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# Variability of mitochondrial DNA control region in the Adriatic populations of European sardine *Sardina pilchardus* (Walbaum 1792)

# Varijabilnost kontrolne regije mitohondrijske DNA u jadranskim populacijama srdele *Sardina pilchardus* (Walbaum 1792)

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## ABSTRACT

This study found exceptionally high haplotype diversity within the sampled European sardine populations in the Croatian Adriatic Sea using mtDNA analysis with each individual possessing a unique haplotype. This suggests a high degree of genetic variation within the Croatian sardine populations. Despite high haplotype diversity, the analysis of molecular variance (AMOVA) revealed low genetic differentiation between the two sampled locations (Ližnjan and Dugi Otok). The majority of genetic variation (92.82%) was found within populations, rather than between them ($F_{st}$ = 0.072). The median-joining network also did not show a clear separation between the two locations' haplotypes. The low genetic differentiation and lack of clear separation in the haplotype network suggest high gene flow between the Ližnjan and Dugi Otok populations. This might be attributed to the proximity of the two sampling sites and the high dispersal potential of sardine larvae. While overall genetic differentiation was low, the presence of unique haplotypes in each individual and slight variations in haplotype frequency between locations hints at the possibility of local adaptation or micro-evolutionary processes influencing the sardine populations. Further investigation with larger sample sizes across a wider geographic area is needed to explore this possibility.

**Keywords:** Clupeidae, molecular markers, haplotype diversity, genetic variation

## SAŽETAK

Ova studija, koristeći mtDNA analizu, utvrdila je iznimno visoku haplotipsku raznolikost unutar ispitanih populacija srdele u Jadranskom moru u Hrvatskoj, pri čemu svaka jedinka ima jedinstveni haplotip. To ukazuje na visoki stupanj genetske varijabilnosti unutar hrvatskih populacija srdele. Unatoč visokoj haplotipskoj raznolikosti, analiza molekularne varijance (AMOVA) pokazala je malu genetsku diferencijaciju između dvije lokacije (Ližnjan i Dugi otok). Većina genetske varijabilnosti (92,82%) pronađena je unutar populacija, a ne između njih ($F_{st}$ = 0,072). Median-joining network također nije pokazala jasnu separaciju između haplotipova s dva lokaliteta. Mala genetska diferencijacija i nedostatak jasne separacije u haplotipskoj mreži sugeriraju visoku izmjenu gena između populacija Ližnjana i Dugog otoka. To se može pripisati blizini dvaju mjesta uzorkovanja i visokom potencijalu rasprostiranja ličinki srdele. Iako je ukupna genetska diferencijacija bila mala, prisutnost jedinstvenih haplotipova u svakoj jedinki i blage varijacije u frekvenciji haplotipova između lokaliteta ukazuju na mogućnost lokalne adaptacije ili mikroevolucijskih procesa koji utječu na populacije srdele. Za daljnje istraživanje ove mogućnosti potrebna su daljnja istraživanja s većim uzorcima na širem geografskom području.

**Ključne riječi:** Clupeidae, molekularni markeri, raznolikost haplotipova, genetska varijacija

## INTRODUCTION

The European sardine (*Sardina pilchardus* (Walbaum 1792)) is a small pelagic fish whose main distribution area is in the Mediterranean Sea and the eastern Atlantic Ocean from Iceland and the North Sea to the coast of Senegal, with some marginal populations in the area around Madeira, the Azores and the Canary Islands (Whitehead et al., 1988). The sardine belongs to the Clupeidae family and is the most abundant fish species in the Adriatic Sea (Sinovčić, 2000; Cingolani et al., 2003; Mustać and Sinovčić, 2012). In Croatia, it is widely distributed in the coastal areas and in the channels, less so in the open sea, but most abundant in the central and northern parts. While adults mostly reside in coastal waters and perform distinct vertical movements on a daily basis, juveniles are an integral part of the plankton in the open water (Santos et al., 2006). One of the main characteristics of this species is the annual migration related to the location of spawning and feeding. Thus, sardines migrate to spawning areas in the fall, where they remain until early June, and then return to coastal areas in search of food, where they remain until the end of the fall (Stratoudakis et al., 2007). The sardine has been intensively researched concerning its ecology and biology (Gamulin, 1975; Sinovčić et al., 2004; Mustać and Sinovčić, 2007; Dulčić and Glamuzina, 2006). Fishing is one of the most important activities in the Croatian coastal areas and islands. Sardine fishing in the Croatian part of the Adriatic began in the 10th century in the area of Dugi otok (Mustać and Sinovčić, 2007). Pelagic fishing officially dates back to 1532 and was the economically most important form of fishing in the Croatian area around Zadar (Basioli, 1962, 1974, 1984; Mustać and Sinovčić, 2010). Fishing is still an important source of income and employment at the local level, thus directly contributing to stopping the depopulation of Croatian islands. The sardine fishery takes place in spring and fall-winter. According to legal regulations, sardines are caught from January to March and from October to December (NN 126/2021). The sardine is the most economically important fish species in the Adriatic Sea. A prerequisite for fisheries management is the assessment of the fish stock. The share of sardines in the total catch is high, as expected since sardines have always been the main fishing species in the coastal and the island areas of Croatia. The Common Fisheries Policy statistics published in 2022 (European Commission, 2022) contain the data for 2021, which are further broken down and show that in Croatia, European sardines accounted for 69.8% of the total catch and European anchovies accounted for 12.4 %.

During and after the recorded decline in landings in 2005 for sardines, the authorities responded with a management plan and regulations regarding the sardine stock with proposed measures that would help the recovery of the stocks. It also remains to be seen how long the sardine stock can maintain its harvest quota as feed fish for tuna farming and fattening for export from Croatian waters (Carpi et al., 2017).

Considering the important role that sardine plays in the Croatian economy, the problem of managing the sardine stocks and catch while maintaining the natural balance in the Adriatic Sea is a recurring one. Identification of fish stocks is the first step in the management and conservation of biodiversity (Waldman, 2005). The development of marine fisheries depends on planning the fishing intensity that allows for balanced and sustainable fishing in the long term, which requires a responsible and rational approach to the management of marine bioresources. Mitochondrial DNA (mtDNA) sequence analysis has become a powerful tool for fish management. It is frequently used in phylogenetic and phylogeographic studies together with nuclear markers, despite differences in factors such as adaptive introgression, demographic disparities, sex-biased asymmetries, and different mutation rates. The mtDNA is an extremely valuable genetic marker in population and evolutionary biology (Rand, 2001). It is relatively easy to isolate pure homologous sequences because they are present in large numbers of copies, up to 1,000, per cell. The simple organization, maternal inheritance, and lack of recombination with a high level of mutation make mtDNA an ideal marker for studying the structure of populations (Pereira, 2000) as well as for studying evolution, including

migrations, introductions, and population bottlenecks (Harrison, 1989). When observing the structure of a population, mtDNA is very useful, whether it is differences between species (interspecific divergence) or within a single species (intraspecific divergence). As a molecular marker, mtDNA allows discrimination of population structure and monitoring of intraspecific variation in individual, geographically separated populations (Neigel and Avise, 1993). Finally, complete mtDNA genome sequences are available in public databases; primers can therefore be designed for amplification and sequencing of any species that has a published mtDNA genome (Folmer et al., 1994).

The uniqueness of individuals within a population is a consequence of them having different nucleotide sequences represented by different haplotypes. Therefore, this study aimed to evaluate the potential of genetic differentiation of sardines off the Croatian coast by analyzing the mitochondrial control region. Comparison of the obtained results with the previously submitted databases will allow a more comprehensive characterization of the genetic structure of Croatian sardine populations. It is expected that this research will contribute to the knowledge of sardine populations in order to maintain important links between the economic and biological indicators of the Adriatic Sea.

## MATERIALS AND METHODS

A total of 54 individuals were collected by commercial fishermen from wide areas of two locations: off Ližnjan (44.52342° N 14.11146° E) and off Dugi Otok (44.05204° N 14.42058° E; Figure 1) from October 2020 to June 2022.


**Figure 1.** Map of European sardine sampling sites in the Adriatic Sea, marked as black stars symbol

Samples were immediately extracted and preserved in absolute ethanol. Total DNA was isolated from muscle tissue transferred into the laboratory and stored at -20 °C. DNA extraction and sequencing were performed by Macrogen (Macrogen Inc., The Netherlands).

Mitochondrial DNA (mtDNA) variation analysis was performed on a partial sequence of the D-loop region. The analysis utilized the entire dataset, consisting of 54 individuals (6 from Ližnjan and 48 from Dugi Otok), to assess mitochondrial control region (D-loop) diversity and construct a haplotype network.

A fragment of the mitochondrial control region was PCR amplified using the LN20 and HN20 primers. Standard PCR amplification was conducted by the Macrogen sequencing service (Macrogen Inc., The Netherlands). The mitochondrial sequences were manually checked and assembled using FinchTV ver. 1.5.0 (Geospiza Inc.) and aligned with the ClustalW algorithm (Thompson et al., 1997) implemented in MEGA X ver. 11.0.13 (Tamura et al., 2021). The final alignment of 1003 base pairs (bp) was used to generate haplotypes in DnaSP ver. 6.0 (Librado and Rozas, 2009).

Evolutionary relationships between haplotypes were analyzed by a Median-Joining network (Bandelt et al. 1999) constructed with NETWORK ver. 5.0. (Fluxus Technology Ltd.). The weights of the characters' values were set to 10, while the parameter epsilon, which specifies a weighted genetic distance to the known sequences in the dataset, was set to 0 to obtain a sparse spanning network. To determine the amount of genetic variability partitioned within and among populations, an analysis of molecular variance (AMOVA) was performed in Arlequin 3.5.2.2. Population differentiation analyses were conducted with Arlequin 3.5.2.2. (Excoffier and Lischer, 2010) and P-values were estimated. Differences were considered statistically significant at $P < 0.05$. To determine the amount of genetic variability partitioned within and among populations, Analysis of Molecular Variance (AMOVA) was performed in Arlequin 3.5.2.2. and the significances of both the F statistics and variance components were assessed with 1000 permutations.

## RESULTS

The sequenced size of the PCR products was 1003 bp at the 5′ end of the D-loop region for the 54 individuals; namely, 6 Ližnjan and 48 Dugi Otok. Fifty-four different haplotypes were discovered for the D-loop region in two *S. pilchardus* populations, so each individual had its own haplotype. All new haplotypes generated from both data sets were submitted to GenBank under the accession number OR676769-OR676722). The total haplotype and nucleotide diversity were 1.000 ± 0.004 and 0.017 ± 0.0007, respectively (Table 1). A higher value of haplotype diversity was detected in the Ližnjan population (Hd = 1.000; π = 0.019). For the complete dataset of 54 individuals, 148 variable polymorphic sites were identified, with a total of 161 mutations.

Relationships between individuals which included 54 sequences of European sardine with a total length of 1003 bp from Croatia were displayed by median-joining network (Figure 2). The forty-eight D-loop haplotypes from Dugi Otok were compared with six D-loop haplotypes from Ližnjan, with the ClustalW algorithm. In the median-joining network, all 54 individuals have their own unique haplotypes. The median-joining network did not reveal separation between Ližnjan and Dugi Otok haplotypes.

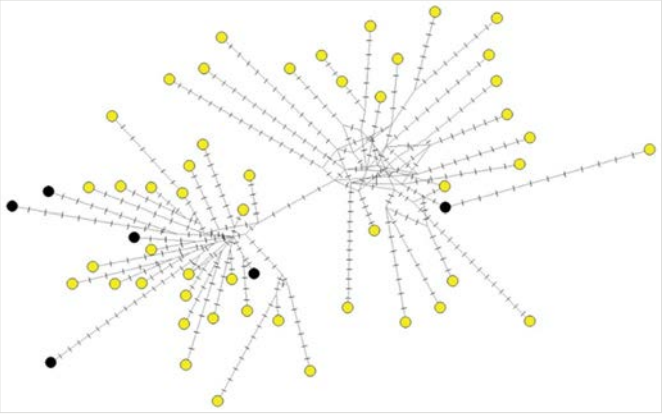

**Figure 2.** Median-joining (MJ) network of the partial D-loop region of European sardine from Croatia (the black circles represent the haplotypes from Ližnjan (northern Adriatic Sea), while the yellow circles show the haplotypes from Dugi Otok (mid-Adriatic Sea). The lines represent mutation positions).

**Table 1.** Diversity of partial D-loop of European sardine populations (Croatia)

| Population | Location | Date | N | h | *S* | Hd ± SD | π ± SD |
|---|---|---|---|---|---|---|---|
| Ližnjan | 44°52'342"N 14°11'146"E | October 2020 | 6 | 6 | 49 | 1.000 ± 0.096 | 0.019 ± 0.003 |
| Dugi Otok | 44°05'204"N 14°42'058"E | June 2022 | 48 | 48 | 130 | 1.000 ± 0.004 | 0.017 ± 0.001 |
| Total | | | 54 | 54 | 148 | 1.000 ± 0.004 | 0.017 ± 0.0007 |

N - number of sequences used for analysis, h - number of haplotypes, *S* - number of segregating sites, Hd - haplotype diversity, π - nucleotide diversity, SD - standard deviation

**Table 2.** Measures of population differentiation for European sardine based on the partial D-loop region using molecular analysis of variance approach

| Source of variation | df[1] | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|---|
| Among populations | 1 | 15.481 | 0.656 $V_a$ | 7.180 |
| Within populations | 52 | 441.167 | 8.484 $V_b$ | 92.820 |
| Total | 53 | 456.648 | 9.140 | |
| Fixation index | $F_{st}$ = 0.072 | | | |

[1] $P < 0.05$, $F_{st}$: $P < 0.05$

Percent variation among populations ($V_a$) and within groups ($V_b$) is indicated The significance of differentiation among and within populations was tested by 1000 permutations.

For population differentiation estimates, the D-loop nucleotide sequence for each individual was used. The AMOVA analysis showed that most of the genetic variation was present within samples (92.82%) and 7.18% of genetic heterogeneity was apportioned among them (Table 2). The total $F_{st}$ value was $F_{st}$ = 0.072.

## DISCUSSION

The interbreeding units that have the ability to reproduce independently represent separate populations. To determine the genetic structure of a population, it is important to recognize the degree of connectivity between samples (Avise, 2000). Due to the planktonic way of life, many marine species have a high dispersal ability in the early life stages, resulting in reduced intraspecific differentiation and smaller genetic structure (Cowen and Sponaugle, 2009).

Terrestrial and freshwater populations are easily defined due to the presence of barriers that prevent crossing. Marine populations have no obvious barriers, but they can be defined as a group of fish that are exploited for fisheries in a particular area (Hellberg, 2009). When planning the management of a particular fish species, it is important to define the genetic stock that represents intraspecific groups of randomly mating samples. These samples respond independently to fisheries exploitation for reproductive isolation (Caballero-Huertas et al., 2022).

Populations in the marine environment may consist of localized subpopulations that are relatively independent and have different ecological and genetic characteristics (Dyrynda et al., 1997). However, there are mechanisms, such as geographic distance, that influence the differentiation of populations, i.e., the increase in genetic variation among marine populations (Riginos et

al., 2011). According to research on the morphological characteristics of the head and the number of gill rakers, two subspecies *S. p. pilchardus* and *S. p. sardine* were established in sardine. This was also confirmed by genetic analyses based on significant pairwise haplotype frequency differences (Atarhouch et al., 2006). Based on differences in morphometric and meristic characters, and supported by local migration patterns of sardine populations, two subpopulations were thought to be present in the Adriatic Sea, inhabiting Adriatic areas north and south of the Jabuka Pit (Alegrìa-Hernàndez et al., 1985). However, Tinti et al. (2002) suggests that they belong to a large self-recruiting population. The genetic structure of sardines was previously investigated with different molecular markers, alloenzymes (Chlaida et al., 2006, 2009; Laurent et al., 2007), microsatellites (Gonzalez and Zardoya, 2007; Baibai et al., 2012; Kasapidis et al., 2012; Ruggeri et al., 2012) and mtDNA polymorphism (Carvalho et al., 1994; Tinti et al., 2002; Sarmaşik et al., 2008; Imsiridou et al., 2019). In the majority of these studies, weak genetic differentiation was determined, which was explained by the dispersion and high level of migration of the adult stages of this pelagic species over a large geographical area (Laurent et al., 2007). Most of the genetic variation was found within populations, and the total $F_{st}$ value ($F_{st}$ = 0.07177) has a higher value compared to previous findings (Imsiridou et al., 2019).

In this study samples from Croatia were also mutually compared as: 6 Ližnjan (north Adriatic) D-loop haplotypes with 48 Dugi Otok (mid-Adriatic) D-loop haplotypes. All haplotypes were unique. We explain the divergence between these populations in an otherwise highly distributed species by natural selection acting on genetic variation. Namely, when the dispersal of larvae is high, there is practically no capacity for population divergence by random processes, so significant genetic divergence between populations must be induced by selection (Nielsen et al, 2009). 44 sampled haplotypes do not show a significant separation from Ližnjan to Dugi Otok. It can be explained by the proximity of the two locations and because the populations were in contact. It was established that three haplotypes from Croatia showed a significantly higher number of mutations (Figure 2). However, the marked difference between the haplotypic structures of the samples may indicate a deeper genetic divergence between the Croatian sardine populations. This may be explained by reduced gene flow between samples and fragmentation of sardine populations at this larger geographic scale, as previously suggested by Ramon and Castro (1997). The lack of profound genetic differences in the sardine population has already been noted (Carvalho et al., 1994) and suggests that there are no strong environmental or hydrographic factors acting as substructuring forces in the Croatian sardine population.

## CONCLUSION

The findings highlight the need for careful management strategies that account for the high gene flow and potential for local adaptation. While the populations appear genetically homogenous at a broad scale within the Croatian Adriatic, management plans should consider potential localized variations to ensure sustainability. Further research to assess genetic diversity across a larger geographic scale is necessary to ensure robust management practices.

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