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Molecular Taxonomy Study of the Genus *Alosa* H. F. Linck, 1790 (Actinopterygii: Clupeidae) along Bulgarian Black Sea Coast Based on Cytochrome b Sequences

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Abstract: The family Clupeidae is believed to be represented by nine species in the Black Sea. However, this view is not generally accepted and the genus *Alosa* is one of the most challenging groups from taxonomic point of view. The phylogenetic relationships among three shad species (*Alosa caspia, Alosa immaculata* and *Alosa tanaica*) from Bulgarian marine waters were studied base on cytochrome b sequences. Totally, one hundred samples of the genus *Alosa* were examined. Five individuals with the most possible identification as *Alosa tanaica* formed a clade positioned basally to the other two species. *A. caspia* and *A. immaculata* formed a clade but being clearly distinct from one another. The results confirm that Cyt b sequences can be used for distinguishing species of the genus *Alosa* occurring in the Black Sea.

Key words*:* *Alosa*, Clupeidae, Black Sea, PCR, cyt b

Introduction

The Clupeidae is one of the commercially important fish families. It consists of mostly marine species but some of them are freshwater and anadromous (Svetovidov 1964, Whitehead 1985, Gaudant 1991). All Black Sea shads are listed in IUCN Red List as vulnerable (Dobrovolov et al. 2012). Numerous studies of genetic structure and species identification have been carried out (Whitehead 1985, Economidis 1986, Faria et al. 2006, Dobrovolov et al. 2012, Turan et al. 2010, 2015, 2018**,** Chiesa et al. 2016, Vernygora et al. 2018, Bani et al. 2019). Despite their importance, little is known about the phylogenetic relationships within the genus *Alosa* H. F. Linck, 1790 (Chiesa et al. 2016, Bani et al. 2019, Turan et. al. 2015, 2018).

Rapidly evolving mitochondrial DNA genes have been shown to be a powerful tool for the assessment of validity and phylogenetic relationships of anadromous and marine fishes (Meyer 1993, Bernatchez & Wilson 1998, Turan et al. 2009). The pattern of maternal inheritance and the rapid rate of evolutionary changes of mtDNA compared to nuclear DNA make it a suitable tool to accomplish genetic studies (Faria et al. 2006, Turan et al. 2009, 2010, 2018).

In Bulgarian part of the Black Sea, the genus *Alosa* is represented by four species: *Alosa immaculata* (Bennett, 1835), *A. caspia* (Eichwald, 1838), *A. maeotica* (Grimm, 1901) and *A. fallax* (Lacepede, 1803) (Yankova et al. 2014). Drensky (1934) described *Alosa bulgarica* as an endemic species for the Bulgarian Black Sea tributaries south from Burgas. This taxon, also listed as *A. caspia bulgarica* by various authors, was recognised as a synonym of *A. tanaica* (Grimm, 1901) (see Kottelat 1997) but Stefanov (2007) believed that its specific morphology and distinct distribution might indicate that it could be an endemic species and more data were needed to clarify its status. As reported by the World Register of Marine Species, *Alosa bulgarica* is not valid name and the accepted name for this taxon *Alosa tanaica* (Grimm, 1901). The applications of molecular and genetic methods are necessary for precise taxonomical identification *Alosa* spp. Low values of genetic distance (0.038) were found between *A. immaculata* and *A. caspia*. The genetic distance between these two species and the other *Alosa* spp. was found to be higher (0.066) (Dobrovolov et al. 2012). Molecular data demonstrated that the differences between *A. caspia* and *A. immaculata* are less than 0.8% (Faria et al. 2006). *A. caspia*, *A. f. nilotica*, *A. immaculata* and *A. tanaica* exhibited close genetic similarity and *A. maeotica* showed the highest genetic differentiation within the genus *Alosa*. The nucleotide divergence of *A. maeotica* from the other congeners is about 0.01 (Turan et al. 2015). *A. immaculata* showed closer genetic relationships to *A. f. nilotica* and *A. caspia* than to *A. tanaica* (Turan et al. 2015). Bowen et al. (2008) used mtDNA sequence analysis and also found the lowest genetic distance between *A. immaculata* and *A. f. nilotica*.

Animal mitochondria contain 13 protein coding genes (Zhang & Jiang 2006). Cytochrome b (cyt b) is commonly used as a region of mitochondrial DNA for determining phylogenetic relationships among animals due to their variability (Bani et al. 2019, Chiesa et. al. 2016, Turan et al. 2010, 2015, 2018, Vernygora et al. 2018). Cytochrome b is very conservative and considered useful in determining relationships at the lower taxonomic levels such as subfamilies, genera, subgenera and species (Freeland 2005, Faria et al. 2006).

The aim of the present study was to assess cyt b as a marker for identification of the species of the genus *Alosa* in the Black Sea.

Materials and Methods

Taxa studied

Shad specimens were collected during the period 2017-2019 from Bulgarian coastal areas (Fig. 1). The species were initially distinguished morphologically using the identification keys by Drensky (1951), Peshev & Boev (1962) and Karapetkova & Zhivkov (2010). One hundred samples of individuals of the genus *Alosa* were analysed; these included *A. caspia* – 34, *A. immaculata* – 61 and *A. tanaica* – 5 (Fig. 1, Table 1). The samples were placed individually into plastic bags and kept frozen at –20ºC. Muscle tissue was taken from each individual in the field or in the laboratory and stored in 96% ethanol.

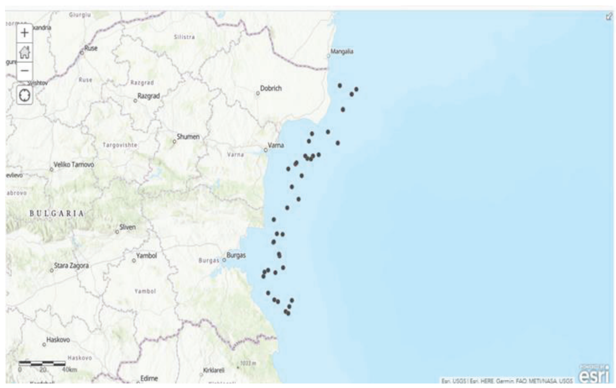

Fig. 1. Samples sites along the Bulgarian Black Sea coast

Table 1. Localities and numbers of the samples analysed.

Species	Localities	Number
Alosa immaculata	Primorsko, Ahtopol, Durankulak, Emine, Sozopol, Burgas Bay, Byala, Nesebar, Varna, Shabla	61
Alosa caspia	Ahtopol, Primorsko, Durankulak, Sozopol, Emine, Byala, Cape Kaliakra, Nesebar	34
Alosa tanaica	Ahtopol, Emine, Durankulak	5

Table 2. List of the primers used to obtain Cyt b sequences.

Name	Sequence 5’-3’	Tm
Cyt b Fw 1	5’- CMCTVCTTAAAATYGCAAACCA-3’	57.6°C
Cyt b Fw 2	5’-GCCCCCTCTAACATTTCTGC-3’	56°C
Cyt b Rev1	5’ –AGGGCRAGBACTCCKCCWAGTTT-3’	59.7°C
Cyt b Rev2	5’- GCAAAHAGHAAGTAYCACTCTGG - 3’	56.5°C

Molecular analyses

DNA was extracted from fish muscle tissue using DNeasy Blood & Tissue kit following the enclosed standard protocol. The isolated DNA was quantified spectrophotometrically by its absorption at 260 nm and the quality was controlled by electrophoresis on 1% agarose gel. For the list of primers used to obtain Cyt b sequences, see Table 2.

Approximately, 150 mg DNA template was taken from each sample and mixed in 200 µl PCR tube with 1 µl of each primer (10 mmol.L-1 concentration), 25 µl PCR master mix (Fermentas) 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 µL of loading dye loaded onto 1.5% 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 a DNA ladder (Fermentas GeneRuler). The PCR products were visualised by UV light and the bands were isolated from the agarose by QIAquick Gel Extraction Kit following the original protocol. The PCR products were isolated from the agarose by QIAquick Gel extraction kit according to the original protocol, and then sent for sequencing to Eurofins-GATC, Germany (Fig. 3).

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 Cyt b using the algorithm of Altschul et al. 1997. The multiple alignments of obtained sequences and phylogenic analyses were performed using MEGA 7.2 (Kumar et al. 2016). The phylogenic tree was built by MEGA 7.2 software using maximum likelihood algorithm with 500 times bootstrapping.

Results

First an online blastn algorithm was used to compare the obtained sequences with those annotated in NCBI database. The results demonstrated high similarity between the annotated Cyt b sequences (E value 3e-110) for *Alosa* spp. The variability in the Cyt b sequences of our 100 samples were analysed and only part of the sequences was demonstrated in order to improve the visualisation (Fig. 2).

The nblast comparison between isolated by us sequences with those annotated in NCBI Genbank

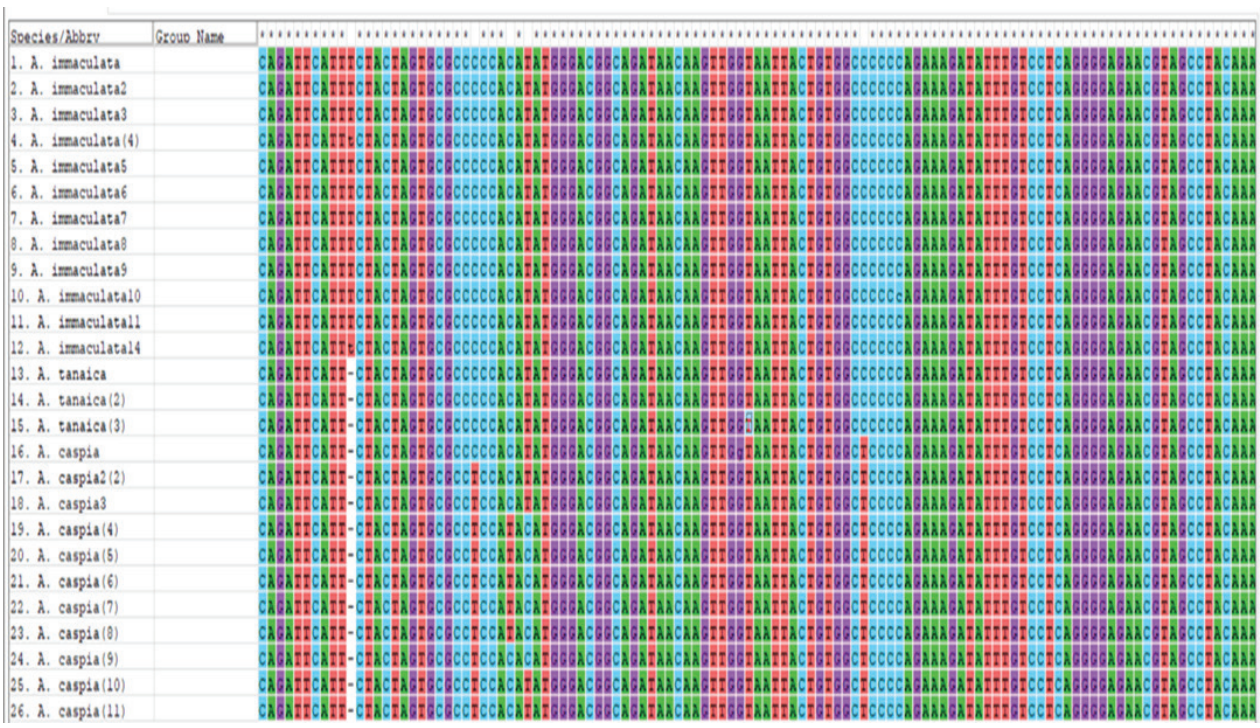

Fig 2. An alignment of Cyt b gene sequences of *Alosa* spp. based on Clustal W algorithm

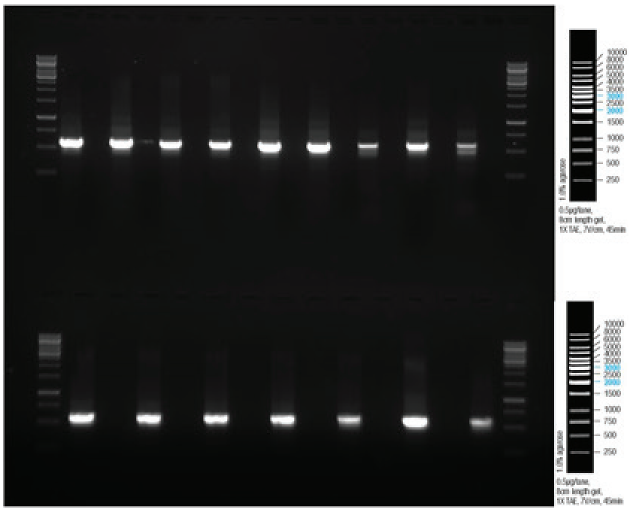

Fig. 3. PCR products visualised by UV light

database resulted in 98 % identity with e-value 0.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou et al. 1987). Bootstrap consensus tree from 500 replicates (Felsenstein et al. 1985) was used to visualise the relationships among the studied taxa. The percentage of replicate tree, in which the associated taxa clustered together, was shown next to the branches. Evolutionary distances were computed using the Maximum Composite Likelihood (Tamura et al. 2004) and presented in numbers of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1).

Discussion

Analysing the phylogenetic tree, we come to the conclusion that *A. immaculata* has closer genetic relationships with *A. caspia* than with *A. tanaica* (Fig. 4). This is in agreement with the studies by Faria et al. (2006), Bowen et al. (2008) and Turan et al. (2015). The problem about the phylogeny of the genus *Alosa* needs further studies. Additional molecular markers should be used to evaluate the phylogenetic relationship among *Alosa* spp. They are extremely vulnerable to anthropogenic changes, especially related of their spawning grounds (Faria et al. 2006). More conservation measures should be taken to increase the stocks of these species, especially the rare species such as *Alosa maeotica* and *Alosa tanaica*.

The obtained results confirm that Cyt b sequences can be used for distinguishing species of the genus *Alosa*.

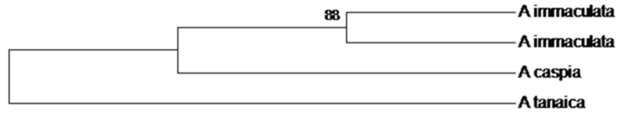

Fig. 4. Phylogenetic tree based on evolutionary relationships of taxa using the Neighbor-Joining method in cyt b sequences

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