Article

Surveying Shared Marine Resources at a Regional Scale: Connectivity and Differentiation of Round Sardinella in Eastern Mediterranean

Alice Ferrari [1,†], Giusy Catalano [1,†], Valentina Crobe [1,*], Alessia Cariani [1], Emre Keskin [2,3,4,5], Pierluigi Carbonara [6], Loredana Casciaro [6], Reda M. Fahim [7], Sharif Jemaa [8], Savaş Kılıç [4,9], Myriam Lteif [8], Abdalnasser S. H. Madi [10], Hatem H. Mahmoud [7], Süleyman Öztürk [4,9], Yaser O. Shtaya [11] and Stefano Lelli [10]

1 Department of Biological, Geological & Environmental Sciences (BiGeA), University of Bologna, 40126 Bologna, Italy; alice.ferrari6@unibo.it (A.F.); giusy.catalano2@unibo.it (G.C.); alessia.cariani@unibo.it (A.C.)
2 Evolutionary Genetics Laboratory (eGL), Department of Fisheries and Aquaculture, Agricultural Faculty, Ankara University, Ankara 06135, Türkiye; keskin@ankara.edu.tr
3 AgriGenomics Hub (AgriGx), Animal and Plant Genomics Research Innovation Centre, Ankara 06135, Türkiye
4 Biodiversity Research Association (BAD), Ankara 06135, Türkiye; kilicsavas@tarimorman.gov.tr (S.K.); suleyman.ozturk@tarimorman.gov.tr (S.Ö.)
5 Aquaculture Research and Application Center, Ankara University (ASAUM), Ankara 06135, Türkiye
6 COISPA—Tecnologia & Ricerca, Via dei Trulli 18/20, 70126 Bari, Italy; carbonara@fondazionecoispa.org (P.C.); casciaro@coispa.eu (L.C.)
7 College of Fisheries and Aquaculture Technology, Arab Academy for Science, Technology and Maritime Transport, Alexandria 1029, Egypt; reda.fahim@fao.org (R.M.F.); hatemhanafy@hotmail.com (H.H.M.)
8 National Centre for Marine Sciences (NCMS), National Council for Scientific Research (CNRS-L), Jounieh P.O. Box 189, Lebanon; sharif.jemaa@cnrs.edu.lb (S.J.); myriamlteif@hotmail.com (M.L.)
9 Mediterranean Fisheries Research Production & Training Institute, Kepez Unit, Antalya 07192, Türkiye
10 General Fisheries Commission for the Mediterranean (GFCM), Food and Agriculture Organization of the United Nations (FAO), 00193 Rome, Italy; abdalnasser.madi@fao.org (A.S.H.M.); stefano.lelli@fao.org (S.L.)
11 Ministry of Agriculture, Nablus Directorate, Nablus P400, Palestine; yaser1971@hotmail.co.uk
* Correspondence: valentina.crobe2@unibo.it
† These authors contributed equally to this work.

Abstract

The round sardinella (*Sardinella aurita* Valenciennes, 1847) is a widely distributed migratory pelagic fish inhabiting the Mediterranean Sea and the eastern Atlantic coasts. The species is heavily exploited and represents a valuable resource for global fisheries. In the Mediterranean area, uptakes of round sardinella are particularly high in the Ionian and Levant regions, where landings have shown fluctuating yet significant peaks in recent decades. Given its migratory nature, understanding the connectivity among populations is crucial for delineating appropriate fishery management units. Previous studies employing morphometric, meristic, and molecular analyses have yielded mixed results regarding population structuring. Here, the genetic differentiation among Eastern Mediterranean *S. aurita* populations was investigated using a multi-marker approach: the mitochondrial cytochrome c oxidase subunit I (COI), cytochrome b (CytB), control region (CR), and 16S ribosomal RNA (16S rRNA), and seven species-specific nuclear simple sequence repeats (SSRs). Overall, the results indicate high genetic diversity coupled with weak population structuring across the Eastern Mediterranean. These analyses aim at clarifying stock boundaries towards supporting sustainable management strategies at a regional scale for this ecologically and economically important species.

Keywords: GSAs; responsible management; stock units; Levant Sea; commercial species

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Key Contribution: By employing a multi-marker approach combining mitochondrial and nuclear DNA, this study supports the overall genetic homogeneity of round sardinella across the Levantine area, with only exceedingly weak regional clustering of Lebanese samples.

1. Introduction

The growing need and interest in wild fishery resources within the perspective of a sustainable management strategy are leading to deepening the knowledge of commercial species at different scales, even testing different scenarios of demographic fluctuations according to climate shifts [1,2]. Small pelagic fisheries represent an important contribution to the wide field of social-economic resources, especially for those onshore countries deeply dependent on marine resources [3,4]. This is the case of the Eastern Mediterranean Sea, where shared and transboundary fish stocks pose particular challenges for management, as national exploitation strategies must be aligned with the biological connectivity of marine populations. Among the commercially interesting species, sardines from the genus *Sardinella* Valenciennes, 1887 are one of the most important wild resources for local fisheries [5].

In particular, the round sardinella, *Sardinella aurita* Valenciennes, 1847, distributed throughout the Atlantic Ocean and in the Mediterranean Sea, has a migratory behaviour mainly driven by water temperature [6] and reaches the highest population densities in Western Atlantic and Western Africa, where three main stock units are inhabiting its waters [7]. It exhibits a preference for clear saline water with optimal temperatures around 23 °C [8]. This temperature inclination justifies its migratory behaviour, characterized by a southward movement during the winter and a northward one during the summer in most oceans and seas [9]. Most importantly, this migratory pattern aligns with the gonadal maturation of the species, as highlighted in the findings by Sabates et al. [10]. Such temperature-driven migrations, coupled with pelagic spawning and dispersive early-life stages, can blur population boundaries and complicate the identification of discrete stock units.

Round sardinella is found mostly in large schools composed by individuals differing in body shape according to the geographical area and other several factors. As a matter of fact, Bremer et al. [11] described different ecotypes in Senegal and in Venezuela and revealed that species of small pelagic fishes have similar schooling behaviour even though they are in areas very far from each other. This combination of phenotypic variability and apparent behavioural similarity raises questions about the extent to which the observed morphological differences reflect underlying genetic structuring [12].

Currently, *S. aurita* is not included in the IUCN Red List of Threatened Species and holds a classification of "Least Concern". However, the current status of exploitation of this species across its geographical distribution should encourage the establishment of precise monitoring and management plans. Indeed, like other small pelagic fish, round sardinella represents a valuable resource for the global economy and food consumption. About 250,000 tons of round sardinella have been globally caught in 2022 (https://www.fao.org/fishery/statistics-query/en/capture/capture_quantity; accessed on 4 July 2025), a small measure considering the 650,000 tons collected in the 90s off Western Africa only [13]. Moreover, round sardinella is one of the most exploited fish species in the Mediterranean Area, especially in the Ionian (37.2.2.) and Levant (37.3.2.) FAO division fishing areas, where, despite a fluctuating trend, we had a peak in catch between 2006 and 2009, with almost 21,000 tons, followed by another peak between 2018 and 2020, with about 17,000 tons

of catch [14]. These pronounced fluctuations further emphasize the need for biologically informed stock assessment frameworks capable of capturing population connectivity at regional scales [15].

Due to their migratory behaviour, the extent of intermingling among populations from different countries across the geographical range plays a crucial role in delineating fishery management units. Like other pelagic species, the eggs and larvae of these organisms are likely to be passively transported by ocean currents [16]. These currents may guide abundant propagules to coastal regions in plankton, the latter serving as a nursery where they attain the size of primary adult fish stocks [16]. From a population genetic perspective, such processes can promote extensive gene flow and reduce genetic differentiation, even across large geographic distances [17,18].

Recent and exhaustive analyses both on the genus *Sardinella* and on the species *S. aurita* included a wide geographical range and represented an important reference for those interested in the management of the resource [19]. Following Palumbi [20,21], setting fishery management strategies requires the understanding of fish stock boundaries and the knowledge of fishery experts on the biological differences and genetic processes of local groups of species.

To examine the population structure, different methods can be employed, relying on body morphometrics, meristic characteristics, and otolith shape, as demonstrated by Ferhani et al. [22], who identified significant differences among sardinella stocks from different areas through analyses of body and otolith shape. Alternatively, mitochondrial and nuclear markers can be employed. For instance, studies conducted on *Sardinella longiceps* Valenciennes, 1847 in the Indian Ocean used neutral markers such as microsatellites [23], while protein electrophoresis was applied to *S. aurita* specimens collected along the Florida coast in the USA [24]. These studies revealed the absence of genetic evidence supporting geographic population structuring in these species. However, most previous genetic studies relied on a limited number of markers or single-marker approaches, potentially constraining their power to detect subtle or fine-scale population structure. Determining the genetic variability of those groups can be useful to unravel the population structure, to verify the migration patterns (physical or genetic in terms of gene flow) across far or recent times, and to estimate the population size [25].

In the framework of the interdisciplinary approach adopted by the FAO-EastMed FISH-BONE Project (Finding Stock Units with Boundaries in Eastern Mediterranean, 2021) and with the support of the General Fisheries Commission for the Mediterranean (GFCM), the genetic difference between several samples from the Eastern Mediterranean Sea was estimated by using different molecular markers (the mitochondrial cytochrome oxidase subunit I, COI; cytochrome B, CytB; control region, CR; the rRNA 16S; and seven species-specific nuclear simple single repeats, SSRs) to unravel stock delimitation and differentiation inferences.

Given the pelagic nature and high dispersal potential of *S. aurita*, populations in the Eastern Mediterranean might be genetically homogeneous. However, previous studies have reported morphometric and otolith differences among populations [11,22], suggesting that subtle genetic differentiation might exist in the area. In this context, we aim to assess patterns of genetic differentiation among round sardinella populations in the Eastern Mediterranean by integrating multiple mitochondrial and nuclear markers with complementary analytical frameworks, testing the hypothesis of panmixia against the alternative of weak but detectable genetic structure.

2. Materials and Methods

A total of 390 samples of *S. aurita* were collected within the framework of the FISHBONE Project in the Eastern Mediterranean Sea, including the Ionian, Aegean and Levantine seas during 2019–2020 (Figure 1, Table 1 and Tables S1). Sample sizes varied among locations due to differences in specimen availability and landing conditions. Sampling stations were treated as geographic populations for population genetic analyses rather than discrete biological populations due to the high dispersal potential and migratory behaviour of *S. aurita*.

Muscle tissue (about 1 cm^3) was collected from fresh specimens using sterile scalpels and tweezers and immediately preserved in 96% ethanol. The ID-labelled vials containing the tissue were stored at −20 °C until further processing. Biometric data were collected, sex determined, and otoliths extracted and stored. Samples were then transferred to the genetic hubs in Turkey (Evolutionary Genetics Laboratory eGL at the Department of Fisheries and Aquaculture of Ankara University) and Italy (Laboratory of Biodiversity & Evolution of Marine Animals, BEAM, University of Bologna) for laboratory analyses.

Total genomic DNA (henceforth gDNA) was extracted from all the samples using the Sigma-Aldrich, St. Louis, MO, USA GenElute gDNA Miniprep following the manufacturer's instructions, with the exception of the samples collected in Turkey (GSAs 22 and 24) for which the kit Qiagen, Hilden, Germany DNeasy Blood & Tissue Kit was used. The extraction outcomes were assessed through electrophoresis run on agarose gel (1%), and only samples that yielded high-quality DNA were subsequently amplified by PCR.

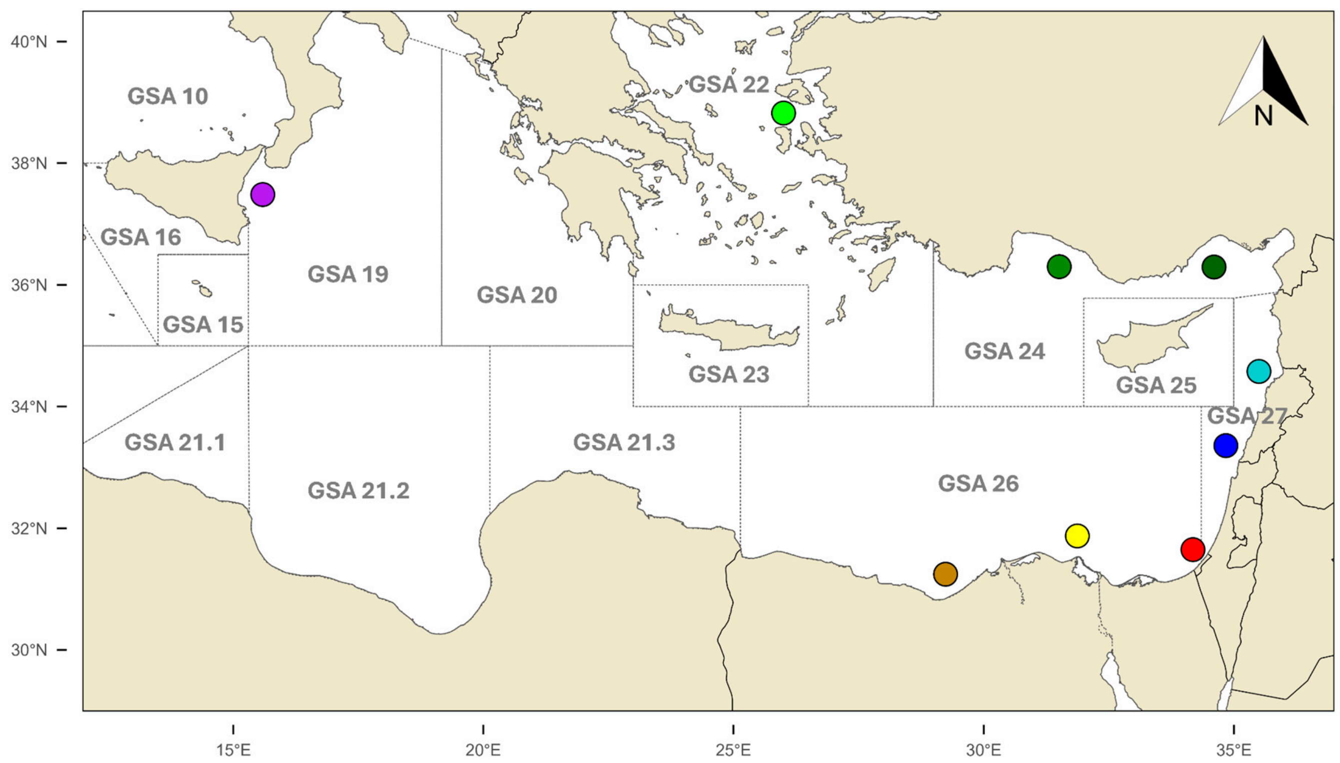

Figure 1. *Sardinella aurita* sample distribution in the Eastern Mediterranean Sea.

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Table 1. Details of population samples analyzed in the present work, with their respective country and GSAs of provenance, sampling locations, year, and relative codes. "*N* (tot)" refers to the total number of individuals sampled in each location in this study. Of these "*N* (COI)", "*N* (CytB)", "*N* (CR)", "*N* (16S)", and "*N* (SSRs)" refer to the COI, CytB, CR, and 16S individuals sequenced and genotyped in the present work.

Country	GSA	Location Code	Sampling Location	Year	*N* (tot)	*N* (COI)	*N* (CytB)	*N* (CR)	*N* (16S)	*N* (SSRs)
Italy	19	GSA 19	Catania	2020	21	17	20	18	19	21
Türkiye	22	GSA 22	Izmir	2019	30	30	28	30	30	29
	24	GSA 24a	Antalya	2019	30	29	30	30	30	30
	24	GSA 24b	Mersin	2019	30	30	29	29	27	28
Egypt	26	GSA 26b	East of Nile Delta	2019	50	49	49	45	50	38
	26	GSA 26c	West of Nile Delta	2019	50	48	49	32	43	39
Lebanon	27	GSA 27b1	North Lebanon	2019	50	45	49	44	50	41
	27	GSA 27b2	South Lebanon	2019	50	47	49	44	47	41
Palestine	27	GSA 27a	All coasts	2019	79	66	69	70	76	37

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2.1. Mitochondrial DNA

Between 10 and 20 ng of gDNA were used to amplify a 668 bp fragment of the mitochondrial cytochrome oxidase subunit 1 (COI) using the primer pair FishF2/FishR2 [26], a 431 bp fragment of the cytochrome B (CytB) using CytBH/CytBL [27,28], a 560 bp fragment of the control region (CR) using CRA/CRE [29], and a 586 bp fragment of the ribosomal 16s using 16S-ar/16S-br [30] (see Supplementary Table S2). PCR conditions of amplification are reported in the Supplementary Text S1. PCR outcomes were evaluated on a 2% agarose gel and preserved at 4 °C until purification. All PCR products for Sanger sequencing were purified using the ExoSAP-IT TM Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Monza, Italy) following the manufacturer's protocol.

The sequence data were obtained using the same primers used for the amplification by EGL and by the external provider Macrogen Europe (Milan, Italy).

The electropherograms were manually inspected and edited in MEGA v.12 [31]. Sequence alignments were conducted using the ClustalW algorithm [32] implemented using the same software. The available sequences were retrieved for *Sardinella aurita* from the NCBI database (https://www.ncbi.nlm.nih.gov; accessed on 30 December 2024) for each genetic marker targeted and added to the original dataset. When possible, the retrieved data had different geographic origins (i.e., Spain, Greece, Turkey, and Israel) to properly assess intraspecific variability and compare it with our data (Table S3). The newly obtained sequences have been deposited in the NCBI GenBank database (accession numbers available upon acceptance).

The DnaSP v.6 software [33] was used to compute the number of haplotypes (Nh), the number of polymorphic (V) and parsimony-informative sites (P), and the haplotype (Hd) and nucleotide diversity (Pi), which were estimated for each of the newly obtained datasets. The software was also used to estimate Tajima's D statistics in each sampling location in order to verify the stability of geographic populations.

Genetic distance matrices among sequences across geographical samples were calculated with MEGA using the best fitting models identified by JModelTest [34].

The phylogeographic relationship of species haplotypes was inferred with the Median joining (MJ) algorithm implemented in the PopART software v.1.7 [35].

For each mitochondrial DNA marker dataset (COI, CytB, CR, and 16S), estimates of genetic differentiation were computed as pairwise fixation indices (ΦST) based on the haplotype frequency distribution analysis (equivalent to an F analysis [36]) corrected for inter-haplotype sequence divergence using ARLEQUIN v.3.5 [37]. Statistical significance of fixation indices was tested through a permutation test (20,000 iterations) and through applying the Bonferroni correction [38].

2.2. Nuclear DNA

Seven microsatellite loci (SSRs) developed by Ma et al. [39] were amplified individually using singleplex PCRs with optimized annealing temperatures and protocols. For the subsequent analysis, the PCR products were combined into two marker mixes (see Supplementary Table S2). PCR conditions of amplification are reported in the Supplementary Text. The separation of PCR products of individual samples was carried out on a 3% agarose gel electrophoresis. The fluorescently labelled DNA fragments carrying NED, FAM, PET, VIC, and LIZ fluorochromes were mixed with the GeneScan 500 LIZ (Applied Biosystems®, Foster City, CA, USA) internal size standard and separated using the Applied Biosystems®, Foster City, CA, USA 3130xl Genetic Analyzer.

Technical replicates were randomly chosen among samples and across areas, and they were included in each amplification and genotyping reaction for an internal calibration [40,41] to estimate repeatability and error rates resulting from PCR amplifi-

cation. This step is necessary to assess the congruence of results between single-locus amplifications and sampling areas and the fragment analysis where size shifts or allele miscalling might occur. A total of 15 technical replicates were issued from the same DNA source but were processed independently for all loci.

Raw data derived from fragment analysis were imported in GeneMarker v.2.7.4 (SoftGenetics, LLC, State College, PA, USA), and the peak calling was performed after the creation of marker-specific panels for the analysis. After obtaining a matrix with individual genotypes, the Micro-checker v.2.2 software [42] was used with default settings to infer the presence of null alleles (NA), PCR stuttering, and large allele drop-out and determine the frequencies of null alleles across populations and loci. Based on the results of Micro-checker regarding the presence of NA and/or the departure from HWE, genetic diversity and population differentiation tests were investigated.

Overall, the average proportion of missing genotypes per locus in the complete dataset was 9% and varied from 3% (SARA 3, SARA 4, and SARA 8) to 21% and 23% (SARA 9 and SARA 7, respectively), thus a final dataset including 304 individuals and seven loci was analyzed to infer allele frequencies, mean number of alleles per locus, expected and observed heterozygosity values, and polymorphism index using the GENETIX software package v. 4.05 [43]. Allelic richness was estimated using FSTAT v. 2.9.3 [44]. The same software was used to calculate the coefficients of inbreeding (Fis). GENEPOP v.4.2 [45,46] was used to evaluate the linkage disequilibrium among pairs of loci across all populations and the percentage of private alleles [mean frequency of private alleles]. Departures from Hardy–Weinberg equilibrium (HWE) for each locus and population were determined using Fisher's [47] exact test as implemented in the same software. The Markov chain Monte Carlo (MCMC) approximation involved 10,000 dememorization steps, 1000 batches, and 10,000 iterations per batch. Probability tests were also conducted, and the relative values were corrected for multiple testing at alpha 0.05 using the Bonferroni correction.

For population differentiation analyses, pairwise Fst following Weir and Cockerham's model [48] was calculated in ARLEQUIN with 1000 permutations and alpha 0.05 using a Jackknife procedure.

The adegenet and ade4 packages [49] for R [50] were used to perform a discriminant analysis of principal components (DAPC) to identify and describe genetic clusters between sample groupings (9 sampling locations) to infer the genetic structure of *S. aurita* considering differently balanced sample sizes. The same principle was followed to perform a principal coordinates analysis (PCoA) to explore and visualize similarities or dissimilarities of data with GenAlEx v. 6.5 [51].

To investigate the underlying population genetic structure in each dataset, we used a Bayesian clustering algorithm implemented in STRUCTURE v. 2.3.4 [52], incorporating the admixture model, alleles frequencies correlated, and sampling location as a LOCPRIOR. For all datasets, we performed 25 independent runs for each K (1–10) with 1,000,000 iterations and a burn-in of 100,000 generations. Cluster matching and permutation were performed using CLUMPAK [53], while the most likely value for K was estimated from the mean log probability of the data using four alternative statistics (medmedk, medmeak, maxmedk, and maxmeak) carried out using STRUCTURESELECTOR [54].

3. Results

Total gDNA was successfully extracted from all the samples collected and was suitable for the following analyses.

3.1. Mitochondrial DNA

Overall, PCR was successful for all the markers applied to the samples considered (389/390 for COI; 388/390 for CytB; 389/390 for CR; 387/390 for 16S). Amplicon sequencing, editing, and aligning was also successful, since a total of 361 sequences over 389 were aligned for COI, 372 sequences over 388 were aligned for CytB, 342 sequences over 389 were aligned for CR, and 372 sequences over 387 were aligned for 16S.

Overall, the genetic diversity across the newly sequenced and analyzed samples (this study only) with four mitochondrial markers are reported in Supplementary Table S4. The number of mutations ranged between 21 (16S) and 83 (CR), the latter showing the highest haplotype diversity (Hd; 0.706 (±0.120) in Italy (GSA 19) and 0.950 (±0.029) in Egypt (GSA 226c)). Tajima's D values were negative in all the sampling locations analyzed. Also, the values were not significant for the Turkish geographical samples (both GSAs 22 and 24). In general, negative and significant estimates (* $p < 0.05$, ** $p < 0.01$) indicated an excess of low frequency variants, usually a symptom of recent expansion of the populations.

After retrieving additional sequences from public data repositories, the final dataset for COI included 397 sequences (605 bp long), the one for CytB included 379 sequences (767 bp long) and the 16S final alignment included 404 sequences (526 bp long). No other CR sequences were included, as the one available sequence was too short to be reliably comparable (306 bp compared with our 418 bp).

The percentage of genetic distances measured for each marker within geographical samples is reported in Supplementary Table S5 (see datasheets "COI-16S") and revealed that the most homogeneous population sample, for instance, according to the COI marker, is Turkey (GSAs 22 and 24a; 0.020%), while the most heterogeneous one is Greece (0.230%), followed by Lebanon (GSA 27b1; 0.190%).

The percentage of genetic distances measured for each marker between pairs of geographical samples is shown in Supplementary Table S5 (see datasheets "COI-16S") and revealed that the lowest distance (0.011%), for the conservative COI marker was measured between Tunisia (GSA 13) and Türkiye (GSAs 22 and 24b), whilst the highest percentages were observed between Greece and Italy (0.235%), followed by the Greece and Lebanon sampling locations (GSA 27b1 and GSA 27b2), with 0.201% and 0.192% of genetic distance, respectively. A similar distance was estimated between Palestine and Israel, with 0.238% of genetic distance when considering the CYTB marker. When considering the most variable marker (CR), the highest genetic distance was observed between the Lebanon GSA 27b1 and GSA 27b2 (0.860%).

The single-gene haplotype MJ networks obtained from the final datasets appeared concordant for the COI, CytB, CR, and 16S markers (Figures 2 and S1–S3) in showing star-like frameworks, with the most common haplotypes shared by almost all the samples of *Sardinella aurita* collected in the Mediterranean Sea, crowned with less common haplotypes. Clusters of fewer individuals sharing the same haplotype, down to one individual displaying only a different variant, are separated from two to 18 mutational steps. In detail, Figure 2 referred to the COI marker and showed one main haplotype where all the sampling locations considered were represented by 86% of the sequences (except for one individual from the Atlantic Bay of Biscay). A second haplotype, differing by two mutations, was also shared by all the geographic samples (except from Greece, Tunisia, and Egypt–North of Suez Channel) but at a smaller scale (3.5% of the sequences). The third haplotype included fewer samples (2.1%) from Turkey (GSA 22), Egypt (both sampling sites 26b and 26c), Lebanon (both sampling sites 27b1 and 27b2), and Palestine. From the latter, one-individual variants were diverging with two (Turkey GSA 24a and Palestine) to 15 mutations (Bay of Biscay in the Atlantic Ocean, Lebanon, both 27b1 and 27b2, and Italy, Catania).

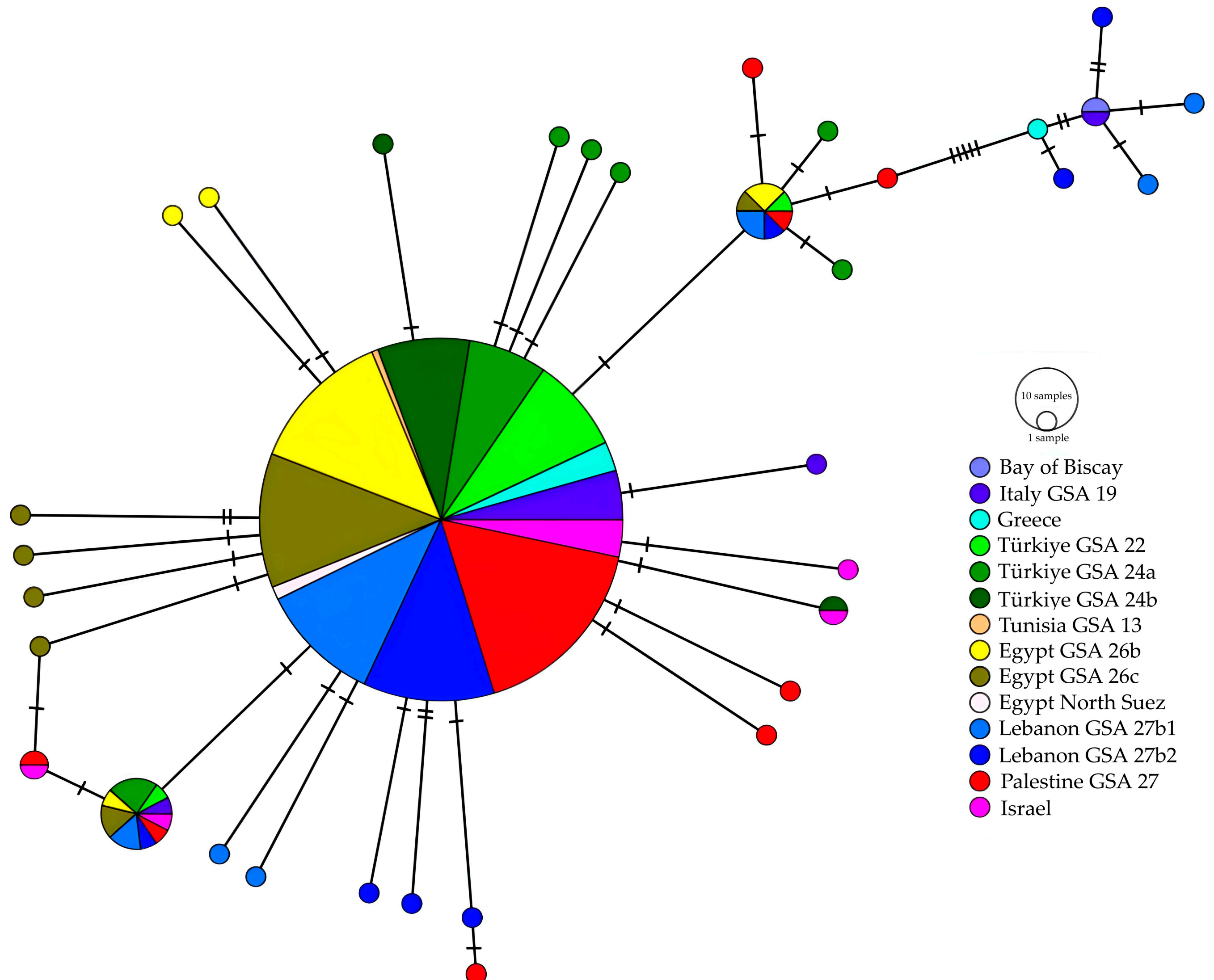

Figure 2. Median joining network of *Sardinella aurita* haplotypes identified across the COI dataset (605 bp). The size of each circle is proportional to the number of samples within each haplotype. Unsampled haplotypes are represented with black dots and single mutational steps are represented as continuous lines. The colours are consistent with those shown in Figure 1.

The high ΦST values measured on the COI dataset using the Kimura 2P model resulted as non-significant, indicating a lack of genetic differentiation among population samples of the geographical areas considered (Supplementary Table S6, "COI" datasheet). According to the CytB marker, all pairwise comparisons with Spain, Greece, and Israel showed null ΦST values. On the other hand, Turkey (GSA 24b) appeared weakly differentiated from all the other samples with low but significant ($p < 0.05$) pairwise ΦSTs (0.00025–0.037; Supplementary Table S6, "CytB" datasheet). Lebanon (GSA 27b2) appeared dimly and significantly different from Egypt (GSA 26b; ΦST = 0.012) and Lebanon (GSA 27b1; ΦST = 0.038), while Israel differed slightly from Spain and Greece ($p < 0.05$). The overall ΦST values measured on the CR dataset resulted as low and non-significant ($p = 0.273$), as the large majority of the pairwise comparisons except for the comparison between Turkey (GSA 24b) and Egypt (GSA 26c; ΦST = 0.0227, $p < 0.05$), which appeared weakly differentiated (Supplementary Table S6, "CR" datasheet). The same evaluations in the ΦST indices calculated on the 16S final dataset suggested Greece as the most different sample ($p < 0.05$),

while GSA 26b resulted significantly different from GSA 26c (ΦST = 0.001, $p < 0.001$) and Lebanon (GSA 27b1; ΦST = 0.030, $p < 0.05$; Supplementary Table S6, "16S" datasheet).

3.2. Nuclear DNA

The amplification of the seven species-specific simple single repeats (SSRs) isolated in *Sardinella aurita* was successful for most of them. The internal calibration designed on technical replicates (PCR amplification and genotyping) aimed at ensuring the accuracy of genotyping from a technical standpoint and the reading of raw data. For all the samples considered and across all the plates considered, the result of the electropherogram analysis yielded the same outcome, confirming both the initial readings and the absence of batch effects during the analysis process.

The overall results obtained on 304 genotyped individuals showed that all SSRs were highly polymorphic with the number of alleles ranging from 20 (SARA 7) to 47 (SARA 9) across all geographic populations considered. Furthermore, deviation from the Hardy–Weinberg equilibrium was significant for the SARA 5 locus (Supplementary Table S7). The allelic richness highlighted that the most variable geographic population was the GSA 19 (with an average value of 20.27 in SARA 9; Supplementary Table S8), while a lower polymorphism was detected in GSA 27b1, corresponding to North Lebanon (7.83 in SARA 8; Supplementary Table S8).

The results of the exact testing of the genetic differentiation between population pairs revealed low (0.015 in the comparison between GSA 27b1, North Lebanon and GSA 26c-Eastern and Western Nile Delta; p-value < 0.05) but significant Fst values when comparing GSA 27b1 (North Lebanon) with all the other geographic populations, except for GSA 24a, GSA 24b, and GSA 26b, corresponding to Antalya, Mersin, and East of Nile Delta geographic areas (Supplementary Table S9).

The results from the DAPC analyses involved nine geographical populations. The DAPC plot showed scattered points and no clear sign of differentiation between locations (Figure 3).

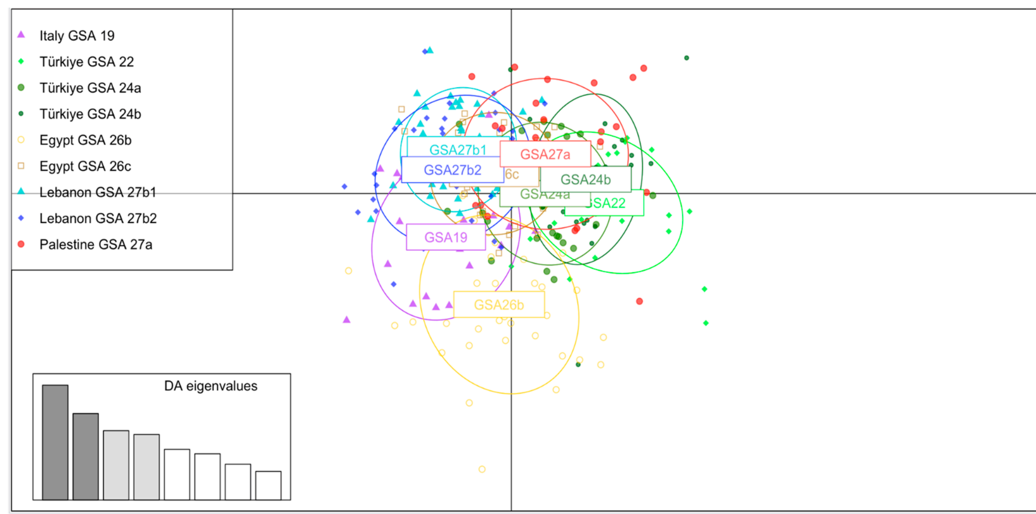

Figure 3. Results of DAPC analysis performed on 304 individuals and seven microsatellite loci. The colours are consistent with those shown in Figures 1 and 2.

Partial inconsistencies on the genetic divergence among samples on a geographical basis were highlighted in the results of PCoA where, for instance, GSA 26b and GSA 26c (East and West Nile delta, respectively) appeared dissociated (Figure 4).

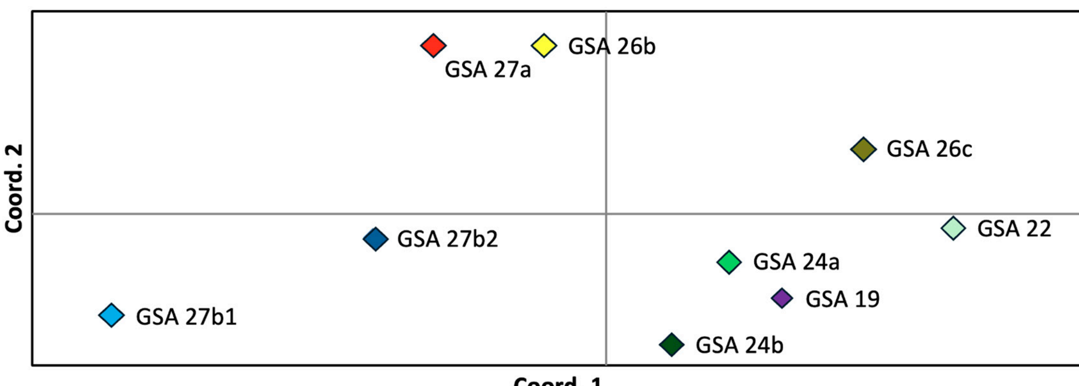

Figure 4. Results of PCoA performed on 304 individuals and seven microsatellite loci. The colours are consistent with those shown in Figures 1–3.

The outputs of the STRUCTURE analysis with STRUCTURESELECTOR did not provide clear-cut evidence of the most likely number of clusters. In particular, the function DeltaK presents the highest values in correspondence of K = 4 (Figure S4). Results from K = 1 to K = 10 were assessed with CLUMPAK. The barplot obtained for the clustering with K = 2 supported a slight separation of the GSA 27b1, and a slight discrimination interested GSA 27b2 and GSA 27a while clustering with K = 3 (Figure 5). Overall, these results indicate slight population differences, but STRUCTURE does not support the presence of discrete management units across the studied area.

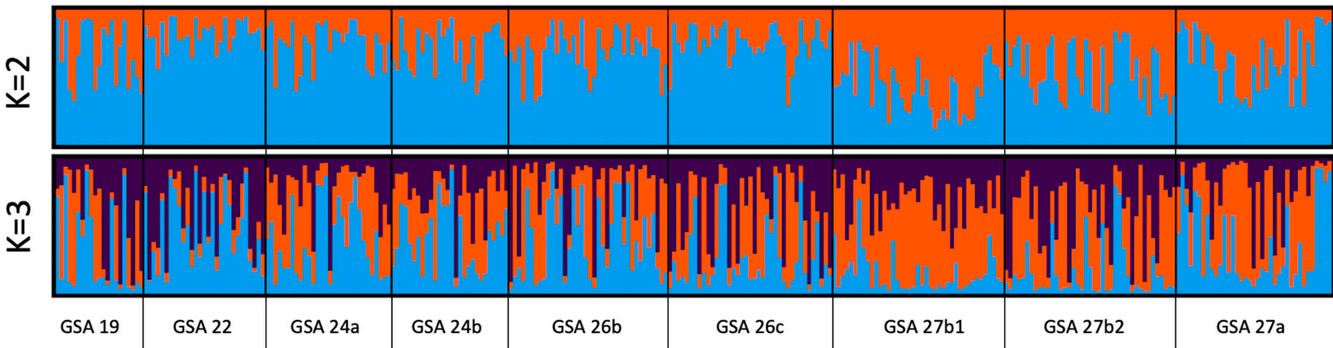

Figure 5. Barplot of the Bayesian clustering analysis performed with STRUCTURE based on seven SSRs. The results are shown for K = 2 and K = 3. Each vertical bar represents an individual, and colours indicate belonging to a group identified by the Bayesian analysis.

4. Discussion

Considering the commercial importance of *S. aurita* and the pronounced fluctuations in its abundance observed over recent decades, considerable efforts have been devoted to the assessment of its genetic variability since the early development and the application of molecular techniques, ranging from protein electrophoresis to restriction enzymes, mitochondrial DNA markers, and neutral nuclear loci [12,19,38,55–58]. Despite this extensive body of work, uncertainty remains regarding the spatial scale at which biologically meaningful stock boundaries can be detected, particularly in regions characterized by high connectivity and complex oceanographic dynamics [59].

On the one hand, the choice of molecular markers and their level of variability is crucial for accurately identifying fish stock units and genetic differentiation patterns [60–63]. On the other hand, molecular approaches alone do not represent a flawless or exhaustive

method, as their resolution and interpretative power are inherently influenced by marker-specific mutation rates and modes of inheritance, which may lead to different signals of population structure. Previous attempts to combine morphological and genetic techniques in *S. aurita* [24], together with subsequent recommendations advocating a holistic framework for stock identification and adaptive management strategies [64], highlight the need for integrative approaches. In this framework, combining mitochondrial markers reflecting historical demography with highly polymorphic nuclear loci sensitive to contemporary gene flow provides a more powerful means of detecting subtle population structure. More recently, comprehensive analyses at both genus and species level across wide geographic scales have provided valuable references for fishery management [19,65], although otolith chemistry and shape analyses remain effective tools for addressing specific questions related to life-history shifts under global change scenarios [7,66–68].

In this context, the present study provides new insight into the genetic variability of round sardinella from the Levantine area of the Mediterranean Sea, integrating mitochondrial and nuclear markers. The analyses based on four mitochondrial markers (COI, CytB, CR, and 16S) consistently revealed low levels of genetic differentiation across most Mediterranean populations of *S. aurita*. For all mitochondrial markers, the prevalence of negative Tajima's D values, together with the star-like topology of haplotype networks and the dominance of one or a few haplotypes, represents a signature of recent demographic expansion. Such patterns have been reported in small pelagic fishes with large effective population sizes and high dispersal potential, where lineage sorting may be slow and regional differentiation weak or transient [69]. Our results are consistent with recent findings from the northeastern Mediterranean Sea, which reported low levels of genetic differentiation among *S. aurita* populations based on the COI data, despite the presence of localized differences in nucleotide diversity and statistically significant pairwise comparisons involving specific coastal areas [70].

Despite the very first successful application of the panel developed by Ma et al. [39] to round sardinella from the Mediterranean, which generated a robust dataset supported by internal calibration based on technical replicates and revealed more alleles than previously reported in the literature, the analyses of seven species-specific microsatellite loci uncovered a high level of nuclear genetic diversity across the sampled populations. All loci were highly polymorphic, with allele numbers ranging from 20 (SARA 7) to 47 (SARA 9), confirming their strong discriminatory power for population genetic analyses. A significant deviation from HW equilibrium was detected only at the SARA 5 locus, and it indicates that, at nuclear level, most populations conform to Hardy–Weinberg expectations, consistent with a generally high level of gene flow.

Pairwise genetic differentiation based on Fst values was generally low, in line with the high dispersal potential of the species [10,71]. Significant Fst values emerged when comparing GSA 27b1 (North Lebanon) with most other geographic locations, including GSA 19 (Italy), GSA 22 (Türkiye), and GSA 26c (Western Nile Delta), although differentiation remained weak in magnitude. A similar pattern of very low genetic differentiation at neutral loci, with sporadic significant pairwise differences between geographically close populations, has also been observed in other regions of the species' range, such as the Gulf of Guinea, where microsatellite analyses highlighted differences between Ghanaian and Ivorian coastal samples against a background of broad genetic homogeneity [57]. The heterogeneous pattern of weak but occasionally significant Fst values involving GSA 27b1 suggests spatially variable connectivity within the eastern Mediterranean, possibly influenced by regional oceanographic features or localized dispersal dynamics, in line with predictions from larval retention and exchange models [72].

The multivariate analyses provided additional insight into these patterns. The DAPC, together with the results of the PCoA, showed overlapping clusters and scattered individual assignments with no clear geographic segregation, which highlighted partial inconsistencies in geographic structuring and reinforced the idea of a largely connected genetic system.

These results highlight that, while each sampling station represents a geographic population rather than a discrete biological population, analyzing multiple populations is relevant to detect subtle spatial structure and local deviations from panmixia, which may inform regional management decisions.

Also, the individual-based analysis conducted using the Bayesian clustering further supported the presence of a weak population structure.

Overall, the microsatellite data depict *S. aurita* as a species characterized by high nuclear genetic diversity and weak but detectable fine-scale population structure when considering Fst comparisons. In contrast, mitochondrial markers mainly reflect shared demographic history and large-scale connectivity across the basin. Nuclear markers reveal localized deviations from panmixia, particularly involving North Lebanon and parts of the Nile Delta, suggesting that subtle spatial heterogeneity may occur despite substantial connectivity. Together, these results indicate that round sardinella in the Eastern Mediterranean functions largely as a connected evolutionary unit at the spatial scale investigated. From a management perspective, the absence of strong and consistent genetic structuring does not support major stock subdivision across the basin. However, the localized signals detected by microsatellites highlight areas where fine-scale processes may operate and where a precautionary approach could be considered. It should also be noted that higher-resolution genomic approaches (e.g., SNP-based analyses) may provide additional insights into subtle or adaptive differentiation (e.g., Corti et al. [73]). Therefore, genetic evidence should be integrated with ecological and fishery data to support informed management decisions in the Eastern Mediterranean.

5. Conclusions

Evidence produced to date on *S. aurita* from the Levantine area indicates a generally highly connected population inhabiting an environment characterized by permeable or intermittent physical barriers, with only weak differentiation observed in the North Lebanon area. However, this subtle signal was not consistently supported across the methodologies applied. Inferences based on more powerful molecular tools, such as SNPs or larger panels of species-specific microsatellite loci (see Chlaida et al. [74]) combined with larval dispersal simulations informed by oceanographic models, otolith shape analyses, and morphometric approaches may enhance the detection of population structure within the Levantine region and improve the identification of biological units. These units represent the fundamental building blocks of fishery management and marine resource conservation [75]. Taken together, our results highlight the importance of continued integrative monitoring to detect emerging or fine-scale structuring, which is relevant for adaptive and ecosystem-based fishery management.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11030175/s1, Text S1: Molecular methods; Table S1: Details of individual samples analyzed in the present work with their respective country and GSAs of provenance, sampling locations, geographic coordinates, year, relative codes, and biological traits; Table S2: Details of primers employed for DNA amplification of both mtDNA and nuDNA. Primer sequence, annealing temperature and reference are reported. * represents optimized annealing temperature used in PCR reaction; Table S3: Details of public sequences and mtDNA marker analyzed in the present work with relative BOLD id and or NCBI accession number. When available, the date of collection, country and GSAs of provenance, sampling locations, and reference are reported;

Table S4: Gene diversity indexes of the geographic samples of Sardinella aurita. N: number of samples, V: variant sites; P: parsimony informative sites; Nh: number of haplotypes, Hd: haplotype diversity, Pi: nucleotide diversity, SD: standard deviation, n.d.: non-computable, * $p < 0.05$, ** $p < 0.01$. Acronyms are consistent with Table 1; Table S5: COI genetic distances observed within (a) and between (b) geographical samples; Table S6: Pairwise fixation indices (below diagonal) and corresponding p-values (above diagonal) for each pair of comparison for each mitochondrial marker alignment. In bold, significant values for $p < 0.05$. Acronyms are consistent with Table 1; Table S7: Details of the genetic diversity of round sardinella. Number of alleles, Hexp: expected heterozygosity; Hobs: observed heterozygosity, Mean AR: mean allelic richness, Fis: inbreeding coefficient, HWE: Hardy–Weinberg equilibrium; in bold, significant results; * significant results after the Bonferroni correction (alpha: 0.0000793); Table S8: Allelic richness estimated per each locus and sampling location; Table S9: Pairwise Fst values estimated in nine geographic locations and seven SSRs. p-values are reported above the diagonal. In bold, significant results at alpha: 0.05; Figure S1: Median joining network of Sardinella aurita haplotypes identified across the CytB dataset; Figure S2: Median Joining network of Sardinella aurita haplotypes identified across the CR dataset; Figure S3: Median joining network of Sardinella aurita haplotypes identified across the 16S dataset; Figure S4: Absolute value of derived delta K (ΔK) for each cluster number (K) from one to 10.

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Institutional Review Board Statement: We declare that the samples of *Sardinella aurita* individuals analyzed in the present work were obtained from commercial and scientific fisheries. The activity was conducted in accordance with the European Union's Common Fisheries Policy (Regulation (EU) No 1380/2013 of the European Parliament and of the Council of 11 December 2013). The fishing operations involved the harvest of fish through standard practices, which inherently result in mortality. No experimental procedures were conducted on live animals; therefore, ethical approval was not required.

Data Availability Statement: The newly obtained sequences have been deposited in the NCBI GenBank database; accession numbers for COI: PZ148869–PZ149229; 16S: PZ149278–PZ149649; Cytb: PZ155526–PZ155897; CR: PZ155898–PZ156239. The microsatellite data that support the findings of this study are available as a genotype matrix from the corresponding author upon request.

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