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Molecular Analysis of Shad Species (Pisces: Clupeidae) from Bulgarian Black Sea Waters

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Abstract: The prevailing view is that the family Clupeidae is represented by nine species in Bulgarian Black Sea waters. However, this concept is not widely accepted. In particular, the genus *Alosa* is notable for its taxonomic uncertainties. The application of molecular techniques will facilitate the accurate identification of species. This study aimed to assess the applicability of the mitochondrial DNA gene NADH1 for distinguishing Bulgarian Black Sea Clupeidae species and to study their phylogenetic relations. To this end, genomic DNA was extracted from 48 representatives of the genus Alosa and 23 representatives of the genus Sprattus. The entire NADH1 gene was sequenced on both sides. The bioinformatic analysis of the obtained results indicates the distribution of three groups in the genus Alosa and four groups in the genus Sprattus. The *Alosa* groups are represented by three different taxa: *Alosa immaculata* (Bennett, 1835), *Alosa caspia* (Eichwald, 1838), and *Alosa species*. The four Sprattus groups are constituted by subpopulations of the single stock. The results, which highlight the potential of the mitochondrial NADH1 gene as a molecular marker, could pave the way for future studies on the biodiversity and phylogenetic relations of Clupeidae species in the Black Sea.

Key words: *Alosa*, *Sprattus*, mtDNA gene $NADH_1$

Introduction

The family Clupeidae includes mostly anadromous/marine and fewer freshwater fish species, which have a worldwide distribution and reach the highest diversity in the tropics (Whitehead 1985, Gaudant 1991). According to numerous sources, there are still some tiny discrepancies in the number of known genera and species within the family, varying from 50 genera and 150 species (Karapetkova & Zhivkov 2010) to 66 genera and 216 species (www.fishbase.org). The family Clupeidae along the Bulgarian Black Sea coast includes five genera with nine species (Karapetkova & Zhivkov 2010) but, according to Yankova et al. (2014), the family actually consists of five genera and eight species. The correct species determination, especially in the genus *Alosa*, by the methods of classical morphology is challenging due to interspecific morphological similarities, complex population structures, and intraspecific phenotypic plasticity (McDowal 2001, 2003, Alexandrino et al. 2006), and interspecies hybridisation (Faria et al. 2004, 2006, 2011, 2012, Jolly 2011, 2012, Czesny et al. 2012, McBride et al. 2014). However, the power of molecular techniques, as demonstrated in this study, serves as a powerful tool for phylogenetic studies. In this respect, single nucleotide polymorphisms (SNPs) are regarded as potentially useful

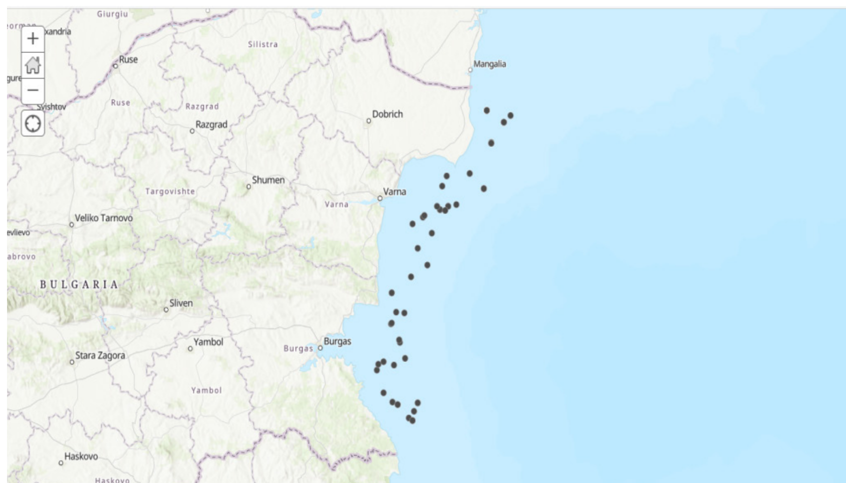

Fig. 1. Map of Bulgarian coastal line representing the sites of the capture of studied individuals.

genotyping markers in taxonomic and phylogenetic analyses. The intensive development of sequencing technologies has made it possible to analyse specific genomic regions in many individuals. Because of that, sequencing represents a powerful approach to detecting SNPs and assigning individuals to a specific group of genomic variants (haplotypes). One of the most appropriate techniques for taxonomy is analysing the mitochondrial DNA (mtDNA). The reasons for that are: mtDNA has more copies than nuclear DNA; it can indicate the degree of intraspecific variability (Bembo et al. 1995); it degrades more slowly and has a ten times faster evolutionary rate than nuclear DNA (Arif & Khan 2009). Mitochondrial protein-coding genes such as ND1 dehydrogenase subunit 1 (ND1), cytochrome b (Cyt b), cytochrome oxidase subunit 1 (COI), etc., are informative markers for genetic analysis. They have been used for precise taxonomic fish species identification, including the Clupeidae family from the Black and Azov Seas (Turan et al. 2001, 2015, Vernigora 2020). For example, some authors reported that some individuals defined as *Alosa caspia* (Eichwald, 1838) by morphological traits do not match the taxonomic clustering by molecular markers – Cyt b and COI (Chiesa et al. 2016, Vernigora 2020). Other authors used the Cyt b gene to distinguish two closely related taxa: *Alosa alosa* (Linnaeus, 1758) and *Alosa fallax* (Lacepede, 1803) (Bentzen et al. 1993, Alexandrino et al. 1996). The Cyt b and ND1 genes were also selected as markers for phylogeny in eight species of the genus Alosa (Chapman et al. 1994, Nolan et al. 2003, Faria et al. 2006). Until now, the analyses of phylogenetic relationships within the genus *Alosa* from the Bulgarian coast of the Black Sea were carried out based on allozymes (Dobrovolov et al. 2012). Despite these efforts, the molecular taxonomic studies of shad species near the Bulgarian coast are insufficient (Boyadzhieva-Doychinova et al. 2012; 2020).

Materials and Methods

Taxa studied

The forty-eight analysed specimens from the genus Alosa were captured near the Bulgarian coast of the Black Sea – from Durankulak in the north to Rezovo in the south (Fig. 1). The species determination was carried out using morphological traits according to the determination keys of Drenski (1951), Peshev & Boev (1962), Svetovidov (1964) and the updated descriptions provided by Kottelat & Freyhof (2007), Karapetkova & Zhivkov (2010). The newest morphological data available (http://www.FAO.org and http://species-identification.org) was subsequently applied to confirm the correctness of the initial determination. A complete list of the samples and their locations is provided in Table 1.

DNA preparation

Samples from all the species were stored in 96% ethanol, and total genomic DNA was extracted from the fish's caudal fin using a DNeasy Blood & Tissue kit (Qiagen) following the enclosed standard protocol. The quantity of the isolated DNA samples was evaluated spectrophotometrically, and its integrity was confirmed by electrophoresis on 1% agarose gel.

Primers

The primers for the present study were designed according to the original idea of Faria et al. (2006) within the flanking regions of the ND1. The Fw

Table 1. List of samples and locations of the genus *Alosa*.

Species	Localities	Number
Alosa caspia (Eichwald, 1838)	Byala, Burgas Bay, Primorsko, Durankulak, Sozopol, Cape Emine, Kavarna, Nesebar, Obzor, Tsarevo	Total – 24
Alosa immaculata (Bennett, 1835)	Durankulak, Cape Emine, Cape Kaliakra, Kavarna, Burgas Bay, Nesebar, Sozopol, Primorsko, Obzor, Saint Vlas, Tsarevo	Total – 21
Alosa immaculata 44 (Bennett, 1835)	Cape Emine	
Alosa immaculata caspia 45	Sozopol	
Alosa species	Byala, Nesebar	Total – 3

primer AAGTCCTACGTGATCTGAG anneals within the 16S rRNA region, and the ND1 Rev primer CGTTCAGGCACAGCTCC anneals within the tRNA-Ile and tRNA-Gln region. Although the sequenced fragment was 1321 bp, only 860 bp sequences corresponding exclusively to the ND1 gene were considered in the analysis.

The primers were ordered from Eurofins-GATC, Germany. They were dissolved in DNase-free water to 100 µmol/L stock solutions, and 10 µmol/L working aliquots were prepared before use

PCR reaction conditions

Exactly 2µl DNA template from each individual was mixed in a 200 µL PCR tube with 1µL of each primer (10 µmoL/L^{-1}), 25µL PCR master mix, and 21µL DNase-free water. PCR amplification was run on a TC-512 THERMAL CYCLER with the following conditions: DNA melting at 95°C – 5 min, followed by 35 cycles of 95°C – 15s; 54°C – 15s; 72°C – 2 min. A final extension at 72°C for 7 min was performed. PCR products were mixed with 7,5 µL 6x Loading dye, loaded onto 1% agarose gel containing 0,5 mg/mL ethidium bromide covered with 1x TBE buffer, and separated by applying an electrical current of 7 volts per cm. The size of PCR products was determined by comparison with a DNA ladder (Fermentas Gene Ruler Cat No N3200S) 1kb – 5 µL and visualised by UV light – 260 nm. The PCR products were isolated from the agarose by QIAquick Gel extraction kit (Qiagen) according to the original protocol and sent to Eurofins – GATC, Germany for bilateral sequencing.

Data analysis

The NCBI BLASTN tool was applied to confirm that the isolated sequences belong to the ND1 gene. The multiple alignment was performed using Geneious Prime 2024.0.2 software (Kearse et al. 2012) and the Geneious algorithm.

A maximum parsimony (MP) analysis was conducted using PAUP 4.0a (build 169) with a heuristic search strategy and 1000 bootstrapping replications (resamplings). Consistency (CI) and retention (RI) indices were calculated with the Phangorn package in R 4.3.2.

The Bayesian inference (BI) tree was constructed using MrBayes 3.2.6 (Huelsenbeck & Ronquist 2001) implemented in Geneious Prime 2024.0.2. A Markov chain Monte Carlo (MCMC) algorithm was applied with the following analysis parameters: Chain length/Number of cycles: 1.1×10^6; heated chains: 4; heated chain temperature: 0.2. The Markov-chain sampling frequency was set at 200, with 10^5 samples being discarded before calculating the summary statistics. As a result, a 50% majority rule consensus tree was generated.

In Geneious Prime 2024.0.2, a default Geneious Tree Builder procedure was used to carry out the neighbour-joining (NJ) analysis based on a bootstrap resampling method with 1000 replicates.

PhyML 3.3.20180214 (Guindon & Gascuel 2003, Guindon et al. 2010) was implemented to carry out the maximum likelihood (ML) analysis with settings allowing the simultaneous optimisation of tree topology, branch length, and substitution rate.

Results

The amplified PCR products were visualised on an agarose gel, and the molecular weight ladder determined the amplicons' size corresponding to the expected. We used the BLASTN algorithm (Altschul et al. 1997) to compare the newly sequenced ND1 gene for all the analysed samples with those

Table 2. SNP positions after performing Geneious alignment of the ND1. The total number of specimens is shown in brackets. The grey table cells reflect the single nucleotide polymorphisms. Identical sites are 88,5%, and the pairwise % identity is 98,9%. *SNPs represented in single samples within the analysed groups.

SNP Position	352	358	370	439	460	466	494	498	514	520	523
A. immaculata (19)	A	G	G	T	A*/G	T	G	T	T	C	A
A. caspia (24)	A	G	G*/A	C	G	T	G	T	T	T	A
A. immaculata 44 (1)	A	G	G	T	G	T	A	C	T	C	A
A. imm. caspia 45 (1)	A	G	A	C	G	T	G	T	T	T	A
A. species (3)	G	A	A	T	A	C	G	T	C	C	G
Consensus	A	G	A	C	G	T	G	T	T	T	A

SNP Position	525	531	538	539	561	568	571	592	634	730	757
A. immaculata (19)	A	G	A	C	T	G	G	A	A	A	A
A. caspia (24)	A	G	A	C	T	A*/G	G	A	A	G	**G**
A. immaculata 44 (1)	**C**	**A**	**C**	**A**	**A**	G	G	A	A	**G**	A
A. imm. caspia 45 (1)	A	G	A	C	T	G	G	A	A	G	A
A. species (3)	A	G	**G**	C	T	**A**	**A**	**G**	**G**	A	A
Consensus	A	G	A	C	T	G	G	A	A	A	A

annotated in the NCBI database. The returned results demonstrated high similarity between them and the annotated. The annotated sequences of the ND1 gene that we used were: Acc.No DQ419797; DQ419792; DQ419794 for *A. immaculata* (Bennett, 1835); Acc.No DQ419793 for *A. caspia* (Eichwald, 1838). The newly obtained sequences for all 48 specimens, with fragment lengths of 860 bp, were deposited to GenBank under accession numbers PP391086-PP391130 and PP421162- PP421164.

The alignment of the analysed sequences showed several single-nucleotide polymorphisms (SNPs), and their positions are presented in Table 2. The Alosa samples assessed in this study were morphologically determined to belong to three species: *A. immaculata* (Bennett, 1835), *A. caspia* (Eichwald, 1838), and *A. tannic* (Grimm, 1901). The molecular data from our bioinformatic analysis confirmed that three groups are formed in the genus Alosa for the samples collected from the Bulgarian coast of the Black Sea.

The first group includes 24 taxa from *A. caspia* species (Table 1). They all have C instead of T at position 439, T instead of C at position 520, and G instead of A at position 757 (Table 2). Three subgroups are observed within this group. The first one includes ten samples (No 2, 5, 11, 23, 4, 6, 10, 14, 24, 16) caught in the central and southern Black Sea. The second subgroup is represented by only five samples (No 1, 3, 7, 20, and 22) from the central Black Sea region. The third subgroup includes nine samples (No 19, 12, 15, 8, 13, 17, 18, 9, and 21) caught from the central and southern Black Sea.

The second group includes 21 representatives of the species *A. immaculata* (Table 1). We observe SNPs at three positions – 370 with G instead of A, 439 with T instead of C, 520 with C instead of T. Three subgroups are formed within this group – the first one includes eight individuals (No 25, 29, 30, 31, 35, 40, 41, and 43), caught from the Central and Southern Black Sea; the second subgroup consists of 6 samples (No 26, 28, 32, 33, 38, 39), collected only from the Southern Black Sea area. The third subgroup includes five individuals (No 27, 34, 36, 37, and 42) – caught from the Northern and Central Black Sea. Within this group, the molecular data showed two individuals (*A. immaculata* 44 and 45) with a slightly different pattern of SNPs (Table 2).

The third group, which we named Alosa species (*Alosa* sp. No 1, 2, and 3), has a large number of SNPs compared to the other two groups. The SNPs are in eleven positions: 352; 523; 538; 592; 634 – G instead of A; 358; 460; 568; 571 – A instead of G; and 466; 514 – C instead of T (Table 2).

The obtained alignment was further used to construct a consensus tree by applying four different methods (Fig. 2). The MP analysis in PAUP 4.0a yielded a consensus tree with a Fitch score of 73 (CI = 0.87671, RI = 0.9357). All characters were of type "unord", and gaps were treated as "missing" data. Forty variable characters were defined as parsimony-uninformative, while 22 were accepted as parsimony-informative.

When MrBayes was used, all BI runs produced highly reproducible phylogenetic inferences. The

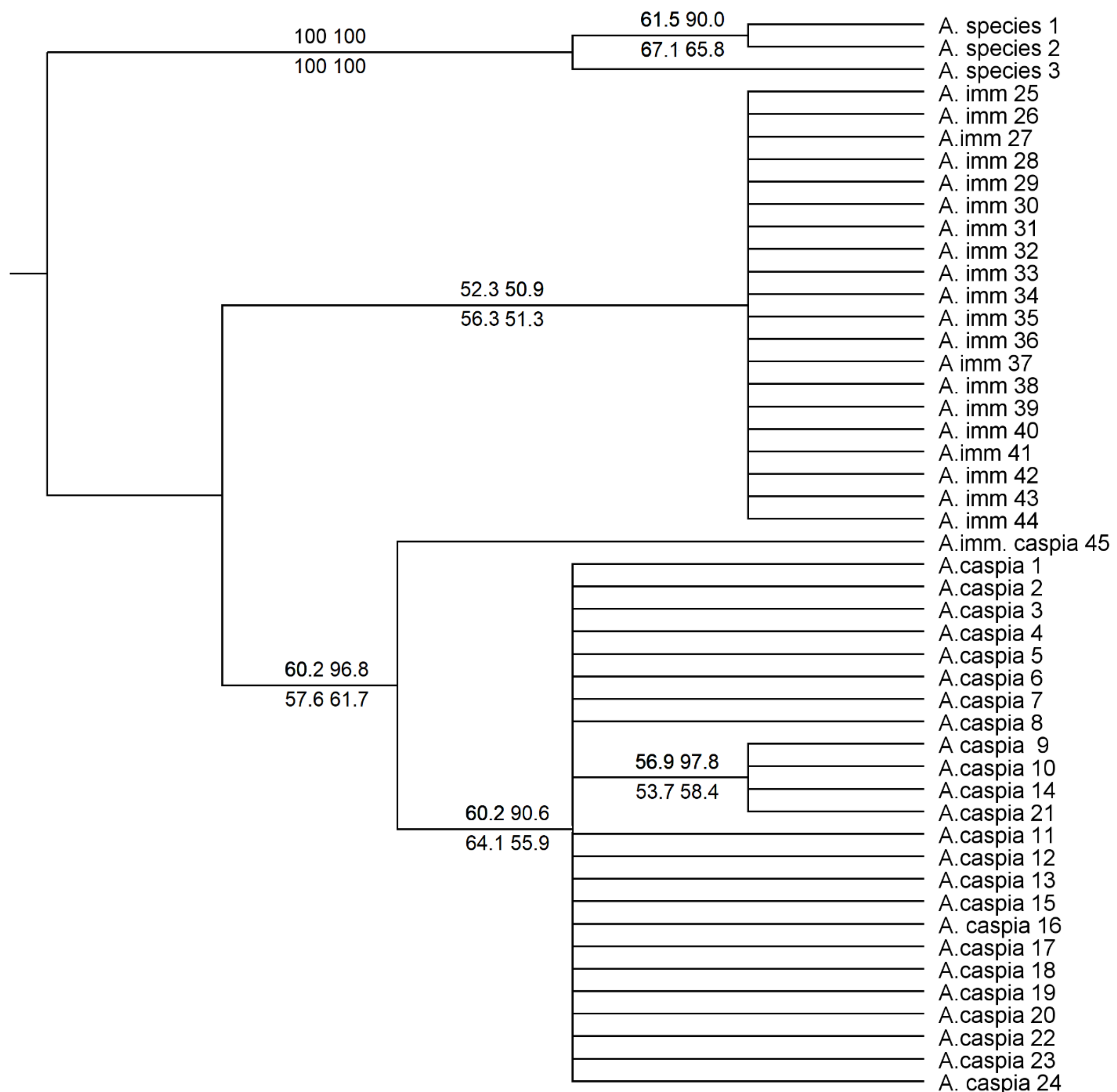

Fig. 2. A consensus tree based on the sequencing data of the ND1 gene of the genus *Alosa*. Support values (over 50%) are shown for MP (above left), BI (above right), NJ (below left), and ML (below right). The cladogram shows that the analysed samples form three groups: *Alosa caspia*, *A. immaculata* and *Alosa* sp.

log-likelihood (lnL) estimates ranged from -1732 to -1707, with auto-correlation time (ACT) values ranging from 1323 to 1484 and adequate sample sizes between 666 and 748.

The cladogram reconstruction using the NL method resulted in two larger clusters consistent with the morphologically determined species.

The maximum ML analysis in PhyML resulted in a consensus tree with an lnL of -1668 and a composite log-likelihood of -55786. All nodes have high bootstrap support. The parsimony was estimated at 70, a result highly concordant with the one obtained in PAUP 4.0a.

Generally, the four different phylogenetic approaches produced highly concordant tree topologies, with the largest clades formed by *A. caspia* and *A. immaculata* being well supported and a smaller outlying group of three individuals denoted as *Alosa species.*

Discussion

During the last decades, mtDNA has enjoyed increasing interest as genetic markers, and SNPs in mitochondrial genes have become common markers for identifying individual differences in genome studies.

In this work, we performed a sequence analysis of the ND1 gene in different shad species. A phylogenetic tree was constructed to investigate the relationships among individuals from several Black

Sea locations. The results from the bioinformatics analysis showed three separate groups of *Alosa* species. Each group differs from the other by a specific set of SNPs and the morphological traits of most of them, but not all confirmed their taxonomic affiliation. In the present study, we identified specimens that were morphologically determined to belong to one taxon, but the molecular data for the ND1 gene didn't confirm this. In thirteen out of 48 specimens from the *Alosa* genus, we found discrepancies between the morphological and molecular characteristics. This observation is concordant with numerous other studies of the genus *Alosa* (Chiesa et al. 2016, Bani et al. 2019, Vernigora 2020). The first group consists of 24 individuals of the species *A. caspia* (Eichwald, 1838) (Acc. No PP391086- PP391107, PP421163, and PP421164), which harbours SNPs at three places (Table 2).

The second group consists of 21 individuals of the species *A. immaculata* (Bennett, 1835) (PP391108 - PP391127 and PP421162) with SNPs at three positions (Table 2) – one individual sample within this clade – *A. imm. Caspian No 45 (Acc. No PP391127)* from Cape Emine is particularly interesting because it fell into both *A. immaculata* and *A. caspia,* according to the obtained molecular data. Another intriguing sample – *A. imm.* No 44 (Acc. No PP391126) also differs from the *A. immaculata* clade. It has SNPs in several positions and still belongs to this clade. In addition, some single samples from the two major *Alosa* clades at some positions showed single nucleotide polymorphisms with a pattern specific to the members of the other clades. These findings could represent specific haplotypes not annotated in sequence databases so far and could be particularly interesting in future studies of the *Alosa* genus.

The third group, the Alosa species, forms a separate clade and consists of only three samples (Acc. No PP391128 – PP391130). These individuals were determined morphologically as *A. tanaica* (Grimm, 1901). However, since we could not find any annotated sequences of the ND1 gene for the species *A. tanaica* (Grimm, 1901) in the NCBI database, we named them *Alosa species*. We assume they might be the species *A. tanaica*, which could be found in other Black Sea countries but not in the areas of Byala and Nesebar. (Yankova et al. 2014). Another possibility is that these individuals represent a new mitochondrial haplotype or taxon from the Black Sea and may reflect another mt DNA lineage (Faria et al. 2006). However, there are insufficient data to confirm its species affiliation. Comparing their sequences with those annotated in the NCBI database showed 99% similarity to the species *A. fallax* and *A. immaculata*. All these data indicate that further investigation of the entire *Alosa* genus is required.

Conclusion

This article examines the diversity of the genus Alosa in Bulgarian Black Sea waters using the ND1 gene as a molecular marker. Our phylogenic analysis differentiated three major subclades within the genus Alosa: *Alosa immaculata* (Bennett, 1835), *Alosa caspia* (Eichwald, 1838), and *Alosa species*. Further efforts are required to clarify Alosa diversity in Bulgarian Black Sea waters. Whole genome sequencing will help with a more accurate phylogenetic review.

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