DNA barcoding and nutritional analysis as a tool for promoting the market of inland fish species

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Abstract

The increasing world market demand for seafood requires an expansion of product categories available to consumers. Inland fish are usually considered having unmarked taste and are less appreciated by consumers; thus, they have low commercial value. Therefore, the marketing of the lake's fresh and processed fish is limited to the local market and consumers are currently uninformed and mistrustful about these species. In this study, six different fish species were caught in the Fondi lake (Lazio, central Italy): *Anguilla anguilla*, *Tinca tinca*, *Carassius gibelio*, *Cyprinus carpio*, *Micropterus salmoides*, *Chelon ramada*. All the samples were subjected to nutritional and DNA barcoding analysis. Moisture, protein, fat, carbohydrates, ash, and sodium content were measured. As regards the fatty acids profile, the most abundant were MUFAs with the highest value in *Anguilla anguilla* (45.97%). Oleic acid (C18: 1 n9 cis) was particularly high in *Cyprinus carpio* (55.46%). The fraction of polyunsaturated fatty acids (PUFA) revealed a higher DHA content (C22: 6 n3) in *Anguilla anguilla* than the other species (>12 %) while *Chelon ramada* presented both higher EPA content (C 20: 5 n3) and total fraction of omega 3 PUFAs. Concerning molecular analysis, a 655 bp fragment of cytochrome C oxidase subunit I (*COI*) gene was successfully used for the identification at the species level using both BOLD and BLAST public databases. The present study gives the basis for improving the knowledge and promoting inland fish' market and traceability along the supply chain.

Introduction

The increase of the world market demand for fish products is due to the growing public awareness and consumers' expectation concerning food security and quality (FAO, 2020). Fish is considered a high nutritional quality food with beneficial effects on human health for the favourable composition of proteins, minerals, vitamins, and essential fatty acids (FA) (Linhartová *et al.*, 2018). In particular, it contains high levels of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, that were demonstrated to prevent coronary artery disease, cancers, and diabetes (Adámková *et al.*, 2011; A. Watters and M. Edmonds, 2012; Linhartová *et al.*, 2018). Besides, DHA has been considered essential for brain and eye development during pregnancy and early childhood (Özogul *et al.*, 2007). The need to satisfy the growing market demand caused an over-exploitation of marine fish stocks and the massive importation from other countries (FAO, 2020). The longer supply lines of the fish chain promote the occurrence of different forms of fish frauds (Reilly, 2018) mainly regarding species substitution of expensive species with less valuable ones coming from abroad and mislabelling (Ceruso *et al.*, 2019; Mascolo *et al.*, 2019). In order to reduce the importation, strengthen the local economy, and improve the market of national products, it would be important to expand the knowledge about local seafood categories between consumers.

Inland fisheries, including fish captures in lakes, rivers, streams, canals, reservoirs, and other land-locked waters (FAO, 2018), are rapidly expanding on a global scale. In 2018, world total capture fisheries production in inland waters recorded their highest-ever catches, at over 12 million tonnes, ten of which are represented by freshwater fish (Aquaculture, 2020). Even though it has been proved that the provided source of essential FA is equivalent to marine fish (Özogul *et al.*, 2007; Linhartová *et al.*, 2018), the effective variety of inland species that can be caught is not properly exploited. In fact, in the common scenario, freshwater fish mainly coming from lakes are often considered having unmarked taste and are less appreciated by consumers, thus they have low commercial value (Özogul *et al.*, 2007; Linhartová *et al.*, 2018).

The current study aimed to improve the knowledge about the quality of some inland fish through their nutritional analysis and species identification in order to promote the trade and the traceability of species with market potential. Different inland specimens belonging to edible species (D.M. MIPAAF, 22 September 2017) have been caught in the Fondi lake, the largest coastal lake of Lazio, central Italy. In the lake there are more than 25 species of fish, some typical of freshwater and others of salt and brackish water, because of the communication with the sea through the canals of Sant'Anastàsia and Canneto (http://www.cittadifondi.it/?page_id=560). In order to provide a full view of inland fish categories of the Fondi lake, both freshwater fish such as *Cyprinus carpio*, *Tinca tinca*, *Carassius gibelio*, *Micropterus salmoides*, euryhaline species such as *Anguilla anguilla*, and a typically marine but under-consumed fish such as *Chelon ramada*, were evaluated in this study.

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Materials and methods

Fish sampling

Six fish species caught in the Fondi Lake (Lazio, central Italy, Figure 1) were collected: European eel (*Anguilla anguilla*, Linnaeus 1758), Tench (*Tinca tinca*, Linnaeus 1758), Prussian carp (*Carassius gibelio*, Bloch 1782), Common carp (*Cyprinus carpio*, Linnaeus 1758), Largemouth bass (*Micropterus salmoides*,

Lacepède 1802), Thinlip grey mullet (*Chelon ramada,* Risso 1827). All the species were provided by local fishermen. Fish species were frozen at -20 °C and transported in a sealed box to the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II. The species were classified based on their anatomical and morphological characteristics and then stored at -80°C until nutritional and molecular analysis was performed.

Nutritional analysis

After preliminary desquamation and manual filleting of fish species, muscle tissues were subjected to chemical analysis according to the A.O.A.C. Official Method of Analysis (Association of Official Analytical Chemists Inc., Arlington, VA, USA, 2000). All tests were done in duplicate for each sample. The protein content was calculated with the Kjeldahl method (*method 991.15*); the lipid fraction was obtained by gravimetric method (*method 960.30*); the moisture was assessed by drying fish samples in an oven (105°C for 24h) (*method 950.46*); finally, the ash content was obtained using a muffle furnace at 600°C (*method 923.03*).

The remaining percentage from the analysis of the above-mentioned parameters was considered as a quote of carbohydrates. The sodium content was determined by argentometric titration with colorimetric indicator.

For the fatty acid profile, a transesterification with 2.5% sulfur: methanol solution (H_2SO_4: MeOH) was used (Watts and Browse, 2002). The fatty acid methyl esters (FAMEs) of the total fat content were analyzed by capillary gas chromatography, using a GC equipped with a flame ionization detector (FID) and a capillary column (Watts and Browse, 2002). The qualitative characteristics assessment of the lipid fraction was carried out by evaluating the atherogenicity index (AI) and the thrombogenicity index (TI) (Ulbricht and Southgate, 1991; Garaffo *et al.*, 2011). These values were calculated on the basis of the relative percentages of acidic fractions, according to the following formulas:

$$AI = (C12:0 + 4 \times C14:0 + C16:0) / [(\Sigma MUFA + \Sigma PUFA\ (n\text{-}6) \text{ and } (n\text{-}3)]$$

$$TI = (C14:0 + C16:0 + C18:0) / [(0.5 \times \Sigma MUFA + 0.5 \times \Sigma PUFA\ (n\text{-}6) + 3 \times \Sigma PUFA\ (n\text{-}3) + (n\text{-}3)/(n\text{-}6)]$$

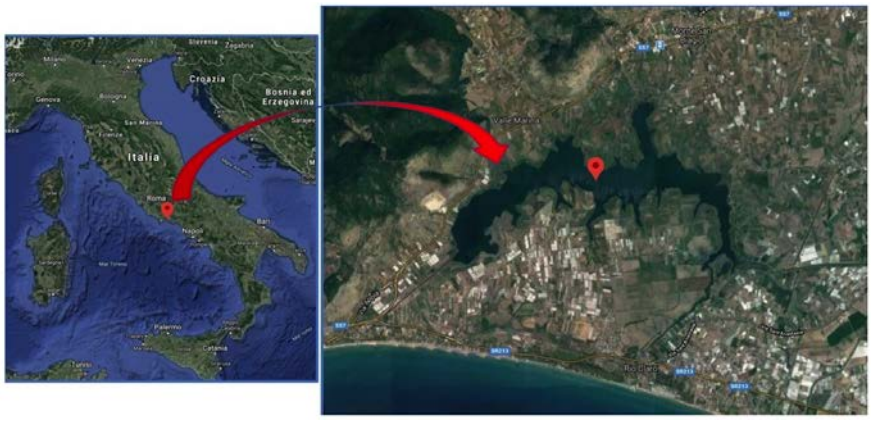

Figure 1. Fondi lake, Lazio, Italy.

Total genomic DNA extraction

Total genomic DNA was extracted from muscle tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA concentration and purity were measured at the ratios of 260/2809 nm and 260/230 nm using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Electrophoretic analysis in 1% agarose gel was performed to check the DNA quality.

Primers selection, PCR amplification and sequencing

A literature investigation was initially performed in order to compare fish's universal primers used for the amplification of a long amplicon length (LAL) fragment from the mitochondrial cytochrome oxidase subunit I (*COI*) gene. All the universal primers considered in this research were reported in Table 1.

The complete mitogenome sequences of the selected freshwater fish species were provided by GenBank: *Anguilla anguilla* (NC_006531.1, Minegishi *et al.*, 2005), *Tinca tinca* (NC_008648.1, Saitoh *et al.*, 2006), 2006), *Carassius gibelio* (NC_014177.1, Liang et al., 2010), *Cyprinus carpio* (NC_001606.1, Chang et al, 1994), *Micropterus salmoides* (NC_008106.1, Broughton et al, 2006), *Chelon ramada* (complete mtDNA not reported). In order to find the most well-matched primer sequences suitable for species identification, *COI* nucleotide sequences of the six species were aligned and analysed using MEGA X (Kumar *et al.*, 2018).

PCR amplifications were carried out in a 2720 Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol reported in Table 2 (Giusti *et al.*, 2017,

Table 1. List of universal primers for fish species compared in this study.

Primer name	Primer sequences	Amplicon length (bp)	Reference
F (LCO1490)	GGTCAACAAATCATAAAGATATTGG	658	Folmer *et al.*, 1994; Hebert *et al.*, 2003;
F (HC02198)	TAAACTTCAGGGTGACCAAAAAATCA		Giusti *et al.*, 2017
H (FISHCOILBC)	CTCAACYAATCAYAAAGATATYGGCAC	655	Handy *et al.*, 2011;
H (FISHCOIHBC)	ACTTCYGGGTGRCCRAARAATCA		Giusti *et al.*, 2017
COIF-ALT	ACAAATCAYAARGAYATYGG	658	Mikkelsen *et al.*, 2005;
COIR-ALT	TTCAGGRTGNCCRAARAAYCA		Giusti *et al.*, 2017
FishF1	TCAACCAACCACAAAGACATTGGCAC	655	Ward *et al.*, 2005; Hubert *et al.*, 2008; Lakra *et al.*, 2016
FishF2	TCGACTAATCATAAAGATATCGGCAC	655	Ward *et al.*, 2005
FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	655	Ward *et al.*, 2005; Hubert *et al.*, 2008; Lakra *et al.*, 2016
FishR2	ACTTCAGGGTGACCGAAGAATCAGAA	655	Ward *et al.*, 2005

modified). PCR products were confirmed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized via ultraviolet transillumination with Universal Hood II Gel Doc System (Bio-Rad, USA). The amplicons were assessed by comparison with the standard marker GeneRuler 50 bp (Thermo Fisher Scientific, Waltham, MA, USA) and then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Amplified products sequencing was carried out by Bio-Fab Research (Rome, Italy). The obtained sequences were aligned using ClustalW integrated into MEGA X (Kumar *et al.*, 2018).

Data analysis

All the gene sequences were submitted to both BLAST analysis (GenBank) and Identification System (IDs) Species Level Barcode Records of BOLD.

The highest identity percentages obtained within the first 100 top match records by BLAST and ID's query were recorded. Species identification with BLAST analysis was based on maximum scores with matching sequences corresponding to >98% identity and coverage, and alignment values E=0.0. As for BOLD, specimens were considered identified at species level when the matches showed at least 98% identity with the reference sequences (Barbuto *et al.*, 2010, Armani *et al.* 2015). The results obtained using molecular analysis were then compared to the morphological and anatomical identification of the caught fish species made by experts.

Results

Nutritional analysis

The chemical composition of each species collected in this study is presented in Table 3.

The percentage of protein (11.8-18.9%), carbohydrates (0.1-0.9%) and ash (0.9-2.8%) varied between species. *Tinca tinca*, *Carassius gibelio* and *Cyprinus carpio* were the species with the highest levels of protein (>17%) while *Anguilla anguilla* showed the lowest content (11.8%). Different values were also found in the lipid content and moisture: in particular *Tinca tinca* was found to be the species with the lowest fat content (0.5%) whereas *Anguilla anguilla* exceeded 23%.

The results of fatty acid composition showed that the class of monounsaturated fatty acids (MUFA) is the most represented, with the highest value in *Anguilla anguilla* (45.97%). Within the MUFA fraction, the highest percentage of acidic content is given by oleic acid (C18: 1 n9 cis), particularly high in *Cyprinus carpio* (55.46%).

The saturated fatty acid (SFA) fraction was not very different between species (21-27%). However, within the class, there are differences relative to single fatty acids. A higher content of myristic acid (C14: 0) and stearic acid (C18: 0) was found in *Chelon ramada* (4.39%) and *Carassius gibelio* (6.70%). As regards the fraction of polyunsaturated fatty acids (PUFA), *Anguilla anguilla* presented a higher DHA content (C22: 6 n3) than the other species (>12%) while *Chelon ramada* presented a higher EPA content (C20: 5 n3) (~12%) and a higher total fraction of omega 3 PUFAs. The increase in this value is mainly due to a higher content of alpha-linoleic acid (ALA - C18: 3 n3) and stearidonic acid (18: 4 n 3).

Total genomic DNA extraction and primers selection

From all the fish species, a good quantity of DNA (~ 40 ng/µl) was extracted. The spectrophotometric analysis confirmed high yield and quality, with ratios of A260/A280 nm and A260/A230 nm >1.8 in all the samples. After the comparison of sequences and the evaluation of the number of matches, two pairs of primers were selected: F

Table 2. Amplification protocols and programs for each couple of primers selected in this study.

	F	H
Buffer 10x	2 µl	2 µl
MgCl2	1.5 mM	1.5 mM
dNTPs	200 µM	200 µM
Taq polymerase	1.25 U	1.25 U
BSA	25 ng/µl	25 ng/µl
DNase free water	up to the final volume achievement (FV)	up to the final volume achievement (FV)
Primers concentration	200 nM	300 nM
Total DNA	~40 ng/µl	~40 ng/µl
Taq activation	94°C for 3'	94°C for 3'
N. of cycles	35	45
Denaturation	95°C for 1'	94°C for 30''
Annealing	50°C for 1'	55°C for 20''
Extension	72°C for 30''	72°C for 40''
Final elongation	72°C for 7'	72°C for 10'

Table 3. Nutritional values (%) of sampled fish species for 100 g of muscle tissue.

	Anguilla anguilla	*Tinca tinca*	*Carassius gibelio*	*Cyprinus carpio*	*Micropterus salmoides*	*Chelon ramada*
Moisture	61.5	78.5	72.5	71.5	80.5	73.4
Protein	11.8	17.9	18.9	17.5	14.7	15.8
Fat	23.7	0.5	7.1	8.4	3.3	6.8
Carbohydrates	0.25	0.9	0.1	0.2	0.1	0.7
Ash	2.1	1.4	0.9	1.7	0.9	2.8
Sodium	0.65	0.8	0.5	0.7	0.5	0.5

(LCO1490 - HCO2198) and H (for_ FISHCOILBC - rev_ FISHCOILBC). Melting temperature (Tm), nucleotide composition, secondary structure, self-annealing, and inter-primer binding, were verified using Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The primers' set is reported in Table 4.

PCR amplification and sequencing

The "H primer set" successfully amplified the target region of *COI*, giving high-intensity bands (Figure 2). The "F primer set" failed to bind to the target DNA of fish samples, requiring an implementation of the amplification protocol provided by the literature. At first, annealing temperature was increased from 40°C up to 55°C but there was no amplification of the *COI* fragment. Therefore, the temperature was set to 50°C, giving sequences length of ~655 bp, corresponding to 100% amplification of the expected amplicons. PCR products were all sequenced with a 100% rate.

Data analysis

As for BOLD ID's results, *Anguilla anguilla*, *Tinca tinca*, *Cyprinus carpio* and *Micropterus salmoides* were identified at the species level with a 100% match whereas *Carassius gibelio* and *Chelon ramada* were identified only at genus level, because of the high sequence similarity. In particular, *Carassius gibelio* presented a 100% match with both *Carassius gibelio*, *Carassius auratus* and *Carassius carassius*, whereas *Chelon ramada* presented a match of 100% similarity with *Chelon saliens* and 99.84% similarity with *Mugil cephalus*. It has been possible to identify this last two fish at species level using BLAST analysis, with the maximum score with matching sequences corresponding to 100% identity and coverage, and alignment values E=0.0. The combination of BLAST and BOLD ID's results, compared to morphological features, confirm the expected species identity.

Discussion

In 2019, the EU was the world's second-largest trader of fishery and aquaculture products after China (EUMOFA, 2020). Italy is one of the largest markets for these products in Europe, and the country covers most of the demand through imports (Eurofish International Organisation; MIPAAF, 2020).

Inland fish products still play a marginal role in this scenario. The marketing of the lake's fresh and processed fish is generally limited to the local market.

The nutritional analysis carried out in this study showed that *Tinca tinca*, *Carassius gibelio* and *Cyprinus carpio* presented a good level of protein and quite low-fat content (Table 3) compared to marine fish products (Özogul *et al.*, 2007; Fernandes *et al.*, 2014). In particular, *Tinca tinca* was the species with the highest protein content (17.9%) and the lowest fat percentage (0.5%). Therefore, this species could be considered as an interesting alternative to marine fish such as *Merluccius merluccius* (17% protein and 0.3% fat) (CREA, 2016) in low fat diet designed to reduce calorie intake, maintaining high levels of protein. As concern fatty acid profile, oleic acid, is particularly expressed in *Cyprinus carpio*. There is a direct relationship between the consumption of SFAs in the diet and the risk of cardiovascular diseases due to the increase in the blood of cholesterol levels associated with LDL (low-density lipoprotein) (Briggs *et al.*, 2017; Wang *et al.*, 2017). The European Food Safety Authority (EFSA) recommends replacing SFAs in the diet with an equal amount of MUFA to reduce blood levels of LDL cholesterol (EFSA, 2010). Among the PUFAs, the omega 3 series play an important role in the prevention of serious human diseases, particularly long-chain ones, such as EPA and DHA (A. Watters and M. Edmonds, 2012; Briggs *et al.*, 2017), whose values are best expressed in *Anguilla anguilla* and *Chelon ramada*, respectively. The quality of the lipid fraction evaluated on the basis of indices of atherogenicity (AI) and thrombogenicity (TI) proved to be remarkable in all the species analysed, in particular *Anguilla anguilla*, *Chelon ramada* and *Cyprinus carpio*. It is important to note that nutritional values of these species are beneficial in comparison with a lot of imported freshwater or marine fish species. Particularly, *Pangasius hypophthalmus*, *Oncorhynchus mykiss* and *Oreochromis niloticus* showed a SFA content of 42.18%, 25.69% and 38.94% respectively (Luczynska *et al.*, 2014). Furthermore, also

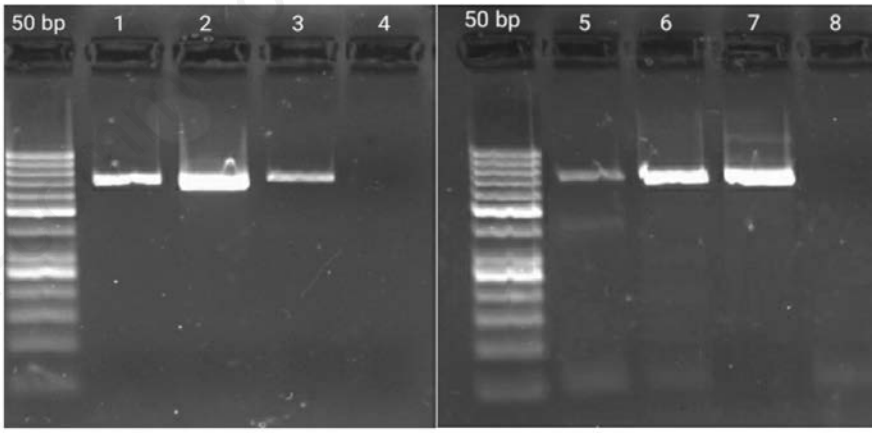

Figure 2. PCR amplification of COI fragment with "H primer set". Electrophoresis on 2% agarose gel. Control ladder: 50 bp. Lane 1: *Chelon ramada*. Lane 2: *Cyprinus carpio*. Lane 3: *Carassius gibelio*. Lane 5: *Anguilla anguilla*. Lane 6: *Tinca tinca*. Lane 7: *Micropterus salmoides*. Lane 4, 8: negative control.

Table 4. Selected primers resulting suitable for the identification of the species *Anguilla anguilla, Tinca tinca, Carassius gibelio, Cyprinus carpio, Micropterus salmoides, Chelon ramada*.

N°	Primer name	Amplicon length (bp)	5'-3' sequence	Tm °C	CG%	nt	A	T	C	G
F	LCO1490	658	GGTCAACAAATCATAAAGATATTGG	56.4	32.0	25	11.0	6.0	3.0	5.0
	HC02198		TAAACTTCAGGGTGACCAAAAAATCA	58.5	34.6	26	12.0	5.0	5.0	4.0
H	for_ FISHCOILBC	655	CTCAACYAATCAYAAAGATATYGGCAC	61.2	38.9	27	11.0	5.5	7.5	3.0
	rev_ FISHCOILBC		GTGCCYATATCTTTYTGATTYGTTGAG	61.2	38.9	27	4.0	12.5	4.5	6.0

some marine fish such as *Gadus morhua* and *Platichthys flesus* presented a higher SFA content (32.77% and 28.72%, respectively) (Luczynska *et al.*, 2014) compared to the examined species (21-27%). Considering the favourable unsaturated fatty acids amounts (especially MUFAs) of our studied specimens, they could be included in diets aimed at controlling cholesterolemia.

Our results reveal that *COI* barcode was successful in identifying the selected specimens, proving to be a powerful tool for species identification. In particular, *Carassius gibelio* is difficult to differentiate from its congeneric species *Carassius carassius*, also in the whole fish because of their high morphological similarities (Guardone *et al.*, 2017). Molecular tools are also particularly important when a fish product loses its anatomical features after industry processing (Ceruso *et al.*, 2020).

The choice of the "H primers set" was found to be effective for the amplification of all the selected species. As regards the "F primers set", recent studies show an amplification rate in fish species of just 34.7%, with no amplification in some freshwater fish such as *Cyprinus carpio* (Giusti *et al.*, 2017). Our results demonstrated that increasing the annealing temperature from 40°C to 50°C, the expected amplicon is obtained for all the six species.

BLAST and BOLD analysis of the *COI* sequences make it possible to recognize and identify a vast number of fish at the species level. Both databases should be used for more accurate sequence comparison and analysis.

Conclusions

The consumer is currently accustomed to the stronger flavour of marine species, at the expense of inland ones, also because of their higher availability on the market. The present work showed that the nutritional profile of some freshwater fish could be equivalent to marine fish species, especially regarding the fatty acids content. Furthermore, the establishment of a DNA barcoding protocol to identify inland fish could assess their traceability along the supply chain, as defined by Regulation (EU) 1169/2011 and Regulation (EU) 1379/2013, enforcing food safety system and contributing to a more transparent and safer fish market.

Inland fish, especially species with high nutritional properties, have interesting market potential and can represent a valid and sustainable alternative to the over-exploitation of fish stocks, and importations improving the national and the local economy. The change in eating habits and the use of a flavoursome way of cooking able to enrich the unmarked taste of inland species could direct the consumer towards a more informed purchase of the local product, taking into account also the lower price compared to the most commercialized marine fish species.

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