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The globalized fish Industry: Employing DNA-barcoding and NIRS technology to combat counterfeiting and safeguard traditional agro-food products

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ABSTRACT

Traditional food plays a crucial role in showcasing the culture and heritage of its producers. The identification of the Traditional agro-Food Product (TFP) "*Seppia bianca di Chioggia*", which is derived from Italian traditional cuttlefish, was performed through genetic analysis utilizing mitochondrial cytochrome *c* oxidase I subunit gene (COI) sequences and near-infrared spectroscopy (NIRS). The set samples were obtained from the shores of Chioggia (Adriatic Sea), and a comparison was made with cuttlefishes collected from Cesenatico (Adriatic Sea) and the coastal regions of France (Atlantic Ocean). The primary objective was to differentiate the TFP from samples sourced from nearby production areas and distant geographic locations that are commonly sold in Italy. A total of 368 cuttlefishes were analysed for NIRS data, whereas a subset of 75 samples were randomly selected and analysed for COI sequences to assess cuttlefish origin and TFP certification. Genetic and NIRS models were then tested on the same set of samples using a machine learning approach through the hold-out validation method. Additionally, an external validation was performed using a new set of cuttlefishes (labelled fished in shore of Chioggia or in Atlantic Ocean) collected from the daily fish market located in Chioggia (Venice, Italy). Both technologies showed excellent accuracy (100%) in discriminating fishing origin between the Adriatic Sea and Atlantic Ocean. However, the genetic information was not sufficient to differentiate cuttlefishes between the closest fishing areas within the Adriatic Sea (balanced accuracy = 50–83.5%) and did not provide information about the production system (TFP certification), while it showed the highest performance of genetic membership assignment (≥96%) among untreated cuttlefishes collected in the fish market. However, the NIRS model completely differentiated (accuracy = 100%) samples according to the specificity of the treatment nature (simple skinned products vs. TFP preparations), while it was unable to differentiate cuttlefish origin in untreated samples collected in the fish market (accuracy = 50%). The present results demonstrated that NIRS represents a rapid, fast, green and nondestructive approach to support on-site, practical inspection to authenticate and promote TFP protection that mirrors a niche product as a result of the synergy between natural resources and cultural heritage.

1. Introduction

Traditional food can be considered representative of the culture and heritage of the producers, and it supports the rural population and improves customary small productions with social and ecological benefits (Cafiero, Palladino, Marcianò, & Romeo, 2020; Moscatelli et al., 2017). The enhancement of the "tradition" through foods is an explicit aspect embedded in some food certifications such as Traditional Specialty Guaranteed (TSG) and Traditional agro-Food Product (TFP; Mipaf, 1999). Although TSG is recognized at the European level, it is not strictly linked to a specific geographical area, contrary to TFP, which mirrors a niche product related to some restricted Italian regions as a result of the synergy between natural resources and culture. Indeed, food products that have distinct geographical connections with quality certifications tend to be regarded as higher in quality by consumers. This is largely attributed to the perceived advantages of regional products, including

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their lower environmental impact and higher levels of safety and freshness. These factors contribute to the positive evaluation of such foodstuffs by discerning consumers that show the willingness to pay more in support of regional production, promoting improved environmental sustainability (Balogh, Békési, Gorton, Popp, & Lengyel, 2016; Giraud & Halawany, 2006; Hassoun et al., 2020).

Traditional Agro-Food Product certification aims to safeguard the interdependence among environmental aspects and conditions, traditional techniques of production and social aspects (rural heritage and culinary uses). Moreover, the TFP certification endorses and supports the needs of the "from farm to fork" strategy of the "European Green Deal" roadmap (European Commission, 2020).

Traditional Agro-Food Product recognition is assigned to a specific food when "methods of processing, preservation and maturing are consolidated over time, homogeneous for the whole territory concerned, according to traditional rules, for a period of no less than 25 years" (Mipaf, 1999). Among the 5.632 Italian TFPs, 2.7% (i.e., 153) refer to preparations and special husbandry techniques of fish, molluscs and crustaceans. The Veneto region (Northeast Italy) stands out for having the highest TFP in fishery preparations and productions (n = 21 TF P; Mipaaft, 2019).

A relevant TFP recognition is attributed to a prepared cuttlefish named "*Seppia Bianca di Chioggia*", which is characterized by a specific process of fishing, production and preparation. In particular, cuttlefishes are fished in the North Adriatic Sea (FAO division fishing area 37.2.1) by adopting a specific technique (pots); after reception, the process consists of washing with purified brackish water followed by manual evisceration (removal of the viscera and bone) of the molluscs. As the final step, cuttlefish are placed back in steel tanks with treated lagoon water, and insufflation with air causes the phenomenon of flesh swelling and curling of the tentacles (40–50 min). This exclusive treatment with purified brackish water confers a particular value on the finished products, as this water resembles the salinity and microelement composition of the natural environment in which the mollusc lived (Veneto, 2014).

Cuttlefish and cephalopods representing the largest volume of domestic supply within Europe (totalling 247,540 tons), demonstrating a significant 21% increase from 2010 to 2019 (FAOSTAT: https://www.fao.org/faostat/en/#data/FBS). However, to meet consumer demand, Italy is the main worldwide importer of fresh cephalopods (squid and cuttlefish; 13.907 tons; FAO, 2018). However, the over-exploited fisheries and the complexity of the global supply chain offer distributors and final retailers several opportunities to falsify certification and traceability documents (the current unique traceability method in the EU) for the sake of greater profit. Moreover, many labelling regulations concern only wholesalers, not restaurants that could deceive customers. This behaviour hurts local fishing businesses and contributes to making fish the second most faked product in Europe (European Parliament and Council, 2011; Pramod, Nakamura, Pitcher, & Delagran, 2014; Sterling & Chiasson, 2014). Plus, food that is recognized for its quality is worth more: Certified items are usually sold for 2.07 times the price of regular items. For fish, shellfish, and crustaceans, certified products are valued at 1.35 times more than those without certification (AND-International, 2019). In addition to the environmental implications related to over- or underrepresentation of catching statistics (Kroetz et al., 2020), mislabelling seafood products can directly affect consumer health, causing reputational damage to competent authorities and to honest food business operators. Therefore, a reliable authentication system is essential for correct labelling to ensure the safety and confidence of consumers and conjunctively to protect local certified productions.

Currently, as reported in the recent review of Cardin et al. (2022), analytical procedures applied for origin authentication purposes involve targeted (i.e., DNA-based, multielemental and isotope analysis) and untargeted (i.e., near infrared spectroscopy, Fourier transform infrared) methodologies that could support the Enforcement of Regulation (EU) 1169/2011 (European Parliament and Council, 2011) for food labelling compliance and fraud detection. Among targeted analyses, the DNA barcoding approach is largely applied for species determination of marine organisms (Cermakova et al., 2023; Imtiaz, Mohd Nor, & Md.Naim, 2017). Although many studies using DNA profiling methods are available for seafood traceability (Gopi et al., 2019), there is a lack of use of this mitochondrial marker to differentiate different geographical origin areas in samples of the same species. This process usually involves the application of several genetic markers, such as the mitochondrial cytochrome *c* oxidase I subunit gene (COI). This gene was reported as suitable for accurate species identification of many marine organisms, including cephalopods (Drábková et al., 2019; Pérez-Losada, Nolte, Crandall, & Shaw, 2007). Although molecular approaches show important advantages, such as accuracy, sensitivity, and high reproducibility, these methods are not able to reveal environmental changes, harvesting periods, storage conditions, and manufacturing process effects on the product. Previous studies found differentiation among cuttlefishes with TFS certification from Chioggia (FAO fishing area 37.2.1) to other origins, such as the Mediterranean Basin (FAO 37.1/37.2) and Atlantic Ocean (France, FAO 27.7), through multielement profile evaluation (Varrà, Husáková, Patočka, Ghidini, & Zanardi, 2021), whereas David Wells et al. (2021) found that cuttlefish fished in the North Est Ocean had higher $\delta^{15}N$ values than Mediterranean Sea cuttlefishes.

Near-infrared spectroscopy (NIRS), on the other hand, has been recently acknowledged as a valuable untargeted analysis for assessing the freshness (physical status) of fishery products during official control, as specified by the Commission Implementing Regulation (EU) 2022/2503 (UE Reg. 2022/2503, 2022). Furthermore, NIRS has already demonstrated its effectiveness in evaluating the traceability of cephalopods (Currò et al., 2021). To the best of our knowledge, there is currently a gap in research regarding the potential of NIRS to discern fish products specifically for the purpose of quality certification recognition. In view of this, the present study aimed to assess an identification system that combines targeted (DNA barcoding) and untargeted (NIRS) analysis for traceability and authentication of TFP brand protection using the niche product "*Seppia Bianca di Chioggia*" as a model.

2. Materials and methods

2.1. Cuttlefish sampling

From December 2020 to July 2022, a total of 368 fresh cuttlefish (*Sepia officinalis*) fished in the division 37.2.1 of the subarea of the Adriatic Sea (https://fish-commercial-names.ec.europa.eu/fish-names/fishing-areas/fao-area-37_en) and in the division 27.7 d of the subarea of the Atlantic Ocean (https://fish-commercial-names.ec.europa.eu/fish-names/fishing-areas/fao-area-27_en) were sampled for traceability purposes (Table 1). In detail, cuttlefishes of the Adriatic Sea were collected close to the shores of Chioggia (Adriatic–Chioggia) and of Cesenatico (Adriatic–Cesenatico), whereas the Atlantic Ocean samples exclusively originated from France's coasts (FAO subarea 27.7 d). This set of samples was collected to test the classification capability between the closest (approximately 140 km; Cesenatico-Chioggia) and farthest sites (Adriatic Sea-Atlantic Ocean).

All cuttlefish samples, regardless of the fishing origin, were processed according to the same company procedures used for TFP "*Seppia*

Table 1
Geographic origin, correspondent FAO fishing area, and the number of cuttlefish samples considered for NIRS and DNA dataset included in the study.

Geographic Origin	FAO Fishing areas	NIRS	DNA barcode
Atlantic Ocean	27.7 d	289	30
Adriatic Sea	37.2.1	79	45
	Adriatic-Cesenatico	16	15
	Adriatic-Chioggia	63	30

Bianca di Chioggia", which consist of washings with purified brackish water and manual evisceration (removal of the skin, viscera and bones) and storage on ice for research purposes. Every cuttlefish was sampled within 12 h from arrival and following company procedures for spectral (NIRS) and genetic purposes. After NIRS analysis, a small portion of arm muscle tissue was excised and placed in Eppendorf tubes with 90% ethanol for further DNA extraction. The flow chart of the study pipeline is reported in the Supplementary file (S1).

2.2. NIRS data collection

The NIRS measurement was carried out after the usual company procedures (skin, gut and bone cuttlefish removal, and storage on ice) on the whole and refrigerated sample (0–2 °C). A PoliSPEC NIR (NIR tech; ITPhotonics, Breganze, Italia) NIRS portable spectrophotometer was used to collect cuttlefish spectral data (902–1680 nm, every 2 nm) through a round scanning window (3.2 cm^2). The individual spectrum of each cuttlefish was the result of the average scans collected continuously for 5 s at a 10-msec integration time. Spectral data were recorded in reflectance (R) units and successively converted into absorbance units as log (1/R) using poliDATA 3.0.1 software (ITPhotonics, Breganze, Italia).

2.3. DNA extraction and COI amplification

Prior to DNA extraction, the samples were carefully removed from ethanol and allowed to undergo a drying process. DNA isolation from the muscle samples was then performed utilizing the QIAGEN DNeasy Blood & Tissue Kit in strict adherence to the manufacturer's recommended protocol under sterile conditions. The resulting isolated DNA was subjected to quantification and assessment of quality using a NanoDrop™ spectrophotometer. To amplify a 700 bp fragment from the 5′ region of the COI gene, PCR was conducted employing the universally recognized primer pair LC01490 and HC02198, as originally described by Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994). The amplification products were subsequently visualized through gel electrophoresis using a 1% agarose gel. For sequencing, the forward direction of the purified amplicons was chosen, and the sequencing process was entrusted to a reputable contract sequencing facility (BMR Genomics, Padova, Italy), with LCO1480 serving as the sequencing primer. All obtained DNA barcodes were meticulously examined to ensure a minimum length of 700 base pairs (bp). To confirm the precise identification of the newly generated sequences as belonging to the *Sepia officinalis* species, comprehensive BLAST searches against the publicly accessible BOLD (Barcode of Life Data system) database (www.boldsystems.org) were carefully executed.

2.4. Sepia officinalis origin sets

A total of 368 individual fresh cuttlefish samples (n = 24 batches) were collected to consider wide sample variability to meet the requirements of NIRS identification analysis, and a large dataset is needed to develop the classification model. To thoroughly evaluate the identification sensitivity of NIRS technology, the data samples were assessed in two spectral datasets to build two identification classification models. In the first dataset (global set), samples were labelled according to the FAO fishing areas (37.2.1, Adriatic Sea, n = 79; 27.7 d, Atlantic Ocean, n = 289), while the second dataset (local set) aimed to assess the authentication of local cuttlefish considering three fishing spots in which France (n = 289) corresponds to the FAO fishing area 27.7 d, whereas the Adriatic Sea was spilt in Adriatic–Chioggia (n = 63) and Adriatic–Cesenatico (n = 16) samples.

A subset of 20% of the samples (n = 75) previously analysed using the NIRS device were randomly selected for genetic characterization of the mitochondrial COI gene. Specifically, 30 cuttlefish samples (n = 10 samples per batch) were considered from France (FAO area 27.7 d), whereas for the Adriatic Sea (37.2.1), 30 (n = 10 samples per batch) and 15 samples (n = 1 batch) were related to the Adriatic–Chioggia and Adriatic–Cesenatico coasts, respectively.

2.4.1. Calibration sets

The global and local datasets were divided into a training set for building classification models and into a testing set for evaluating and validating the developed models. Specifically, the training set for NIRS analysis consisted of 353 samples, whereas the COI analysis training set had 60 samples. The models were validated using hold-out validation, where the dataset was further split into a training set (70%) for repeated cross-validation (with 10 folds and 5 repetitions) and a testing set (30%) with samples selected to maintain proportional representation among sites. Further details are depicted in Fig. S2 of the supplementary material.

2.4.2. Validation sets

To evaluate and compare the identification performance of the NIRS and DNA barcode methods, a hold-out and an external validation were performed. The sets used for the hold-out and for external validation were the same for both analytical approaches to compare their classification performances.

In detail, the hold-out set comprised 15 samples (6 from Adriatic–Chioggia, 3 from Adriatic–Cesenatico, and 6 from the Atlantic Ocean) randomly chosen from the 20% sample of the DNA barcode set. Further details are depicted in Fig. S2 of the supplementary material.

Instead, the set tested in the external validation was derived from 10 new samples of *Sepia officinalis* acquired from the Chioggia daily fish market from two retailers. In detail, 5 of them were commercially sold and labelled as originating from the Atlantic Ocean, while the remaining 5 were designated as originating from the Adriatic–Chioggia Sea. To partially obtain sample homogeneity across sets, all market cuttlefish underwent manual evisceration, skinning and washing using running water by the fishmongers; this procedure is different from the preparation of cuttlefish with TFP marks, which are characterized by washing using purified sea water and air insufflation (Veneto, 2014). Cuttlefish samples were transported in portable refrigerators at 4 °C to the Department of Comparative Biomedicine and Food Science of the University of Padova to perform the NIRS analysis within 2 h and genetic analysis within 24 h from purchase.

2.5. Unsupervised graphical algorithms

Data analysis was carried out by using R software, version 3.2.5 (R Core Team, 2016). In detail, principal component analysis (PCA) was adopted as a descriptive tool for graphical data visualization to discover the patterns of 3D data distribution as reported in Weiner (2017).

2.6. DNA barcode analysis

The sequences were assembled and trimmed in Ugene v. 43 following the instructions in Okonechnikov, Golosova, & Fursov (2012). The multiple alignment of sequences was carried out using the Muscle algorithm implemented in Ugene software. To augment the dataset, two publicly available sequences of *Sepia elegans* and *Sepia bertheloti* (accession numbers KM517939.1 and MN977190) were included as outgroups. A neighbour joining (NJ) cluster analysis was then performed using MEGA XI (Koichiro, Glen, & Sudhir, 2021) to construct a graphical representation of nucleotide divergence patterns based on K2P distances. Moreover, to visualize the geographic diversity of populations, haplotype networks were designed using the median joining network algorithm in PopArt software (Leigh & Bryant, 2015). Haplotype statistics and diversity measures were computed using the Pegas package in the R programming language.

2.7. Supervised classification algorithms

The support vector machine (SVM) was modelled using the caret package of R for both methodologies (NIRS and COI).

In particular, to investigate the NIRS classification capability, SVM-Linear was applied to the global and local sets (Currò et al., 2021, 2022; Espiñeira & Santaclara, 2016). The training sets (global and local sets, both n = 353) were used for the repeated cross-validation (setting number = 10 and repeats = 5). In detail, the 'one-against-one' approach for multiclass (classes >3) classification was adopted, in which k (k − 1)/2 binary classifiers were trained, and a voting scheme was used to find the appropriate class; the C-value (Cost) in the linear classifier and the radial basis function sigma were customized, adopting a grid search. After the completion of the model training process, the classification method was executed to generate results on the sub set and on market set used for hold-out (global and local sets) and for external validation (market set), respectively. Confusion matrices were employed in both validations as robust tools for a comprehensive evaluation of the method's performance, encompassing metrics such as accuracy, sensitivity, and specificity (Bisutti et al., 2019; Velez et al., 2007).

To investigate the DNA barcode identification capability, the SVM classification algorithm was trained using a set of 80% (n = 60) from global and local sets. Using a Monte Carlo procedure with K-fold cross-validation, a set of known loci and individuals were randomly sampled in multiple iterations to train the classification algorithm, while the remaining known loci and individuals were used to iteratively test the accuracy of the predictive model. To perform the population assignment in the hold-out (global and local subsets) and in external validation (market set), the assignPOP package, as a supervised machine learning method, was applied to evaluate the discriminatory capability collected in the global and local sets. Membership probability values, defined as the proportion of cluster members in the entire dataset, were estimated from k-fold cross validation.

3. Results and discussion

(Mathew, 2022; Carvalho, Palhares, Drummond, & Gadanho, 2017), and the current practices for fraud estimation are generally problematic in the application or interpretation of the results. Indeed, verifying the geographical origin of fish and seafood requires intricate, cross-disciplinary approaches due to the combined impact of environmental factors and genetics on the final characteristics of these products (Abou-gabal et al., 2022). Generally, COI analysis has been applied as a method for genetic geolocation to trace the origin of individuals or populations, which suggest particularly relevant in identifying food fraud (Kim et al., 2015; Spies, Gaichas, Stevenson, Orr, & Canino, 2006). Although genetic analysis provides precise, sensitive, and unbiased results, it is costly and requires the use of chemical reagents. Additionally, such a method cannot be performed on-line and *in situ*, and its application and interpretation of the results demand specialized skills. However, NIRS represents a fast and realistic approach to apply in every phase of the supply chain to investigate food authenticity, especially when a product is identified by a quality mark. The NIRS approach offers support in real time among final consumers, producers and official controllers to prevent commercial fraud, recognize the relevant value of local production and protect their inherent tradition. The results will be described first based on the potential of identification between the Adriatic Sea and Atlantic Ocean fishing areas for the NIRS and COI techniques (paragraphs 3.2 and 3.3, respectively) using hold-out validation. Then, in the second part, the performance of such techniques on Local (using hold-out and external validations) and Traditional Food Product (using the hold-out validation) identifications will be described in paragraphs 3.4 and 3.5, respectively.

3.1. Graphical spatial distribution of spectral features

In this study, PCA was performed as an exploratory analysis to discover grouping and trends among NIRS spectral data according to the FAO areas and fishing sites. The spatial distribution of samples was mainly explained by the first 3 PCs accounting for 99.6% of the variance (PC1 = 70.1%; PC2 = 27.5%; PC3 = 2.0%). Promising classification outcomes are expected considering the clusterization observed both in NIRS for the global and local sets, as depicted in Figs. 1 and 2, respectively. In the scores plot shown in Fig. 1, the samples collected from the Adriatic Sea and the Atlantic Ocean exhibit a clear differentiation, forming two distinct groups. On the other hand, despite the close proximity of the samples in the local dataset, a notable clustering based on the three fishing sites is evident (Fig. 2). This similarity in spatial distribution can likely be attributed to either the strong interdependence of variables within each group or the extensive dispersion of sample data points.

3.2. NIRS analysis: cuttlefish classification according to the Adriatic Sea or Atlantic Ocean

The identification of fishing geographic origin products by combining infrared spectroscopy with chemometric approaches has been investigated by several authors. In detail, Liu et al. (2015) classified tilapia fillets from four different Chinese regions through chemical composition prediction, obtaining a range of correct identification from 72 to 85%. In the research conducted by Ghidini et al. (2019) successfully classified (sensitivity of 98%; specificity and accuracy both of 99%) minced semifinished and finished salted anchovies (*Engraulis encrasicholus*) according to geographical origin (Morocco, Spain, Tunisia or Croatia). In another study, Guo et al. (2018) reported a complete identification (accuracy = 100%) in sea cucumber (*Apostichopus japonicus*) among nine origins in China. The classification according to geographical origin in cuttlefish has already been assessed in an earlier study by Currò et al. (2021), in which an overall accuracy of 92% was observed by combining NIRS with the SVM model to classify cuttlefish according to five different FAO fishing areas (NE Atlantic Ocean, EC Atlantic Ocean, E Indian Ocean, WC Pacific Ocean, and the Adriatic Sea). However, the present study focused exclusively on fresh cuttlefish sold in Italy as local or imported to deeply define the fishing origin and to investigate the possibility of assessing the TFS certification. In the present study, a large number of fresh cuttlefishes caught per site (n = 289 and n = 79 samples per Atlantic Ocean and the Adriatic Sea, respectively) were considered to improve the variability of the fresh product with different origins but with the same treatment process to evaluate the NIRS capability to identify the real TFP. Indeed, the SVM model with the Linear Kernel (Table 2) differentiated cuttlefishes according to fishing origin and TFP certification even with unbalanced data among classes, similar to previous studies (Currò et al., 2021). The performance of the model was evaluated considering the overall accuracies and the ability to classify sample origin correctly as belonging (sensitivity) or not belonging (specificity) to a specific class. In particular, excellent classification performance (accuracy = 100%) was observed considering the two FAO fishing macro areas (Atlantic Ocean and the Adriatic Sea; Table 2) and the three fishing sites (Atlantic Ocean, Adriatic–Cesenatico and Adriatic–Chioggia coasts; Table 3). The highest accuracies of classification observed between the two macro areas and among the sites could be attributed to the differences in cuttlefish composition due to the variability in water environments and food sources of the considered fishing sites (Gopi et al., 2019; Saito, Ishihara, & Murase, 1997; Varrà, Husáková, et al., 2021). The findings highlight that despite treating all cuttlefish samples as TFPs, the implementation of NIRS technology enables the accurate identification and differentiation of the genuine "*Seppia Bianca di Chioggia*" TFP, fished specifically from the Chioggia fishing area, as opposed to the other fishing regions.

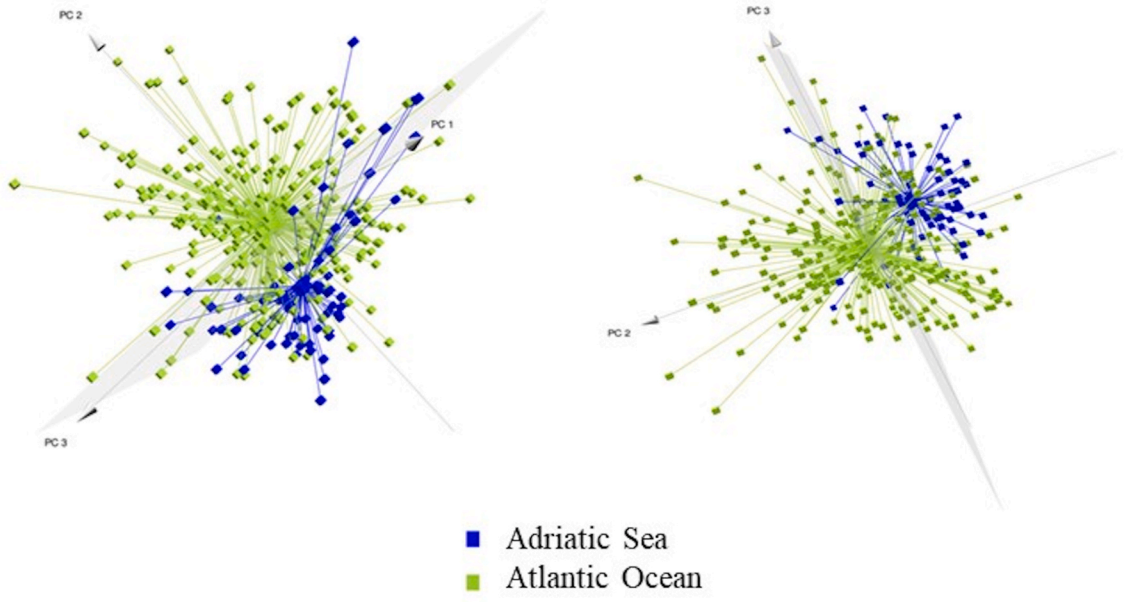

Fig. 1. Principal component score plot for PC1, PC2 and PC3 of cuttlefishes fished from the two FAO fishing area Atlantic Ocean (27.7; green cubes) and the Adriatic Sea (37.2.1; blue cubes). Images depict the same 3D model from two angles.

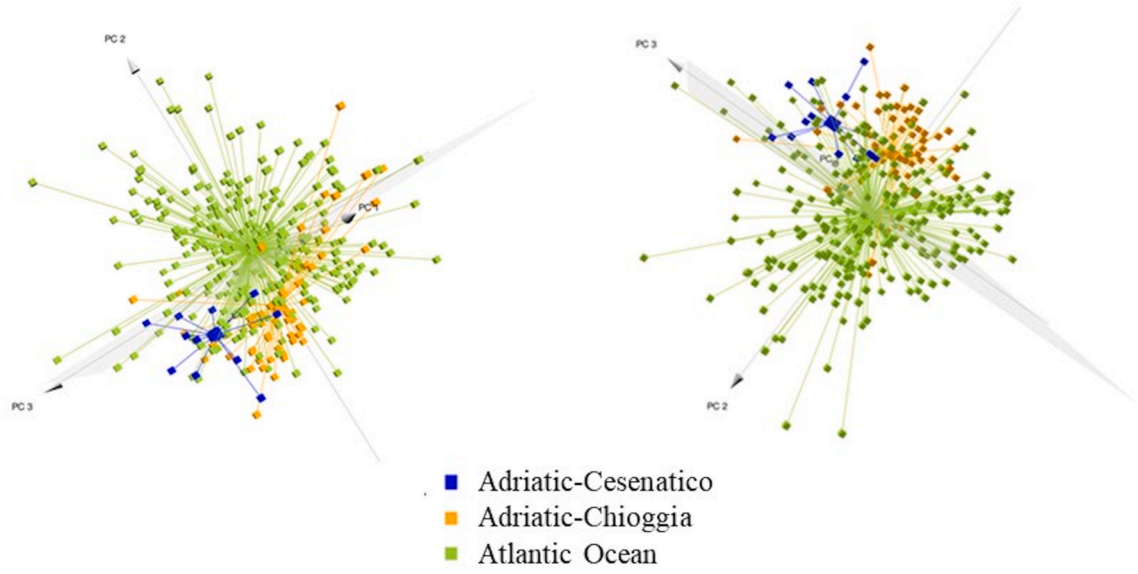

Fig. 2. Principal component score plot for PC1, PC2 and PC3 of cuttlefishes fished from Atlantic Ocean (green cubes) Adriatic-Cesenatico (blue cubes) and Adriatic-Chioggia (yellow cubes). Images depict the same 3D model from two angles.

3.3. Genetic analysis of COI: cuttlefish classification according to the Adriatic Sea or Atlantic Ocean

Concerning the genetic analysis, a total of 75 samples from the Atlantic Ocean and Adriatic Sea were successfully sequenced and examined. Fig. 3 summarizes the results of the genetic analysis. In particular, Fig. 3 depicts the median-joining haplotype network. Each circle represents a unique haplotype, and its size reflects the number of individuals expressing that haplotype. Crosshatches indicate the number of nucleotide differences between haplotypes. Focusing on the population structure of *Sepia officinalis* between the Atlantic Ocean and Adriatic Sea, the haplotype network from the mitochondrial marker COI showed a total of 18 haplotypes. Of these, 10 haplotypes grouped under the Atlantic Ocean cluster, which is equivalent to 55% of the total haplotypes identified. The remaining 8 haplotypes grouped under the Adriatic Sea, with most of them shared between the two Adriatic Sea sample sites, Chioggia and Cesenatico (Fig. 3 84% for all sequences. Population connectivity was grouped into two main clusters, with no shared haplotype between the Atlantic and Adriatic clusters. The NJ tree based on COI showed that Atlantic lineages tended to cluster together, separated from the cluster formed by the Adriatic samples (Fig. 3).

Therefore, the population structure is clearly comprised of two main distinct clusters, seen both in the haplotype network and in the phylogenetic tree. This result suggested a useful application of COI sequences for the attribution of local provenance, albeit limited to the comparison between the Atlantic Ocean and the Adriatic Sea, which are separated by a geographical barrier to migration/gene flow. These data suggested segregation between two major populations (Atlantic Ocean vs. Adriatic

Table 2
Performance of classification of linear SVM according to the global set considering the two fishing areas in hold-out validation. The number of samples (N) used in the test set were the same for infrared spectroscopy (NIRS) and genetic analysis.

NIRS	Predicted Classes (N)	
	Atlantic Ocean	Adriatic Sea
Atlantic Ocean	6	0
Adriatic Sea	0	9
Sensitivity (%)	100	100
Specificity (%)	100	100
Balanced accuracy (%)	100	100
Genetic		
Atlantic Ocean	6	0
Adriatic Sea	0	9
Sensitivity (%)	100	100
Specificity (%)	100	100
Balanced accuracy (%)	100	100

Table 3
Performance of classification of linear SVM according to the local set considering the three fishing areas in hold-out validation. The number of samples (N) used in the test set were the same for infrared spectroscopy (NIRS) and genetic analysis.

NIRS	Predicted Classes (N)		
	Atlantic Ocean	Adriatic-Cesenatico	Adriatic-Chioggia
Atlantic Ocean	6	0	0
Adriatic-Cesenatico	0	3	0
Adriatic-Chioggia	0	0	6
Sensitivity (%)	100	100	100
Specificity (%)	100	100	100
Balanced accuracy (%)	100	100	100
Genetic			
Atlantic Ocean	6	0	0
Adriatic-Cesenatico	0	0	3
Adriatic-Chioggia	0	0	6
Sensitivity (%)	100	0	100
Specificity (%)	100	100	67
Balanced accuracy (%)	100	50	83.5

Sea). Isolation by distance (IBD) was proposed as the main factor involved in the segregation of cuttlefish populations (Atlantic Ocean (NEA) and Mediterranean Sea (MS; Pérez-Losada et al., 2007). This genetic isolation could be related to geographical barriers, differences in environmental conditions and peculiar biological traits of sepia officinalis, such as their attitude towards short migrations and their reproductive habits as benthic eggs (Pérez-Losada et al., 2007). Conversely, these results also confirmed previous findings on the cuttlefish population of the Atlantic Ocean that was defined by a considerable degree of genetic variation with a valuable genetic exchange between local populations (Wolfram, Mark, John, Lucassen, & Pörtner, 2006).

3.4. NIRS and COI analyses: Local product identification

The capability of techniques to identify the local product was performed first on the local set using the hold-out validation and then using the market samples through the external validation for both techniques.

Considering the counterfeiting problems, the present results on NIRS showed pros and cons of these fast technologies. Although cuttlefish samples had identical treatments and originated from the same fishing area (Adriatic Sea; FAO fishing subarea 37.2.1), the NIRS technology combined with the SVM model demonstrated a clear capability to differentiate samples fished from the Chioggia and Cesenatico shores, proving complete sensitivity, specificity and balanced accuracy (100%). This is probably due to differences in the diet and in the environmental conditions (elemental composition). This distinction suggests that NIRS could be applied and included in the fishery supply chain by food business operators and food control authorities first to verify and demonstrate the authenticity of the product origin. Moreover, the present study represents a valuable and faster alternative of screening to classify cuttlefish without sampling destruction compared to the studies of Varrà et al. (2021) and David Wells et al. (2021). Regrettably, the NIRS classification model applied in the external validation on the market set yielded unsatisfactory results; specifically, the confusion matrices revealed that the samples from the fish market were randomly assigned (accuracy = 50%) between classes (data not shown). This implies that the classification model failed to accurately distinguish and assign the samples to their respective origin classes, leading to unreliable origin distinction. Such a result was expected and explainable by the differences in the training set composed of cuttlefish treated as TFPs (washing in stainless steel tanks with clean seawater and air insufflation) and used to distinguish samples cleaned and nontreated as TFPs collected from the fishery market (gutted, skinned and washed using running water).

According to the COI sequence analysis, the SVM model used for the classification of individuals at their geographic origin was very sensitive, with a membership probability of 94% for the Atlantic basin (Table 4). Regarding the Adriatic Sea, all the samples from Chioggia and Cesenatico were assigned a greater probability to Chioggia, with an average membership probability of 75% (Table 4). This is probably because Chioggia and Cesenatico samples are genetically indistinguishable or due to the greater number of samples considered for Chioggia sites. Regarding the fish market samples, all the individuals were correctly assigned to their geographical origin as indicated on the related label, showing a membership probability between 96% and 98% for all samples (Table 4). Some studies on cuttlefish have shown the absence of a population structure within the Adriatic basin (Drábková et al., 2019; Garoia et al., 2004; Pérez-Losada et al., 2007). Moreover, in other geographic areas of the Mediterranean Sea, it also seemed difficult to define a genetic classification between populations, and other methods could be useful in providing more in-depth information for the local structure of the populations (Turan & Yaglioglu, 2010). However, the problems related to genetic information could be overcome by the application of different tandem technologies (e.g., other untargeted approaches, food-profiling approaches or specific local markers).

3.5. NIRS and COI analyses: Traditional Food Product identification

To confirm the misclassification that is dependent on the divergences in treatment, a further investigation was performed to evaluate whether the NIRS approach can reveal the differences between treated and untreated cleaned cuttlefishes to check the authentication of the TFP certification. In detail, the dataset of samples processed as TFP (n = 368) and non-TFP (n = 10) cuttlefishes was investigated using the SVM linear model and considering a hold-out validation (n = 264 samples for the training set and n = 114 in the testing set, maintaining the proportionality of 70% and 30% of data between the sets). The results obtained were remarkable, exhibiting a flawless performance with a 100% achievement in sensitivity, specificity, and accuracy (S3 in Supplementary material). Conversely, COI analyses evaluating the genetic

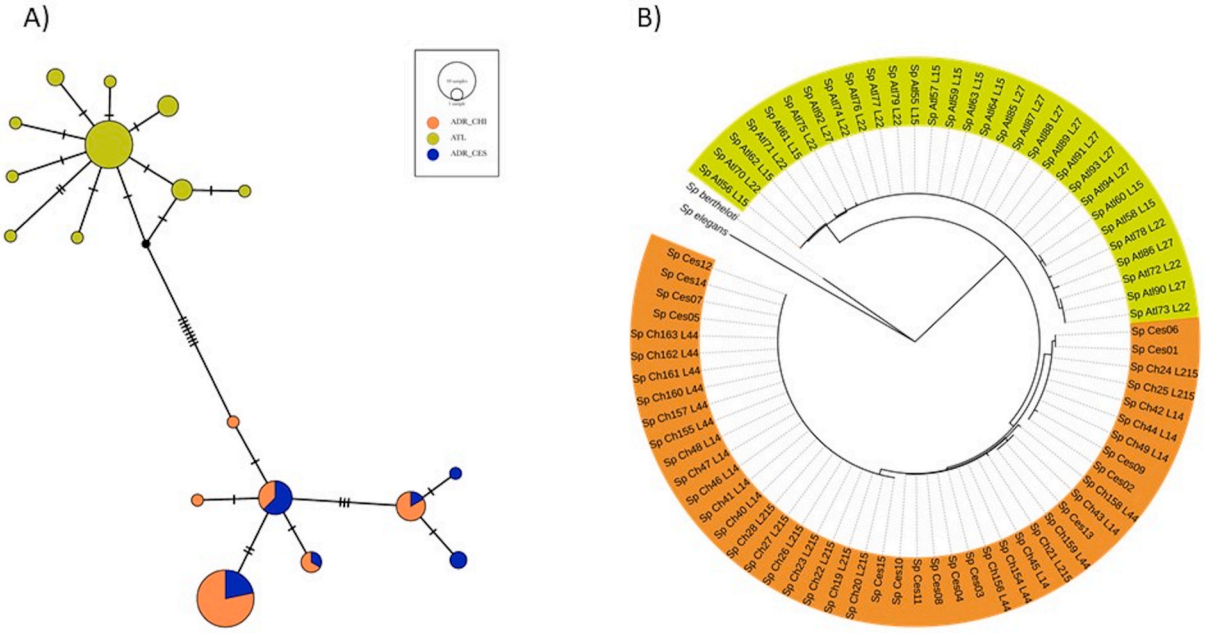

Fig. 3. Population net (PopArt, TCS network) of Atlantic (green circles) and Adriatic samples (orange circles for Chioggia samples and blue circles for Cesenatico samples) of *Sepia officinalis* (A). Phylogenetic tree of the *Sepia officinalis* samples inferred from a mitochondrial COXI gene alignment (B). The Adriatic cluster is highlighted in orange and the Atlantic cluster in green.

Table 4
Genetic Membership assignment (%) of global, local set in hold-out and samples from Chioggia fish market in external validation.

Reference classes	Genetic Membership assignment (%)- hold out validation		
	Atlantic Ocean	Adriatic-Cesenatico	Adriatic-Chioggia
Atlantic Ocean	96	–	4
Adriatic Sea	6	–	94
	Genetic Membership assignment (%)- hold out validation		
Atlantic Ocean	94	2	4
Adriatic-Cesenatico	4	26	70
Adriatic-Chioggia	4	21	75
	Genetic Membership assignment (%)- external validation		
Atlantic Ocean	98	–	2
Adriatic-Chioggia	4	–	96

sequences were not affected by the TFP process of cuttlefishes. This outcome highlights the robustness and reliability of the methodology employed in accurately distinguishing and classifying TFP cuttlefish to preserve the strong connection between territoriality and tradition. This approach supports efforts to combat counterfeiting and mislabelling; otherwise, it promotes the farm-to-fork strategy safeguarding the heritage and tradition associated with local products.

4. Conclusions

This study highlights the challenges posed by counterfeiting and mislabelling in the fish sector, particularly in the context of globalization and the farm-to-fork strategy. Cuttlefishes collected from the Atlantic Ocean, Adriatic–Chioggia and Adriatic–Cesenatico shores were processed according to the treatment procedure of TFP in a business plant and were investigated through genetic analysis (COI sequences) and near-infrared spectroscopy (NIRS) to identify the unique fishing origin (Adriatic–Chioggia) related to the "*Seppia Bianca di Chioggia*" TFP mark. The hold-out approach was applied in the genetic sequences and in the spectroscopic data showing accurate identification of fishing origin in both approaches. Nevertheless, contrary to the NIRS approach, no genetic differences were observed between Adriatic–Cesenatico and Adriatic–Chioggia cuttlefishes. Moreover, the genetic and NIRS identification models were also externally tested on samples collected on the fishery market and recognized as non-TFP cuttlefishes to assess the classification capability according to the origin. Notable genetic identification was observed, whereas NIRS technology, utilizing a model developed with TFP-treated samples, exhibited a high level of specificity, which resulted in its inability to accurately identify the origin of untreated samples (non-TFP) collected by the fish market. Otherwise, the NIRS approach in this study showed excellent distinction capability of cuttlefishes from the fish market and business plant due to the differences in treatment. Thus, NIRS technology offers a fast and feasible alternative to genetic analysis, especially when quality marks and specific treatment are involved. Integrating genetic analysis and NIRS can help combat counterfeiting. Further research can explore the application of NIRS to other fish species and improve classification models to protect local productions and the relative's traditions and cultural heritage.

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CRediT authorship contribution statement

Sarah Currò: Investigation, Data curation, Writing – original draft, Conceptualization, Methodology. **Massimiliano Babbucci:** Investigation, Data curation, Writing – original draft, Writing – original draft, preparation, Conceptualization, Methodology. **Paolo Carletti:** Supervision, Writing – review & editing. **Luca Fasolato:** Investigation, Writing – original draft, Writing – original draft, preparation, Conceptualization, Methodology. **Enrico Novelli:** Supervision. **Stefania Balzan:** Writing – review & editing, Conceptualization, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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