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Towards the authentication of European sea bass origin through a combination of biometric measurements and multiple analytical techniques

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1 **Toward the Authentication of European Sea Bass Origin** 2 **through a Combination of Biometric Measurements and** 3 **Multiple Analytical Techniques**

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12 **ABSTRACT:** The authenticity of fish products has become an imperative issue for authorities
13 involved in the protection of consumers against fraudulent practices and market stabilization. The
14 present study aimed to provide a method for authentication of European sea bass (*Dicentrarchus*
15 *labrax*) according to the requirements for seafood labels (Regulation 1379/ 2013/EU). Data on
16 biometric traits, fatty acid profile, elemental composition, and isotopic abundance of wild and
17 reared (intensively, semi-intensively, and extensively) specimens from 18 southern European
18 sources ($n = 160$) were collected, clustered in six sets of parameters, and then subjected to
19 multivariate analysis. Correct allocations of subjects according to their production method, origin,
20 and stocking density were demonstrated with good approximation rates (94, 92, and 92%,
21 respectively) using fatty acid profiles. Less satisfying results were obtained using isotopic
22 abundance, biometric traits, and elemental composition. The multivariate analysis also revealed that
23 extensively reared subjects cannot be analytically discriminated from wild subjects.

24 **KEYWORDS:** seafood labeling, *Dicentrarchus labrax*, sea bass authentication, analytical
25 fingerprinting, stocking density, fatty acid profile, isotope analysis, elemental composition

26 **1. Introduction**

27 The demand for sea products for human consumption is in continuous expansion, with aquaculture
28 gaining importance in compensating for the deficit of global capture fisheries; (1) as a result, an
29 increasing number of new farmed species have entered the marketplace over the past decade. Public
30 interest in food quality and origin, in particular, the awareness of health benefits derived from fish
31 consumption, has strongly increased in recent years. Differentiation of seafood products may
32 influence consumer preferences, especially concerning discrimination between wild and farmed
33 fish. Actually, consumer choice between wild and reared products seems to be strongly affected by
34 beliefs resulting from stereotypes; for instance, cultured fish are usually associated with the use of
35 antibiotics and growth promoters, while wild products are considered tastier and healthier than
36 reared fish. (2) Thus, regulatory interventions aim to avoid mislabeling or substituting wild fish
37 with farmed specimens and to mitigate risks to consumer confidence and health.
38 In the European context, as a result of the variety of sources of fish products (from seawater or
39 freshwater; from intensive, semi-intensive, or extensive rearing), Regulation (EU) 1379/2013 (3)
40 was issued to give consumers clear and accurate information on the main features and traceability

41 of fish products. Because wild fish are generally put on the market at a higher price, there may be a
42 temptation to falsely label farmed products to sell them at a better price. The removal of specific
43 external traits during product processing makes the identification of fish species difficult, and the
44 risk of fraudulent substitution of sought-after species with less valuable species grows. Accidental
45 or intentional mislabeling may also occur due to the global production of seafood, which allows the
46 supply of the same products from different areas 4. Moreover, strong competition among the main
47 producing countries in the Mediterranean region (Italy, Greece, Spain, and Turkey, above all)
48 demands detailed characterization and differentiation of sea bass quality. (5) Verifying the
49 authenticity of fish products is therefore imperative, to check correspondence with the label,
50 establish the real commercial value, avoid unfair competition, and ensure consumer protection
51 against fraudulent practices.

52 European sea bass (*Dicentrarchus labrax*) represents one of the main aquaculture fish products in
53 the European Union (EU). (6) European sea bass is a demersal opportunistic species, inhabiting
54 coastal waters (down to about 100 m depth) and shallow waters, such as estuaries, lagoons, and
55 tidal flats. European sea bass is intensively farmed in floating cages or in inshore ponds and reared
56 semi-intensively or extensively in brackish lagoons. (7) The different rearing systems and feeding
57 regimes in sea bass farming may affect flesh quality, especially in terms of fat level and
58 composition, (8) which necessarily influences the quality of the product. (9,10) Wild sea bass feed
59 in inshore and estuarine waters, which offer dietary fatty acids (FAs) from the fauna of those areas,
60 whereas commercial feeds contain quite different FAs, typically fish products obtained from the
61 open oceans. (4) The lipid content of the flesh also influences the ratios of the stable isotopes of
62 carbon (C) and nitrogen (N); the former varies according to the nature of the dietary C sources as
63 well, while the latter depends upon the trophic position of the diet of the subjects. (11)
64 According to some authors, the employment of chemometric techniques, in support to a documental
65 routine, would be a useful tool to improve transparency and the trust of consumers in the food trade.
66 To date, the most promising approach to characterize a fish product seems to be the application of
67 different analytical techniques on the same matrix, followed by multivariate analysis of the data
68 obtained. (12) The multivariate analysis applied to the FA profiles, obtained using gas
69 chromatography, (4,10,12,13) nuclear magnetic resonance (NMR), (14) or near-infrared
70 spectroscopy (NIRS), (15) has been previously used to authenticate wild and farmed sea bass as
71 well as the geographical origin. Other marine species have also been authenticated by employing
72 stable isotope analysis (16) or coupling the stable isotope ratio with multi-element analyses, (17) to
73 assess origin and production method and even characterize the species. Biometric parameters are
74 often used to authenticate sea bass type of production, because they are the easiest, quickest, and
75 cheapest techniques and do not require laborious or expensive equipment or expert knowledge or
76 specialization. (12) Elemental composition of fish flesh is generally used as a chemical signature of
77 the particular water body where the subject has grown, because marine fish incorporate different
78 trace elements, from the environment and the diet, into their skeletal tissues and organs. (18) The
79 variation on elemental compositions has also been investigated in otoliths of different fish species,
80 to distinguish wild from farmed environments; however, divergent results were obtained as a result
81 of an interannual variability in elemental composition of otoliths, which confounded the origin
82 determinations of subjects. (19) The use of molecular genetic markers is considered by Brown et al.
83 the most suitable and informative tool for discrimination of wild and farmed fish, because they are
84 not influenced by the age of the subject. (20) Unfortunately, these techniques are among the most
85 expensive and time-consuming currently available, making them unavailable to many sectors. (19)
86 Previous studies have assessed the capacity of different analytical techniques to distinguish wild
87 from farmed sea bass (10) and to determine their geographical origin, (4) although these
88 preliminary investigations examined a limited number of sources and/or subjects, sampled in a
89 limited time span. The findings of these studies highlighted the difficulty in characterizing the
90 farmed subjects, (10) suggesting the need to introduce new variables and apply this method on a

91 wide range of fish samples from various geographical locations, (4) considering the interseasonal
92 variability for improving the robustness of the models. (10)
93 Prompted by all of these considerations, the present study aimed to provide a method for
94 authentication of the origin of European sea bass specimens by combining data from a number of
95 chemical analyses. Being one of the main European aquaculture products, European sea bass (*D.*
96 *labrax*) was chosen as a result of the availability of multiple product typologies in the
97 Mediterranean market. For the first time, a vast number of samples have been analyzed, from
98 sources properly scattered during a 1 year sampling among the southern European area, from both
99 aquaculture and fishery sectors; moreover, a “stocking density factor” has been introduced as a
100 variable in the multivariate statistical analysis.

101 **2. Materials and Methods**

103 **2.1. Sampling Design**

104 Wild and farmed European sea bass subjects were sampled from 18 different Italian and foreign
105 sources. Batches of 10 specimens were ordered from every source, equally split into two
106 “macroseasons” (autumn–winter, AW; spring–summer, SS) to guarantee a perfect balance (equal
107 number of specimens from each macroseason for each source). The geographic distribution for each
108 sampling site is reported in [Figure 1](#). Wild subjects ($n = 45$) came from four main areas, three in the
109 Mediterranean Sea and one close to the French Atlantic coast. Intensively reared subjects (IR; $n =$
110 85) were collected from fish farms equipped with either floating or submersible cages; in two cases,
111 sea bass belonged to semi-intensive farms (SIR; $n = 20$), and in a single case, they came from an
112 extensive farm (ER; $n = 10$). [Table S1](#) of the Supporting Information reports the respective Food
113 and Agriculture Organization of the United Nations (FAO) fishing area, subarea, and division for
114 each fishing site, country of origin, locality of sea bass farms, as required by Regulation (EU)
115 1379/2013, equipment features, and stocking density for breeding. Because the studies of Xiccato et
116 al. (5) and Carbonara et al. (21) suggested that stocking density in production influences fish
117 composition, an additional categorical variable was used, taking one of the following labels: “0” for
118 wild specimens, “1” for extensively farmed specimens (up to 0.0025 kg m^{-3}), “2” for semi-
119 intensively farmed specimens (up to 1 kg m^{-3}), and “3” for intensively farmed specimens (up to 30
120 kg m^{-3}). Stocking density of production was recently recognized as a potential chronic stress factor
121 in several species of fish; high density of rearing can adversely affect the quality of the farmed
122 product, as a result of a negative effect on the fish growth rate and survival and feeding rates.
123 (21,22) Source allocation according to stocking density was based on the statements of individual
124 farmers, through the compilation of a special form attached to every consignment.

125

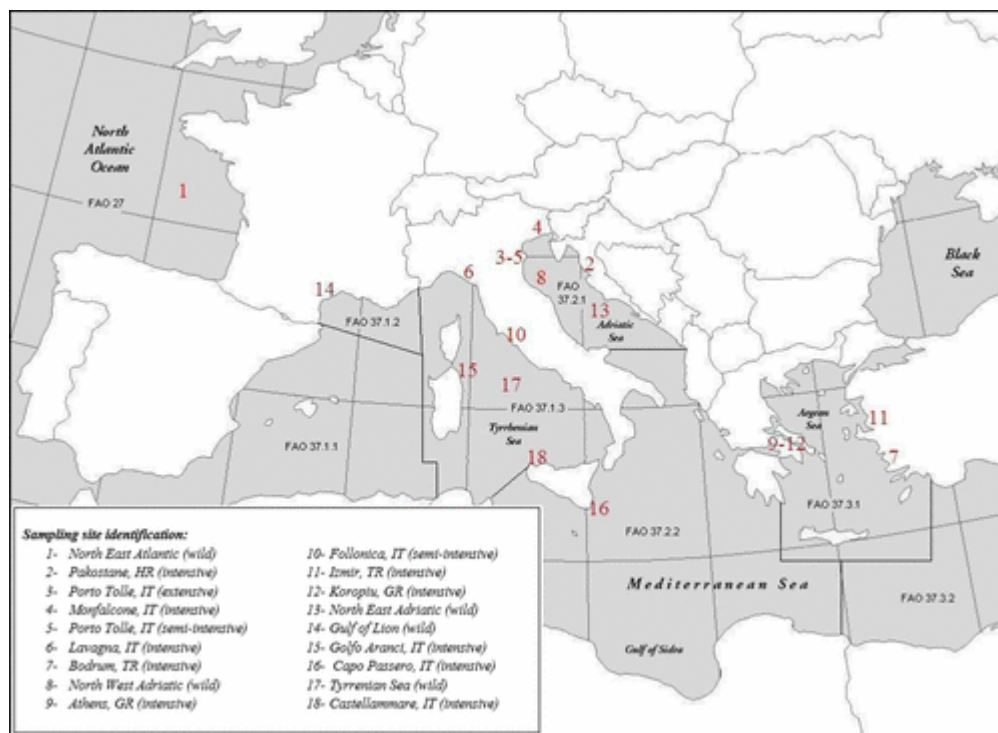
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128

129

Figure 1



131

132 Figure 1. Geographical distribution of the sampling sites, including the pertinent FAO fishing areas,
 133 subareas, and divisions.

134 2.2. Biometric Traits and Sample Preparation

135 Each of 10 subjects from every batch was analyzed following the United States Environmental
 136 Protection Agency (U.S. EPA) procedure for fish sampling. (23) Every subject, after measurement
 137 of weight and length, was eviscerated, and data on the weight of eviscerated fish, viscera, liver,
 138 gonads (if any), and perivisceral fat were recorded. Wild and reared subjects, both national and
 139 foreign, were purchased from large-scale retail trade, and they were of commercial size. Fillets from
 140 each subject were homogenized by means of a food processor (Multiquick System ZK 100, Braun,
 141 Kronbergim Taunus, Germany) and quartered by hand twice; thereafter, samples were put into
 142 plastic bags, compacted, then frozen, and stored at -20°C . The complete description of the
 143 procedure is reported in section 2.2 of the Materials and Methods in the Supporting Information.

144 2.3. Moisture Determination, Lipid Analysis, and FA Profile

145 The moisture content of flesh was measured following AOAC procedure 950.46B, (25) according
 146 to which 10 g of homogenized flesh was put into a porcelain crucible and heated at $100\text{--}102^{\circ}\text{C}$ for
 147 18 h to determine dry weight before calculating the moisture.

148 The extraction of total lipids (TL) was performed in duplicate for each sample, following a
 149 procedure proposed by Bligh and Dyer, (26) and, adopting some adjustments, briefly described as
 150 follows: around 4 g of homogenized fillets was put into a tube immersed in an ice bath and mixed
 151 with 18 mL of a solution of 1:1 chloroform/methanol for 1 min using an Ultra-Turrax T25 (IKA-
 152 Werke GmbH & Co., Staufen, Germany), and then 12 mL of a solution of 1:1 chloroform/deionized
 153 water was added to the sample and mixed. After centrifugation (4000 rpm at 4°C for 10 min), the
 154 lower phase containing chloroform and dissolved TL was separated and passed through a layer of

155 anhydrous sodium sulfate. A total of 1 mL of the lipid extract was completely desolvated on a hot
156 plate at 50 °C; the lipid residue was weighed; and the percentage of TL was calculated.
157 Quantification of phospholipids (PL) in TL was carried out by colorimetric determination of
158 phosphorus, following the procedure reported by Marinetti. (27) Briefly, 5 µg of TL-extracted
159 sample was mineralized with perchloric acid at 250 °C. Afterward, ammonium heptamolybdate and
160 1-amino-2-hydroxy-4-naphthalene sulfonic acid were added to the samples; they were kept at 100
161 °C for 7 min, until a colored complex was obtained. The absorbance of this complex was measured
162 at 830 nm to estimate the amount of phosphorus in the samples; PL quantification was obtained by
163 multiplying the calculated amount of phosphorus by 25.
164 Neutral lipids (NL) were separated from PL following a normal-phase solid-phase extraction (SPE)
165 method, described by Bayır et al., (28) which uses 12 mL Strata SI-1 silica (55 µm, 70 Å) columns,
166 with 2 g of substrate (Phenomenex, Torrance, CA, U.S.A.). Briefly, after equilibration with 3 mL of
167 chloroform, silica columns were loaded with the TL sample and diluted in 3 mL of chloroform.
168 After that, the NL fraction was separated by charging the cartridge with 3 mL of chloroform 8
169 times. After vacuum drying of the column, PL were eluted with four aliquots of 3 mL of methanol
170 and then four aliquots of 3 mL of a solution of 3:7 (v/v) chloroform/methanol.
171 Fatty acid methyl esters (FAMES) from TL, NL, and PL were obtained by transmethylation
172 involving sulfuric acid as the catalyst, following the method proposed by Christie. (29) Briefly,
173 samples were dried under a gentle nitrogen stream and diluted with 100 µL of toluene and 1 mL of
174 the methylating solution of 1% sulfuric acid in methanol (96% purity). Samples were kept at 50 °C
175 in a heater for 12 h. Then, 1 mL of 5% NaCl buffer and 900 µL of hexane were added to the
176 samples, which were successively vortex-mixed and centrifuged for 10 min at 2000 rpm. The
177 supernatant containing FAME was then collected and injected into a Varian 3380 gas
178 chromatograph (Agilent Technologies, Palo Alto, CA, U.S.A.) fitted with a CP-8200 Varian
179 autosampler, a split injector set at 230 °C, and a flame ionization detector system set at 300 °C.
180 Chromatographic separation of FAME was attained by means of a 30 m × 0.32 mm (inner diameter)
181 × 0.25 µm (film thickness) fused silica-bonded phase column (DB-23, J&W Scientific); the oven
182 temperature was programmed from 150 to 230 °C at a rate of 5 °C min⁻¹, with a final isotherm. FAs
183 were identified by comparing the retention times of unknown FAME to those of known FAME
184 standard mixtures, with their content being reported as a percentage of the sum of FAME (%
185 FAME) of TL, NL, and PL, respectively. The complete description of the procedures for moisture
186 determination, lipid analysis, and FA profile is reported in section 2.3 of the Materials and Methods
187 in the Supporting Information.

188 **2.4. Determination of Macro- and Microelements and Toxic Elements**

189 Mineralization was performed by adding 6 mL of nitric acid (67% purity, Ultrapure Merck,
190 Darmstadt, Germany) and 2 mL of hydrogen peroxide (31% purity, Ultrapure Merck) to 1 g of
191 sample, through a MULTIWAVE 3000 microwave system (PerkinElmer). Detection of the
192 elements was obtained by means of Optima 2100 inductively coupled plasma atomic emission
193 spectrometry (ICP-OES, PerkinElmer, Waltham, MA, U.S.A.). For determination of the
194 macroelements, a Meinhard cyclonic spray chamber was employed, with radial viewing
195 configuration. Microelements and toxic elements were determined with a CETAC U5000 ultrasonic
196 nebulizer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) in axial view configuration. The
197 complete description of the procedure is reported in section 2.4 of the Materials and Methods in the
198 Supporting Information.

199 **2.5. Stable Isotope Analysis**

200 Determination of C and N isotopic composition in sea bass muscle was carried out through the
201 procedure described below: 0.07 ± 0.01 and 0.7 ± 0.1 mg for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses, respectively,

202 of freeze-dried fillets (<0.6 mm grain size) were analyzed by means of an EA/NA-1100 elemental
 203 analyzer with CHN configuration (Thermo Finnigan), in helium continuous flow mode and coupled
 204 to a Finnigan Delta Plus XP mass spectrometer. Sample isotope ratios were then calculated
 205 according to international reference standard V-PDB for C and atmospheric nitrogen for N. The
 206 complete description of the procedure is reported in section 2.5 of the Materials and Methods in the
 207 Supporting Information.

208 **2.6. Quality of Analytical Data**

209 Analyses of samples were performed in duplicate, and data were constantly monitored by means of
 210 standard reference materials (SRMs). During analysis, each SRM was processed in duplicate 3
 211 times, following the same procedures. The accuracy and precision of the measurements, calculated
 212 on the basis of repeated analysis of SRMs, was $\pm 0.2\%$. The descriptions of the SMRs used are
 213 reported in section 2.6 of the Materials and Methods in the Supporting Information.

214 **2.7. Data Processing and Statistical Analysis**

215 MATLAB software was used for data processing, principal component analysis (PCA), and sample
 216 classification through discriminant analysis (MATLAB “classify” function embedded into ad-hoc-
 217 built scripts to apply cross-validation procedures and estimate classification performance).

218 Every source was characterized using biometric parameters and compositional characteristics of the
 219 entire sea bass fillets (as the edible part), grouped into the following sets of variables: biometric
 220 parameters, FA composition of TL, FA composition of NL, FA composition of PL, elemental
 221 composition, and isotopic abundances (Table 1). The whole set of measures, provided by each
 222 analytical technique, was statistically analyzed to obtain an “analytical signature” for every source;
 223 first of all, average, range, standard deviation, and coefficient of variation were calculated for each
 224 source. Therefore, for the automatic classification of samples, mono- and multivariate statistical
 225 analyses were employed, with the aim of predicting the production method (wild or farmed sea
 226 bass), geographical origin (FAO fishing subarea for wild subjects and country of origin for farmed
 227 subjects), and so-called “stocking density” factor.

228 Table 1. Description of the Sets of Variables Used To Classify Sea Bass in the Multiparametric
 229 Analysis_a

biometric parameter	FA composition of TL	FA composition of NL	FA composition of PL	elemental composition	isotopic abundance
total length	moisture	N 14:0	P 14:0	As	$\delta^{13}\text{C}$
body weight	TL	N 15:0	P 15:0	Ca	% C
eviscerated weight	14:0	N 16:0	P 16:0	Cd	$\delta^{15}\text{N}$
viscera weight	15:0	N 17:0	P 17:0	Co	% N
liver weight	16:0	N 18:0	P 18:0	Cr	
gonad weight	16:1 ω -7	N 16:1 ω -7	P 16:1 ω -7	Cu	
perivisceral fat weight	17:0	N 18:1 ω -9	P 18:1 ω -9	Fe	
left fillet weight	18:0	N 18:1 ω -7	P 18:1 ω -7	Hg	
right fillet weight	18:1 ω -9	N 20:1 ω -11	P 20:1	K	

biometric parameter	FA composition of TL	FA composition of NL	FA composition of PL	elemental composition	isotopic abundance
skinned left fillet weight	18:1 ω -7	N 20:1 ω -9	P 22:1	Mg	
skinned right fillet weight	18:2 ω -6	N 20:1 ω -7	P 18:2 ω -6	Mn	
fillet skin weight	18:3 ω -3	N 22:1 ω -9	P 20:2 ω -6	Na	
frame weight	18:4 ω -3	N 18:2 ω -6	P 20:4 ω -6	Ni	
condition factor	20:1 ω -9	N 20:2 ω -6	P 22:4 ω -6	P	
carcass yield	20:1 ω -7	N 20:4 ω -6	P 22:5 ω -6	Pb	
viscerosomatic index	20:2 ω -6	N 22:4 ω -6	P 18:3 ω -3	S	
hepatosomatic index	20:4 ω -6	N 22:5 ω -6	P 18:4 ω -3	Se	
gonadosomatic index	20:4 ω -3	N 18:3 ω -3	P 20:4 ω -3	Zn	
% fat	20:5 ω -3	N 18:4 ω -3	P 20:5 ω -3		
fillet with skin	22:1 ω -11	N 20:4 ω -3	P 22:5 ω -3		
fillet without skin	22:4 ω -6	N 20:5 ω -3	P 22:6 ω -3		
% frame	22:5 ω -6	N 22:5 ω -3	P SFA		
% skin	22:5 ω -3	N 22:6 ω -3	P MUFA		
gutting yield	22:6 ω -3	N SFA	P ω -6		
skinning yield	SFA	N MUFA	P ω -3		
skinning + boning yield	MUFA	N PUFA ω -6	P PUFA		
	PUFA	N PUFA ω -3	P PUFA/SFA		
	PUFA/SFA	N PUFA	P MUFA/SFA		
	MUFA/SFA	N PUFA/SFA	P ω -3/ ω -6		
	ω -3	N MUFA/SFA	P ω -6/ ω -3		
	ω -6	N ω -3/ ω -6	P EPA + DHA		
	ω -3/ ω -6	N ω -6/ ω -3	P EPA/DHA		
	ω -6/ ω -3	N EPA + DHA	P DHA/EPA		
	EPA + DHA	N EPA/DHA	P 20:1 + 22:1		
	EPA/DHA	N DHA/EPA	P ARA/EPA		
	DHA/EPA	N 20:1 + 22:1	P EPA/ARA		
	20:1 + 22:1	N ARA/EPA	P LN/ARA		
	ARA/EPA	N EPA/ARA	P LN/ALA		
	EPA/ARA	N LA/ARA			
		N LA/ALA			

231 FA, fatty acid; TL, total lipids; NL, neutral lipids; and PL, polar lipids.

232 Analyses of single parameters were carried out for each classification target, to determine if
233 different groups of subjects were significantly different from each other. To compare wild and
234 farmed sea bass, Student's *t* test was applied, accepting as significant *p* values of ≤ 0.05 ; to compare
235 wild subjects from different FAO fishing subareas, farmed subjects from different countries, and
236 sea bass classified according to stocking density, one-way analysis of variance (ANOVA) was
237 conducted, accepting as significant *p* values of ≤ 0.05 , plus a post-hoc Bonferroni correction as a
238 result of the high number (175) of parameters analyzed. Multiparametric analyses (PCA and
239 classification) were conducted on each aforementioned set of variables separately. The PCA
240 procedure was applied to give an overview of the ability of selected parameters to discern samples
241 according to the production method, geographical origin, and stocking density by visual inspection
242 of the parameter space. The aim of the multivariate statistical analysis was to assess the
243 classification performance of each set of variables and to determine an optimal and restricted
244 parameter set for sample classification. For these purposes, samples were categorized according to
245 the quadratic discriminant classifier. The "leave-one-out cross-validation" procedure allowed for
246 the assessment of method robustness during classification. This validation method consists of
247 removing from the whole sample set, one at time, every single subject, which is going to be
248 classified on the basis of information concerning all of the other samples. (30)

249 **3. Results and Discussion**

250
251 Each sample was processed to collect data about biometric characteristics and chemical
252 composition (moisture, lipid content, FA composition, macro- and microelements, and isotopic
253 abundance). Data obtained are reported in Table S2 of the Supporting Information.

254 **3.1. Biometric Traits**

255 Weight and length of wild subjects analyzed in this study were similar to those reported in other
256 studies. (4,8,24) The condition factor is considered a good indicator of the dietary condition of fish
257 species; (32) likewise to what was reported in other works, (10,12,33,34) significant differences in
258 condition factor and hepatosomatic index were found among subjects grouped according to the
259 stocking density ($p < 0.05$). However, unlike the findings of Fasolato et al., (10) perivisceral fat
260 weights in cultured samples from the present study were not significantly higher than wild samples
261 (see Table S2 of the Supporting Information). Several biometric traits measured in sea bass from
262 this investigation were significantly different ($p < 0.001$) among subjects from different rearing
263 systems; the diversity among groups is especially marked if comparing IR subjects to sea bass from
264 other rearing systems (wild, ER, and SIR): weights of eviscerated samples, viscera, fillets (with and
265 without skin), skin, and frame of IR subjects are significantly lower compared to other groups of
266 subjects. Further parameters, such as percentages of frame, fillet and skinned fillet yields, skinned
267 and boning yields ($p < 0.001$), liver weights, and percentages of fat and skin ($p < 0.05$) were
268 significantly different too. ER subjects were found to be more similar to wild specimens than to
269 farmed specimens in viscera weights and percentages of fat and skin (see Table S2 of the
270 Supporting Information). Culture conditions are known to influence biometric traits of subjects; the
271 feeding strategy (different feed ingredients and feeding regimes) and the stocking density (affecting
272 swimming activity and feed intake) might influence fat deposition in liver and fillets. (4)
273 With regard to reared subjects, significant differences were found in percentage of fat ($p < 0.001$)
274 and perivisceral fat ($p < 0.05$), with sea bass from Turkey those with higher levels; significant
275 differences were also found in eviscerated weights and fillet (with and without skin) weights ($p <$
276 0.05). These findings may be the results of diverse feeding strategies adopted by farmers in

277 different countries; furthermore, differences in genetic strain might also be responsible (see [Table](#)
278 [S2](#) of the Supporting Information).

279 **3.2. Moisture, Lipid, and FA Profile**

280 Levels of TL (3.72 ± 2.1 g/100 g of wet weight), PL, and FA composition of wild subjects were
281 similar to those reported in similar studies. ([4,8,24,31,35,36](#)) A high maximum value of TL (7.63
282 g/100 g) was likely due to the high lipid content of subjects from source 8, probably from
283 commensals at a nearby intensive plant. In accordance with similar investigations, results from this
284 study indicate that wild and farmed sea bass differ significantly for moisture content, ([8,35](#)) TL ($p <$
285 0.001), and FA profile ($p < 0.05$). ([4,8,35](#)) A high lipid content in the diet of IR subjects results in
286 higher flesh fat levels; in fact, the TL content and total monounsaturated fatty acids (MUFA) are
287 typically higher in farmed subjects, while the total saturated fatty acids (SFA), polyunsaturated fatty
288 acids (PUFA), and ω -3/ ω -6 ratios are lower than those in wild subjects. ([4,8,10,13,35,37](#)) In this
289 study, though, MUFA was not significantly different among the production methods (see [Table S2](#)
290 of the Supporting Information).

291 Concerning PUFA, ω -3 levels (28.86 ± 7.47 g/100 g of wet weight) in wild subjects were similar to
292 those reported in previous studies, ([8,35,36](#)) while ω -6 levels (6.21 ± 2.63 g/100 g wet weight) were
293 slightly lower; ([10,24,37,38](#)) therefore, the ω -3/ ω -6 ratio (5.95 ± 3.82) was higher than those in the
294 literature, representing a significantly discriminative parameter for the production method ($p <$
295 0.001). Indeed, ω -3 PUFA are generally predominant in wild sea bass, while ω -6 PUFA are more
296 abundant in farmed subjects. The increasing use of vegetable oils in feed formulation seems to
297 depress lipogenic activity in farmed subjects and reduce flesh SFA, especially in terms of palmitic
298 acid (16:0); in confirmation of this, a study conducted on farmed sea bass found a negative
299 correlation between SFA and flesh TL. ([10](#)) Moreover, the substitution of fish oil with vegetable
300 lipid sources leads to an increase in C₁₈ FAs, such as 18:1 ω -9 [oleic acid (OA)], 18:2 ω -6 [linoleic
301 acid (LA)], and 18:3 ω -3 [α -linolenic acid (LNA)]. At the same time, feeding with plant oils reduces
302 the 20:4 ω -6 [arachidonic acid (ARA)], 20:5 ω -3 [eicosapentaenoic acid (EPA)], and 22:6 ω -3
303 [docosahexaenoic acid (DHA)] levels in flesh. ([9,10](#)) As a result of the limited capacity of marine
304 fish to convert LA and LNA to longer chain and more unsaturated FAs, they accumulated
305 unchanged in farmed subjects; on the other hand, as a result of the low content of ARA in
306 commercial feed, wild sea bass are significantly richer in this FA than farmed sea bass. ([4,10,13](#))
307 High variability in EPA and DHA levels in wild and farmed fish has been previously observed,
308 probably caused by diet, season, and location; ([32](#)) aquafeeds available in the market present high
309 heterogeneity in terms of FA composition, while the FA profile of wild fish depends upon the
310 availability of food, season, and site of catch. ([12](#))

311 The TL content of fillets from SIR sea bass was higher than that from ER subjects (9.00 ± 2.34 and
312 4.40 ± 1.53 g/100 g of wet weight, respectively), and the FA profile was similar to that for IR
313 subjects: high in ω -6 PUFA (in particular, LA), low in ω -3 PUFA levels (in particular, DHA), and,
314 consequently, with a low ω -3/ ω -6 ratio. SIR specimens were also closer to IR specimens for NL and
315 PL composition, while ER subjects were more similar to wild subjects. The similarity in TL
316 between ER and wild specimens was also confirmed by Orban et al.; ([35,39](#)) however, in this
317 investigation, ER subject profile was higher in SFA and MUFA, similar in ω -6 PUFA, and lower in
318 ω -3 PUFA compared to findings from this study. The FA profile of SIR, instead, was in line with
319 that reported by Trocino et al. ([40](#)) (see [Table S2](#) of the Supporting Information).

320 The TL content of fillets from IR sea bass differed significantly ($p < 0.001$) among specimens from
321 different countries as well as the profile of almost all FAs: SFA, MUFA, ω -6, ω -3, and ω -3/ ω -6
322 ratio. Equivalent levels of EPA and DHA were found in Italian IR as well as for Greek and Croatian
323 farmed subjects; a predominance of the DHA content over EPA was instead detected in IR from
324 Turkey. With regard to the FA composition of NL, Turkish IR sea bass were higher in MUFA and
325 lower in SFA and ω -3, while Italian IR were higher in ω -6 compared to subjects from other

326 countries. With regard to the FA composition of PL, Croatian IR sea bass showed higher levels of
327 SFA (especially palmitic acid) compared to other countries, while fillets from Turkish IR contained
328 higher levels in MUFA and lower levels of ω -3 and ω -6 (see [Table S2](#) of the Supporting
329 Information).
330 Data from the literature about IR sea bass, not distinguishing by country of origin, present wider
331 ranges for TL (as well as in terms of SFA, MUFA, PUFA ω -3, and PUFA ω -6) and higher DHA
332 levels than those obtained in this study. (9,39,40) The NL profile of IR sea bass from this
333 investigation was higher in terms of PUFA than those published in the literature, (41,42) while the
334 PL profile was comparable (see [Table S2](#) of the Supporting Information).

335 3.3. Trace Elements

336 Elemental composition analysis showed that wild sea bass had higher levels of macro- and
337 microelements compared to those reported in the studies of Žvab Rožič et al., Alasalvar et al., and
338 Fuentes et al. (6,8,13) Toxic elements never exceeded the limits set by the European Regulations for
339 sea bass muscle, i.e., 0.50 mg kg⁻¹ for Hg, (43) 0.05 mg kg⁻¹ for Cd, (44) and 0.30 mg kg⁻¹ for Pb.
340 (45) Limits for As have not yet been regulated, but levels found in five subjects from the Atlantic
341 source (see [Figure 1](#)) were alarming (1.07–2.30 mg kg⁻¹ muscle). The presence of toxic elements in
342 the environment is mainly affected by anthropogenic activities; therefore, levels of trace elements
343 are generally lower in farmed subjects compared to wild subjects as a result of a lower age when
344 caught and controlled feeding (46) (see [Table S2](#) of the Supporting Information).

345 The elemental composition for ER subjects was similar to that for wild subjects and comparable to
346 the data available from the literature about K, Ca, Mg, and Se. (39) In comparison to those found
347 for ER specimens analyzed by Orban et al., (39) lower levels of Na, P, and Cr and higher levels of
348 Zn and Fe were detected as well as a minor contamination by toxic elements, because they found
349 very high Hg concentrations as a result of environmental geological features. The elemental
350 composition for all IR subjects was similar to those reported in the literature, (6,13,39,46,47)
351 although no data were published on S. The only exception was for Cu, which was found at a higher
352 concentration compared to the levels reported in the available literature (see [Table S2](#) of the
353 Supporting Information). With regard to toxic elements, very high Pb levels were detected in two
354 subjects from source 2 (see [Figure 1](#)) collected in the spring/summer season (data not shown),
355 which increased the mean above the legal limit.

356 Marine fish incorporate different trace elements, from the environment and the diet, into their
357 skeletal tissues and organs, forming a chemical signature of the particular water body where the
358 subject has grown. (18) Wild populations of sea bass from the Mediterranean move among various
359 coastal habitats; thus, it is difficult to detect substantial differences in trace elemental signatures
360 among wild subjects. On the contrary, aquaculture makes fish remain static in one location, and
361 then a distinct elemental composition is likely to appear. (12) However, contrasting results have
362 been reported within the published studies; (8,18,46) feed is a factor that may explain this
363 variability: mineral levels are extremely variable in fish feeds as a result of differences in raw
364 ingredients used in diet formulation, the addition of specific macro- or trace mineral premixes
365 (enriched with Cu, Fe, Zn, Mn, Co, Cr, and Mg), the potential presence of contaminants in feed
366 ingredients, (18) or the use of metal-based antifoulants to protect the cage nets. (12) Results from
367 this study revealed that levels of P and Se were determinant characters for distinguishing the sea
368 bass production method and geographical origin, Na and Cu were significantly different ($p < 0.05$)
369 among sea bass grouped for production methods, while K and S differed significantly ($p < 0.05$)
370 among geographical locations (see [Table S2](#) of the Supporting Information).

371 3.4. Isotopic Abundances

372 Data on isotopic abundance from this study were comparable with those from the available
 373 literature; (4,10,18,48) they revealed a slight difference in the ranges of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values
 374 between wild and farmed subjects and similar levels in Italian and foreign IR sea bass (see Table S2
 375 of the Supporting Information).
 376 Stable isotope analysis is described to be a powerful tool in the analysis of trophic relationships in
 377 aquatic environments. C and N are the principal elements composing living organisms; they spread
 378 throughout the marine food web, enriching the isotopic signatures of marine vertebrates. The stable
 379 isotope ratio of C varies according to the nature of the dietary C sources, while stable isotopes of N
 380 depend upon the trophic position of the diet of the subjects. CO_2 assimilated by terrestrial plants,
 381 which are major constituents of commercial feeds, is less enriched with $\delta^{13}\text{C}$ than carbon sources in
 382 the natural diet of wild marine fish. As a result, the flesh of wild marine fish generally presents
 383 higher $\delta^{13}\text{C}$ values than the flesh of farmed fish. The trophic level of fish feed formulations affects
 384 the $\delta^{15}\text{N}$ levels in the flesh of farmed sea bass, which may result in significant differences in $\delta^{15}\text{N}$
 385 values in the flesh of farmed and wild sea bass, (11) as reported in previous similar studies.
 386 (4,10,15) According to Fasolato et al., variability in $\delta^{15}\text{N}$ seems to be more informative of the
 387 geographical origin of fish, in the case of differences in feed formulations among each area or
 388 country of provenance, rather than geological or environmental influences. Moreover, a variability
 389 in the lipid content can alter tissue $\delta^{13}\text{C}$ values and may be misinterpreted as dietary or habitat
 390 shifts. (10)

391 **3.5. Classification of European Sea Bass Sources toward Sea Bass Labeling**

392 Table 2 shows the results of the multivariate analysis: the percentage of subjects correctly allocated
 393 in relation to production method (farmed/wild), origin (FAO fishing subareas for wild or country of
 394 origin for farmed subjects), and stocking density, employing the selected sets of variables. For a
 395 correct implementation of the discriminant analysis for the classification of subjects, data
 396 introduced in the multivariate analysis had been scaled for fish size (considered as length), because
 397 it was found to be correlated with some of the selected set of parameters (mainly biometric
 398 parameters and elemental composition).

399 Table 2. Percentage of Sea Bass Correctly Classified According to Production Method, Origin
 400 (FAO Fishing Areas for Wild Specimens and Country of Origin for Farmed Specimens), and
 401 Stocking Density, Obtained by Multivariate Analysis Employing the Selected Set of Variables^a

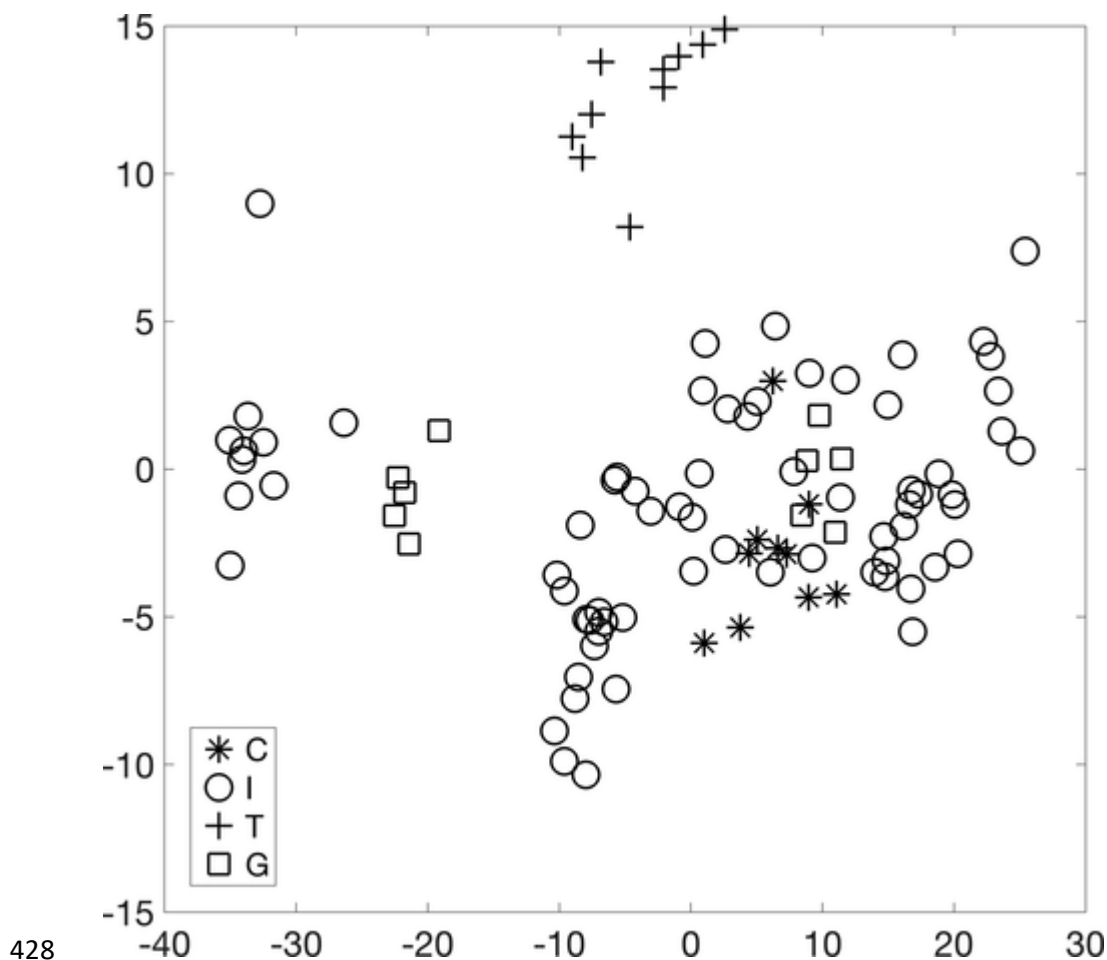
set of variables	production method	origin		
		wild	farmed	stocking density
biometric parameters	82	58	64	72
FA composition of TL	93	69	81	87
FA composition of NL	94	73	73	87
FA composition of PL	92	64	92	92
elemental composition	79	56	58	66
isotopic abundances	91	44	60	77

402 ^a
 403 FA, fatty acid; TL, total lipids; NL, neutral lipids; and PL, polar lipids.

404 FA compositions of TL, NL, and PL and isotopic abundances were the best performing sets of
 405 variables for the classification of sea bass ($n = 160$) according to production method (farmed/wild),
 406 providing percentages of correct allocation over 90%. The worst performing set of variables was
 407 elemental composition, which reduced the percentage of subjects correctly classified, even for those
 408 with 100% correct allocation by other sets. Among all of the sources, number 3 (ER subjects; see

409 Figure 1) had the worst performance for allocation according to production method, with subjects
410 generally being scattered among wild sea bass.
411 Examining the parameter “origin”, in terms of FAO fishing subareas for wild subjects ($n = 45$), the
412 highest percentage of subjects correctly allocated was obtained by the set of variables FA
413 composition of PL (see Table 2). All of the other sets of variables showed performances for correct
414 allocation always below 70%, with isotopic abundance having the poorest performances (44%).
415 With regard to farmed sea bass, once again, FA composition of PL provided the best performance
416 for classifying subjects according to country of origin ($n = 115$), followed by FA composition of
417 TL, while biometric parameters, isotopic abundances, and elemental composition gave a low
418 percentage for correct allocation (<64%). In general, farmed subjects were better classified than
419 wild subjects by the tested set of variables.
420 The PCA applied for the discrimination of European sea bass according to their origin confirmed
421 that it was impossible to obtain a clear separation of subjects based on this parameter, even when
422 combining multiple sets of variables together. In the PCA plot for farmed subjects classified
423 according to their country of origin (Figure 2), sea bass from Turkey were separated from the other
424 subjects for FA composition of NL; the PCA plot for wild sea bass classified using FA composition
425 of PL (Figure 3) showed a slight but detectable separation between subjects from Mediterranean
426 FAO fishing subareas and those caught in the Atlantic Ocean (from source 1; see Figure 1).

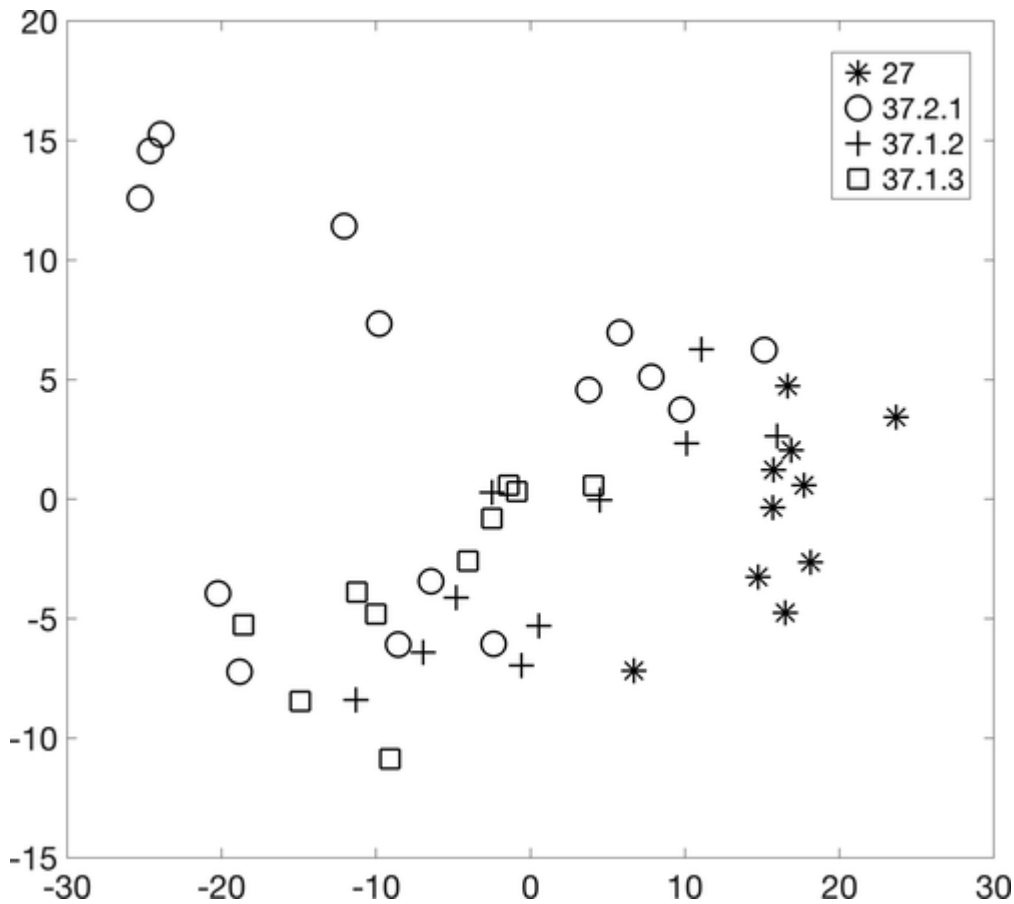
427 **Figure 2**



429 Figure 2. Scatterplot of PCA performed on farmed sea bass ($n = 115$) classified according to the
430 country of origin and obtained using “FA composition of NL” as a set of variables. C, Croatia; I,
431 Italy; T, Turkey; and G, Greece.

432

Figure 3



433

434 Figure 3. Scatterplot of PCA performed on wild sea bass ($n = 45$) classified according to FAO
435 fishing subarea of origin and obtained using “FA composition of PL” as a set of variables. FAO
436 fishing subarea 27 is in the Atlantic Ocean, the others are in the Mediterranean Sea.

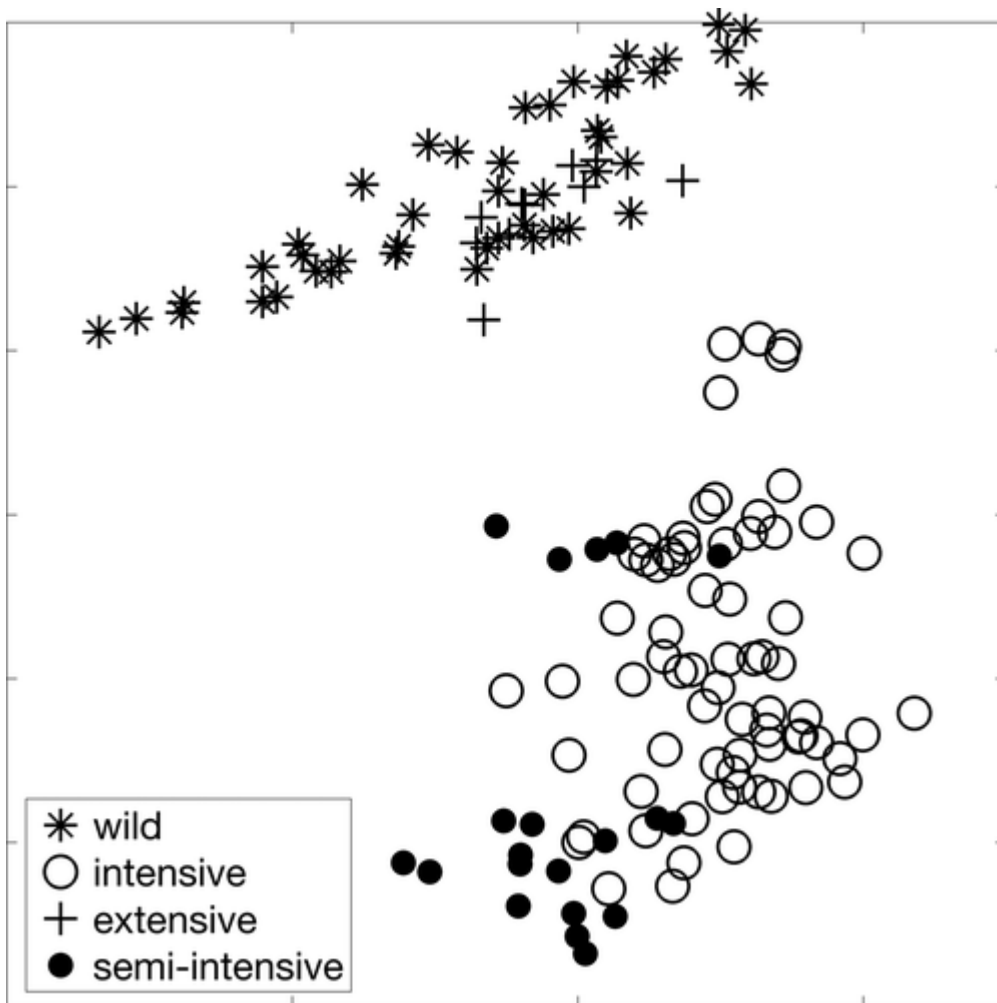
437 Concerning the classification parameter “stocking density”, the best performing set was FA
438 composition of PL (92%), while FA composition of TL and NL provided slightly lower
439 percentages. Biometric parameters and isotopic abundances showed performances for correct
440 allocation around 70%, and the lowest percentage was obtained by employing the elemental
441 composition set. The most evident visual separation was attained in the PCA plot (Figure 4)
442 obtained by combining three sets of variables: FA composition of TL, FA composition of PL, and
443 FA composition of NL. In this plot, the population of sea bass was separated into two big groups:
444 SIR + IR and ER + wild, with ER specimens allocated in the demarcation zone between wild and
445 SIR + IR.

446

447

448

449

Figure 4

451

452 Figure 4. Scatterplot of PCA performed on 160 specimens of sea bass (85 from intensive rearing, 20
 453 from semi-intensive rearing, 10 from extensive rearing, and 45 caught in the wild) and using three
 454 sets of variables: “FA composition of TL”, “FA composition of PL”, and “FA composition of NL”.

455 The efficacy of FA composition for discriminating between wild and farmed fish is strongly
 456 supported by several studies; (4,49) many authors have managed to classify cultured and wild
 457 European sea bass fillets on the basis of lipid profile and FA composition. (4,8,10,13,35,37)
 458 According to reports in the literature, LA, LNA, ARA, and DHA generally provide the greatest
 459 contribution to the characterization of farmed and wild sea bass. (4) In fact, Fasolato et al. (10)
 460 found significantly higher levels of LA, LNA, and OA in farmed subjects, while ARA and DHA
 461 were characteristic of their wild counterparts. (8,37) These results could be due to several factors,
 462 including feed type and availability, feed ingredients (usually high in fat and terrestrial plant oils,
 463 such as LA), and reduced activity of farmed fish. (13,37) Moreover, because ARA, EPA, and DHA
 464 are considered to be essential for marine species, (4) high levels of these FA and low levels of LA
 465 are considered by many authors to be a specific marker for wild status. (4,8,12,13) Unlike the
 466 results reported by other authors, (4,10) in this study, OA did not provide any contribution toward
 467 the characterization of farmed and wild sea bass. The FA profile represented an interesting factor to
 468 identify sea bass origin, FAO fishing subarea (for wild subjects), and country of origin (for farmed
 469 subjects), especially when focusing on PL, which provided the best performance.
 470 Relative isotopic abundance, however, did not give such satisfying results, performing excellently
 471 in classifying the production method but not as well for other label parameters (see Table 2).

472 Fasolato et al. (10) identified $\delta^{13}\text{C}$ as one of the most powerful variables for describing the wild
473 cluster. Stable isotopes of C and N are useful for differentiating among sea bass production methods
474 (4,10) but not for distinguishing the country of origin. (13) Elemental composition obtained by
475 means of ICP–OES was not suitable for classifying subjects according to production method, FAO
476 fishing subarea, or country of origin. Likewise, literature data do not support the difference in
477 mineral composition between the flesh of wild and farmed sea bass; (8,18,46) it seems that neither
478 the origin of the fish nor the feeding system has any effect on this parameter, except for the Ca
479 content. (13) Biometric parameters, despite their poor performance (see Table 2), can be considered
480 a suitable set of variables for the authentication of the sea bass method of production as a result of
481 the easy, rapid, and cheap data collection. (12)

482 In conclusion, this study examines in depth the power of analytical and biometric parameters to
483 classify fish products according to the requirements for seafood labeling cited in Regulation (EU)
484 1379/2013. This is the first study conducted on a vast number of samples, collected during a 1 year
485 sampling from multiple sources properly scattered among the central and east–southern European
486 area, from both aquaculture and fishery sectors. Moreover, for the first time, an attempt was made
487 to support the already-mentioned labeling criteria with stocking density, with additional voluntary
488 information envisaged by Regulation (EU) 1379/2013.

489 Results from the multivariate analysis showed that good rates of correct identification of sea bass in
490 terms of production method (farmed/wild) could be obtained using all sets of variables tested in this
491 study. FA composition of PL is the most performing set, providing high rates of correct
492 classification of sea bass according to the stocking density factor and the country of origin of
493 farmed subjects. Moreover, sample classification according to stocking density, combining the three
494 sets of variables, not only pointed out a clear separation between wild and farmed subjects but also
495 highlighted an interesting result: the impossibility to distinguish SIR from IR subjects and ER from
496 wild subjects (Figure 4). The simple discrimination of farmed and wild subjects may incorrectly
497 classify ER subjects, allocating them among wild subjects. This aspect has not often been
498 considered in similar previous studies, yet it is a key factor for correct classification of sea bass for
499 labeling purposes. From a legislative point of view, this fact should be kept in consideration for
500 responsible classification of sea bass, because it might induce the mislabeling of ER specimens.
501 From a nutritional point of view, this study emphasized the ER product, highlighting its analytical
502 similarity to wild-caught sea bass (especially for the lipid profile).

503 It would be interesting to carry out a sensory evaluation, to verify if the analytical and nutritional
504 similarity between ER and wild sea bass might be sensorially noticed. Future studies may validate
505 the findings of this study using alternative or improved chemometric methods and even extend the
506 investigation to other fish species with a different fat content, to improve the informative value of
507 the FA profile for fish authentication.

508 **Supporting Information**

509 Description of European sea bass sources (Table S1), means, standard deviations (SD), and
510 ANOVA *p* values of data obtained in the study (Table S2), histogram plots of Pearson’s correlation
511 coefficients of the various parameter classes with biometric parameter “length” (Figure S1), all
512 PCA plots and loading plots grouped by set of parameters (Figures S2–S7), and extended version of
513 the Materials and Methods

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