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

Iolanda Venuti,1 Marina Ceruso,1 Giuseppe Palma,2 Giorgio Smaldone,3 Tiziana Pepe1
1Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples; 2Assiottica Italia, Rome; 3Department of Agricultural Sciences, University of Naples Federico II, Naples, Italy

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 (C20: 5 n3) and total fraction of omega 3 PUFA. 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 (Aquatulture, 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’Anastasia 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.

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, Linnaeus 1817), European perch (Perca fluviatilis, Linnaeus 1758), and European catfish (Silurus glanis, Linnaeus 1758).
Lacepède 1802), Thinlip grey mullet (Chelonus 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 colormetric indicator.

For the fatty acid profile, a transesterification with 2.5% sulfur: methanol solution (H$_2$SO$_4$: 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 × C14:0 + C16:0) / [(ΣMUFA + ΣPUFA (n-6) and (n-3)]

**TI =** (C14:0 + C16:0 + C18:0) / [(0.5 × ΣMUFA + 0.5 × ΣPUFA (n-6) + 3 × ΣPUFA (n-3) + (n-3)/(n-6)]

**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 260/280 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), 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 (LC01490)                  | GTGCACAAATCTATAAGATATGG | 658              | Folmer et al., 1994; Hebert et al., 2003; |
| F (HOR218)                   | TAAACCTCCAGGTTGACCACAAAAATCA | 655              | Giusti et al., 2017; |
| H (FISHCOILBSC)             | CTCAACATCTAAAGATATGGCAAC | 655              | Handy et al., 2011; |
| H (FISHCOIHBC)              | ACTTCYGGGTTGRCRAARATCA | 655              | Giusti et al., 2017; |
| COIF-ALT                    | ACAATATAYAGAYATGG | 658              | Mikkelsen et al., 2005; |
| COIR-ALT                    | TCTAGGRTGCRAARATCA | 655              | Giusti et al., 2017; |
| FishF1                      | TCAACCAACACACGAGATAGTGGCA | 655              | Ward et al., 2005; Hubert et al., 2008; Lakra et al., 2016 |
| FishF2                      | TGACTATCATAAAAATGATGGCAC | 655              | Ward et al., 2005; |
| FishR1                      | TAGACTGCTGAGGGTGCCGAAATGCA | 655              | Ward et al., 2005; Hubert et al., 2008; Lakra et al., 2016 |
| FishR2                      | ACTTCAGGTTGACGAGAATCAGAA | 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 concentration of polyunsaturated fatty acid (PUFA), was found to be the species with the lowest fatty acids (0.5%) whereas 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/A230nm ≥ 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:

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

|            | F                        | H                        |
|------------|--------------------------|--------------------------|
| Primer     | 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 final volume achievement (FV) | up to 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.**

| Species                | 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           |
The combination of BLAST and BOLD features, confirm the expected species ID’s results, compared to morphological and coverage, and alignment values E=0.0.

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 low sequence similarity. 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 (EUOMOA, 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.](image-url)
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 cholesterol.

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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