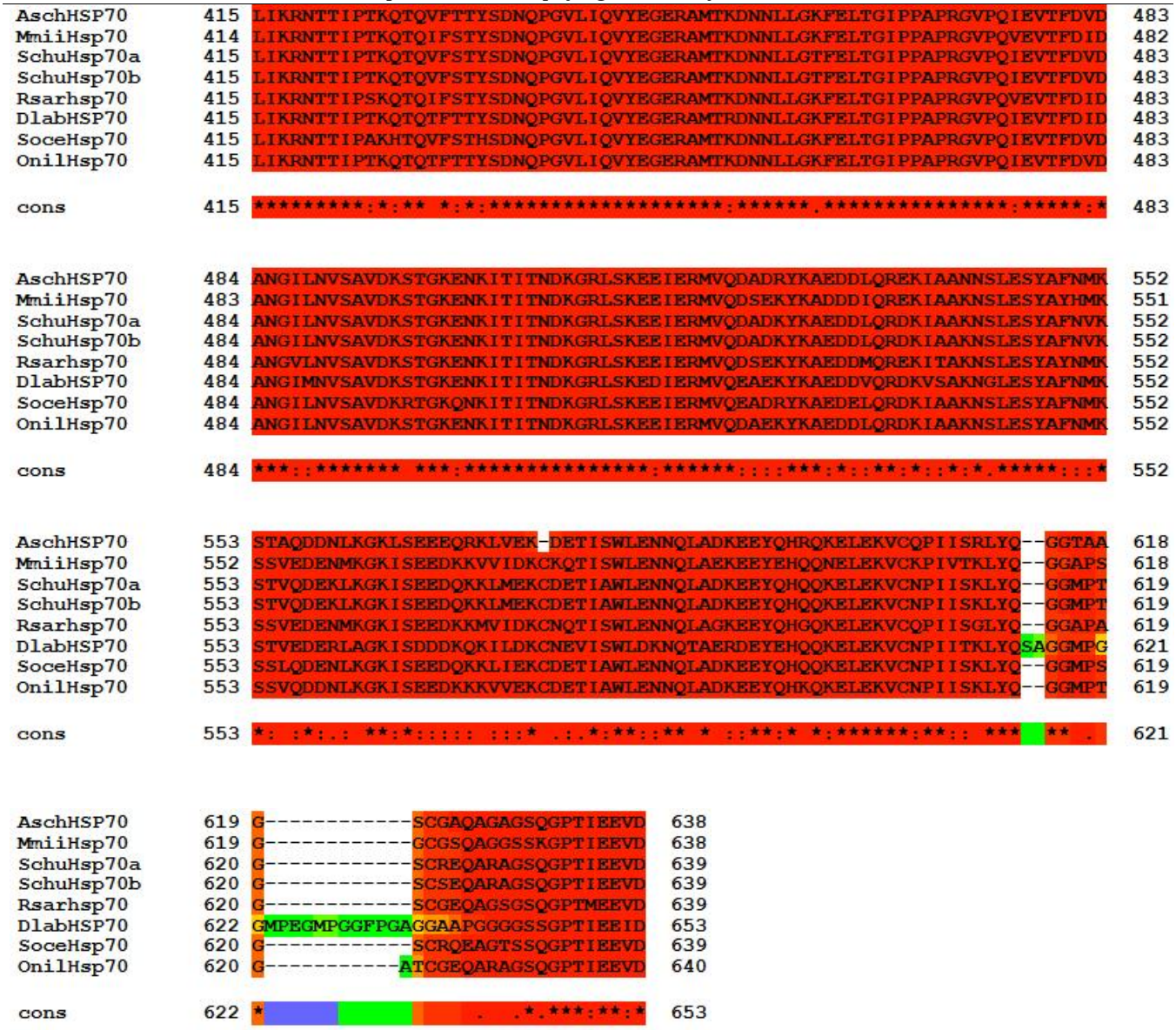

Figure 2 The Hsp70 amino acid sequences alignment generated by using T-coffee software; AschHSP70 - *Acanthopagrus schlegelii* Hsp70, MmiiHsp70 – *Miichthys miiuy* Hsp70, SchuHsp70a – *Siniperca chuatsi* Hsp70a, SchuHsp70b – *Siniperca chuatsi* Hsp70b, Rsarhsp70 – *Rhabdosargus sarba* Hsp70, DlabHSP70 – *Dicentrarchus labrax* Hsp70, SoceHsp70 – *Sciaenops ocellatus* Hsp70, OnilHsp70 – *Oreochromis niloticus* Hsp70; **.** and **:** show one and two different amino acid in the corresponding alignment position, respectively; ***** shows that all the amino acids are identical in the corresponding alignment position.

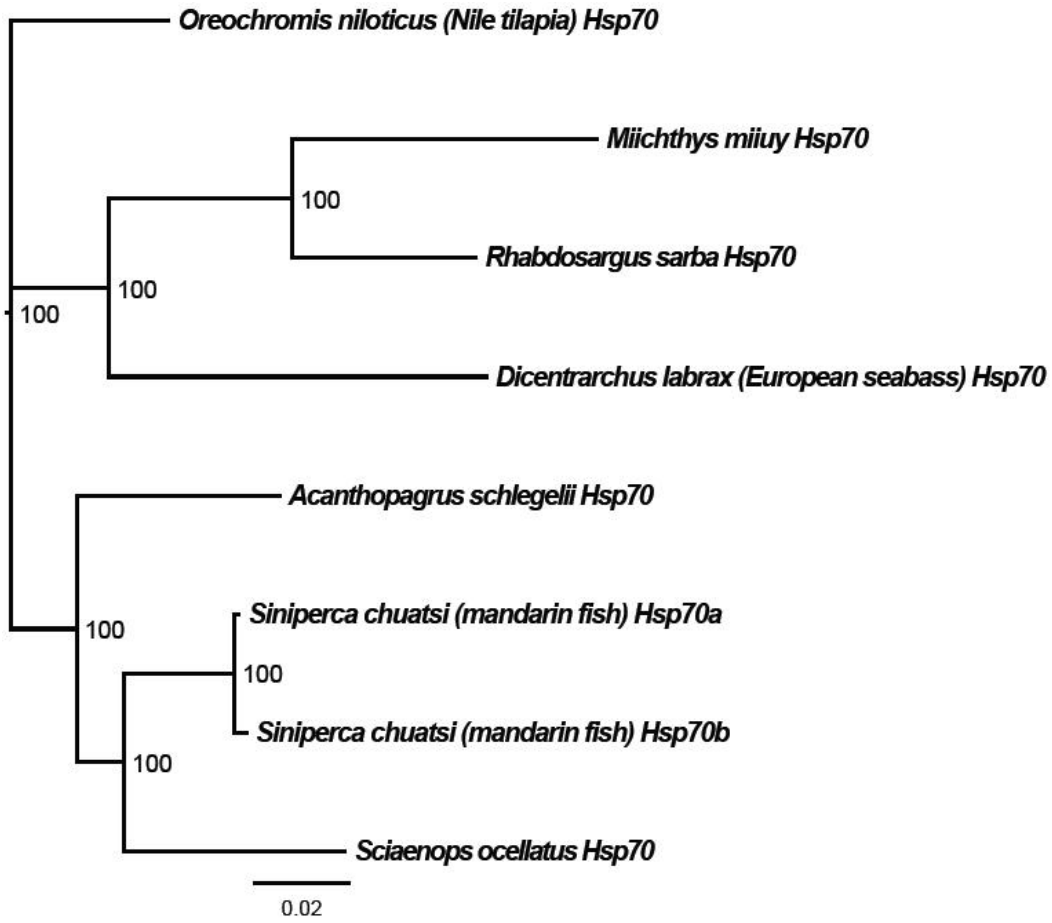

Figure 3. Phylogenetic relationships among Hsp70 amino acid sequences using BI method (arithmetic mean = -3535.337; harmonic mean = -3544.737). Posterior probability values higher than 50% are indicated on each node. The scale for branch length (0.02 substitution/site) is shown below the tree.

The comparison of the phylogenetic trees showed that the best resolved phylogenetic tree was the amino acid sequences based phylogenetic tree (Figure 3). The nodes of this phylogenetic tree were supported by higher baesian posterior probability values than the nodes of coding cDNA sequences based phylogenetic tree. Generally, the cDNA based phylogenetic trees are more resolved than the amino acid based phylogenetic trees, but this was not that case. However, the outgroup sequence was separated by the other sequences in each of the phylogenetic tree, which were grouped in three and two clusters in the coding cDNA based and amino acid sequences based phylogenetic tree, respectively. We compared these phylogenetic analyses results with the taxonomic data of NCBI Taxonomy database (www.ncbi.nlm.nih.gov/Taxonomy/). In the amino acid sequences based phylogenetic tree the Hsp70 sequences of *M. miiuy*, *R. sarba* and *D. labrax* were grouped together, but only *M. miiuy* and *D. labrax* are members of *Percomopharia incertae*, *R. sarba* is a member of *Spariformes*. The other cluster was composed by Hsp70 sequences of *A. schlegelii*, *S. chuatsi* and *S. ocellatus*. Only *A. schlegelii* is a member of *Spariformes*. In these comparisons emerged many discordances, which indicated that these organisms Hsp70 phylogeny didn't respect organism phylogeny. Kominek and colleagues data [35] revealed that the evolutionary history of this highly conserved and ubiquitous protein family was surprising complex and dynamic. For this reason, further genomic and proteomic analyses are needed in order to have more information about the evolutionary history of these particular proteins in the analyzed fish species.

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