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Application of Variability in its Sequences for Studying Biodiversity of Black Sea Clupeid Fishes (Pisces: Clupeidae)

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Abstract: According to many authors, in Black sea Clupeidae family is represented by 9 species. However, this concept is not commonly accepted. For instance some molecular data demonstrated that the differences between *A. caspia* and *A. immaculata* are less than 0.8%, which could means that they were very recently split into two different species and still may have hybridization. The aim of this study was to assess applicability of ITS1 markers for distinguishing among Black sea Clupeidae species and to study their phylogenic relations. For this purpose ITS1 regions were isolated from four common representatives of the family in Bulgarian coastal areas. The representatives of *A. caspia* and *A. immaculata* formed one joint cluster but within it both species were clearly separated. The samples of *S. sprattus* and *S. pilchardus* formed clearly distinct clusters. Several SNPs were found in samples from genera *Sprattus* and *Alosa* suitable for population genetics investigations. No SNPs were found in samples of genus *Sardina*. The obtained results demonstrated suitability of ITS1 sequences for studying biodiversity and phylogenic relations of Clupeidae species in Black sea.

Key words: *Alosa*, Black sea, Clupeidae, ITS, Phylogeny, *Sprattus*.

Introduction

Clupeidae family consists of mostly marine fishes but some of them are freshwater and anadromous (Gaudant 1991, Whitehead 1985). Their bodies are usually elongated, round to laterally flatten. Most of them feed on small planktonic animals. Size of adults range from 2 to 75 cm but usually is up to 40 cm (Whitehead 1985, Drenski 1951, Karapetkova, Zhivkov 2010, Peshev, Boev 1962). This is one of the most important families of commercial fishes. The representatives of Clupeidae subjects of commercial fishing are herrings, sardines, shads, sprat, menhadens (Faria *et al.* 2006). According to Karapetkova, Zhivkov (2010) in Black sea Clupeidae family is represented by 9 species: *Alosa immaculata, Alosa caspia nodrmanni, Alosa caspia bulgarica, Alosa maeotica maeotica, Alosa fallax nilotica, Stpattus sprattus, Clupeonella cultriventis, Sardinella aurita,* and *Sardina pilchardus*. However, this concept is not accepted by all authors and often is subject of debate. For instance some molecular data demonstrated that the differences between *A. caspia* and *A. immaculata* are less than 0.8%, which could mean that: 1) they are not two different species or 2) they were very recently split into two species and hybridization between them still is possible according to Faria *et al.* (2006). Also in most of the recent

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publications and databases only *A. caspia* (Eich) is accepted as a species, while *A. caspia nodrmanni* and *A. caspia bulgarica* are indicated either as synonyms or as subspecies (http://species-identification.org/). On the other hand the presence of teeth in palatine and vomer in Black sea *Alosa* species could support the view that they could be different subgenus (Borbori *et al.* 2001). However, probably due to the isolation of Black sea the relationship between Clupeidae species could be much more complex because of a variety of forms that have been described (Mezhzherin, Fenorenko 2005).

The economic importance of Clupeidae species and the need to take adequate measures for the conservation and proper management of fishery resources of the family in Black sea region determine the necessity of more detailed study of biodiversity and phylogenetic relationships among species in this family. A relatively modern and widely used method is based on comparative study of ITS1 of sequences (Booton *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, Booton *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, ITS1 is often believed to evolve without functional constraints. The relatively fast sequence evolution of 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 rRNA gene cluster (Armbruster *et al.* 2000, Armbruster 2001, Armbruster, Korte 2006, BALDWIN 1992, Krieger 2008). The sequence comparison of ITS1 regions have been used to study the family Cichlidae both in Lake Victoria and Lake Edward (Booton *et al*.1999) and hybridization between *Nematalosa japonica* and *N. come* (Clupeiformes: Clupeidae) (Imai *et al.* 2009).

The aim of the study was to assess applicability of ITS1 markers for distinguishing among Black sea Clupeidae species.

Materials and Methods

Taxa studied

Twenty two samples representing four Clupeidae species were examined: *Alosa immaculata* (Benn), *Alosa caspia* (Eich, Ant), *Stpattus sprattus* (L), and *Sardina pilchardus* (Walb). The samples were fished-out from South and North Bulgarian coastal areas (Fig. 1). The species were determined by morphological features using initially the determination key of Drenski (1951) and Peshev, Boev (1962). Updated descriptions provided by Karapetkova, Jivkov (2010) and by newest morphological data available (http://www.FAO.org and http://species-identification.org/) were consequently used to confirm determination. The determination was also independently confirmed by Sen. Assist. Prof. Konstantin Michailov. The list of samples, locations, methods and dates of capturing are summarized in Table 1.

DNA preparation

DNA was extracted from fish muscle tissue using DNeasy Blood & Tissue kit (Qiagen, cat N 69504) 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.

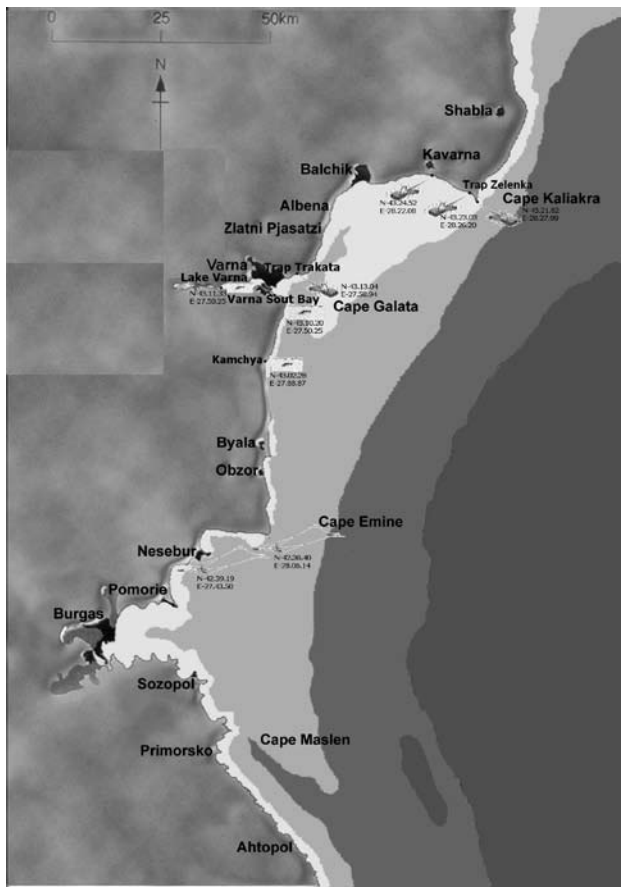

Fig. 1. Map of Bulgarian costal line representing the samples sites.

Table **1.** List of the studied samples – species name, location and method, and dates of capturing

Sample	Species	Locality	Data
1	*Sprattus sprattus* (L.)	Trap Kaliakra 43º21´82´´N, 28º27´99´´E	13.04.2011
2	*Sprattus sprattus* (L.)	Fish Ship Nesebar 42º38´40´´N, 28º06´14´´E	11.02.2011
3	*Sprattus sprattus* (L.)	Fish Ship Nesebar 42º39´19´´N, 27º43´50´´E	19.01.2011
4	*Sprattus sprattus* (L.)	Dragnet Lake Varna 43º11´33´´N, 27º50´25´´E	09.05.2011
5	*Sprattus sprattus* (L.)	Dragnet Lake Varna 43º11´33´´N, 27º50´25´´E	25.05.2011
6	*Sprattus sprattus* (L.)	Dragnet Lake Varna 43º11´33´´N, 27º50´25´´E	27.05.2011
7	*Sprattus sprattus* (L.)	Trap Trakata 43º13´04´´N, 27º58´94´´E	31.05.2011
8	*Alosa caspia* (Eich/Ant.)	Dragnet Lake Varna 43º11´33´´N, 27º50´25´´E	31.05.2011
9	*Sprattus sprattus* (L.)	Dragnet Varna South Bay 43º10´20´´N, 27º55´18´´E	01.04.2011
10	*Alosa caspia* (Eich/Ant.)	Dragnet Varna South Bay 43º10´20´´N, 27º55´18´´E	01.04.2011
11	*Sprattus sprattus* (L.)	Dragnet Varna South Bay 43º10´20´´N, 27º55´18´´E	01.04.2011
12	*Alosa immaculata* (Benn)	Trap Kaliakra 43°21´82´´N, 28º27´99´´E	09.06.2011
13	*Sardina pilchardus* (Walb)	Trap Kaliakra 43°21´82´´N, 28º27´99´´E	21.06.2011
14	*Sardina pilchardus* (Walb)	Trap Kaliakra 43°21´82´´N, 28º27´99´´E	14.06.2011
15	*Alosa immaculata* (Benn)	Trap Kaliakra 43°21´82´´N, 28º27´99´´E	21.06.2011
16	*Alosa immaculata* (Benn)	Trap Kaliakra 43°21´82´´N, 28º27´99´´E	22.06.2011
17	*Alosa immaculata* (Benn)	Dragnet Kamchya 43º02´28´´N, 27º88´87´´E	15.06.2011
18	*Sprattus sprattus* (L.)	Dragnet Varna South, Bay 43º10´20´´N, 27º55´18´´E	01.04.2011
19	*Sprattus sprattus* (L.)	Fish Ship Nesebar 42º10´12´´E, 27º51´31´´N	01.02.2011
20	*Alosa immaculata* (Benn)	Trawl Fishing Kavarna 43º24´52´´N, 28º22´08´´E	11.02.2011
21	*Alosa immaculata* (Benn)	Trawl Fishing Kamchya 43º02´28´´N, 27º88´87´´E	28.01.2011
22	*Alosa immaculata* (Benn)	Trawl Fishing Kamchya 43º02´28´´N, 27º88´87´´E	28.01.2011

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 μL PCR tube with 1 μL of each primer (10 mmol.L^{-1} concentration), 25 μL PCR master mix (Fermentas, Cat N 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 PCR amplification was carried-out by using the following program: initial DNA melting at 94 ^{0}C – 5 min; next 35 cycles of 94 ^{0}C – 45 s; 58 ^{0}C –45 s; 72 ^{0}C – 2 min 30 s and final extension at 72 ^{0}C for 10 min. PCR products were mixed with 7.5 mL of loading dye (Fermentas, Cat N 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 ladder (Fermentas GeneRuler Cat N SM0311). The PCR products were visualized by UV light.

PCR product isolation, cloning and sequencing

The PCR products were isolated from the agarose by QIAquick Gel extraction kit (Qiagen, Cat N 28704) according to the original protocol, and then used for U/A cloning by applying Qiagen PCR Cloning Kit (Cat N 231224). The ligation reactions were mixed with 250 μL freshly prepared competent bacterial (*E. coli*-TOP 10 – Invitrogen) cells. The plasmids containing PCR products were isolated using QIAprep Spin Miniprep Kit (Qiagen, Cat N 27104) and sent for sequencing to MWG – Biotech AG, Frankfurt, Germany.

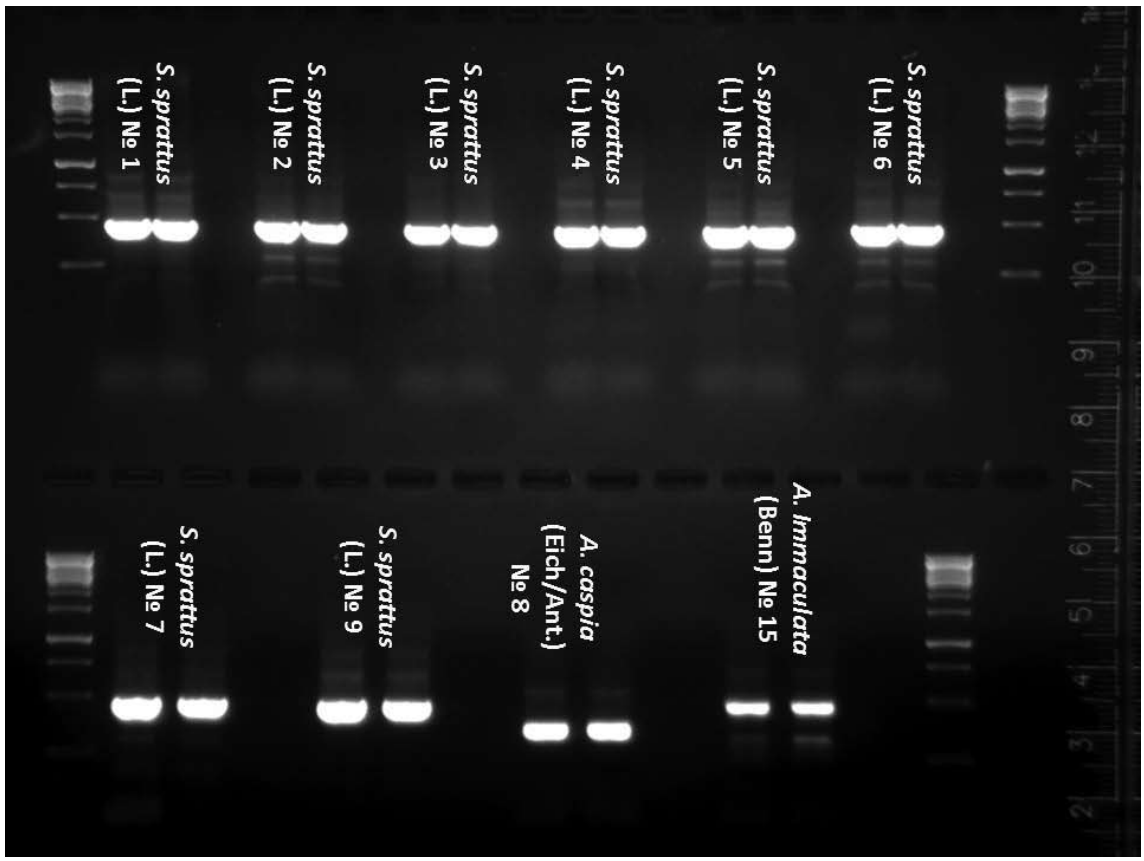

Fig. 2. ITS1 products amplified by primers ITS1 Fw and ITS1 Rev were separated by 1% agarose gel and visualized by UV light. The PCR products size was determined using 1 Kb Fermentas GeneRuler (Cat. N SM0311)

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). The obtained results were visualized by Tree Viewer (v. 1.6.6).

Results and Discussion

The suitability of 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 ^{o}C. The optimal amplification of the ITS1 region was achieved with 150 ng (2 μL) DNA template and annealing temperature 55 ^{o}C (Fig. 2). 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. The returned results demonstrated high similarity between them and the annotated ITS1-5,8S-ITS2 regions of *Clupea pallasii* (Acc. N *AB375624*; E value 3e^{-81}).

Several single nucleotide polymorphisms (SNPs) were identified in isolates sequences. The positions of SNPs in the samples of *S. sprattus* are presented on Fig. 3A. Substantial variability was found on relative position 98. Four samples (1, 7, 9, 11) captured in Varna bay possess there cytosine, while samples 2, 3, and 19 from the Southern Black sea cost possess on this position guanine. Interestingly the samples captured from Lake Varna (4, 5, 6) possess there thymine. The samples from Southern Black sea possess also SNP on position 136 – instead of guanine they have adenine. The third SNP is on position 139 and can not be connected with geographic location. It was found in the samples 2, 3, 5 and 9 and is transition G → A.

In samples of *A. immaculata* (Fig. 3b) only one SNP was found on relative position 15 – two samples: 12 and 21 possess there cytosine, just like samples of *A. caspia*. However the differences between the two species could be clearly seen in the area with relative positions 140-160.

No SNPs were found in samples of genus *Sardina*.

The degrees of differences between studied species 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, 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 (Fig. 4) was build by Tree Viewer (v. 1.6.6) software.

The results presented on the general phylogenic tree (Fig. 4) allowed us to distinguish very well different fishes by genus and species. It displayed high similarity (nearly 100%) between the studied samples of *A. immaculata,* which clustered together. Probably the captured samples belong to one population that migrate along the see coast during food foraging. The same is true for the samples of *S. pilchardus*. They both were captured in Varna bay, so probably they belong to one population.

The representatives of *A. caspia* and *A. immaculata* formed one joint cluster, but species were clearly separated within it. Both samples of *A. caspia* did not form a homogeneous group. However, more samples are needed to draw conclusion about the population structure of this species.

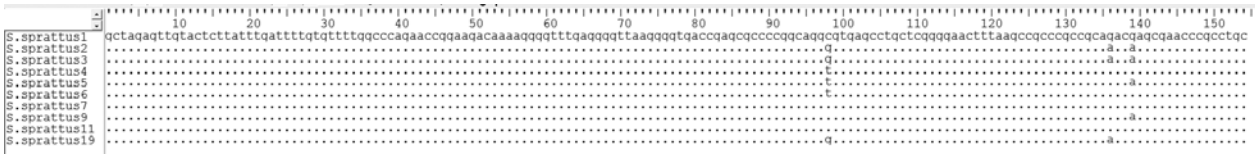

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Fig. 3. Single nucleotide polymorphism in the isolated ITS regions. Positions and types of SNPs in the samples of *Sprattus sprattus* are presented on Fig. 3a. The SNPs in genus Alosa are presented on Fig. 3b.

The samples of *S. sprattus* formed clearly distinct and well clustered group. Two sub-clusters could be observed. The first one comprises of the sprat samples collected from Southern coastal regions: N2, N3 and N19, while the second consist of samples from Northern regions including those caught in Lake Varna. Probably this division corresponds with the existence of at least two populations in our sprat resources.

Conclusions

The ITS1 region is one of the most popular sequences for phylogenetic analyses at the generic and infragenerec levels in animals. In this study we identified two SNPs that are suitable for distinguishing sprat from Southern and Northern Bulgarian Black sea coastal areas. More samples are needed to identify similar SNPs in genera *Alosa* and *Sardina*.

In general the obtained results demonstrated suitability of ITS1 sequences for studying biodiversity and phylogenic relations of Clupeidae species in Black sea. In our future investigations we are planning to use them together with some single-copy nuclear genes. The usage of other genes along with ITS sequences would provide a possibility to correlate the observed changes with certain phenotype traits.

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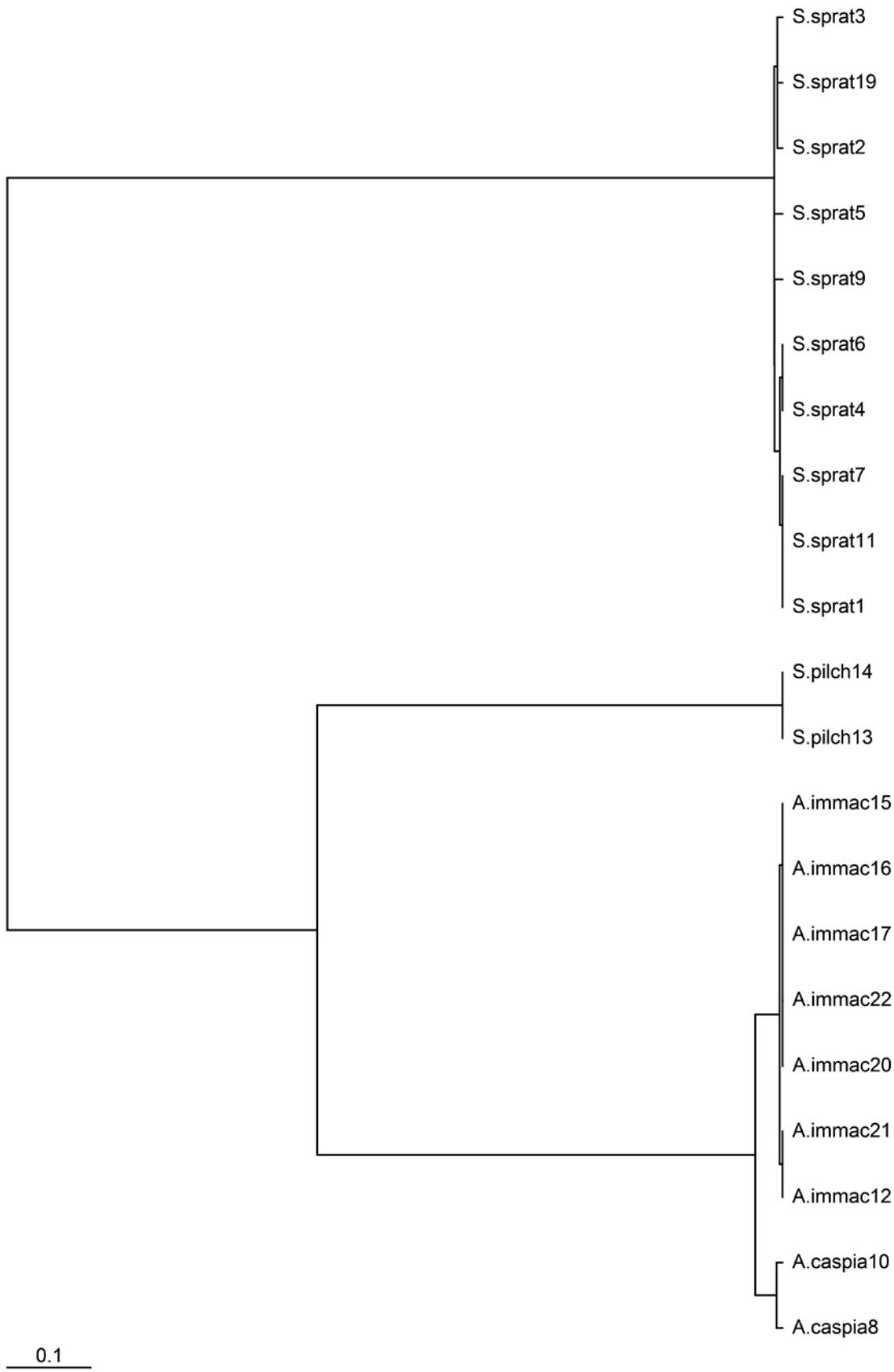

Fig. 4. General phylogenic tree based on data about differences in ITS1 sequences of the studied species. A. immac = *Alosa immaculata* (Benn); A. casp = *Alosa caspia* (Eich); S. sprat = *Sprattus sprattus* (L); S. pilch = *Sardina pilchardus* (Walb). More details are provided in Table 1. For simplicity some overlapping samples were excluded.

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