

ANALYSIS OF MITOCHONDRIAL DNA IN ADRIATIC SARDINE (*Sardina pilchardus*): A PRELIMINARY STUDY ¹

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ABSTRACT

To determine the phylogenetic history of sardines researchers use mitochondrial DNA (mtDNA). Mitochondrial DNA contains highly informative polymorphic sites. The cytochrome b (cyt b) gene has been especially used by investigators because of its sufficient point mutation rate, enabling discrimination of closely related fish species, and determination of interspecific variability in pelagic fish species for population identification. Scientific surveys based on meristic, morphometric, and reproductive data suggested that two subpopulations of sardines coexisted in the Northern-Middle Adriatic Sea. Mitochondrial DNA analysis of sardines from the Adriatic Sea has never been conducted in the Republic of Croatia. This study included 14 sardine samples from three separate locations, as well as 30 different haplotype sequences retrieved from GenBank. The aim of this study was to determine the number of haplotypes in sardine populations from the Adriatic Sea, as well as to determine differences between populations. Mitochondrial DNA sequence analysis revealed 11 different haplotypes of sardines from the Adriatic Sea. The differences between samples from different locations were not scientifically significant, and the results of this study indicate homogeneity in the Adriatic sardine stock. However, three parsimony informative mutations found in our samples produce a clearly visible distinction between the Adriatic sardine samples and samples obtained from the seas adjacent to the Adriatic. This distinction of the Adriatic sardine samples can have applications in fisheries and fishery product quality testing.

Key words: fish / Adriatic sardine / *Sardina pilchardus* / molecular genetics / mitochondrial DNA / phylogeny cytochrome b gene / haplotypes

1 INTRODUCTION

Sardine (*Sardina pilchardus*, Walb., 1972) fishing on the Adriatic Sea coast began more than a thousand years ago. Since 1970's scientific research based on meristic, morphometric and reproductive data has been conducted in order to determine the population structure of the Adriatic sardine. Alegria-Hernandez *et al.* (1986) concluded that there are two subpopulations of sardines in the Northern and Middle Adriatic Sea that are distinct by the number of gill rakers, length structure, head length and vertebral number. Except for the work done by Tinti *et al.* (2002), mitochondrial DNA (mtDNA) of

the Adriatic sardine has not been effectively studied in the Republic of Croatia, nor has it been compared to that of sardines from the surrounding seas. In the last 30 years, mtDNA was often used to estimate evolutionary and demographic history of populations and closely related species (Tinti *et al.*, 2002). As mtDNA is normally inherited exclusively from the mother, it is very useful because it represents a direct maternal lineage which can be traced far back in time. This, combined with its rapid mutation rate, makes it useful for studying intra- and interspecific phylogenetic relationships (Zhang and Hewitt, 1996; Snoj, 1997).

The purpose of this study was to compare a

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359-bp fragment of the cytochrome b (*cytb*) gene of sardines from three different locations on the Adriatic Sea coast (Northern, Middle and Southern), along with samples from the Ionian Sea, Aegean Sea, Black Sea, Sea of Marmara, Mediterranean Sea, the European coast of the Atlantic Ocean and other closely related fish species (*Sardinops melanostictus*, *Sardinops sagax*, *Sardinops caeruleus*, *Sardinella maderensis*, *Sardinella aurita*, *Engraulis encrasicolus*, *Engraulis japonicus*, *Clupea harengus*, *Sprattus sprattus*). We aim to determine intra- and interspecific phylogenetic relationships along with the number of haplotypes present in the Adriatic Sea sardine population.

2 MATERIAL AND METHODS

Sardine samples were obtained directly from fishing vessels upon their return to the harbor in Mali Lošinj, Zadar and Dubrovnik (Fig. 1). This study was performed using a total of 14 samples of lateral muscle tissue of Adriatic sardines which were stored in 70% ethanol and kept on ice during transportation to the laboratory where samples were stored at -20°C .

Total genomic DNA was extracted from 25 mg of

lateral muscle tissue using the Sigma-Aldrich Gen Elute™ Mammalian Genomic DNA Miniprep Kit. A 359-bp fragment of the *cytb* gene was amplified using universal primers L14841 and H15149 published by Kocher *et al.* (1989). Approximately 50 ng of template DNA was amplified in 25 μL reaction mixture containing GoTaq Green Mastermix and 0.2 μM of each primer. The thermal cycle profile consisted of an initial denaturation step (94°C , 2 min) followed by 35 cycles of denaturation (94°C , 30 s), annealing (48°C , 30 s), and extension (72°C , 60 s), and a final extension holding step (72°C , 10 min).

Amplified DNA was purified through electrophoresis on 1% agarose gels and eluted using the QIAquick Gel Extraction Kit. DNA was sequenced on an ABI PRISM 3100 – Avant Genetic Analyzer with the same primers used for amplification.

The 313-bp *cytb* sequences were aligned using the MEGA 4.0 program (Tamura *et al.*, 2008). In MEGA we created the neighbor-joining phylogenetic tree, using the bootstrap method (500) phylogeny test and Kimura 2-parameter substitution model. We also created files ready for importing into DNAsp 5.10.00 (Rozas *et al.*, 2009), which was used for mtDNA haplotype analysis. The median-joining network was drawn last using Network 4.516 (Forster *et al.*, 2009).



Figure 1: Sampling locations (1 – Mali Lošinj, 2 – Zadar, 3 – Dubrovnik)

Table 1: Haplotypes (mtDNA *cytb* sequences) of Adriatic sardine samples compared to a reference sardine mtDNA sample (Lavoue *et al.*, 2007; AP009233.1)

Haplotype	14755	14795	14800	14825	14848	14875	14876	14883	14896	14947	15004	15019	15052	15061	15085
AP009233.1	T	T	C	A	C	A	C	G	A	G	T	A	C	G	C
H_1	.	.	.	C	.	.	T	A	A	A
H_2	.	.	T	A	A	A
H_3	C	.	A	A	A
H_4	G	G	A	A	A
H_5	G	.	.	.	A	A	A
H_6	.	.	.	C	A	.	.	A	A	A
H_7	A	A	A
H_8	C	.	A	.	.	A	A	A
H_9	T	A	A	A
H_10	A	.	.	A	A	A
H_11	C	C	.	.	.	C	T	A	A	A

3 RESULTS AND DISCUSSION

MtDNA analysis of the 313-bp fragment of the *cytb* gene revealed 12 polymorphisms and 11 different haplotypes (Table 1) which combined with low bootstrap values (Fig. 2) points to a homogeneous population.

Of the 12 polymorphisms present in the Adriatic sardine samples, 8 are singleton, while 3 are parsimony mutations. Since we used the same primers and some of our sampling locations overlap (Mali Lošinj, Fig. 1), the results of this study are comparable to those of Tinti *et al.* (2002). Clearly visible differences can be seen between Adriatic sardines, sardines from neighbouring seas and related species (Fig. 2)

The median-joining network (Fig. 3) shows that the most common haplotype (H_7) is shared between samples from this investigation and samples from Tinti *et al.* (2002). All other haplotypes are derived through 1 to 2 nucleotide substitutions from haplotype H7 (Fig. 3).

However, even though the same primers were used in both studies, our usable 313-bp fragment is longer than the usable 307-bp fragment from Tinti *et al.* (2002) and our sequences are not entirely overlapping, which effectively means that because of this dislocation our sequences differed in approximately 20–30 sites. Our samples contain 3 polymorphic sites (Table 1 – 15052, 15061, 15085) which when compared to the reference sample and sardine samples from the surrounding seas produce a clearly visible distinction between the Adriatic sardine samples and samples from neighboring seas (Fig. 2).

This finding holds potential significance because it shows it is possible to distinguish Adriatic sardines from

those existing in the surrounding seas, which can, with further investigation, have applications for fisheries and fishery product quality testing.

The results of this investigation go towards confirming the results and conclusions made by Tinti *et al.* (2002), meaning specifically that the sardine population in the Adriatic Sea is homogeneous. This conclusion rejects the hypothesis based on morphometric and meristic methods presented by Alegria-Hernandez *et al.* (1986) that the Jabuka trench serves as a barrier which separates two sardine subpopulations, one with predominantly larger and another with predominantly smaller individuals. These and other differences found by her team can be attributed and are most likely caused by the differing ecological conditions in which these fishes thrive.

The possible reason for the sardine population homogeneity could be the relatively small size of the Adriatic sea (138.595 km²), which greatly diminishes the possibility of finding significant genetic differences between sardines from different localities, especially using a mitochondrial marker. Since the Adriatic Sea is quite shallow in the north (<50 m), this area was dry during the last ice age and was recolonised app. 8000 years ago. This period of time would not be sufficient to produce significant genetic differences within a population residing within this confined area, even if there is a geographic formation which serves as a genetic material barrier. Kochzius and Blohm (2005) performed a similar study in the Red Sea Aqaba Bay and have also did not find a significantly different population structure.

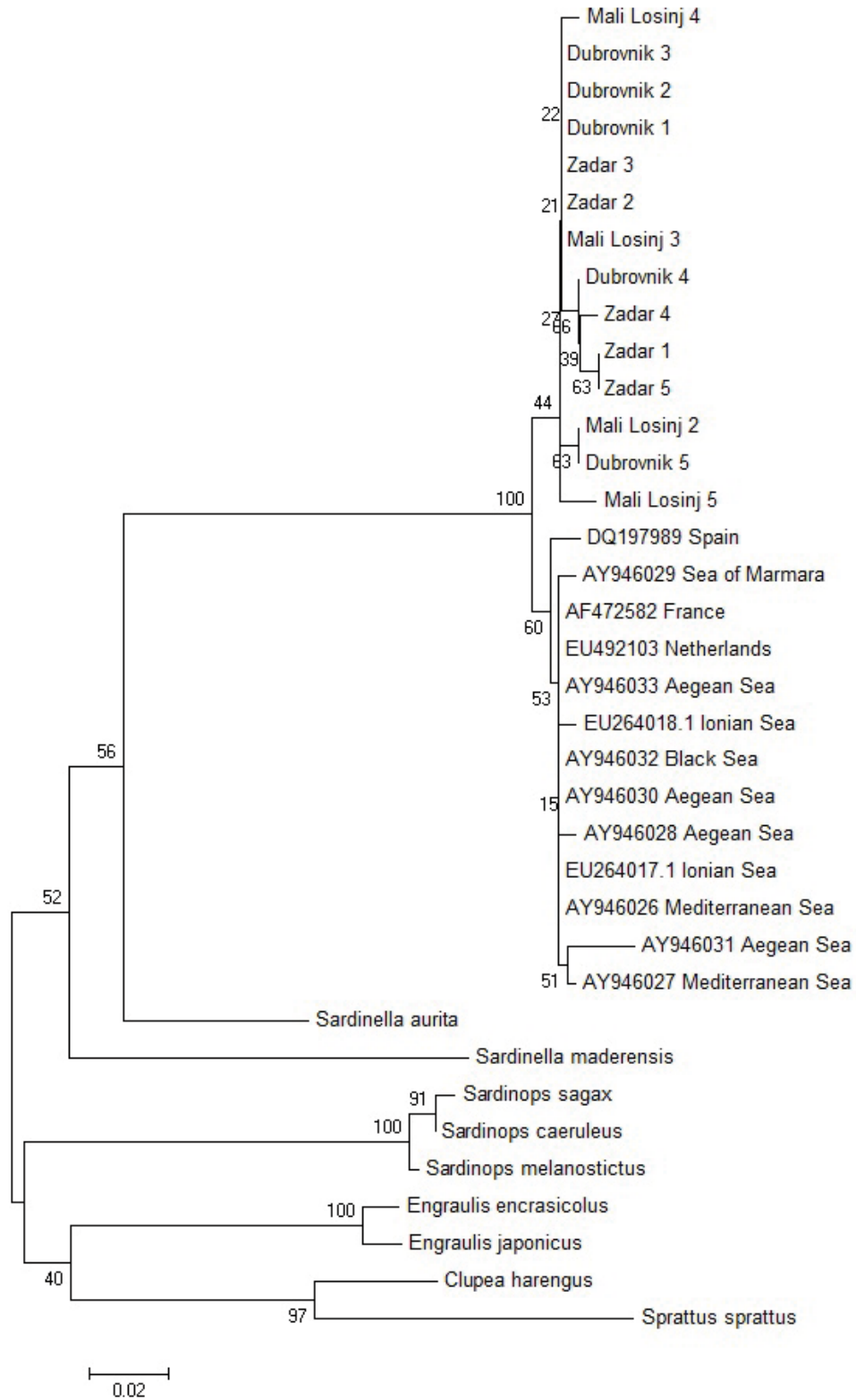


Figure 2: Neighbour-joining dendrogram of 14 Adriatic sardine samples along with samples from Ionian, Aegean, Black Sea, Sea of Marmara and Mediterranean Sea, the coasts of Netherlands, France and Spain and samples from related species (*Sardinella aurita*, *Sardinops sagax*, *Sardinops caeruleus*, *Sardinops melanostictus*, *Engraulis encrasicolus*, *Engraulis japonicus*, *Clupea harengus*, *Sprattus sprattus*)

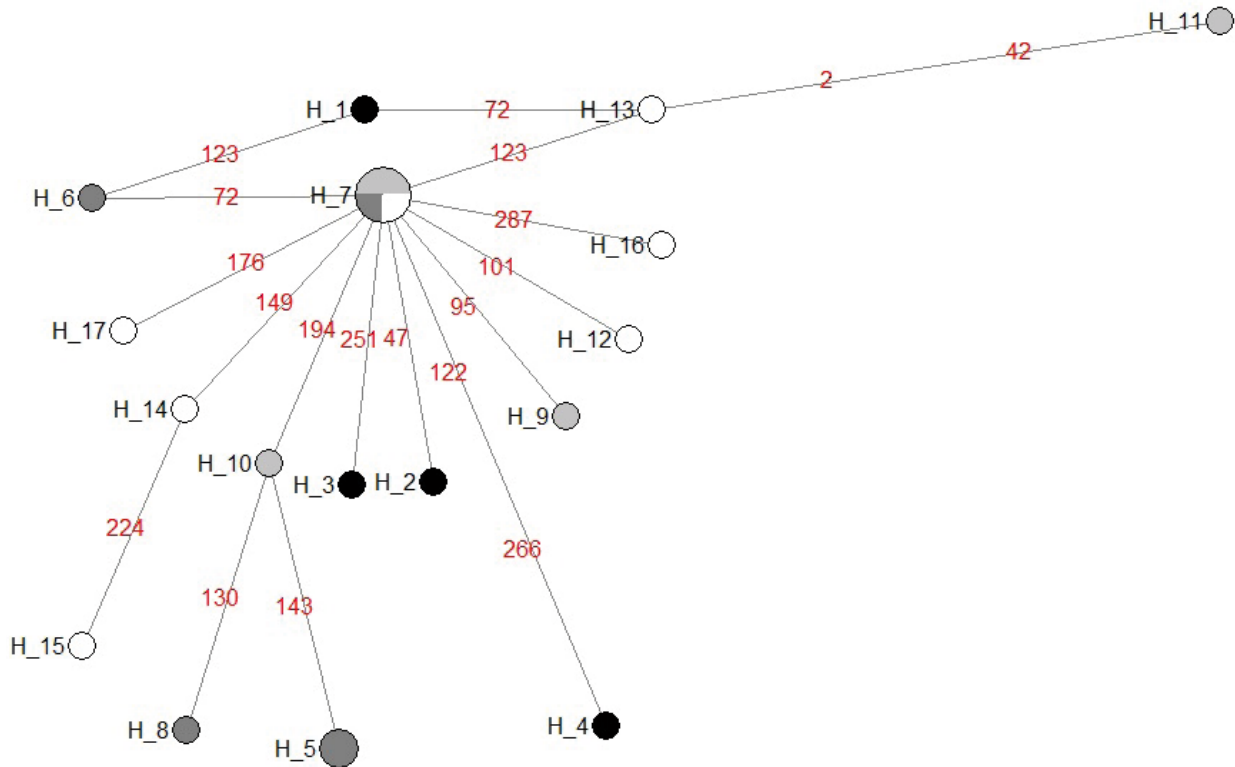


Figure 3: Median-joining network of Adriatic sardine samples from this study and from Tinti *et al.* (2002)
(Legend: ■ Mali Lošinj, ■ Zadar, ■ Dubrovnik, □ Tinti haplotypes)

4 CONCLUSIONS

The analysis of the 14 sequences obtained, along with results and analysis from the comparable study Tinti *et al.* (2002), point to a homogeneous population of sardines within the Adriatic Sea. The investigated population contains 11 haplotypes, the most common haplotype being haplotype 7. Our samples contain 3 polymorphic sites which when compared to sardine samples from the surrounding seas produce a clearly visible distinction between the Adriatic sardine samples and samples from neighboring seas. This holds potential significance because it shows it is possible to use a mitochondrial marker to distinguish Adriatic sardines from those existing in the surrounding seas, which can, with further investigation, have applications for fisheries and fishery product quality testing. However, we would like to point out that this was a pilot study made without any considerable financial support (Master thesis), so the scope of this investigation, including the number of locations and the sample size was limited. In the future, this study should be revisited and include a greater sample size which would result in a more robust study from which more significant conclusions could be drawn.

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