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Stock Discrimination of Gilthead Seabream (*Sparus aurata* Linnaeus, 1758) through the Examination of Otolith Morphology and Genetic Structure

George Geladakis [1,2,*], Costas Batargias [1], Stylianos Somarakis [3] and George Koumoundouros [2]

[1] Biology Department, University of Patras, 26504 Rio, Patra, Greece; cbatargias@upatras.gr
[2] Biology Department, University of Crete, 70013 Heraklion, Crete, Greece; gkoumound@uoc.gr
[3] Institute of Marine Biological Resources and Inland Waters (IMBRIW), Hellenic Centre for Marine Research (HCMR), 71500 Heraklion, Crete, Greece; somarak@hcmr.gr
* Correspondence: geladakisg@upatras.gr

Abstract: Reliable stock identification constitutes an integral component of effective fishery management. Current methods for the identification of putative stock units comprise the analysis of both phenotypic and genetic variability. The present study examined the spatial variation in otolith morphology (shape and asymmetry) and genetic composition of 395 wild-caught Gilthead seabream (*Sparus aurata*) specimens, collected from the Aegean and Ionian Seas (eastern Mediterranean) between 2014–2018. The degree of scale regeneration (SRD, % of regenerated scales) was used as an indicator to assess the potential presence of aquaculture escapees in the wild-caught samples. Otolith shape and asymmetry analyses showed a phenotypic discrimination between northwestern Aegean and Ionian Gilthead seabream populations. Genetic analyses of nine microsatellite markers revealed higher levels of genetic variation in the wild compared with samples obtained from aquaculture farms. Despite the absence of genetic structure among the wild-caught seabream populations, a low but statistically significant genetic differentiation was found between reared fish and fish collected in the field. The SRD was considered effective in detecting the presence of aquaculture escapees that may have escaped in either early or late rearing phases.

Keywords: otolith shape plasticity; bilateral asymmetry; aquaculture escapees; wild fish; microsatellites; population subdivision

Key Contribution: The combined analysis of otolith and scale morphology proves adequate for distinguishing wild Gilthead seabream stocks: uncovering additional phenotypic variability resulting from the presence of aquaculture escapees in natural populations.

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1. Introduction

Gilthead seabream (*Sparus aurata* Linnaeus, 1758) is a highly valuable finfish for both fisheries and aquaculture industries in the Mediterranean Sea. Following FAO [1], global capture and aquaculture production of this species in 2020 was approximately 8646 tonnes and 282,073 tonnes, respectively. It is a coastal euryhaline marine fish with a natural distribution that extends along the eastern Atlantic coasts and the Mediterranean Sea, up to the Black Sea [2]. Previous studies have explored the natural genetic structuring of wild seabream populations across its entire distribution range using different genetic markers, including allozymes, mtDNA, microsatellites, and, recently, SNIPs (e.g., [3–8]). However, depending on the type and number of markers used, the existing literature on the genetic structuring of wild Gilthead seabreams has produced contradictory results with no evidence of simple isolation by distance among populations (e.g., [3,6,9,10]). Studies in both the Atlantic and Mediterranean Seas, at a variety of geographical scales, have indicated either an absence of genetic structure or a weak to strong population subdivision within

and among different geographical areas (e.g., [7,8,11–18]). The long pelagic larval stage (up to 50 days at 17–18 °C in Gilthead seabreams [2,19]) is likely to contribute to migrant exchange among fish populations and seems to be one of the main reasons for the slight genetic differentiation and lack of geographic patterning among wild stocks in various fish species, including Gilthead seabreams (e.g., [11,12,20–23]). Additionally, accidental escapes of reared seabream, mainly due to technical or operational failures [24–26], or the release of gametes into the wild through spawning within sea-cages [27] may have impacted the genetic structure of local populations, potentially resulting in the introgression of foreign DNA into the wild gene pool. This hypothesis is further supported by the ability of reared escapees to survive in the natural environment for extended periods of time and to compete effectively for natural resources [28–30].

Except for genetic markers, a variety of methods and approaches have been proposed to discriminate wild fish stocks (namely self-reproducing fish groups that are available for exploitation in a given area, with the members of each group having similar life history characteristics [31]), including the analysis of phenotypic variability of different morphological traits of fish, such as body shape or otolith and scale morphology (e.g., [32–37]). Those traits are highly applicable in detecting differences in environmental conditions that fish experienced during their lifetime, particularly in the cases of marine species that breed in the open sea (e.g., Gilthead seabreams), where there are fewer barriers to dispersal and a small amount of gene flow between populations is sufficient to maintain the genetic homogeneity [12,20,22,38]. Previous studies have detected differences in body shape or in otolith and scale morphology between wild or between wild and reared Gilthead seabream populations of the western and eastern Mediterranean Sea [39,40], between fish of the wild and of a farmed origin in Greece [41–43] as well as between wild, reared, and farm-associated seabream populations across the Adriatic Sea [16,44–46].

Otoliths are calcified structures found in the inner ears of teleosts and involved in hearing and balance systems [47]. They consist of a calcium carbonate material that is deposited continuously on an organic matrix throughout the fish's lifetime. Unlike scales and bones, otoliths are metabolically inert (i.e., once deposited, otolith material is unlikely to be resorbed or altered) and contain reliable fingerprints that provide information for the entire life cycle of the fish [48–50]. The otolith shape results from the combined expression of its genotype and the conditions of the growing environment [51–58]. However, it also depends on the fish's developmental stage, age, body size, sex, or sexual maturity status (e.g., [50,51,59–64]). At the individual level, bilateral differentiation in biomineralization processes induce morphological differences between the left and right pair of otoliths. At the population level, otolith fluctuating asymmetry (FA) refers to the random deviations from perfect bilateral symmetry induced by differences between the left and right body sides [65]. Due to the high functional value of the otolith structures in fish balance and hearing [66,67], otolith FA is considered a sensitive indicator for examining the effects of environmental stress and/or environmental heterogeneity on the health status of wild fish populations (e.g., [68–72]).

In the present study, we investigated the genetic population structure and spatial variation in the shape and asymmetry of otoliths in wild-caught Gilthead seabreams sourced from the Aegean and Ionian Seas (eastern Mediterranean). To control for the potential presence of aquaculture escapees among wild-caught individuals, we used the degree of scale regeneration (SRD, % of regenerated scales), which has been shown to be significantly higher in reared fish [40–42,73,74], as an indicator to exclude potential aquaculture escapees (as previously demonstrated by [41]) in the comparisons of otolith morphology (shape and asymmetry) and genetic composition between wild-caught fish of different geographical origins.

2. Materials and Methods

2.1. Sample Origin and Scale Examination

A total of 395 Gilthead seabreams were used for this study (Table S1). All fish were caught in the field from the North (Kavala), West (Maliakos Gulf), and East (Kalymnos) Aegean Sea; from different areas of the Ionian Sea (Patraikos Gulf, Central Ionian Sea, Corfu); as well as from the Messolonghi lagoon (Western Greek coast) between 2014–2018 (Tables S1 and S2, Figure 1). In the Ionian Sea, fish were collected with an experimental bottom trawl onboard the R/V PHILIA. In the Aegean Sea, the samples were obtained onboard small scale fishing vessels (netters), and in the Messolonghi lagoon, samples were obtained from the seasonal (autumnal) trap fishery, which take place during the mass seaward migrations of the species from the lagoon to the open sea, prior to the spawning season [75]. Depending on geographical origin, the fish samples were assigned into four groups: northwestern Aegean (NWA), East Aegean (EA), Ionian (I), and Messolonghi (M). Given that coastal lagoons exhibit large fluctuations in abiotic parameters [76], the study also aimed to investigate the effect of the specific environmental conditions of the Messolonghi lagoon during the period when it serves as a nursery ground for the fish [77] on the otolith morphology of wild Gilthead seabreams.

Figure 1. Map of the sampling area showing the locations of wild-caught fish. (1) Maliakos gulf; (2) Kavala; (3) Kalymnos; (4) Corfu; (5) Central Ionian; (6) Patraikos; (7) Messolonghi lagoon.

In the laboratory, fish were measured for standard length (SL, tip of snout to base of the central caudal lepidotrichia) and the largest pair of otoliths (*sagittae*) were extracted from each specimen. All otoliths were cleaned in 75% ethanol and individually photographed against a dark background with reflected light using a digital camera (Lumenera INFINITY1-5C, Teledyne Lumenera, Ottawa, Ontario, Canada) attached to a stereoscopic microscope (Olympus SZ61, Olympus Europa SE and Co., KG, Hamburg, Germany). A sample of 20 scales was then randomly removed from the left side of each fish, within the region between the anterior dorsal fin and the lateral line. Scales were photographed and examined for the presence or absence of a regenerated nucleus. The degree of scale regeneration (% regenerated scales) (SRD) was then calculated for each specimen (e.g., [74,78]). Within each geographic group, in order to assess potential temporal changes in SRD, Kruskal–Wallis and Mann–Whitney U tests were performed between the SRD of fish collected in different years.

In the present study, to minimize any effects of the possible presence of aquaculture escapees in the examined samples, the SRD was used to assign wild-caught fish into one of three predetermined groups: (a) fish with SRD lower than 30% (L30), (b) fish with SRD between 31–60% (31–60), and (c) fish with SRD higher than 60% (M60). Following Geladakis et al. [41,74], wild-caught seabreams with SRD higher than 30% (50% of fish specimens, Geladakis et al. [41]) had an increased possibility of including fish that escaped from net pens at various phases of the rearing process.

Two additional Gilthead seabream samples from two commercial cage farms, one from the northwestern Aegean (NWAR, n = 30) and another from the Ionian Sea (IR, n = 28), were also included in this study to serve as reference points for the genetic analysis of wild-caught specimens.

2.2. Otolith Shape Analysis

To ensure consistent orientation between otoliths from both sides of the body, the images of right otoliths were horizontally flipped. Using conventional image analysis techniques, all captured images were transformed to binary (ImageJ software version 1.3 k, U. S. National Institutes of Health, Bethesda, MD, USA [79]) and further analyzed by using the ShapeR package [80], an open software in R (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria [81]). Otolith outlines were automatically extracted from the images and each otolith shape was then represented by a two-dimensional coordinate matrix (x, y) for further analysis. Primarily, four univariate morphological descriptors were calculated for each otolith outline: surface area (O_S, mm^2), perimeter (O_P, mm), maximum length (O_L, mm), and maximum depth (O_D, mm). Based on the criterion of 98.5% accuracy for otolith reconstruction, a normalized elliptic Fourier approach was used to create a total of 12 harmonics, each one composed of four coefficients [80]. Otolith shape data were standardized and aligned with respect to size by omitting from the analysis the first three harmonic coefficients (resulting in 45 coefficients out of the initial 48). In order to adjust otolith shape with respect to allometric relationships with fish standard length (SL), those Fourier coefficients exhibiting a significant interaction ($p < 0.05$) between groups and fish SL were also excluded from the analysis [80].

Elliptic Fourier data of wild-caught individuals were first analyzed for differences between fish of different origin (NW. Aegean, E. Aegean, Ionian, Messolonghi) and SRD levels (L30, 31–60, M60) using multivariate nonparametric analysis of variance (PERMANOVA [82]). PRIMER-E software (version 6, Plymouth Routines in Multivariate Ecological Research, Plymouth, UK [83]) with the PERMANOVA add-on was used to transform the elliptic Fourier coefficients to a Euclidean distance matrix. PERMANOVA was performed on the partitioned sums of squared Euclidean distances to generate the distribution of the pseudo-F statistic using unrestricted permutation of raw data (9999 permutations [84]). Respective p-values were also estimated for the statistical significance of the tests. To evaluate the arrangement and differentiation among groups in multivariate space, the Euclidean distance matrix was subjected to canonical analysis of principal coordinates (CAP [85]). The determination of the number of PCO axes (m) was guided by the calculation of the minimum misclassification error based on the "leave-one-out" approach. An a priori hypothesis regarding distinct sample groups was tested using CAP by obtaining a p-value through 9999 permutations [83].

2.3. Otolith Bilateral Asymmetry

Eight traits were used to assess bilateral asymmetry: four univariate morphometric descriptors (O_L, O_D, O_S, O_P) and four high-amplitude harmonics (H_2–H_5) (i.e., low-order harmonics, which describe the main outline characteristics of otolith contour [86]). Amplitudes were derived from the elliptic Fourier coefficients of each harmonic descriptor

without adjustment for fish standard length (SL) [41]. The following equation, as described by Tort [86], was used to calculate the amplitude of harmonic n for each otolith:

$$amp_n = \frac{1}{2}\sqrt{a_n^2 + b_n^2 + c_n^2 + d_n^2}$$

Primarily, all traits were assessed for the type of bilateral asymmetry, namely fluctuating asymmetry, directional asymmetry, or antisymmetry [69,87]. Fluctuating asymmetry (FA) is considered ideal when the distribution of right minus left measurements (R-L) has a zero mean and follows a normal variation. On the other hand, directional asymmetry (DA) is characterized by a significantly skewed R-L distribution while antisymmetry displays either bimodal or platykurtic distribution characteristics [88]. According to Palmer and Strobeck [89], conventional skew and kurtosis statistics are considered powerful tests to examine departures from normality as they provide additional insights into the variation between sides. One-sample *t*-tests were performed to determine whether the R-L distribution of the otolith traits displayed skewness (g1) and kurtosis (g2) equal to zero [90]. To examine the potential correlation between asymmetry and fish size, a straightforward regression analysis was conducted by assessing the natural logarithm of the absolute bilateral difference (ln | R-L |) in relation to size (SL) for each trait [69,87].

To quantify the degree of otolith asymmetry, the variance of the bilateral difference (FA_1 = var(R-L)) was computed for each trait and fish group [91]. Bonferroni-corrected F-tests were applied to test for significant differences in FA_1 among the different groups [88].

2.4. Microsatellite DNA Analysis

A small piece of caudal fin tissue was cut from each fish and stored in 99.9% ethanol. Total genomic DNA was extracted by Chelex-100 (5%) following the DNA isolation protocol as described in [92]. Fish DNA was genotyped with the following nine microsatellite markers: D67, SAGT1, VBC003, Bd10, ELD10, BMAP54, SGAT32, VBC160, SAGT41 [93–96]. For the simultaneous amplification of the nine microsatellite loci, multiplex PCRs were run in 13.3 µL total volume reactions containing 4.5 µL of KAPA2G Fast Multiplex PCR mix (Kapa Biosystems, Potters Bar, United Kingdom), ~50 ng template DNA, and Primers with a final concentration of 0.1–0.5 µM. PCR cycling conditions consisted of an initial 95 °C denaturation step for 3 min, followed by 35 cycles of 30 s at 94 °C., 30 s at 56 °C, and 30 s at 72 °C, with a final elongation step at 72 °C for 45 min. The ABI3500 automated sequencer (Applied Biosystems, Carlsbad, CA, USA) was used to separate microsatellite fragments while to determine the size of alleles, the produced electropherograms were analyzed using STRand software (version 2.4.110, Veterinary Genetics Laboratory, Davis, CA, USA [97]).

The possible presence of null alleles and genotyping errors in the dataset was tested using MICROCHECKER software (version 2.2.3, University of Hull, Hull, UK [98]). Significant deviation from Hardy–Weinberg equilibrium (HWE) for each locus and fish group were tested using GENEPOP (version 4.7.5, University of Montpellier, Montpellier, France [99]). Microsatellite toolkit (version 3.1.1, Animal Genomics Lab, University College Dublin, Ireland [100]) was used to calculate observed (Ho) and expected (He) heterozygosity as well as the mean number of alleles across all loci (A). The mean effective number of alleles across loci (Ae) was calculated using POPGENE (version 1.32, University of Alberta, Alberta, Canada [101]). FSTAT (version 2.9.4, UNIL, Lausanne, Switzerland [102]) was used to calculate inbreeding coefficient (F_{IS}) and allelic richness (Ar) and to perform an exact test for linkage disequilibrium between loci. Global and pairwise F_{ST} values were calculated in Arlequin (version 3.5.2.2, University of Bern, Bern, Switzerland, [103]) using 10,000 permutations to test for statistical significance. All multiple pairwise tests were adjusted using Bonferroni corrections [104].

To further analyze the genetic relationships among wild-caught fish groups, a Bayesian clustering analysis was performed using STRUCTURE software (version 2.3.4, Stanford University, Stanford, CA, USA [105]). The model-based clustering method was run assuming a number of K populations ranging from one to ten (maximum number of

groups examined). For each K, burn-in period was set to 100,000 interactions followed by 1,000,000 MCMC repetitions, using an admixture model without a priori structuring information. STRUCTURE results were analyzed with the CLUMPAK online software (Tel Aviv University, Tel Aviv, Israel [106]) to detect the best K value using the Evano method [107] and DISTRUCT feature [108] to visualize the optimal number of clusters. Finally, two same Structure analyses were also conducted to analyze the genetic relationships between reared and wild-caught fish of the same origin, one for the northwestern Aegean Sea and another for the Ionian Sea.

3. Results

3.1. Degree of Scale Regeneration (SRD)

Significant differences were found in the average degree of scale regeneration (SRD) among samples collected in different years within each geographic group of fish ($p < 0.05$, Kruskal–Wallis and Mann–Whitney U tests) (Figure 2A–D). More precisely, concerning the northwestern Aegean Sea, significant differences in the average SRD levels were observed between samples collected in 2015 and 2017 (Figure 2A). Regarding the Ionian Sea, significant deviations in SRD levels were detected among samples collected in 2014 and those from 2015 and 2018, as well as between samples taken in 2015 and 2017 and between 2017 and 2018 (Figure 2C). The average (±SD) SRD varied among groups from 34.6 ± 22% (East Aegean) to 52.7 ± 33.0% (northwestern Aegean) (Table 1). The proportion of wild-caught fish in the predetermined SRD groups also varied depending on their origin, ranging from 36.8% (northwestern Aegean) to 53.8% (East Aegean) for the group L30, 22.4% (northwetsern Aegean) to 34.6% (Ionian) for the group 31–60, and 13.8% (East Aegean) to 40.8% (northwestern Aegean) for the group M60 (Table 1, Figure S1).

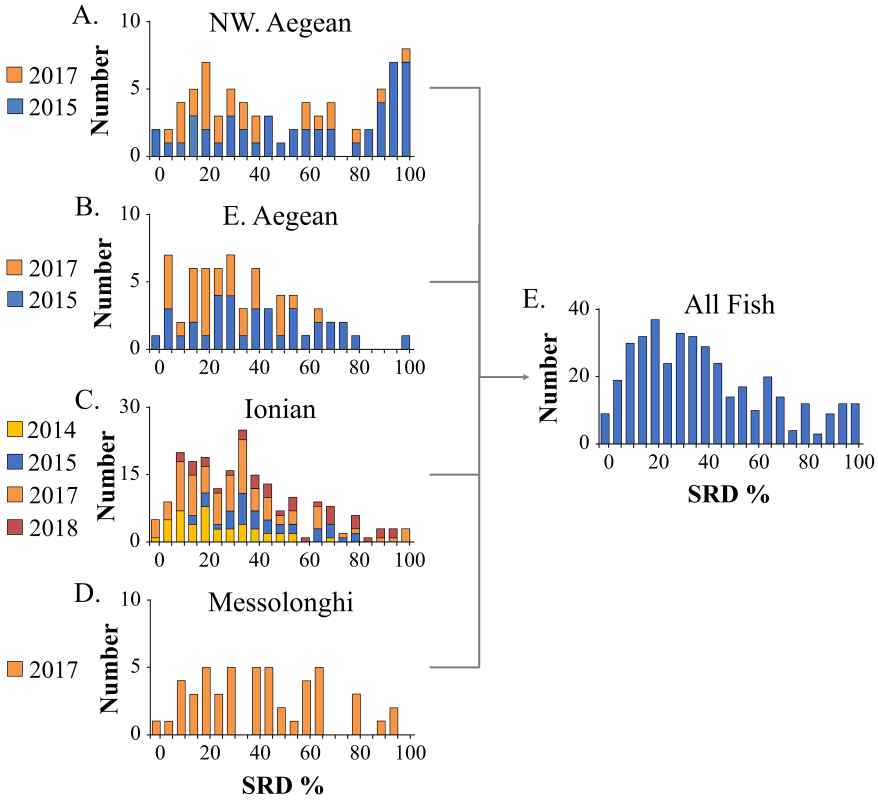

Figure 2. SRD (degree of scale regeneration) frequency distributions of wild-caught fish by geographic group (**A**–**D**) and regardless of fish origin (**E**). 2014–2018, sampling years. Number of fish per year for each geographic group is shown in Table S2.

Table 1. Characteristics of wild-caught fish samples, classified by their geographic origin and according to their SRD level. n: number of fish; SL_{mean}: average standard length (±SD, cm); SL_{range}: minimum and maximum standard length; SRD: average degree of scale regeneration (±SD, %). All: all wild-caught fish; L30, wild-caught fish with SRD ≤ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%.

		NW. Aegean	E. Aegean	Ionian	Messolonghi
All	n	75	65	205	50
	SL_{mean}	19.1(2.0)	18.4(1.2)	17.8(1.9)	17.0(0.5)
	SL_{range}	13.8–23.7	15.5–20.9	13.8–24	16.2–18.5
	SRD	52.7(33.0)	34.6(22.1)	35.8(23.8)	41.6(24.7)
L30	n	28	35	99	22
	SL_{mean}	17.7(1.9)	18.3(1.2)	17.4(1.7)	17.0(0.4)
	SL_{range}	13.8–21.2	16.4–20.9	13.8–22.5	16.3–17.8
	SRD	17.5(9.0)	17.9(9.4)	16.7(8.5)	18.9(8.9)
31–60	n	17	21	71	17
	SL_{mean}	19.6(1.3)	18.6(1.3)	17.8(1.8)	17.0(0.5)
	SL_{range}	15.8–20.8	15.5–20.7	14.2–22.3	16.2–18.1
	SRD	46.8(9.8)	45.7(7.6)	42.0(7.7)	48.2(7.9)
M60	n	30	9	35	11
	SL_{mean}	20.2(1.7)	18.3(0.9)	18.9(2.1)	17.0(0.6)
	SL_{range}	15.9–23.7	16.8–19.8	13.8–24.0	16.4–18.5
	SRD	87.7(12.3)	73.6(11.5)	77.2(12.3)	76.8(12.5)

3.2. Otolith Shape

For both left and right sides of the body, PERMANOVA showed that fish origin had a significant effect on otolith shape (Table 2). The effect of SRD level was not statistically significant for both the left and right otoliths (Table 2). Finally, the interaction between fish origin and SRD was significant for the right but not the left otolith (Table 2).

Table 2. Results of PERMANOVA testing for differences in otolith shape between wild-caught fish of different geographical origins (NW. Aegean, E. Aegean, Ionian, Messolonghi) and SRD levels (L30, 31–60, M60). Different tests were performed for each body side (left, right). Number of permutations = 9999.

Otolith	Source	Type III Some of Squares	df	Mean Squares	Pseudo-F	*p* (perm)
Left	Origin	0.030	3	0.010	5.630	<0.001
	SRD	0.004	2	0.002	1.141	0.296
	Origin:SRD	0.015	6	0.003	1.453	0.054
	Residuals	0.626	357	0.002		
	Total	0.681	368			
Right	Origin	0.057	3	0.019	8.441	<0.001
	SRD	0.004	2	0.002	0.947	0.471
	Origin:SRD	0.023	6	0.004	1.692	<0.01
	Residuals	0.805	359	0.002		
	Total	0.887	370			

For the right otolith, following the PERMANOVA model of interaction between fish origin and SRD, pairwise comparisons showed that fish with low and moderate levels of scale regeneration (L30 SRD and 31–60 SRD) from the northwestern Aegean Sea and the Messolonghi lagoon had significantly different otolith shapes compared to the other geographic groups (Table 3). However, there were no significant differences in otolith shape between fish from the East Aegean and the Ionian Sea (Table 3). In individuals with SRD levels > 60% (M60 SRD), the only significant difference in otolith shape was

observed between fish from the Ionian Sea and those from the northwestern Aegean Sea and Messolonghi lagoon (Table 3). Finally, when we tested for differences in otolith shape between fish with different SRD levels within each geographic location, significant differences were found between fish with M60 SRD and those with L30 or 31–60 SRD in the northwestern Aegean and Ionian Seas (Table S3).

Table 3. Results of pairwise tests (*t*-tests) for differences in otolith shape between fish with different geographical origin (*Right otolith*). L30: wild-caught fish with SRD (degree of scale regeneration) ≤30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60% Number of permutations = 9999. ns, p (perm) > 0.05. * p (perm) < 0.05. ** p (perm) < 0.01. *** p (perm) < 0.001. Number of fish otoliths per group is shown in Table S5.

L30	NW. Aegean	E. Aegean	Ionian	Messolonghi
NW. Aegean	-	*	**	**
E. Aegean	1.534	-	ns	***
Ionian	1.992	1.059	-	**
Messolonghi	2.337	2.327	2.137	-
31–60	NW. Aegean	E. Aegean	Ionian	Messolonghi
NW. Aegean	-	***	***	**
E. Aegean	2.564	-	ns	*
Ionian	3.223	0.896	-	**
Messolonghi	1.978	1.729	2.048	-
M60	NW. Aegean	E. Aegean	Ionian	Messolonghi
NW. Aegean	-	ns	***	ns
E. Aegean	1.303	-	ns	ns
Ionian	2.044	1.117	-	**
Messolonghi	1.397	1.344	1.866	-

The canonical analysis of principal coordinates (CAP) showed that otolith shape differentiation among fish with a different geographical origin increased with the increased level of SRD for both body sides (31–60 and M60; Figure 3). All CAP analyses presented highly significant permutation statistics (for details see Appendix A, Tables S4 and S5).

In the case of the left side of the body, for the fish with an L30 SRD, the first canonical axis (squared canonical correlation of CAP1 $\delta_1{}^2 = 0.776$) separated the geographic groups of the Aegean (NW. Aegean and E. Aegean groups) from those of the Ionian Sea (Ionian and Messolonghi lagoon groups) while the second canonical axis (CAP2) was not related to any meaningful geographical differentiation (Figure 3). In fish with a 31–60 SRD, CAP1 (squared canonical correlation $\delta_1{}^2 = 0.672$) separated the fish group of the NW. Aegean Sea from the other groups, whereas CAP2 was also not related to any meaningful geographical differentiation. In fish with an M60 SRD, CAP1 (squared canonical correlation $\delta_1{}^2 = 0.657$) separated the group of the Messolonghi lagoon from the other geographic groups, and CAP2 separated the groups of the Aegean from those of the Ionian Sea (Figure 3).

In the case of the right side of the body, for the fish with an L30 SRD, the first (squared canonical correlation of CAP1 $\delta_1{}^2 = 0.454$) and second (CAP2) canonical axis did not reveal any meaningful geographical differentiation between groups. For the fish with a 31–60 SRD, CAP1 (squared canonical correlation of CAP1 $\delta_1{}^2 = 0.746$) discriminated the fish group of the northwestern Aegean from the groups of the Ionian and E. Aegean Sea (Figure 3) while CAP2 was related to the differentiation between the group of Messolonghi and the other geographic groups. Finally, in fish with an M60 SRD, CAP1 (squared canonical correlation $\delta_1{}^2 = 0.687$) separated the group of Mesolonghi lagoon from the groups of the Ionian and northwestern Aegean Sea (Figure 3), whereas CAP2 separated the fish of the northwestern Aegean from the fish of the Ionian Sea (Figure 3).

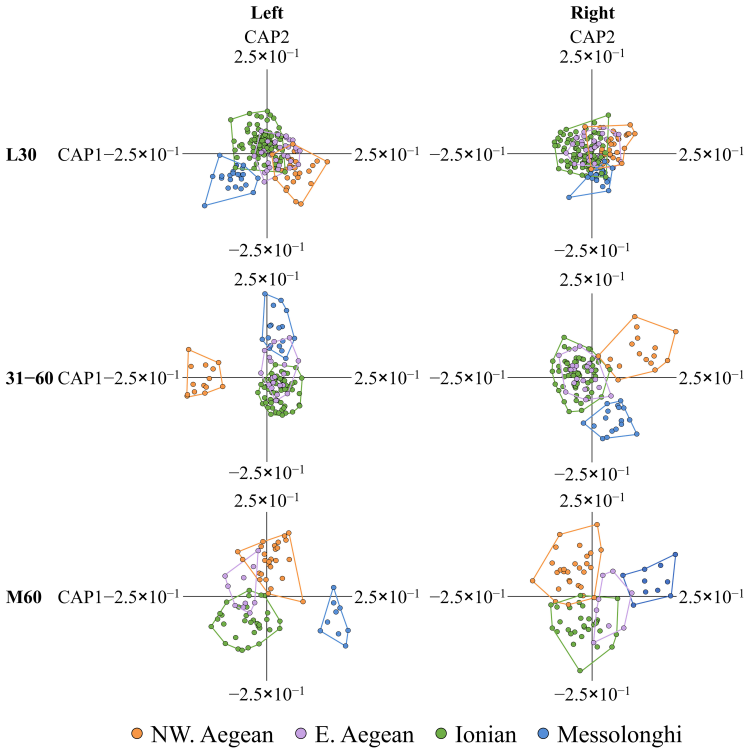

Figure 3. Plots of scores on the first two CAP axes (CAP1-2) to visualize otolith shape differences among geographic groups. L30: wild-caught fish with degree of scale regeneration (SRD) $\leq$ 30%; 31–60: fish with SRD 31–60%; M60: fish with SRD > 60%. Number of fish otoliths per group is shown in Table S5.

3.3. Otolith Bilateral Asymmetry

The asymmetry of the otolith perimeter and harmonic 3 showed a significant correlation with the fish SL ($p < 0.01$, not shown). Thus, a ratio transformation was applied prior to the calculation of the FA_1 index for these two otolith traits, with (R-L) values divided by (R + L)/2 [91]. Antisymmetry was absent, with all kurtosis estimates (g2) being either non-statistically different from zero or significantly positive (Table 4). Interestingly, directional asymmetry was detected for three of the otolith size descriptors, O_S, O_P, and O_L (skew significantly negative, R < L), and the shape descriptors H_2 and H_5 (skew significantly positive, R > L) (Table 4). However, fluctuating asymmetry indices based on signed R-L values (e.g., FA_1) are not biased by skewed distributions [88].

Table 4. Estimates of skew (g1) and kurtosis (g2) of otolith asymmetry values (R-L) for surface (O_S), perimeter (O_P), maximum length (O_L), maximum depth (O_D), and harmonics 2–5 (H_2–H_5). SE: standard error, t_s: t-statistic, n: sample size. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

R−L	n	Skew (g1)	Kurtosis (g2)	SE (g1)	SE (g2)	t_S (g1)	t_S (g2)
O_S	357	−0.417	2.074	0.129	0.258	−3.23 **	8.04 ***
O_P	357	−0.529	2.979	0.129	0.258	−4.09 ***	11.55 ***
O_L	357	−0.294	0.845	0.129	0.258	−2.28 *	3.28 **
O_D	357	0.028	0.159	0.129	0.258	0.22	0.62
H_2	357	2.245	16.111	0.129	0.258	17.37 ***	62.48 ***
H_3	357	0.060	0.993	0.129	0.258	0.47	3.85 ***
H_4	357	0.116	1.553	0.129	0.258	0.89	6.02 ***
H_5	357	0.273	1.297	0.129	0.258	2.11 *	5.03 ***

Otolith asymmetry analysis within each geographic location revealed that the degree of scale regeneration (SRD) had a significant effect on otolith shape fluctuating asymmetry (FA)

in three out of the four geographic groups (northwestern Aegean, Ionian, and Messolonghi). Specifically, fish of M60 SRD exhibited significantly higher FA_1 in the shape descriptors H_2 and/or H_4 when compared to the other SRD categories (L30 and 31–60 SRD; Figure S2).

The geographic origin of the fish had a significant effect on the otolith FA. In individuals with an L30 SRD, depending on the descriptor examined, the fish from Messolonghi lagoon displayed significantly higher (i.e., H_2) or lower (i.e., H_4) otolith asymmetry compared to other groups (i.e., northwestern Aegean and Ionian groups; Figure 4). Additionally, the shape descriptor H_2 exhibited significantly higher fluctuating asymmetry in fish from the Ionian Sea compared to the northwestern Aegean Sea (Figure 4). In individuals with a 31–60 SRD, for the descriptors O_L and H_3, FA_1 was significantly higher in the northwestern Aegean Sea than in the Messolonghi lagoon (Figure 4). Furthermore, in fish with an M60 SRD, the shape descriptor H_2 exhibited significantly higher FA in the Ionian Sea compared to the other geographic groups (Figure 4).

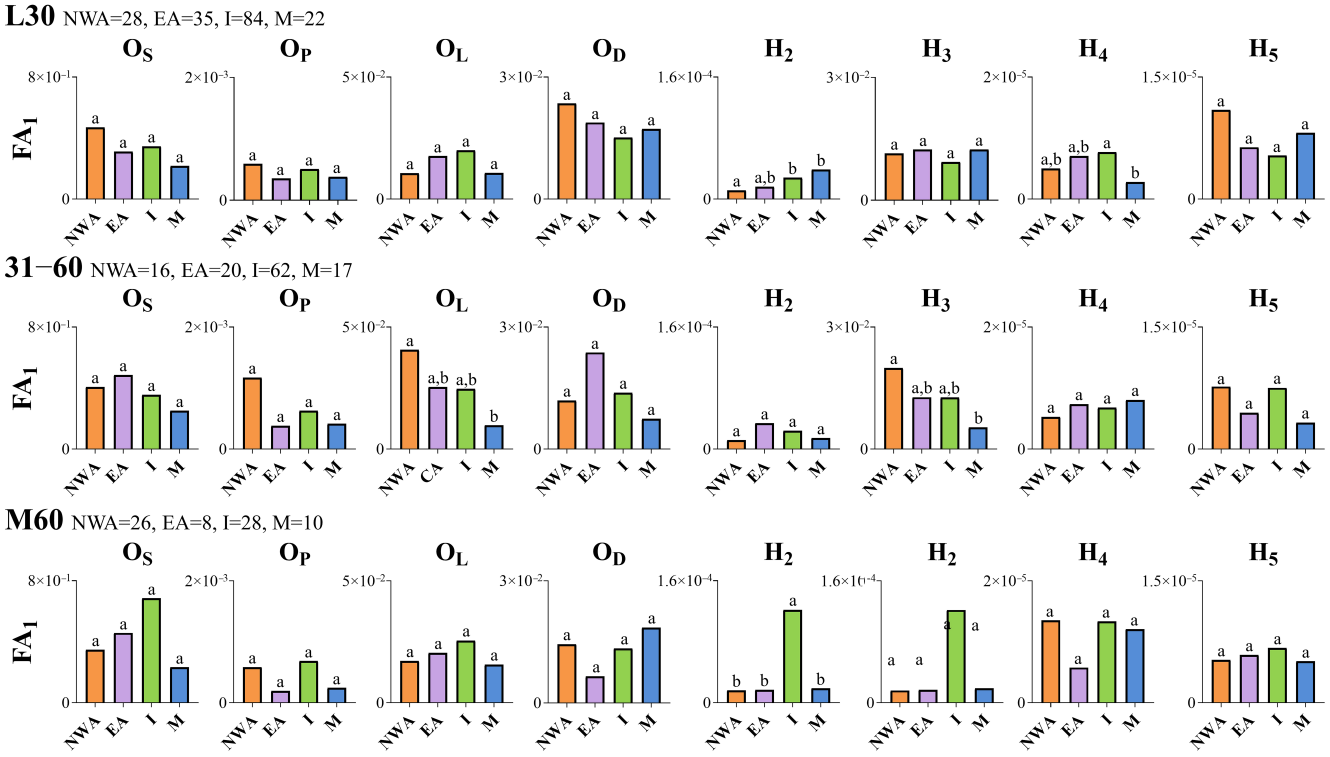

Figure 4. Comparison of the index FA_1 among wild-caught fish of different geographical origins. NWA, northwestern Aegean Sea; I, Ionian Sea; EA, East Aegean Sea; M, Messolonghi lagoon. L30: wild-caught fish with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: fish with SRD 31–60%; M60: fish with SRD > 60%. O_L: maximum otolith length; O_D: maximum otolith depth; O_S: otolith surface area; O_P: otolith perimeter; H_2–H_5: harmonics 2–5. Values without a common letter are statistically different ($p < 0.05$, Bonferroni-corrected F-tests). Numerical values next to the SRD categories represent the number of fish otolith pairs per geographic group.

3.4. Genetic Diversity

A total of 286 wild-caught fish and 55 reared fish were successfully genotyped at 9 microsatellite loci with no missing genotype within the dataset. Micro-Checker did not detect null alleles or genotypic errors using 95% confidence intervals. Between all pairs of loci, there was no consistent evidence of linkage disequilibrium following a strict Bonferroni correction for multiple tests. All fish groups appeared to be in Hardy–Weinberg equilibrium ($p > 0.05$, Fisher's exact test).

Genetic diversity varied among wild-caught fish groups of different geographical origins and degrees of scale regeneration (SRD), ranging from 8.67 to 16.11 in the number of alleles (A), 6.70 to 9.42 in the effective number of alleles per locus (Ae), 8.54 to 9.08 in allelic richness (Ar), as well as 0.85 to 0.92 and 0.85 to 0.88 for the observed (Ho) and expected (He) heterozygosity, respectively (Table S6). Summarizing the results in respect to SRD levels (Table 5), it was obvious that there was a clear negative correlation between the SRD levels and the magnitude of the genetic parameters A, Ae, and Ar of the different groups. Among wild-caught groups, the fish with the lower SRD (L30) exhibited the highest values of the genetic parameters, whereas the fish with the higher SRD (M60) exhibited the lowest values.

Table 5. Summary statistics for genetic variation of wild-caught and reared Gilthead seabreams, grouped according to SRD level and expressed as average values among the different geographic regions. n: fish number; A: average number of alleles; Ae: effective number of alleles; Ar: allelic richness; Ho and He: observed and expected heterozygosity; F_{IS}: fixation index. L30: wild-caught fish with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%; NWAR: reared fish from the northwestern Aegean Sea; InR: reared fish from the Ionian Sea.

Fish Group	n	A	Ae	Ar	Ho	He	F_{IS}
L30	129	14.85 ± 6.09	8.82 ± 4.82	8.77 ± 2.66	0.88 ± 0.02	0.86 ± 0.03	−0.02
31–60	91	13.41 ± 5.52	8.21 ± 4.13	8.79 ± 2.78	0.89 ± 0.02	0.86 ± 0.03	−0.03
M60	66	11.78 ± 5.09	7.98 ± 4.10	8.75 ± 2.91	0.88 ± 0.03	0.87 ± 0.02	−0.02
NWAR	27	10.44 ± 4.33	6.46 ± 2.99	7.39 ± 2.24	0.84 ± 0.02	0.83 ± 0.03	−0.02
InR	28	9.78 ± 3.6	5.43 ± 2.43	7.08 ± 2.03	0.80 ± 0.03	0.79 ± 0.03	−0.01

Reared Gilthead seabreams exhibited lower genetic diversity in A, Ae, Ar, Ho, and He compared to wild-caught counterparts for both regions (northwestern Aegean and Ionian Seas), whereas the reared fish from the Ionian Sea showed the lowest values in all the examined genetic parameters compared to the northwestern Aegean reared fish (Tables 5 and S6).

3.5. Genetic Structure and Individual Assignment

Considering only the wild-caught groups, the global F_{ST} exhibited a null value (−0.00045, $p > 0.05$), indicating a lack of genetic differentiation among geographic groups and SRD levels. This result was supported by the respective pairwise F_{ST} estimations, analyzed either as an origin within SRD groups (Table S7) or vice versa (Table S8).

Taking into account wild-caught (with different SRD levels) and reared groups, global F_{ST} values showed 0.008 ($p < 0.001$) and 0.014 ($p < 0.001$) for the northwestern Aegean and Ionian Sea, respectively, revealing slight indication of genetic differentiation between wild-caught and reared fish. Pairwise F_{ST} analysis revealed statistically significant differences only between reared and wild-caught fish groups (L30, 31–60, M60) for both the northwestern Aegean and Ionian Seas. ($p < 0.01$, Table 6). It is worth noting that the F_{ST} values of the Ionian reared group was almost double that of the northwestern Aegean in all pairwise comparisons.

Clustering analysis for all the wild-caught fish suggested the presence of 6 clusters (K = 6) as determined by the ΔK procedure (Figure 5A). Fish groups were assigned to the six clusters with equal distribution and proportion of individual membership in each cluster (16.7%).

Table 6. Pairwise F_{ST} (lower triangle) and the respective levels of statistical significance (upper triangle) among reared and wild-caught fish from the northwestern Aegean and the Ionian Sea, respectively. L30: wild-caught fish with SRD (degree of scale regeneration) ≤ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%; ns: $p > 0.05$, * $p < 0.05$. Number of fish per group is shown in Table S6.

NW. Aegean	L30	31–60	M60	Reared
L30	-	ns	ns	*
31–60	0.0027	-	ns	*
M60	−0.0072	0.0016	-	*
Reared	0.0160	0.0189	0.0143	-
Ionian [1]	L30	31–60	M60	Reared
L30	-	ns	ns	*
31–60	0.0004	-	ns	*
M60	0.0003	−0.0018	-	*
Reared	0.0399	0.0395	0.0374	-

[1] Ionian group includes wild-caught fish from both the Ionian Sea and Messolonghi lagoon.

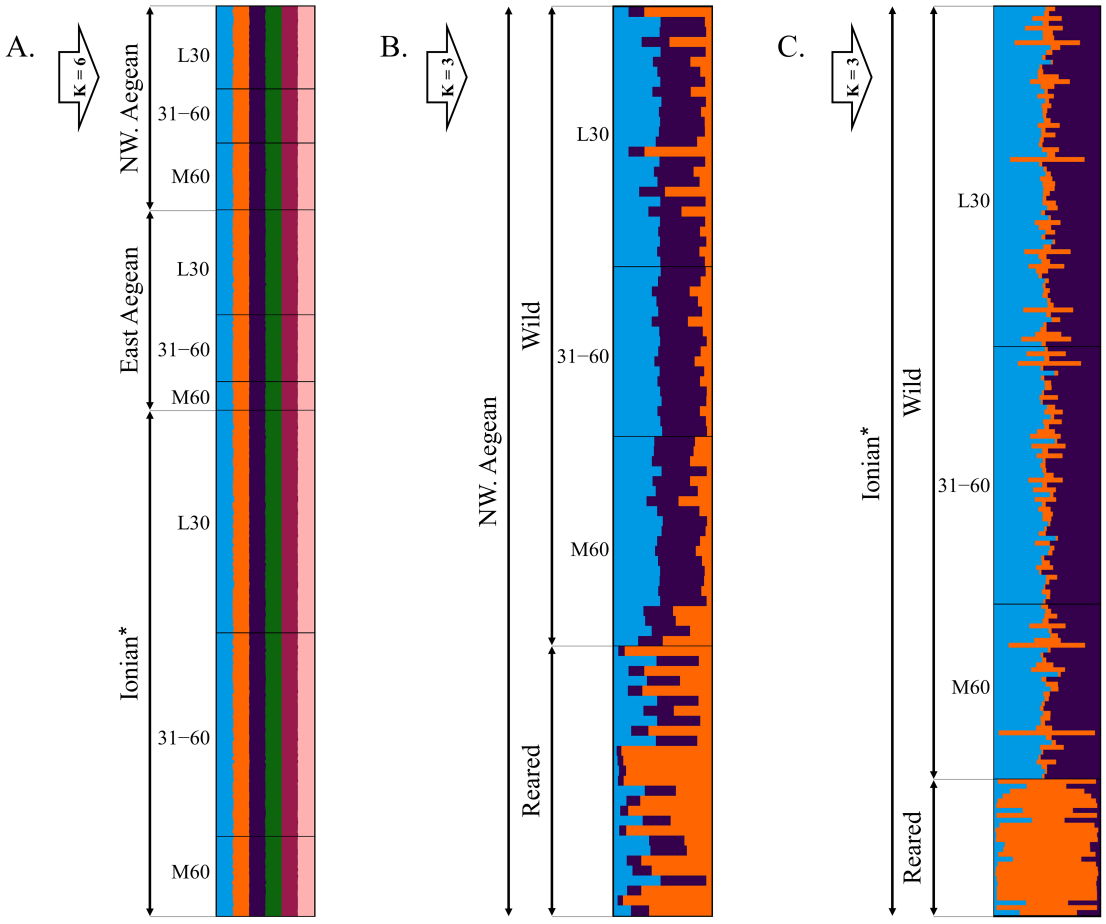

Figure 5. Bayesian clustering analysis of Gilthead seabream genotypes at 9 neutral microsatellite loci. (**A**): all wild-caught fish form six clusters (K = 6); (**B**,**C**): reared and wild-caught fish form three clusters (K = 3) for both the northwestern Aegean (NW. Aegean) and Ionian Seas. Black horizontal lines divide individuals belonging to different groups based on their degree of scale regeneration (SRD) and/or based on their wild or reared origin. L30: wild-caught fish with SRD ≤ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. * Ionian group includes wild-caught fish from both the Ionian Sea and Messolonghi lagoon. Number of fish per group is shown in Table S6.

The results of the clustering analysis for the reared and wild-caught fish from both the northwestern Aegean and Ionian Seas revealed that the optimal number of clusters (K) was three (Figure 5B,C). The wild-caught fish groups of L30, 31–60, and M60 SRD were assigned to two separate clusters, with a total proportion of membership to these clusters ranging from 82.6% to 90.2% for both seas. The third cluster was associated with a reared origin, with an average individual membership proportion of 57% and 82.1%, for the reared fish from the northwestern Aegean and Ionian Sea, respectively (min: 5.1%, max: 97.6%). Additionally, 16 out of 64 northwestern Aegean (7 from L30, 2 from 31–60 and 7 from M60) and 27 out of 159 Ionian (13 from L30, 8 from 31–60 and 6 from M60) wild-caught individuals were not effectively assigned to clusters associated with wild origin, resulting in total individual membership proportions to these clusters of less than 82%.

4. Discussion

In the present study, otolith shape and asymmetry analyses of wild-caught fish with a low possibility of being aquaculture escapees (L30 SRD group) revealed a phenotypic discrimination between northwestern Aegean and Ionian Gilthead seabream populations. However, a genetic analysis indicated an absence of genetic differentiation among the studied geographic fish groups. The otolith shape is a popular tool used for the discrimination among fish populations (e.g., [53,109,110]) with significant value as an indicator of stock identity (e.g., [47,58]). The otolith shape has been shown to be affected by both biotic and abiotic environmental drivers during fish development, including water temperature [60,111–113], habitat depth [114], type of substrate [115], acidification levels [116], food quantity [117], and food composition [55,112]. Geladakis et al. [35] suggested that observed phenotypic variability among wild fish stocks of the European sardine (*Sardina pilchardus* Walbaum, 1792) could be attributed to differences in temperature and food availability between the Aegean and Ionian Seas. In Gilthead seabreams, the otolith shape was significantly different between distant wild populations of Greece and Spain [40] or nearby populations with and without association with aquaculture farms in the Adriatic Sea [46]. The lack of otolith shape differences between fish from the East Aegean and Ionian Seas in this study may potentially indicate that these two geographically distinct seabream populations have experienced common environmental conditions during critical developmental stages. It should be noted here that previous studies on stock discrimination using otolith shape (e.g., [20,118]) have demonstrated that fish sampling conducted over broad temporal scales with unbalanced sample sizes has the potential to influence the results obtained because the prevailing environmental conditions could affect otolith growth rates, thereby inducing otolith shape alterations [51,56,119]. In this study, fish sampling was largely opportunistic, especially in the Ionian Sea, covering several years and different sampling sites and/or seasons. Unfortunately, with the exception of lagoon fisheries, such as the Messolonghi trap fishery, there is no other fishing practice in Greece targeting Gilthead seabreams at sea because the species has low abundance and is only caught occasionally as bycatch of bottom trawls and passive nets. Consequently, in order to ensure an adequate number of fish for the between-areas comparisons, we pooled all samples available from the broad geographic areas considered regardless of year, site, and season of collection. Sampling over broad temporal scales to acquire adequate sample sizes was also the approach followed in several other wild seabream population surveys in the past (e.g., December 2008–March 2009 [17], July 2015–January 2017 [18], October–December in 2015 and 2016 [45,46], July 2009–June 2010 [15,39,40], 2001–2014 [6]. Pooling data over large temporal and/or spatial regimes integrates temporal and/or areal heterogeneity in otolith shape, and the results of the analyses represent average conditions. However, in discriminating putative stocks, adequate and balanced sample sizes may not be possible in synoptic surveys for wild seabream. Thus, given no alternative, the pooling of data is a usual and accepted approach (e.g., [6,15,17,39,40,45,46]). In an attempt to assess the effect on fish otolith shape of sampling at several occasions (different years and/or months), a PERMANOVA analysis was carried out for each of the four geographical areas considered,

with a sampling survey (Year × Month combination) as a fixed factor (analyses not shown). PERMANOVA results and subsequent pair-wise comparisons showed that the effect of the sampling survey was only significant in the northwestern Aegean (for the right otolith) and the Ionian Sea (for both right and left otoliths). However, subsequent pairwise comparisons revealed that the samples that significantly differed in each of these two areas were those with a high proportion of fish with >60% regenerated scales (i.e., two samples: NW. Aegean, November 2015, 36.3% M60 fish; Ionian Sea, February 2018, 76.9% M60 fish [Table S2]). This finding suggests that differences in otolith shape among samples collected in the same geographical area could be primarily attributed to the increased incidence of potential aquaculture escapees in certain samplings rather than a year and/or seasonal effect.

A genetic analysis revealed a higher genetic variation in wild compared to the reared Gilthead seabream populations. In agreement with our results, previous studies that assessed genetic variation using microsatellite markers found similar levels of observed and expected heterozygosity, mean number of alleles, and allelic richness in wild and reared seabream populations throughout Greece [5,14,15,120]. The absence of genetic structuring among the examined seabream samples indicates a genetic homogenization between the Aegean and Ionian Sea populations. Depending on the set of markers used, the existing literature suggests either an absence of genetic structure or a subtle genetic subdivision (F_{ST} = 0.009 [5], F_{ST} = 0.002 [14]). A recent geographically broad study by Maroso et al. [6] within the Mediterranean Sea and Atlantic Ocean revealed a weak genetic subdivision (F_{ST} = 0.007) of wild Gilthead seabreams between the Aegean and Ionian Seas using 1159 genome-wide SNP markers. All these results combined with the absence of discernible physical or ecological barriers along with the long-lasting larval stage of Gilthead seabreams (~50 d [12,19]), enable high dispersal capacity, and support the exchange of individuals between populations of the Aegean and Ionian Seas, ultimately leading to a homogenized genetic pool.

In Greece, there has been an intensive rearing of Gilthead seabreams over the past three decades with rapid expansion in the previous decade [14]. There is a continuous concern that this expansion of aquaculture activity has increased the likelihood of accidental escape events, which could potentially threaten the genetic status of wild fish populations and lead to unknown consequences for their fitness and survival. Reared fish may exhibit large genetic differences from adjacent wild populations mainly due to the different genetic background of broodstocks (i.e., different broodstock origins) or due to the application of selective breeding programs that aim to enhance specific traits [5,15,23]. Our results (F_{ST} = 0.014–0.038) are in agreement with the existing literature, which has mainly shown weak genetic divergence between reared and wild Gilthead seabream populations along its distribution range, with F_{ST}s ranging between 0.006–0.069 [3,5,15,18,121,122]. The genetic admixture of escapees with local wild populations could potentially introduce foreign DNA into the wild gene pool and damage local adaptations, but this stands for species with strong genetic structure like salmonids (F_{ST} = 0.034–0.2, [123–125]). For such species, previous studies have shown that hybrids resulting from crosses between farmed and wild individuals could exhibit lower fitness (e.g., [126]) or relative survival (e.g., [127]) compared to native fish. However, following Sewall Wright's guideline, F_{ST}-values between 0 and 0.05 indicate "no to little genetic differentiation", F_{ST}-values between 0.05 and 0.15 represent moderate differentiation, F_{ST}-values between 0.15 and 0.25 are considered large, and F_{ST}-values above 0.25 show a very large degree of genetic differentiation [128–132]. It is evident that this is not the case for Gilthead seabreams, which exhibit weak genetic structure with no signs of local adaptations in its populations, mainly due to the high dispersal capability of the species (a long-lived pelagic larval stage [12,19,22]).

Scales have been utilized as a cost-effective and easily applicable morphological trait for identifying aquaculture escapees in the wild. In aquaculture facilities, friction and physical collisions among fish are intensified due to handling manipulations (e.g., counting, size-grading, and vaccination) and high population densities within sea-cages, leading to significantly high rates of scale loss [73,133,134]. Lost scales are promptly

replaced by new (regenerated scales), characterized by the presence of a large regeneration nucleus lacking growth *circulii* [74,135,136]. Based on our results on the otolith shape and asymmetry analyses, fish with a high degree of scale regeneration (31–60 and M60 SRD groups) exhibited increased otolith shape variability in comparison to the L30 group. Furthermore, fish with an M60 SRD exhibited significant differences in otolith shape and higher asymmetry levels compared to other SRD groups. In Gilthead seabreams, reared fish (>94% SRD) display distinct otolith shapes and higher asymmetry levels than wild fish [41]. In addition, Geladakis et al. [41] showed that wild-caught fish with SRD levels greater than 30% exhibit intermediate otolith phenotypes between wild fish with lower SRD ($\leq$30%) and reared fish. Since scales are continuously lost during the rearing process [74], we suggest the 60% SRD as a minimum threshold, beyond which wild-caught fish have an increased possibility of including individuals that escaped from net pens near harvest sizes, thus presenting phenotypes closely similar to those of reared fish. Following our results, a positive correlation emerged between SRD levels and the magnitude of otolith shape and asymmetry variation, suggesting that SRD can not only be used as a reliable index for detecting the presence of aquaculture escapees in the wild, but also to provide insights into the timing of fish escape from aquaculture net pens. On the other hand, a negative correlation was revealed between SRD levels and the genetic diversity (present study). Considering the lower levels of genetic diversity of the reared fish, this is in concordance with the proposal of Geladakis et al. [41,74] that the possibility of the presence of escapees increases as the level of SRD increases, as well. Given the utility of the SRD as a tool for identifying the presence of aquaculture escapees in the wild, the observed variations in SRD levels among wild-caught fish collected across the different years within each geographic group could potentially indicate a transient modification in the proportion of escaped individuals in the wild populations over time.

In our study, significant deviations from bilateral symmetry were observed in relation to both the origin within SRD groups and vice versa. These deviations were not restricted to only fluctuating asymmetry (FA), but also included directional asymmetry. Directional asymmetry (DA) refers to a consistent preference of a character toward greater development on one specific body side [137]. In roundfish species, deviations from perfect bilateral symmetry in otolith morphology, expressed as FA or DA, have previously been observed at both early and later stages of fish development (e.g., [41,72,112,113,138–140]. Otolith FA is regularly associated with different environmental stressors, and it is considered a sensitive indicator of fish health, performance, and population fitness [65,72]. On the other hand, otolith DA has been suggested to have a genetic origin and/or be a phenotypic plastic response induced by environmental drivers, such as water temperature, indicating a functional adaptation of fish hearing against environmental perturbations [112,113]. In the current work, a spatial variation was observed in the magnitude of otolith asymmetry (expressed as FA or DA) between different geographic groups. Depending on the descriptor examined, in fish with an L30 and 31–60 SRD, the group from Messolonghi lagoon exhibited significantly higher or lower FA or DA compared to other geographic groups. In fish with an L30 SRD, the Ionian group displayed significantly increased otolith shape DA (i.e., in H_2 descriptor) compared to the northwestern Aegean fish group. This finding is consistent with a previous study by Mahé et al. [141], which also showed that the amplitude of otolith DA in bogue (*Boops boops* Linnaeus, 1758) varied geographically, suggesting the potential use of directional bilateral asymmetry in otolith shape as a new method for stock discrimination. The observed shape differences in the right, but not in the left, otolith of the examined wild-caught individuals may be the underlying mechanism of the DA (the right side larger than the left side concerning the shape descriptor H_2) induced by early environmental conditions, such as temperature [113]. Bilateral differentiation in the variability of otolith shape was also observed in Mahé et al. [140], with significant discrimination between geographical stock units of bogue with respect to the shape of the right otolith but not the left otolith. However, an open question remains regarding the potential mechanism leading to the bilateral differentiation in otolith biomineralization

processes during critical ontogenetic periods of fish and, therefore, the morphological differences between the left and right pair of otoliths.

During the late winter to early spring season (February to March), the Gilthead seabreams from the Ionian Sea temporarily enter the lagoon, which acts as a nursery ground, followed by migration to the open sea for breeding during October to December [76]. The current study indicates that during this brief lagoon period of the Gilthead seabream life cycle, the environmental conditions substantially influence the morphology of their otoliths in terms of both the shape and asymmetry. Coastal lagoons are key ecosystems with increased natural productivity and intensive fishing exploitation by local communities [75]. Fishing activity in these ecosystems is based on the seasonal entrance of young fish in the lagoons, where fish are then captured during their autumn-to-winter offshore migration [76]. The Messolonghi–Etoliko lagoon complex is the largest in Greece and one of the most important coastal lagoon systems in the Mediterranean Sea [142]. Because of the limited depth of the lagoons, meteorological changes rapidly affect the abiotic parameters of the water masses [143]. The interaction between environmental conditions and genetic background could be the underlying mechanisms of otolith shape variability through determination on the relative growth of the different otolith parts (allometry) during fish growth [53,144]. Through the prism of otolith shape plasticity, a brief period of residence in lagoon ecosystems seems to significantly affect the otolith growth trajectories of seabream individuals and, therefore, the resulting otolith morphogenesis.

5. Conclusions

The utilization of diverse methodologies to identify distinct fish stocks enhances our understanding of the attributes that vary among wild populations due to the influence of different environmental and/or genetic factors [31]. Although there is a lack of genetic differentiation among the examined seabream populations, our findings suggest a phenotypic discrimination between the northwestern Aegean and Ionian Gilthead seabream stocks. The combined use of otolith and scale morphology proved to be a useful approach to successfully examine the additive phenotypic variability induced by the presence of aquaculture escapees in the wild seabream stocks.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes8060291/s1. Table S1: Sample origin, year and month of collection. n, number of fish analyzed; Table S2: Sampling information per area, year, month and SRD group. n: number of fish analyzed. SLmean: average standard length (±SD, cm); Table S3: Results of pairwise tests (*t*-tests) for differences in otolith shape between wild-caught fish with different SRD (degree of scale regeneration) levels (Right otolith). L30: wild-caught fish with SRD $\leq$ 30%; 31–60: fish with SRD 31–60%; M60: fish with SRD > 60%. Number of permutations = 9999. ns, P (perm) > 0.05. * P (perm) < 0.05. ** P (perm) < 0.01. *** P (perm) < 0.001. Number of fish otoliths per group is shown in Table S5; Table S4: Average scores along the first two CAP axes (CAP1, CAP2) for wild-caught fish of different geographical origin. L30: wild-caught fish with SRD (degree of scale regeneration degree) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%; Table S5: Cross validation re-classification matrix of the canonical analysis of principal coordinates (CAP) on otolith elliptic Fourier data to reclassify wild-caught fish to their origin. L30: wild-caught fish with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. Different tests were performed for each body side (left, right); Table S6: Summary statistics for genetic variation of Gilthead seabreams per fish group. n: number of fish; A: average number of alleles; Ae: effective number of alleles; Ar: allelic richness; Ho and He: observed and expected heterozygosity; F_{IS}: fixation index. NWA: wild-caught fish from the northwestern Aegean Sea; EA: wild-caught fish from the East Aegean Sea; I: wild-caught fish from both the Ionian Sea and Messolonghi lagoon; L30: wild-caught fish with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. NWAR: reared fish from the northwestern Aegean Sea; IR: reared fish from the Ionian Sea; Table S7: Pairwise F_{ST} (lower triangle) and the respective levels of statistical significance (upper triangle) among wild-caught fish of different geographical origin. L30: wild-caught fish

with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. ns: $p > 0.05$. Number of fish per group is shown in Table S6; Table S8: Pairwise F_{ST} (lower triangle) and the respective levels of statistical significance (upper triangle) among wild-caught fish with different possibility to include aquaculture escapees. L30: wild-caught fish with SRD (degree of scale regeneration) $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. ns: $p > 0.05$. Number of fish per group is shown in Table S6; Figure S1: Pie charts illustrating the percentages of wild-caught fish with different SRD (degree of scale regeneration) levels. L30: wild-caught fish with SRD $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. Number of fish per group is shown in Table 1; Figure S2: Comparison of the index FA_1 among wild-caught fish of different SRD (degree of scale regeneration) levels. L30: wild-caught fish with SRD $\leq$ 30%; 31–60: wild-caught fish with SRD 31–60%; M60: wild-caught fish with SRD > 60%. NWA, northwestern Aegean Sea; EA, East Aegean Sea; I, Ionian Sea; M, Messolonghi lagoon. O_L: maximum otolith length; O_D: maximum otolith depth; O_S: otolith surface area; O_P: otolith perimeter; H_2-H_5: harmonics 2–5. Values without a common letter are statistically different ($p < 0.05$, F-test). Numerical values next to the geographic groups represent the number of fish otolith pairs per SRD category.

Author Contributions: G.G.: Investigation, Formal analysis, Visualization, Writing—original draft preparation. C.B.: Writing—review and editing, Visualization, Supervision. S.S.: Resources, Writing—review and editing, Visualization, Supervision. G.K.: Conceptualization, Resources, Writing—review and editing, Supervision. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The research conducted in this study exclusively involved fish that were captured and promptly sacrificed either for commercial purposes or as part of experimental sampling efforts conducted by the R/V PHILIA (experimental bottom trawl of Hellenic Centre for Marine Research, HCMR). No live animals were utilized in any capacity during our study. As part of our research, otolith measurements and tissue sampling for DNA extraction concerned fish that had already been sacrificed for commercial or experimental sampling purposes. Consequently, we maintain that obtaining Ethics Committee or Institutional Review Board approval is not necessary for the submission of our manuscript.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Regarding the left side of the body, in the case of wild-caught individuals with SRD levels $\leq$ 30% (L30), a selection of m = 32 was determined based on maximum (64.2%) correct reallocations (=35.8% misclassification error, Table S5). The canonical analysis resulted in three canonical axes (the first two are shown, Table S4). The first axis exhibited a squared canonical correlation of ${\delta_1}^2 = 0.776$ and yielded highly significant permutation statistics (trace statistic = 1.452, $p < 0.001$, ${\delta_1}^2 = 0.776$, $p < 0.001$). For wild-caught individuals with SRD levels between 31–60% (31–60), a choice of m = 22 was determined based on the maximum (58.1%) correct reallocations (=41.9% misclassification error, Table S5). The canonical analysis produced three canonical axes (Table S4), with the first axis demonstrating a squared canonical correlation of ${\delta_1}^2 = 0.672$ and highly significant permutation statistics (trace statistic = 1.207, $p < 0.001$, ${\delta_1}^2 = 0.672$, $p < 0.001$). Additionally, for wild-caught individuals with an SRD > 60% (M60), a selection of m = 17 was made based on maximum (64.6%) correct reallocations (=35.4% misclassification error, Table S5). The canonical analysis yielded three canonical axes (Table S4), with a squared canonical correlation of the

first axis $\delta_1^2 = 0.657$ and highly significant permutation statistics (trace statistic = 1.412, $p < 0.001$, $\delta_1^2 = 0.657$, $p < 0.001$).

In the case of the right side of the body, for the fish with an L30 SRD, a selection of m = 23 was made based on the maximum (58.3%) correct reallocations (=41.7% misclassification error, Table S5). The canonical analysis resulted in three canonical axes (Table S4), with the first axis exhibiting a squared canonical correlation of $\delta_1^2 = 0.454$ and highly significant permutation statistics (trace statistic = 0.970, $p < 0.001$, $\delta_1^2 = 0.454$, $p < 0.001$). For the fish with a 31–60 SRD, a choice of m = 31 was made based on maximum (65.8%) correct reallocations (=34.2% misclassification error, Table S5). The canonical analysis yielded three canonical axes (Table S4). The first axis demonstrated a squared canonical correlation of $\delta_1^2 = 0.746$ and highly significant permutation statistics (trace statistic = 1.710, $p < 0.001$, $\delta_1^2 = 0.746$, $p < 0.001$). Furthermore, for the fish with an M60 SRD, a selection of m = 23 was made based on maximum (60.5%) correct reallocations (=39.5% misclassification error, Table S5). The canonical analysis produced three canonical axes (Table S4), with the first axis demonstrating a squared canonical correlation of $\delta_1^2 = 0.687$ and highly significant permutation statistics (trace statistic = 1.564, $p < 0.001$, $\delta_1^2 = 0.\ 687$, $p < 0.001$).

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