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Application of Nonparametric Multivariate Analyses to the Authentication of Wild and Farmed European Sea Bass (*Dicentrarchus labrax*). Results of a Survey on Fish Sampled in the Retail Trade

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The aim of this study was to apply biometric measurements and analyses of proximate composition, fatty acid composition, and ratios of stable isotopes of carbon (δ^{13}C) and nitrogen (δ^{15}N) in muscle tissue to reliably differentiate between wild and farmed European sea bass (*Dicentrarchus labrax*). Farmed (n = 20) and wild (n = 19) European sea bass were purchased between March and May 2008 and used as standard samples. In the same months, a survey was conducted to evaluate the truthfulness of the statements on the labels of European sea bass sold in retail markets (declared farmed n = 34 and declared wild n = 33). In addition, data from the literature (reference) were employed to build the profile type of wild and farmed European sea bass. Primarily, an exploration and comparison of the analytical data of the standard data set based on principal component analysis and permutation test were performed. Afterward, an inferential statistical approach based on nonparametric combination test methodology (NPC) was applied on standard samples to check its suitability in discriminating the production method. This multivariate statistical analysis selected 30 variables on a total of 36 available. The validation of standard fish data set was accomplished by a novel nonparametric rank-based method according to profile type (just 1 misclassification over 39 samples). Both the NPC test and nonparametric rank-based method were then applied to survey fishes using the selected variables with the aim to classify the individual European sea bass as "true farmed" or "true wild". The former test segregated 10 fishes over 33 declared wild, whereas the results obtained by the nonparametric rank-based method showed that 11 of 33 declared wild European sea bass samples could be unquestionably attributed to the wild cluster. Moreover, considering the comparative contribution of profile type, a few surveyed farmed samples were ascribed to the wild cluster.

KEYWORDS: European sea bass; proximate composition; fatty acids; stable isotopes; authentication; nonparametric combination methodology; permutation test

INTRODUCTION

Fish acceptance and choice by consumers are strongly influenced by the awareness of the origin of the fish. Therefore, differentiation in seafood products could have a significant effect on consumer preferences, especially with regard to the distinction between wild and farmed fish (*1*). Consumer judgment is unfavorable to cultured fish because it is frequently associated with antibiotics and the usage of growth promoters (*2*). In addition, wild fishing products are usually judged as being healthier and tastier and of higher nutritional value than reared fish, especially by the elderly (*3*). In intensive marine aquaculture, the high lipid content of the diet modifies the chemical composition of the fish, resulting in a higher fat content that influences the quality of finfish (*4*). Moreover, the incorporation and composition of the fatty acids (FA) found in the intramuscular fat (IMF) strongly depend on the FA profile of the diet. In marine fish, the substitution of fish oil with other vegetable lipid sources leads to a reduction in the 20:5n-3 (eicosapentaenoic acid; EPA) and 22:6n-3 (docosahexaenoic acid; DHA) levels in the liver and flesh (*5*−*7*). In addition, in these fish, EPA, DHA, and 20:4n-6 (arachidonic acid; ARA) are considered to be essential dietary FA due to the marine fish's scarce ability to synthesize them from the C18 precursor unsaturated fatty acid (*8*). Thus, a high ARA amount could be considered a specific marker for wild status (*9*). In contrast, in farmed fish, the plant oil intake leads to an increase in C18 FA in muscle lipids, particularly 18:2n-6 (linoleic acid; LA), 18:3n-3 (α-linolenic acid; LNA), and 18:1n-9 (oleic acid; OA) (*7*).

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Interestingly, in some marine species, the large amount of dietary plant derivatives seems to have no effect on the perceived quality of the flesh (*10*, *11*). However, a change in the fatty acid profile usually decreases the nutritional value of finfish with a reduction in the n-3/n-6 ratio and the n-3 percentage (*7*). Among n-3 highly unsaturated fatty acids (HUFA), EPA and DHA are considered to be nutraceutical compounds involved in a large number of physiological functions pertinent to human health (*12*). Nevertheless, although feeding practices and other aspects of farming management could influence the nutritional value, taste, or flavor of finfish, the common consumer is not usually able to distinguish between caught and reared fish (*3*, *13*).

Commission regulation (EC) 2065/2001 stipulates a minimum amount of information on fish production that must be made available to the consumer (*14*). In particular, the label must contain details on traceability including species, method of production (caught in freshwater or marine water vs farmed), and the area of production. Therefore, reliable new analytical techniques for seafood authentication are required (*15*). The use of fatty acid profile together with fingerprinting of carbon and nitrogen stable isotopes is the most promising method for fish authentication (*15*−*18*). The ratios of the stable isotopes of C and N ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, expressed as $\delta^{13}C$ and $\delta^{15}N$) depend on the lipid content of the fish and the carbon source or the trophic position of the diet (*18*). Normally, wild marine fishes have higher $\delta^{13}C$ values than farmed fishes (*16*, *18*). This is because the carbon source of the natural diets of marine fish (the dissolved inorganic carbon pool, $\delta^{13}C = 0‰$) is more enriched in $\delta^{13}C$ than the atmospheric CO_2 ($\delta^{13}C = -8‰$) used for photosynthesis by the terrestrial plants that can contribute to the commercial feed formulation. Depending on the trophic level of the fish feed with respect to the natural diet, wild fishes can show $\delta^{15}N$ values that are significantly different from those of farmed fishes (*19*).

European sea bass is an opportunistic species, exhibits demersal behavior, and inhabits coastal waters down to about 100 m depth, although it is more common in shallow waters like estuaries, lagoons, and tidal flats. The adult European sea bass predates mainly on fish and benthic crustaceans, even if mollusks and insects can also be found in their stomach (*20*). Juvenile subjects also feed on several soft-body taxa such as *Anellida* and *Phyllodocida* or small crustaceans such as *Copepoda*, *Mysidacea*, *Isopoda*, and *Amphipoda* (*21*−*23*). The European sea bass is farmed intensively in floating cages or in in-shore ponds and extensively in brackish lagoons. In the former, the employment of high nutritional feed is common. The characterization of wild and farmed European sea bass has been described by a wide range of literature data (*24*) that span the variability of chemical and morphological descriptors in relation to sample size, season of fishing or sampling, geographical location of marine area, or different farm management. The performance of the analysis of morphometric traits, proximate composition, and fatty acid profile together with the monitoring of C and N stable isotopes in fish flesh was evaluated to discriminate the production method of European sea bass commercialized in Italy. Samples collected in the retail trade chain (survey samples) were compared to a standard data set and to the reference data.

A multivariate analysis called nonparametric combination (NPC) methodology (*25*, *26*) was employed with the aim to discriminate samples according to the method of production. This inferential technique is based on both permutation tests and nonparametric combination methodology that allow relaxation from the stringent assumptions of parametric methods (such as *t* and *F* tests). NPC permits a more flexible analysis in terms of both the specification of the multivariate hypotheses and the nature of the variables involved. The application of nonparametric statistics, such as permutation test, showed better performances in the case of asymmetric and not normal distribution of variables in comparison with other parametric approaches (*27*). Moreover, the NPC methodology does not need a modeling for dependence among variables and is not affected by the problem of the loss of degrees of freedom when the number of variables is large compared to the sample size (*27*). This approach can be particularly suited for sea bass randomly collected without any sampling plan previously drawn. Farmed and wild samples were collected without any preclusion to sex, age, size, or type of feeding that render the sample quite skewed. On the basis of the results of the NPC test methodology, a novel nonparametric rank-based classification method was applied. This method keeps the same advantages described above, allowing a more robust application than explorative statistics such as principal component analysis. The present study describes the use of two profile types, respectively obtained from wild and farmed analytical data available in the literature (reference data) and here applied as comparative model for the nonparametric rank-based classification.

MATERIALS AND METHODS

Fish Sampling. *Standard Fish*. Wild European sea bass ($n = 19$) from the Mediterranean sea (FAO zone 37.1) and from the Atlantic ocean (FAO zone 27) were sampled from the wholesale fish markets of Venice and Milan during the spring months of 2008.

Farmed European sea bass ($n = 20$) were collected during the same period. Ten subjects were sampled from three intensive farms located in northeastern Italy, and 10 samples were from Greece.

Survey Fish. The collection of survey samples ($n = 67$) was accomplished during the spring months of 2008 in 14 different large-scale retail trade establishments (total $n = 29$ samples, whereof $n = 4$ were declared as wild and $n = 25$ were declared as farmed) and 23 different fishmongers or dedicated fish markets (total $n = 38$ samples, whereof $n = 29$ were declared as wild and $n = 9$ were declared as farmed) located in a few large towns of Italy. Each sample was recorded, and its information was traced according to the requirements of Commission regulation (EC) 2065/2001. A sensory evaluation of freshness was accomplished according to the scheme of EC Regulation 2406/1996 by means of a trained group of panelists.

Morphometric Traits. After collection, fishes were immediately submitted to biometric measurement. Total length (34.6 ± 5.0 and 36.8 ± 9.8 cm for farmed and wild samples, respectively) was taken from the most anterior extremity of the closed mouth to the caudal rays pinched to give the maximum length measurement (TL). Total body weight (472 ± 219 and 561 ± 381 g for farmed and wild samples, respectively) was recorded (BW). The condition factor (KI) was calculated using the formula $KI\,(\%) = 100 \times (BW)/TL^3$. After dissection, liver and perivisceral fat weights were recorded to estimate the hepatosomatic (HSI, liver weight/BW × 100) and celomatic fat (CFI, mesenteric and perinephric fat weight/BW × 100) indices.

Sample Preparation, Proximate Composition, and Fatty Acid Analysis. Whole skinned fillet was homogenized (4000 rpm × 10 s, Retsch, Düsseldorf, Germany) and submitted to analysis. Moisture, crude protein, and ash were estimated following standard methods (*28*). Residual moisture was determined by oven-drying at 103 °C until weight became constant. Crude protein was estimated according to the Kjeldahl method, and total nitrogen was converted to crude protein using the conversion factor 6.25. Ash was measured after sample incineration in a muffle oven at 550 °C for 18 h. The percentage of IMF was determined after cold extraction of lipid matter (*29*). Fatty acid methyl esters (FAME) were obtained according to the method of Christie (*30*) with little modification. Briefly, 20 mg of fat was submitted to acid derivatization with 2 mL of 3 N methanolic HCl (Sigma-Aldrich, St. Louis, MO) in a glass-stoppered vial held at 60 °C for 2 h, during which it was periodically mixed. After mixture dilution with deionized water (2 mL), FAME were extracted with 2 mL of *n*-hexane. FAME (1 μL) were analyzed by a gas chromatograph (Shimadzu Italia Srl, model G17A, Milano, Italy) equipped with an automatic sampler (Shimadzu Italia Srl, model AOC 20i), split mode (split ratio 1:50, temperature of 250 °C), and flame ionization

detector (FID) set at 250 °C. Compound separation was accomplished by an Omegawax 250 capillary column (30 m × 0.25 mm i.d., film thickness = 0.25 μm, Supelco, Bellefonte, PA) using helium 6.0 as carrier gas (flow rate = 1.3 mL min^{-1}). The oven temperature program was from 140 to 220 °C at 4 °C min^{-1} to reach 220 °C and then isothermal at 220 °C held for 25 min. Fatty acids were identified by matching the retention time with that of an external standard mixture. Peak areas were corrected according to experimental FID response factors. The analyses were conducted in duplicate.

Isotope Analysis of Free Fat Matter. An aliquot of the homogenized sample (50 g) was freeze-dried and homogenized again (4000 rpm × 5 s, Retsch) to obtain a comminute and well-homogenized powder. The free fat matter (obtained after repeated washing with the Folch solvent mixture) was subjected to analysis of the stable isotope ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$, which was carried out using an isotope ratio mass spectrometer (Delta plus XP ThermoFinnigann, Bremen, Germany) following total combustion of the sample (≈0.5 mg) in an elemental analyzer (EA Flash 1112 ThermoFinnigan), as described elsewhere (*31*, *32*). The values were expressed in δ‰ (*31*) against international standards (Vienna-Pee Dee Belemnite for $\delta^{13}C$, air for $\delta^{15}N$) and computed against working in-house standards (casein), which were calibrated against international reference materials (*32*). The uncertainty (2σ) of measurements was < 0.3‰ for the $\delta^{13}C$ and $\delta^{15}N$ analyses.

Statistical Analysis. The permutation test was used at first to evaluate the significance of statistical differences between farmed and wild standard fish ($n = 39$). At the same time PCA was applied with the aim to reveal the internal structure of the data. Nonparametric combination (NPC) test methodology (*27*) was then used on standard samples to select the useful variables for the subsequent contrasts among groups (both standard and survey groups). With the purpose of expanding the variability observed for the variables considered, two profile types were built using literature data and employing them as model for a new nonparametric rank-based classification. This multivariate approach based on the Spearman rho coefficient as similarity index toward both profile types permitted to extend NPC test results in order to effectively classify each individual sample to either the wild or farmed cluster.

Significance Test and Explorative Analysis of Standard Fish. The variables considered in this study ($n = 36$) were submitted to permutation test with the aim to compare farmed and wild fish (*27*). This inferential technique is an alternative to the traditional *t* test and ANOVA *F* test. The permutation test is an exact-type inferential procedure, for which no assumption is needed on sample size; the exactness allows us to apply permutation tests to very small sample sizes. It is necessary to emphasize that assumptions regarding the validity of traditional parametric *t* and *F* tests (such as normality and random sampling) are rarely satisfied in practice, so that consequent inferences when not improper are necessarily approximated and their approximations are often difficult to assess. On the contrary, the permutation test is effective with any sample size (*26*).

After that, a first multivariate analysis was conducted by PCA to explore the raw data to highlight the more predictive variables (*16*, *19*). This approach was applied only to the standard samples. To highlight the significance of correlation (Spearman rho) between variables (intramuscular fat vs fatty acids and body weight vs isotopes), pairwise relationships have been tested (SPSS 17.0, Chicago, IL).

Comparison between Standard and Survey Fish via Nonparametric Combination (NPC) Test Methodology. Comparisons among standard farmed, standard wild, and survey European sea bass have been performed using NPC test methodology (*27*). As a general rule, considering a *k*-dimensional hypothesis-testing problem, the NPC solution was processed in two steps. First, a suitable set of *k* one-dimensional permutation tests called partial tests was defined. Each partial test examined the marginal contribution of any single response variable in the comparison between groups. Second, the nonparametric combination of dependent tests into one second-order combined test, which was suitable for testing possible global differences between the multivariate distributions of groups, was performed. NPC test analysis was done with the free software NPC Test R10 (*33*). The first comparison was accomplished between farmed and wild standard fish to discover the nonuseful variables. Afterward, PCA was applied with the goal of finding similarities and differences between each surveyed sea bass and both farmed and wild standards. PCA allowed the classification of those survey samples that were not in agreement with the information reported on the label.

Nonparametric Rank-Based Classification According to Profile Type. The proposed methodology consisted of the following steps.

Farmed profile type and wild profile type were created according to the data reported in the literature (called reference data summarized in Tables 1 and 2 of the Supporting Information, where 26 and 10 references were employed for farmed and wild profile type respectively; other additional information have been provided in **Table 3**). Each profile type considered only those significant variables that have been shown to be useful to discriminate between standard farmed and wild samples. Median was chosen as the statistical descriptor for profile type for both populations. However, there were not enough data to establish values for four variables (KI, HSI, CFI, celomatic fat weight) for the wild profile type, so they were excluded from the data set. The method was then applied to both standard and survey samples.

For each individual sample, two indices that were suitable to measure how "close" or "far" each individual sample was from the farmed or wild profile type were calculated. The two indices were calculated as follows.

Considering the *n*-dimensional pooled data set (farmed and wild samples), the farmed profile type was added to the last row (obtaining $n + 1$ units) and, within each of the *k* significant variables, the rank of each individual sample was calculated (actually, it was a value from 1 to $n + 1$). In this way a $(n + 1) \times k$ rank matrix was obtained, where the ranks related to the farmed profile type were in the last row.

For each *i*th individual sample ($i = 1, ..., n$) the index rho_i (*i*th individual sample, farmed profile type) was calculated, that is, Spearman's rho correlation coefficient between the related vector of *k* ranks from the *i*th individual sample and the vector of ranks of the farmed profile type. The *P* value for testing whether the correlation could be considered as equal to zero was then performed and, if the null hypothesis was not rejected, the estimated correlation coefficient was set equal to zero. In this way, the *n* values estimating how "close" or "far" the *n* samples were from the farmed profile type were obtained.

The procedure described above was repeated considering as confronting profile the ranking provided by the wild profile type.

The *i*th sample was classified as "true farmed" if rho_i (*i*th individual sample, farmed profile type) was greater than rho_i (*i*th individual sample, wild profile type) or as "true wild", otherwise.

The procedure was applied twice, first to the standard and then to the survey samples. In the first case, a validation of the proposed method through the cross-classification with the "true" production mode (standard samples) was performed. In the second case, the classification method to the declared production mode (survey samples) was applied. The survey samples, according to both Spearman rho coefficients, were then classified into one of four categories: declared farmed/classified farmed, declared farmed/classified wild, declared wild/classified farmed, and declared wild/classified wild. To implement the novel proposed rank-based classification method, a suitable routine using the R statistical software was built. These routines are available upon request.

RESULTS

Standard Fish. A total of 36 variables were submitted to the permutation test (**Tables 1−3**). Most of the data were significantly different between the farmed and wild standard groups. Celomatic fat, KI, hepatosomatic index, and celomatic fat index were significantly higher in cultured samples than in wild ones (**Table 1**). All of the parameters of chemical composition were affected by the production method (**Table 2**). Cultured fish had higher intramuscular lipid content (fat) than caught fish, with an expected reduction on moisture. On average, the flesh lipid content of farmed samples was roughly 3-fold higher than that of its wild counterpart. Protein percentage was significantly lower in wild than in cultured fish. Statistical analysis of fatty acid profiles described a higher percentage of C14:0, C18:1n-9, C20:1n-9, C22:1n-11, C18:2n-6, C18:3n-3, C20:2n-6, and C20:3n-3 and a concomitantly lower amount of C16:0, C18:0, C16:1n-7, C18:1n-7, C20:4n-6, C20:5n-3, C22:5n-3, and C22:6n-3 in cultured fish. Total saturated fatty acids (SFA) was higher in wild than in farmed European sea bass, whereas the situation is opposite for total monoenoic FA. Total polyenoic FA were unaffected

Table 1. Morphometric Traits and Indices of European Sea Bass Samples of the Standard Data Set (Mean ± SD and Permutation *P* Values)

	standard data set		
variable[a]	farmed	wild	*P* value
no. of observations	20	19	
celomatic fat (g)	12.9 ± 10.6	0.7 ± 1.0	0.001
KI	1.08 ± 0.11	0.96 ± 0.10	0.001
HSI	2.30 ± 0.96	1.33 ± 0.41	0.002
CFI	2.96 ± 2.04	0.33 ± 0.42	0.001

[a] KI (condition index) = body weight × 100/total length^3; HSI (hepatosomatic index) = (liver weight/BW) × 100; CFI (celomatic fat index) = (mesenteric and perinephric fat weight/BW) × 100.

Table 2. Proximate Composition (Percent) and Fatty Acid Profile (Percent of Total Fatty Acids) of Flesh of Farmed and Wild European Sea Bass of the Standard Data Set According to Permutation Test (Mean ± SD and Permutation *P* Values)

	standard data set		
variable	farmed	wild	*P* value
proximate composition			
no. of observations	20	19	
moisture	72.5 ± 1.7	77.4 ± 1.4	0.001
protein	19.0 ± 0.6	18.1 ± 0.8	0.001
fat	5.9 ± 1.9	2.0 ± 0.8	0.001
ash	1.25 ± 0.06	1.19 ± 0.07	0.001
fatty acid			
no. of observations	20	19	
C14:0	2.9 ± 0.6	2.1 ± 0.8	0.001
C16:0	16.3 ± 1.2	19.4 ± 0.7	0.001
C18:0	3.9 ± 0.5	5.5 ± 0.9	0.001
∑ saturated	23.1 ± 1.3	27.0 ± 0.8	0.001
C16:1 n-7	4.5 ± 0.7	5.3 ± 1.4	0.017
C18:1 n-9	19.8 ± 1.4	14.8 ± 2.8	0.001
C18:1 n-7	2.7 ± 0.3	4.1 ± 0.8	0.001
C20:1 n-9	3.4 ± 1.2	1.8 ± 1.4	0.001
C22:1 n-11	2.5 ± 1.5	1.2 ± 1.8	0.021
C22:1 n-9	0.2 ± 0.1	0.2 ± 0.2	NS[a]
∑ monounsaturated	33.1 ± 4.4	27.5 ± 4.4	0.001
C18:2 n-6	12.9 ± 7.0	1.5 ± 0.9	0.001
C18:3 n-6	0.2 ± 0.05	0.2 ± 0.07	NS
C18:3 n-3	1.7 ± 0.6	0.5 ± 0.2	0.001
C20:2 n-6	0.6 ± 0.2	0.4 ± 0.1	0.002
C20:3 n-6	0.2 ± 0.2	0.2 ± 0.1	NS
C20:3 n-3	0.5 ± 0.2	0.3 ± 0.2	0.001
C20:4 n-6	0.9 ± 0.3	3.6 ± 1.7	0.001
C20:5 n-3	7.9 ± 1.5	9.6 ± 2.1	0.005
C22:5 n-3	1.7 ± 0.4	2.5 ± 0.8	0.002
C22:6 n-3	10.3 ± 2.3	16.5 ± 4.5	0.001
∑ polyunsaturated	37.0 ± 4.8	35.1 ± 4.4	NS
∑ n-3	22.2 ± 3.2	29.3 ± 3.8	0.001
∑ n-6	14.2 ± 6.9	5.5 ± 2.3	0.001
n-3/n-6	2.2 ± 1.5	6.4 ± 3.0	0.001
EPA + DHA	18.2 ± 3.3	26.0 ± 4.1	0.001

[a] NS, not significant $P > 0.05$.

by the production method. Farmed samples showed a reduction in the n-3/n-6 ratio and lower levels of n-3 HUFA percentages in fillets (**Table 2**) and a concomitantly higher percentage of n-6.

The stable isotope ratios of C and N were significantly different between wild and cultured sea bass (**Table 3**). The fat free muscle showed significantly lower δ^{13}C and δ^{15}N values in farmed fish. The explorative statistical analysis according to PCA showed that, on the basis of all variables studied, first (PC1) and second (PC2) factors of the PCA represented 58% of the total variability.

Table 3. Stable Isotope Ratio δ^{13}C and δ^{15}N Values in Free Lipid Matter of Flesh of Wild and Farmed European Sea Bass of the Standard Data Set According to Permutation Test[a]

	standard data set		
variable	farmed	wild	*P* value
no. of observations	20	19	
δ^{13}C ‰ vs V-PDB	−18.1 ± 0.9	−15.0 ± 1.6	0.001
δ^{15}N ‰ vs air	12.5 ± 1.0	15.0 ± 2.9	0.001

[a] Weight was considered as covariate (mean ± SD and permutation *P* values).

Table 4. Factor Loadings of Explorative PCA for European Sea Bass Standard Data Set[a]

variable	PC1	PC2
celomatic fat (g)	−0.642	0.625
KI	−0.279	**0.724**
HSI	−0.678	−0.304
CFI	−0.482	**0.726**
moisture	**0.919**	−0.130
protein	−0.530	0.435
fat	**-0.919**	0.103
ash	−0.569	0.171
C14:0	−0.593	0.113
C16:0	**0.910**	0.099
C18:0	**0.873**	−0.189
∑ saturated	**0.884**	−0.017
C16:1 n-7	0.379	0.605
C18:1 n-9	**-0.817**	0.357
C18:1 n-7	**0.863**	0.319
C20:1 n-9	−0.367	**0.720**
C22:1 n-11	−0.218	**0.782**
C22:1 n-9	−0.136	0.527
∑ monounsaturated	−0.414	**0.835**
C18:2 n-6	**-0.836**	−0.531
C18:3 n-6	−0.426	−0.061
C18:3 n-3	**-0.867**	−0.459
C20:2 n-6	−0.515	−0.475
C20:3 n-6	0.281	−0.276
C20:3 n-3	−0.216	0.687
C20:4 n-6	**0.848**	−0.239
C20:5 n-3	0.571	0.385
C22:5 n-3	0.617	0.016
C22:6 n-3	**0.795**	0.077
∑ polyunsaturated	−0.263	**-0.771**
∑ n-3	**0.872**	0.102
∑ n-6	**-0.768**	−0.606
n-3/n-6	**0.787**	0.545
EPA + DHA	**0.879**	0.298
δ^{13}C ‰ vs V-PDB	**0.765**	−0.398
δ^{15}N ‰ vs air	0.638	−0.050

[a] All of the variables are reported; factor weights >0.7 and < −0.7 are shown in bold type.

The contribution of each variable to the principal components is reported in **Table 4**.

According to the weight of the variables, component 1 was mainly associated with moisture, fat, C16:0, C18:0, ∑ saturated, C18:1n-9, C18:1n-7, C18:2n-6, C18:3n-3, C20:4n-6, C22:6n-3, ∑ n-3, ∑ n-6, n-3/n-6, EPA plus DHA, and δ^{13}C, whereas component 2 was described by CFI, KI, C20:1n-9, C22:1n-11, ∑ monounsaturated, and ∑ polyunsaturated fatty acids. Among quality parameters only CFI, KI, moisture, and fat were contributing descriptors to the PCA.

Results of the NPC test analysis are presented in **Table 5**. Starting from the global *P* value that was equal to 0.015, the standard farmed and wild samples were found to be significantly

Table 5. Partial and Global Permutation *P* Values for the Three Performed Inferential Data Analyses [a]

variable	standard farmed vs standard wild	standard farmed vs survey	standard wild vs survey
fat	**0.010**	1.00	**0.013**
protein	**0.010**	0.995	**0.021**
ash	**0.010**	0.972	0.059
moisture	**0.010**	1.00	**0.013**
C14:0	**0.010**	0.163	**0.013**
C16:0	**0.010**	0.447	0.142
C16:1 n-7	0.122		
C18:0	**0.010**	0.971	**0.013**
C18:1 n-9	**0.010**	0.995	**0.013**
C18:1 n-7	**0.010**	0.972	**0.013**
C18:2 n-6	**0.010**	1.00	**0.013**
C18:3 n-6	0.330		
C18:3 n-3	**0.010**	1.00	**0.013**
C20:1 n-9	**0.010**	0.085	**0.023**
C22:1 n-11	0.094		
C22:5 n-3	**0.018**	0.826	**0.013**
C20:2 n-6	**0.010**	0.971	**0.022**
C20:3 n-6	0.909		
C20:4 n-6	**0.010**	1.00	**0.013**
C20:3 n-3	**0.010**	0.053	**0.050**
C20:5 n-3	**0.047**	0.971	**0.021**
C22:1 n-9	0.909		
C22:6 n-3	**0.010**	0.995	**0.013**
$\sum$ saturated	**0.010**	0.276	0.354
$\sum$ monounsaturated	**0.010**	0.879	**0.021**
$\sum$ polyunsaturated	0.510		
n-3	**0.010**	0.971	**0.013**
n-6	**0.010**	1.00	**0.013**
n-3/n-6	**0.010**	0.995	**0.013**
celomatic fat (g)	**0.010**	0.995	**0.021**
$\delta^{13}C$	**0.010**	0.971	**0.013**
$\delta^{15}N$	**0.024**	0.995	**0.013**
KI	**0.010**	0.97	0.354
HSI	**0.010**	0.971	**0.022**
CFI	**0.010**	0.085	**0.022**
EPA + DHA	**0.010**	0.971	**0.013**
P global	**0.015**	0.071	**0.013**

[a] Partial *P* values have been corrected for multiplicity, and the global *P* values have been obtained using Tippet combining function.

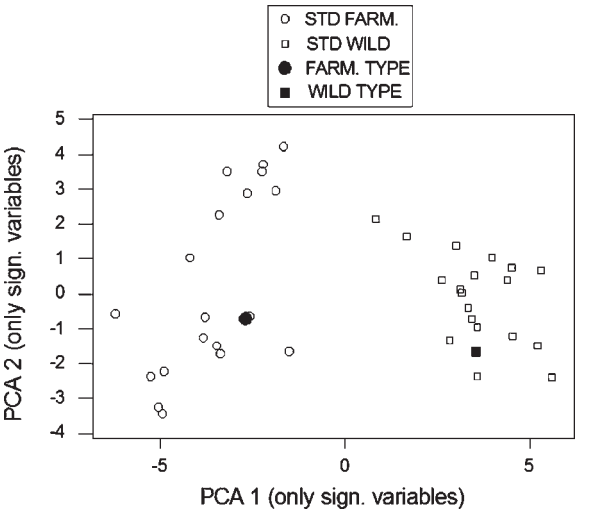

Figure 1. Plot of the first two components of the farmed and wild European sea bass standard samples using only significant variables (n = 30) obtained after NPC analysis between groups. Farmed and wild profile types have been plotted to highlight the visual position of each studied group.

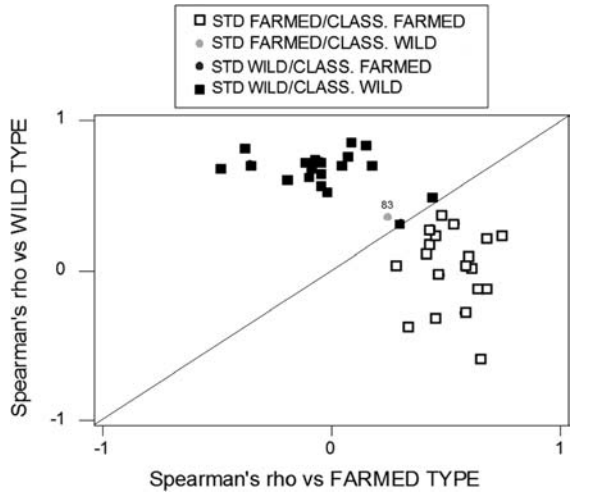

Figure 2. Performance of cross-classification according to nonparametric rank-based classification method. The two Spearman rho correlation coefficients of the standard data set were plotted versus the farmed and the wild profile type. Misclassified farmed sample has been labeled. The solid line (bisector) indicates equal value of Spearman rho correlations with wild and farmed profile type. "83" corresponds to a standard farmed classified as wild.

different (significance α-level equals to 5%). The global multivariate difference can be explained by 30 of the 36 considered variables, which have been denoted by an individual significant *P* value. Due to the so-called multiplicity problem (*34*), individual *P* values have been corrected using the Tippet procedure. The variables selected allowed discrimination between European sea bass production methods. A graphical representation of this result can be seen in **Figure 1**, where the first two PCAs were plotted along with the farmed and wild profile types obtained from the literature (see the Supporting Information for references and profile type description). Both profile types clearly belong to the respective homologous cluster. Rank-based cross-classification performances have been described in **Figure 2**. The percentage of the samples of the standard wild data set correctly classified was 100%, whereas it was 95% for the standard farmed data set, with only one misclassified sample (identified as 83). Most of the standard wild samples evidenced relatively high correlation with wild profile type (rho from 0.5 to 0.9) and at the same time low or no correlation with farmed profile type.

Survey Fish. The freshness evaluation highlighted that almost all of the samples collected were classified as A, which means an intermediate class of freshness. Comparisons between standard farmed versus survey samples and standard wild versus survey samples were carried out considering the 30 significant variables selected in the previous test (**Table 5**). This approach considered only the discriminative information to classify farmed and wild samples. The remaining variables played only a redundant noise effect. Because the global *P* value for the comparison between standard farmed and survey was equal to 0.071, there was no empirical evidence, at the 5% significance α level, that standard farmed and survey belonged to different populations. Most of the samples in the survey population were considered to be similar to standard farmed. In contrast, because the global *P* value for the comparison between standard wild and survey was equal to 0.013, at the 5% significance α level the standard wild and survey samples may come from two different populations. These results are represented graphically in **Figure 3**, where the first two PCA

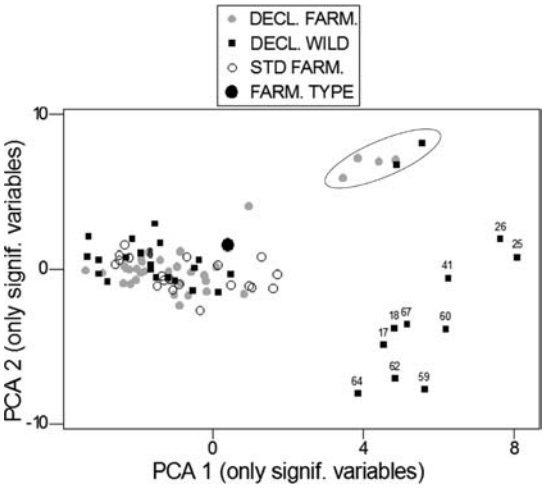

Figure 3. Plot of the first two components describing the contrast between standard farmed and survey samples using only significant variables (n = 30) obtained after NPC analysis between groups. Farmed profile type was also plotted to highlight the visual position of each studied group. The samples far from farmed cluster have been labeled and were considered as wild. The ellipse highlights a farmed subgroup far from the farmed cluster. Numbers highlight only the real wild survey sea bass.

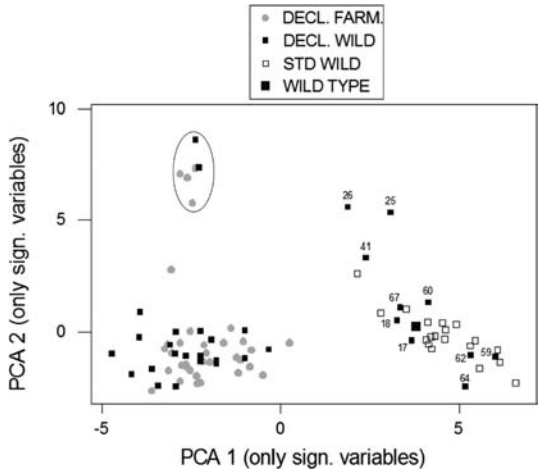

Figure 4. Plot of the first two components describing the contrast between standard wild and survey samples using only significant variables (n = 30) obtained after NPC analysis between groups. Wild profile type was also plotted to highlight the visual position of each studied group. The samples in the wild cluster have been labeled and are considered as wild. The ellipse highlights a farmed subgroup far from the farmed cluster. Numbers highlight only the real wild survey sea bass.

components for the groups of standard farmed and survey are plotted together with the farmed profile type (according to reference data). An identification label was put close to those samples that appeared to be "far" from the standard farmed. **Figure 4** shows the first two PCA components for the groups of standard wild and survey together with the wild profile type. The identification label has been put on samples that appeared to be "close" to the standard wild. These labeled units were, probably, the only genuine wild surveyed European sea bass.

Results of the nonparametric rank-based classification method for survey samples are shown in **Figure 5**, with individual labels for the declared wild/classified wild. According to this classification approach, only 11 samples were clustered into the declared wild/classified wild group. It is worth noting that many of these

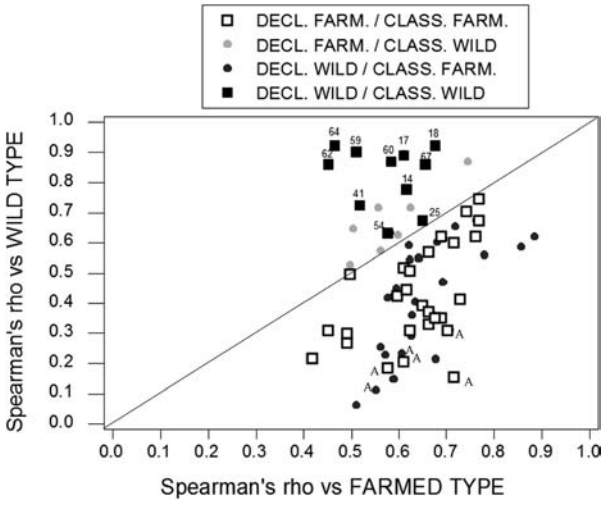

Figure 5. Plot of the two Spearman rho correlation coefficients of the survey samples versus the farmed and the wild profile type. This graph visually represents the novel proposed nonparametric rank-based classification method. The solid line (bisector) indicates equal value of Spearman's rho correlations with wild and farmed profile type. Numbers highlight only the real wild survey sea bass. The subgroup far from the farmed cluster were labeled "A".

labeled samples match those samples also labeled in **Figures 3** and **4**. Only one sample considered to be wild ("26" in **Figures 3** and **4**) was not assigned to the wild group by Spearman's rho correlation (**Figure 5**). On the other hand, two additional samples ("14" and "54") were assigned to the wild group only by the nonparametric rank-based classification (Spearman rho coefficients = 0.77 and 0.63 or 0.62 and 0.58 with wild or farmed profile type, respectively). All samples declared wild/classified wild showed Spearman rho coefficient with wild profile type higher than 0.60; meanwhile, the rho with farmed profile type was <0.69. These 11 "true wild" samples came, respectively, from large-scale distribution (n = 4) and fishmongers/small fish markets (n = 7).

DISCUSSION

A great amount of literature has described the effects of age, rearing condition, starvation, and feed intake on fish body trait variability (*24*). With regard to the condition factor (KI), the values pointed out were lower than those previously noted by other authors (*35*, *36*). This is probably due to a different measurement of maximum squeezed length. Furthermore, caught European sea bass had a lower condition factor (KI), which describes a slender body shape. Commercial feed for sea bass usually contains fish meal and fish oil, maize gluten, soybean and wheat meal (*37*), and variable percentages of vegetable oils. Its proximate composition varies in lipid (from 10 to >20%) and protein (46−53%) content (*35*, *38*). The higher energy intake of farmed subjects explained the significantly higher celomatic fat, IMF, HSI (*24*, *39*) and nitrogen content of farmed fish, which confirm that in European sea bass the primary sites of lipid storage are the liver and perivisceral adipose tissue (*40*). Comparable data on proximate composition were observed by other authors who sampled farmed and wild European sea bass in Greece and in Italy (*9*, *37*). Flesh lipid content in sea bass collected in the spring months in the Aegean sea was nearly 5-fold higher in cultured subjects than in wild ones (*41*). A similar trend for lipid flesh content was observed in the wild European sea bass from southern England, when compared to farmed ones from Scotland and Greece. In contrast, nitrogen content was higher in farmed

compared to wild sea bass (*16*). A similar difference has been recently described in subjects sampled from the Mediterranean area (*42*). The lower protein content of the wild subjects could be related to the reduced availability of nutrients that usually occur in the winter (*43*) and to the lower water temperature that would reduce food intake (*44*). This was also accompanied by a reduction in body lipid reserves and an increase in water content (*45*). The use of fatty acid and isotopic fingerprinting as authentication methodologies to identify the production method has been recently suggested by several authors (*15*−*17*). The present study highlighted that the highest value of SFA was obtained in wild sea bass as had also been described elsewhere (*9*, *16*, *37*). Among SFA, 16:0 and 18:0 were the highest contributing descriptors of the first component of the PCA (**Table 4**). The highest level of saturates may, at least partially, rely on more intense production by lipogenic activity. The natural diet of the European sea bass tends to be similar in terms of 16:0 and richest in 18:0 in comparison to aquaculture's feedstuff, the high energy content of which could depress the lipogenic activity (*40*,*46*,). The increment in the employment of vegetable oils in feed formulation (i.e., rapeseed oil) caused a general reduction in saturated FA, especially 16:0. In addition, the negative relationship observed in the present study between 16:0, 18:0, and IMF ($P < 0.001$) could also contribute to this phenomenon. Monoenoic acids showed the highest mean value in farmed fish, in accordance with other authors (*9*, *37*), whereas the percentages of polyenoic FA were similar between farmed and wild (*37*). Considering the n-6 group, in farmed sea bass the most abundant acid was LA. The flesh content of this fatty acid is generally higher in sea bass fed different terrestrial oil sources (*24*). It must be considered that several vegetable oils could be employed for partial substitution of fish oil in the feedstuff formulation, leading to both LNA and LA being enhanced (*7*). According to the explorative statistic approach of PC analysis some fatty acids such as OA, LA, and LNA together with Σ n-6 and IMF were the highest contributors to the determination of the cultured cluster. The large LA accumulation in intramuscular lipids of sea bass has been considered to be a marker for vegetable feeding ingredients (*5*, *9*). Similar observations on polyunsaturated n-6 have been reported for other marine species traded in the Mediterranean area such as the sea bream (*17*,*24*), turbot (*19*), and sharpsnout sea bream (*47*) and for Atlantic species such as the halibut (*48*). In addition, Özyurt and Polat (*43*) suggested that LA levels in wild Mediterranean sea bass varied during the year, with the maximum value being on average equal to 8.64%. In the present study, the variation in LA percentage found in cultured fish encompassed the seasonal variability of wild sea bass mentioned above. Meanwhile, C16:0, C18:0, ARA, Σ n-3, n-3/n-6 ratio, and δ^{13}C were the most powerful variables of the wild cluster. The ARA percentage in feedstuff is generally $< 1\%$ (*7*, *38*), whereas the natural diet of sea bass (i.e., invertebrates) contains more (*49*). On the other hand, LA, the precursor of the n-6 series, accumulated in the lipids of marine fishes without significant transformation due to their reduced capacity for chain elongation and desaturation (*50*). Interestingly, ARA percentage in aquaculture feeding trials has shown a decrease in the neutral and polar lipids in the juvenile European sea bass that is proportional to its concentration in the diet and the advancement of the feeding period (*51*). The higher percentages of ARA and DHA in wild fish are probably due to a reduction in the total amount of fat with particular regard to the neutral fraction. In the present study, IMF percentage was negatively correlated with ARA and DHA ($P < 0.001$), a remark that corroborates a former observation (*16*). In farmed fish, n-3 HUFA could be restored during a washing-out period with enrichment of the diet with fish oil with little drain of C18 fatty acids previously supplied by vegetable oil containing diets (*7*). Therefore, the use of these markers to distinguish between cultured and wild fish should be considered cautiously and only in relation to fingerprinting that takes into account also other fatty acids.

The δ^{13}C analysis of free-fat muscle showed the existence of a different isotopic pattern between farmed and wild standard samples. The level of δ^{13}C reflects the feed habit of fish: the employment of terrestrial material (with lower ^{13}C content) in the feed of farmed fish explained the significant difference between farmed and wild specimens. A similar trend was described for δ^{13}C of the bulk oil fraction of flesh total lipids of the sea bass (*16*). However, the variability in tissue lipid content can alter bulk tissue δ^{13}C values (*52*) and could be falsely interpreted as dietary or habitat shifts. For this reason, the present study considered only the free-fat extract. The IMF influences particularly the δ^{13}C of fish due to its large depletion in ^{13}C in comparison to other biochemical fractions (*53*). Furthermore, according to Barnes (*54*), variations in lipid content could influence δ^{13}C abundance. The δ^{13}C values observed in the present study were similar to the range described elsewhere for both farmed and wild groups considering only defatted tissues. With regard to δ^{15}N, the higher values in wild fishes can be a consequence of the higher trophic level of the fish feed in the marine natural system. The lipid extraction process should not affect the ^{15}N content of the defatted tissue, unless the extraction process leaks proteins linked to lipids (*55*). Among isotopes, only δ^{13}C was included with other major descriptors in the PC analysis. In fact, shifts in δ^{15}N values ($P < 0.05$) were observed depending on body weight and total length of the standard samples, even if it was shown that the effect of diet is predominant over size, time, and temperature change (*56*). Moreover, some authors have suggested that seasonal changes of δ^{13}C in muscle are small, especially for larger predator fish with a long turnover time of tissue (*57*). On the other hand, the influence of the temperature regime and the seasonality should be taken into account when populations that live at different latitudes are compared. Implementation of the isotope data set of European sea bass traded in Italy considering seasonality will improve the robustness of the model.

The NPC test applied on the standard data set indicated that the majority of the variables were adequate to differentiate between farmed and wild fish. Moreover, NPC test analysis revealed the presence of only six variables that carried redundant information and small discriminant function. The removal of these variables was necessary to improve clustering. Using the variables selected by the NPC test, it was possible to obtain a complete separation of the two clusters studied (farmed and wild standard data set, **Figure 1**). An initial comparison among the parametric and nonparametric approaches suggested a wide range of descriptive variables useful for classification of the fish production method. Other studies based on explorative PCA and related methods such as linear discriminant analysis reported a restricted number of variables useful to resolve problems on wild and farmed fish authentication (*16*, *19*). The use of the NPC test methodology is far from some limits of this approach that need the transformation of variables to obtain normal data distribution. On the other hand, the use of few variables in an explorative PCA could provide a rapid discrimination on a large data set (*16*), but this approach does not consider the wide range of variability in the overall population of commercialized European sea bass. The acquisition of the profile type as a tool for such analysis allowed for the broad comparison between the standard samples and the European sea bass population described in the literature. The degree of similarity (rho) shows the individual location of each analyzed subject in comparison with the variability of the

reference data. The analytical results obtained in the standard data sets were in accordance with the available references, highlighting the robustness of the analytical assessment and the diligence in the sample collection. The performance of the standard data set cross-classification obtained by the nonparametric rank-based methodology was very high (**Figure 2**). Nevertheless, the misclassification of one standard farmed sample could affect the accuracy of the global procedure (analytical and statistical methods) when applied to farmed samples. In contrast, this method correctly classified all standard wild samples. However, the real sensitivity and specificity of the nonparametric ranking method must be evaluated in a wide range of known samples. These preliminary tests indicate the usefulness of this approach to discover mislabeled samples collected during the survey.

According to the NPC test, one of the most noteworthy results of this study was that 23 of the 33 surveyed samples that had been labeled as wild were ascribed to the farmed cluster by the analytical approach adopted (**Figure 3**). Not one of the farmed European sea bass was assigned to the wild cluster (**Figure 4**). As shown in **Figures 3** and **4**, the score plot of the two-dimensional calculated PCs showed also a separation of six samples, four declared farmed and two declared wild, respectively (a third subgroup). These fishes were considered as undetermined samples; they had a high level of monounsaturated fatty acids concomitant with a low percentage of n-3 HUFA ($<4.5\%$), resulting in a subpartition. Nonparametric rank classification (**Figure 5**) of the survey samples determined a few changes on the general picture obtained with the NPC test. The subpartition disappeared because the six samples (**Figure 5**, A) that made it were assigned to the farmed group with Spearman rho ranging from 0.55 to 0.71 with farmed profile type and from 0.11 to 0.31 with wild profile type. This suggested that the variability of the reference data for cultured fish is wider than that concerning the standard data set. Moreover, seven samples were placed in a position defined as declared farmed/classified wild (**Figure 5**). This could be attributed to the ranking approach, where the chemical variables of the survey sea bass were compared with those of the profile type. Therefore, it is not surprising that some farmed fish presenting flesh composition close to that of the wild fish could be shifted toward this cluster. According to the rank classification the results suggested that only 11 of 33 samples provided by the present survey may really be labeled as wild European sea bass. Therefore, this novel classification method has proved to be effective and it provides a validation of the NPC results. However, it was the implementation of the profile type based on chemical data obtained from the literature that really brought to light the power of this analytical approach, albeit with some limits when applied to the classification of farmed fish. Exploitation of the wide reference resources that are growing up and that bear a broad variability in the chemical data can further improve the accuracy in the discrimination of true wild European sea bass from farmed ones adopting the nonparametric rank classification approach.

This study describes a preliminary survey on European sea bass commercialized in Italy. The results obtained in this surveillance action showed that the fraud of substitution (farmed for wild) occurred in two of three samples collected. Analysis of the results separated by type of sale showed that all 4 wild samples collected at large-scale retail trade were correctly labeled, whereas 22 of 29 wild samples collected at fishmongers' or fish markets resulted as mislabeled. Therefore, it seems that the authenticity of valuable fish products such as European sea bass sold at the retail point of the market was far from guaranteed. The employment of chemometric tools in addition to documental routine would be useful for the more structured enterprises to improve transparency and consumers' faith in the food trade.

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Supporting Information Available: Table 1, supplementary data on biometric traits, proximate composition, fatty acids, and stable isotope level of farmed European sea bass drawn from references; Table 2, supplementary data on proximate composition, fatty acids, and stable isotope ratios level of wild European sea bass drawn from references (the data quoted in Tables 1 and 2 have been used to calculate farmed and wild profile type, respectively; profile type was applied as comparative model for the nonparametric rank-based classification); Table 3, summary of the references employed for the construction of sea bass profile type with indication of season or water temperature, geographical area of relative fish sampling, and age or size of fish when available. This material is available free of charge via the Internet at http://pubs.acs.org.

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