MOLECULAR TAXONOMIC STUDY OF HORSE MACKEREL
(Trachurus mediterraneus) USING ITS1 VARIABILITY

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Abstract


Carangidae is a family of marine, mostly predatory fishes, which comprises of species like jacks, pompanos, jack mackerels and scads. Several of them are subject of commercial fishing. In Black Sea Carangidae is represented by two species: Trachurus trachurus and T. mediterraneus. The Mediterranean horse mackerel (Trachurus mediterraneus) is subject of commercial fishing. The economical importance of the horse mackerel and the need of adequate measures for conservation and proper management of its resources in the Black Sea require more information about the population dynamics and biodiversity of that species. In this study the applicability of ITS1 sequences for molecular taxonomy studies of horse mackerel was assessed. For this purpose genomic DNA was isolated from ten samples caught in different Bulgarian coastal areas. It was used as a template for PCR amplification of the ITS1 regions. The results presented on the general phylogenetic tree allowed us to distinguish two subclusters. The first one comprises of the samples caught during the summer in the Northern coastal region and during the winter from the Southern coastal regions, while the second consist of samples captured during the winter and spring from Northern regions. Probably this division corresponds with the existence of at least two populations of Mediterranean horse mackerel (Trachurus mediterraneus) in Bulgarian coastal region that migrate during different seasons from North to South and back.

Key words: Black Sea, Carangidae, horse mackerel, ITS, Phylogeny, Trachurus

Introduction

The family Carangidae (Perciformes), is spread world-wide and consists of about 33 genera and 140 species (Froese and Pauly, 2001). Most of the species are predators that hunt in the open sea or near reefs. The genus Trachurus consists of economically important pelagic fishes. In Europe, it is represented by 3 species: horse mackerel, T. trachurus; Mediterranean horse mackerel, T. mediterraneus; and blue jack mackerel, T. picturatus (Tortonese, 1975; Whitehead et al., 1986; Fisher et al., 1987). T. trachurus has a global distribution including the Mediterranean, the Black and the Marmara Seas, Atlantic, Western Pacific and Indian Oceans. The other 2 species, T. mediterraneus and T. picturatus, have a more restrict distribution – from the Bay of Biscay (France), southward to Morocco and eastward in the Mediterranean Sea, whereas T. mediterraneus inhabits also the Marmara and Black Seas (Fish base online).

In the Black Sea, Carangidae family is represented by two species: Trachurus trachurus and
T. mediterraneus (Drenski, 1948, 1951; Aleev, 1956; Georgiev and Kolarov, 1959, 1962; Stoyanov et al., 1963; Svetovidov, 1964; Valkanov et al., 1978; Sivkov, 2004; Zhivkov et al., 2005; Kapapetkova and Zhivkov, 2006; Raykov and Yankova, 2008; Yankova et al., 2010). Nümann (1956) and Aleev (1957) examined the systematic position of the Black Sea horse mackerel. These authors stated that in the Black Sea the species is represented by four local subpopulations: a south western (Bosporic), a northern (Crimean), an eastern (Caucasian) and a southern (Anatolian). Each subpopulation has its own biological characteristics such as wintering grounds, fat content, spawning patterns, age composition, growth rate, feeding patterns.

The horse mackerel is a migratory species distributed in the whole Black Sea (Ivanov and Beverton, 1985). In the spring, it migrates to the north for reproduction and feeding. In summer, the horse mackerel is distributed preferably in the shelf waters above the seasonal thermocline. In the autumn, it migrates towards the wintering grounds along the Anatolian and Caucasian coasts migration (Ivanov and Beverton, 1985). The horse mackerel matures at age of 1–2 years during the summer, which is also the main feeding and growth season. It spawns in the upper layers, mainly in the open part of the sea as well as near the coast (Arkhipov, 1993). Eggs and larvae are often found in areas with a low productivity and higher salinity (Arkhipov, 1993). Daskalov (1999) has found that horse mackerel recruitment is related to divergence and increased productivity of the sea. Peak spawning in the Bulgarian Black Sea Coast falls between June–August (Georgiev and Kolarov, 1962; Stoyanov et al., 1963; Karapetkova and Zhivkov, 2006; Yankova and Raykov, 2009; Yankova, 2011).

The catches of Black sea horse mackerel were realized by active (bathypelagic trawls and surrounding nets) and passive fishing gears (gill netting, trawl net, trap nets) (Prodanov et al., 1997; Yankova et al., 2010a).

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The economic importance of T. mediterraneus species on one hand and the need to take adequate measures for the conservation and proper management of Black Sea fishery resources on the other determines the need for study of population dynamics and biodiversity of that species.

A relatively modern and widely used method is based on comparative study of the ITS1 of sequences (Bootton et al., 1999). The ITS1 is spacer RNA embedded between two structural components of the ribosome complex e.g. the 18S rRNA and 5.8 S rRNA (Baldwin, 1992; Bootton et al., 1999). The ITS1 rRNA, is also transcribed from the rRNA gene cluster but it is „removed“ during the maturation of the long rRNA precursor molecule. Thus, the ITS1 is often believed to evolve without functional constraints. The relatively fast sequence evolution of the ITS1 helps in the reconstruction of phylogenetic relationships of closely related taxa (e.g., of species or genera). It is relatively easy to isolate and clone ITS1 from PCR amplicons to check for intra-individual nucleotide variation in the rRNA gene cluster (Armbruster et al., 2000; Armbruster, 2001; Armbruster and Korte 2006; Baldwin, 1992; Krieger, 2008).

For the family Carangidae thus far the ITS1 was used to study only tropical species in Southern Japanese Sea.

The aim of the study was to assess the applicability of ITS1 sequences for molecular taxonomy studies of horse mackerel.

Materials and Methods

Samples studied
Ten samples of Trachurus mediterraneus (Aleev) caught in different coastal areas from South and North Bulgaria were examined. The species were determined by morphological features using initially the determination key of Drenski (1951) and Peshev, Boev (1962). Updated descriptions provided by Kapapetkova, Jivkov (2010) and by newest morphological data available (http://www.FAO.org and http://species-identification.org/) were consequently used to confirm determination. The exact locations, methods used to capture the fishes as well as the time of fishing are summarized in Table 1.

DNA preparation
DNA was extracted from fish muscle tissue using DNeasy Blood & Tissue kit (Qiagen, cat № 69504) following the enclosed standard protocol. The isolated
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DNA was quantified spectrophotometrically by its absorption at 260 nm and the quality was controlled by electrophoresis on 1% agarose gel.

**Primers**

Primers designed by University of British Columbia, Nucleic Acid-Protein Service Unit, for ITS1 region (http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/) were tested in this study: ITS1-Fw 5'-GTGGTGCATGGCCGTTCTTA-3', and ITS1 Rev 5'-GCTGCGTTCTTCATCGACGC-3'. The primers were ordered from "Metabion International AG", Martinsried, Germany and upon arrival were dissolved in DNase-free water to 100 mmol stock solutions. Before use, 10 mmol aliquots were prepared.

**PCR reaction conditions**

Approximately 150 ng DNA template was taken from each sample and mixed in 200 mL PCR tube with 1 mL of each primer (10 mmol.L⁻¹ concentration), 25 μL PCR master mix (Fermentas, Cat № K0171) and 21 μL DNase-free water (supplied with the master mix kit). The PCR tubes were places TC-512 THERMAL CYCLER (Techne) PCR apparatus and the PCR amplification was carried-out by using the following program:

- initial DNA melting at 94°C – 5 min;
- next 35 cycles of 94°C – 45 s; 58°C – 45 s; 72°C – 2 min 30 s and final extension at 72°C for 10 min.

The PCR products were mixed with 7.5 mL of loading dye (Fermentas, Cat № R0611), loaded onto 1% agarose gel containing 0.5 mg/ml ethidium bromide (final concentration) covered with 1X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with DNA by comparison with a DNA ladder (Fermentas GeneRuler Cat № SM0311). UV light visualized the PCR products.

**PCR product isolation, cloning and sequencing**

The PCR products were isolated from the agarose by QIAquick Gel extraction kit (Qiagen, Cat № 28704) according to the original protocol, and then used for U/A cloning by applying Qiagen PCR Cloning Kit (Cat № 231224). The ligation reactions were mixed with 250 μL freshly prepared competent bacterial (E. coli-TOP10 - Invitrogen) cells. The plasmids containing PCR products were isolated using QIAprep Spin Miniprep Kit (Qiagen, Cat № 27104) and sent for sequencing to MWG – Biotech AG, Frankfurt, Germany.

**Data analysis**

The gel images were captured by BIO-VISION+3026. WL system (Vilber Lourmat) using four different exposition times and processed by accompanying software. The online nblast analyses were used to confirm that the isolated sequences belong to ITS1 spacer region using the algorithm of Altschul et al. (1997). The multiple alignments of obtained sequences were performed using Vector NTI 10.1 software (Invitrogen) and ClustalW algorithm. The obtained sequences were processed by PHYLIP (Phylogeny Inference Package, version 3.5c by Joseph Felsenstein (c) Copyright 1986–1993 by Joseph Felsenstein and the University of Washington) package using and maximum likelihood with molecular clock algorithm (Felsenstein 1981). Tree Viewer visualized the obtained results (v. 1.6.6).

**Results and Discussion**

The suitability of the primers was initially tested using several different PCR conditions. In order to
achieve optimal amplification we did vary the amounts of DNA template from 50 to 300 ng and annealing temperatures from 48 to 57°C. The optimal amplification of the ITS1 region was achieved with 150 ng (2 μl) DNA template and annealing temperature 52°C (Figure 1). The amplified PCR products were with the expected size (about 350 bp). They were isolated from the gel, cloned in pDrive vectors as described in materials and methods and sequenced.

We used online blastn (Altschul et al., 1997) algorithm to compare the isolated ITS1 sequences with those annotated in NCBI database. For the family Carangidae thus far the ITS1 was used only to study tropical species in Southern Japanese Sea.

Several single nucleotide polymorphisms (SNPs) were identified in isolates sequences. The positions of SNPs are presented on Figure 2. From the 3’ end (resp.
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starting from 5.8 S rRNA), A/G transition was found at relative position 92. Two A/C transversion were found at relative position 183 and 188. These findings suggest that the population of horse mackerel in the Western regions of Black Sea is not completely homogeneous. More careful examination of the SNPs reveals that fishes captured near Varna in the beginning of August 2011 have similar SNPs as those captured close to the Turkish border in December 2011. The SNP positions are 93 and 188. While fishes captured by the end of October 2011 pose similar SNPs like those captured in May, 2012.

The degrees of differences between studied samples were assessed via analyses of obtained sequences with Phylip software package. As the ITS regions are not functional, the evolution of these sequences seem to occur according to the neutral model of Kimura, in which the genetic drift is the major driving force. Natural selection cannot operate on non-functional sequences because they do not have adaptive meaning for the organism, so the changes occurring in such sequences are random and accumulate mutations uniformly with the time (molecular clock) (Gardes and Bruns, 1993; Graur, 2000; Whitehead, 1985). This makes neutral sequences useful markers for phylogeny analyses because the mutation rate reflects the divergence time between populations/species (Bromham, 2008; Graur, 2000; Whitehead, 1985).

Therefore, we have chosen algorithm, which allowed us to estimate phylogenies by maximum likelihood based on molecular clock (Felsenstein 1981). The tree (Figure 3) was build by Tree Viewer (v. 1.6.6) software.

The results presented on the general phylogenic tree (Figure 3) allowed us to distinguish two sub clusters. The first one comprises of the samples caught during the summer in the Northern coastal region and during the winter from the Southern coastal regions, while the second consist of samples captured during the winter and spring from Northern regions. Probably this division corresponds with the existence of at least two populations of Mediterranean horse mackerel (Trachurus mediterraneus) in Bulgarian coastal region that migrate during different seasons from North to South and back.

Conclusions

Although these results are very preliminary yet, the registered variability in ITS1 regions of Mediterranean horse mackerel (Trachurus mediterraneus) will open opportunity to study migratory ways and populations of these important for fishes.

References


