A case study on the labeling of bottarga produced in Sardinia from ovaries of grey mullets (*Mugil cephalus and Mugil capurrii*) caught in Eastern Central Atlantic coasts

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Abstract

The aim of this case study is to show how traditional and molecular methods can be employed to identify the *Mugilidae* species currently used in Sardinia (Italy) to produce the traditional *bottarga* for the processing of their ovaries. A total of six specimens of *Mugil cephalus* (n=3) and *Mugil capurrii* (n=3) were subjected to external morphology and meristic measurements. Subsequently, tissue samples of white muscle and ovaries from three individuals *per* species were underwent PCR-sequencing assay of mitochondrial DNA cytochrome oxidase subunit I (*COI*). The external morphology and meristic characters showed a sufficient level of reliability in the identification between the two species. At the same time, the molecular techniques showed the discriminatory power and confirmed the correct species identification in all the sampling units. DNA barcoding may be an effective aid to traditional taxonomy and can facilitate accurate species identification among the *Mugilidae*.

Introduction

Mugilidae are coastal fishes found in temperate, subtropical and tropical regions within marine, brackish and freshwater habitats worldwide and represent an important food source in several Mediterranean and Atlantic countries. According to FAO's FishStat Plus data sources, the total fishery production of mullets from the Eastern Central Atlantic was over 30 thousand tonnes in 2010 (Harrison, 2016). They are members of the order *Mugiliformes*, represented by a single family, and including officially 62 species belonging to 14 genera (Thomson, 1997). Recently, (Whitfield *et al.*, 2012) it was proposed to recognize 20 mullet genera including 70 species (11 of which belong to the genus *Mugil*). Systematics of *Mugilidae* are still much debated and based primarily on morphological characters, but those classically used in species identification are remarkably similar within this family (Durand *et al.*, 2012) and make quite challenging species identification (Gonzales-Castro and Ghasemzadeh, 2016). Using a mitochondrial gene-based phylogeny as criterion, *Mugilidae* classification was recently proposed (Xia *et al.*, 2016). While not disputing such author's molecular results, Harrison (2016) found that they disagree with morphological analyses, and the nomenclature does not seem to be properly used in commercial and research fisheries communities and by other applied fieldworkers who might not be familiar with the systematic literature. Traditionally, the features of diagnostic value for *Mugilidae* include (Thomson, 1997): the presence or absence of an adipose eyelid (this character is commonly employed to differentiate the genus *Mugil* from remaining genera of the family), the origin of the various fins and the number of fin rays, the linear morphometric measurements of body proportions using the traditional measures employed on fishes (Harrison, 2016).

Mugil cephalus (Linnaeus, 1758) is diffused worldwide and according to FAO is marketed as *flathead grey mullet* and *MUF* (3-Alpha Species Codes). *Mugil cephalus* is an important species for both aquaculture and fisheries, and in many regions of the world it is captured during the spawning migration to harvest the egg roe which is salted and dried prior to be marketed as a delicatessen (Livi *et al.*, 2011). Linnaeus, referring briefly to this species in his *Systema Naturae* (1758), stated: *Botargo italorum ex hujus ovis*. In Italy, the botargo or mullet roe, is traditionally produced from flathead grey mullet's egg roe, and marketed as *bottarga*. This name derives from the Arabic word *batārikh*, which in turn, derives from the greek-byzantine term *ootàrichon* meaning *dried and salted fish eggs*. In the Sardinian language (Italy), is called *butàriga*, preserving a strong assonance with the original Arabic word. Moreover, the technological process of grey mullets bottarga was formerly described in the *Storia naturale di Sardegna* (Cetti, 1777), in which it is reported that several species of mullets are found in the Sardinian Sea, but that: *... as far as I am concerned, having observed these mullets, I have not found any difference indicating that they could be considered different species.* The morphological distinction between the various species belonging to the *Mugilidae* has always been a difficult task because of the extreme homogeneity of the family. In particular, referring to the systematic of *Mugilidae*, in the eleventh volume of the *Histoire naturelle des poissons,* Valenciennes (1836) reported that *...in general, the great similarity between species of this genus makes their distinction one of the most difficult tasks in Ichthyology*. Describing the *Mugil cephalus*, the same author reported that *…precisely aligned with the second dorsal fin is the anal, presenting eight soft rays*. Despite its global spread in both hemispheres, *Mugil cephalus* has a discontinuous distribution. As it will be reported later, the key features regarding its taxonomic status have been raised in many genetic studies, most of which suggest that this scientific name includes a

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species complex (Whitfield *et al.*, 2012) or, in other words, that *Mugil cephalus*, once presented as an example of globally distributed species, is now shown to harbor several cryptic species (Durand *et al.*, 2012). *Mugil capurrii* (Perugia, 1892) inhabits only the Eastern Central Atlantic and according to FAO is marketed as *leaping African mullet* and *MUO* (3-Alpha Species Codes). It was initially described as having the second dorsal fin behind the anal. At a later time, Tortonese (1963) highlighted one of the key features allowing to differentiate it in respect to the *Mugil cephalus*, since his anal fin invariably presented nine soft rays.

Other authors (Trewavas and Ingham, 1972) published a key to the mullets species, with the purpose of supplementing the section on the *Mugilidae* in the fishes checklist of the Northeastern Atlantic and Mediterranean (valid as to genera only within the region). The following key was reported: cleft of mouth as wide as or wider than long = *Mugil cephalus*; cleft of mouth longer than wide = *Mugil capurrii*. In this regard, in the FAO species identification sheets for fishery purposes of the Eastern Central Atlantic, fishing areas 34 and, in part, 47 (Fischer *et al.*, 1981), the same approach for the species belonging to the same genus has been confirmed. Thomson (1997) reported the diagnostic characters of the *Mugilidae* of the world and highlighted that *Mugil cephalus* presents robust body, rounded profile and the pectoral fin reaching the posterior rim of eye or slightly behind when laid forward. On the contrary, *Mugil capurrii* presents slender body, pointed head and a mouth corner on vertical from the anterior rim of eye, in other words, the lip of upper jaw reaching vertical from anterior field of iris. In addition, the pectoral fin does not reach the eye when laid forward. Even though their weakness, these diagnostic characters between these two grey mullets, have recently been confirmed in the FAO species identification guide of the living marine resources of the Eastern Central Atlantic (Harrison, 2016). At present, they are routinely used as distinctive characters in ichthyology practice. New methodologies have been developed in the past few decades, which improved the accurate discrimination of species, for example the geometric morphometrics (González-Castro and Ghasemzadeh, 2016) and the sequencing of nuclear and mitochondrial genes. It is clear that historical methods of identifying fishes, even for practical reasons, are predominantly based on visible morphology. Although modern taxonomy regularly employs many other traits, including internal anatomy, physiology, behavior, geography and lately isozymes and genes, morphological characters remain the cornerstone of taxonomy. However, there are difficulties in relying primarily on morphology when attempting to identify fishes from isolated portions or processed products (Ward *et al.*, 2009). In this regard, it has been long recognized that nucleic acids sequence diversity, whether assessed directly or indirectly through protein analysis, can be used to discriminate species. By using these methods, the identification of Eastern Central Atlantic *Mugilidae* species has been firstly proposed (Trape *et al.*, 2009) by PCR restriction fragment length polymorphism (RFLP) analysis of mitochondrial 16S ribosomal RNA region. The grey mullets from African waters and in particular from southern Mauritania to Senegalese coast are also increasingly represented in international trade of fishing products. Ovaries of large species such as *Mugil cephalus* are specifically exported and due to its commercial importance there is an increasing risk of fraud by substitution (Trape *et al.*, 2009). The molecular techniques could help food inspection in order to reinforce labeling regulations. Furthermore, the demand for mullet roe in many parts of the world has grown considerably in recent decades and elevated the status of grey mullet to be called *grey gold* by fishermen (Whitfield *et al.*, 2012). Several nuclear and mitochondrial DNA regions have been successfully used for the identification of fish species, even though the reliability of such techniques, relies on DNA regions that are highly conserved within the same species and sufficiently variable between species. In previous studies, analysis of random amplified polymorphic DNA (RAPD) and proteins, coupled with both, of above mentioned salted and semi-dried grey mullet ovary product known as *bottarga di muggine* from different geographical origins did not differentiate samples (Barra *et al.*, 2008). In other studies, mitochondrial DNA sequencing was used to identify and distinguish different species of commercially important Mediterranean grey mullets. These *fingerprints* were used to identify the species of several samples of bottarga from *Mugil* genus which, as previously reported, is a well-established traditional product of Sardinia, Italy (Murgia *et al.*, 2002). The demand of consumers to know the origin of food is significantly increasing and novel methods of understanding and assessing consumer trust have been studied to meet this demand. In previous studies, partial *cytochrome b* gene sequences were resolved for *Mugil cephalus* specimens sampled from fourteen different geographic sites (Livi *et al.*, 2011). Other authors (Durand *et al.*, 2012) have provided an initial comprehensive molecular systematic account of the *Mugilidae* using nucleotide sequence variation at three mitochondrial loci (*16S* rRNA, *cytochrome b* and *cytochrome oxidase I*) from fifty-five species.

Currently, the use of a universally accepted short DNA sequence for identification of species (DNA barcoding or Barcode) has been proposed for many groups of animals, both invertebrate and vertebrate, and a fragment of the mitochondrial gene *cytochrome c oxidase subunit I* (*COI*), is the most used sequence of DNA barcoding. In fact, within-species variation for this gene is low if compared with between-species variation. Therefore, species are regularly identified by a particular sequence or by a tight cluster of very similar sequences. The effectiveness of this gene region for species-level identifications was recently validated for application across all fish species (Ward *et al.*, 2009), including *Mugilidae* family (Durand *et al.*, 2017). Accurate and unambiguous identification of fish and fish products, from eggs to adults, is in fact important in many aspects: it would enable retail substitutions of species to be detected, assist in managing fisheries for long-term sustainability, and improve ecosystem research and conservation (Ward *et al.*, 2009).

The aim of this case study was to show how both the traditional and new molecular tools can be employed to identify/discriminate these two species in order to guarantee the proper commercial labeling of mullet roe *bottarga* produced in Sardinia (Italy).

Case Report

Only a small part of the mullet egg roes processed in Sardinia (Italy) originates from fishing in its coastal waters and lagoons. On the other hand, the ability of Sardinian producers to transform grey mullets ovaries imported from different parts of the world in an excellent product is well-known. As a matter of fact, originality of this product is strongly linked to local raw materials. Otherwise, it is well-known that the meticulous choice of good raw material of non-local origin and the use of consolidated processing techniques as traditional salting and drying parameters, leads to a finished product bearing the peculiar characters of *bottarga di muggine*, even though appropriate labelling in terms of ingredients and traceability should be guaranteed. Nowadays, regular supply of frozen raw material, guarantees production all year round. The final products acquire the origin of the country or territory where, pursuant to article 60 of Regulation (EU) No 952/2013, they under-

went their substantial processing or working. However, it is only for the product obtained from ovaries of local *Mugil cephalus* that the matched geographical name *di Sardegna* or *Sarda* can be used, referring in this case to a traditional regional product included in the national list provided by Italian Ministerial Decree n. 350 of 1999 and confirmed with the in force seventeenth revision of 2017. At the end of January 2017, the regional daily newspaper *L'Unione Sarda* published an investigative report on Sardinian products deserving the naming of Protected Designation of Origin (PDO) or Protected Geographic Indication (PGI) on their labels, focusing on bottarga. At present, this regional product is not under the protected geographical status due to *…too little production…* or because of the incapability to *...gather the manufacturers around a table…,* leading to a *...market invaded by mullet egg roes imported from African countries...* In order to guarantee the right of consumers to consciously make their choice when purchasing bottarga on the basis of a proper labelling, the competent authorities decided to conduct regular checks focusing on verification of correctness of geographical and species traceability of all batches of ovaries of grey mullets caught in Eastern Central Atlantic coasts. At the beginning of March 2017, a batch of 3119 kg of frozen fish eggs from Senegal and designated with CN code 0303.9190 of Regulation (EU) No 2016/1821, has been subjected to regular sampling at the Border Inspection Post of Genova (Italy). The entire batch was accompanied by a health certificate reporting *Mugil cephalus* as species of origin and was destined to a processing plant located in the Sardinian province of Oristano. Five sampling units were collected from the whole batch and sent to the Genetics and Immunobiochemistry laboratory of the Zooprophylactic Institute of Turin for further biomolecular analysis. Species identification was performed using *COI* gene as genetic marker and comparing the obtained nucleotide sequences with public DNA databases. Genomic DNA was extracted from all samples using commercial kit based on spin column containing a silica resin (ReliaPrep™ gDNA Tissue Miniprep System, Pomega), and a specific PCR reaction amplified a portion of *COI* gene following the standard protocol described by Ward *et al.* (2005). The reaction was run on GeneAmp® PCR System 9700 (Applied Biosystems, Life Tecnologies), and the amplicons were sequenced according to Sanger's method on both strands using the same PCR primers on ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Life Techonolgies). The obtained sequences were compared with those deposed both in GenBank and in BOLD databases. Based on validation data of the method, the final specimens assignment to species was based on a minimum similarity value of 98%. Out of the five sampling units, three were identified as *Mugil capurrii* and the remaining two as *Mugil* spp. In the latter two cases, it was not possible to define the species, but only a genus level determination analyses revealed a mixture of eggs deriving from different species of *Mugil*. As a matter of fact the sequences of the two samples presented heterozygous mutation sites and the comparison with the databases revealed a significant similarity with different species of *Mugil*. According to these results, an information for attention (iRASFF No 2017/327039 of March 28) was notified. Successively, since the notification of different species identified does not represent any health related issues, and then *does not fall within the scope of the RASFF system*, this information for attention was rejected. Although this topic essentially concerns with fair practices in food trade and protection of consumer interests, including food labeling, it is included in the scope of Regulation (EC) No 882/2004 on official controls and, pursuant to article 35 of Regulation (EU) No 1379/2013, as fishery products can be offered for sale to the final consumer or to a mass caterer only if appropriate labelling indicates: *the commercial designation of the species and its scientific name*.

Therefore, we decided to study in depth the manageability aspects regarding the differential identification of the two *Mugil* species of the Eastern Central Atlantic coasts (*Mugil cephalus* and *Mugil capurrii*), upstream as well as downstream of the supply chain. The availability of vouchered sequences of the other *Mugil* spp. sharing the aforesaid FAO area (*e.g. Mugil bananensis*, *Mugil curema* and *Mugil curvidens*) has not been verified because these species reach too small adult medium lengths (Harrison, 2016) and, therefore, the ovaries

Figure 1. Adult specimens of the Mugilidae family: A = *Mugil cephalus*; B = *Mugil capurrii*.

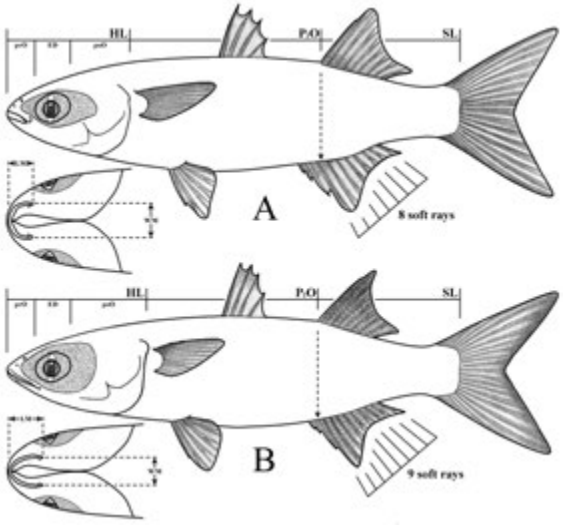

Figure 2. External morphology and usual measures employed in the Mugilidae (full lateral view and ventral view of head): A = *Mugil cephalus*; B = *Mugil capurrii*; SL = standard length; D_2O = 2nd dorsal fin origin; HL = head length; prO = preorbital length; ED = eye diameter; poO = postorbital length; LM = length of mouth; WM = width of mouth.

Figure 3. Lateral and ventral view of mouth in *Mugil cephalus* (A) and *Mugil capurrii* (B).

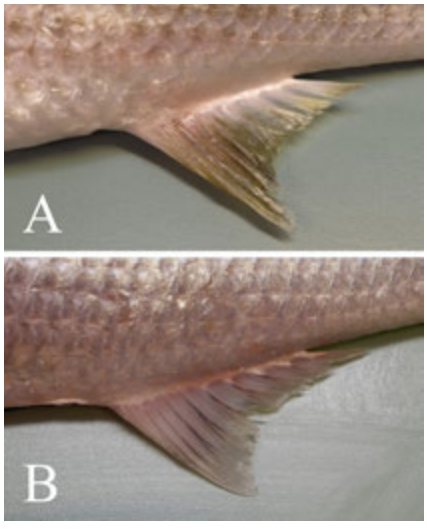

Figure 4. Spiny and soft rays of the anal fin in *Mugil cephalus* (A) and *Mugil capurrii* (B).

cannot be used in the technological process of *bottarga* production. In order to compare with local specimens of *Mugil cephalus*, one of the main Sardinian importer was asked to integrate the next buying of grey mullets ovaries from Mauritania, with a monospecific batch of frozen adult specimens of *Mugil capurrii*. Comparable *Mugil capurri* and *Mugil cephalus* specimens with the same standard length of 40 cm were subjected to external morphology and meristic measurements (Figure 1). Subsequently, the same specimens underwent PCR-sequencing assays as previously described for frozen fish eggs, isolating samples both from fragments of white muscle samples and ovaries of three individuals *per* species. Biomolecular tests showed that three samples of muscle belonged to *Mugil cephalus*, while the others to *Mugil capurii*. Same results were obtained from the analyses of the correspondent ovaries.

Discussion

The results of the present case study highlighted that the external morphology and meristic characters of practical use, if correctly applied by well-trained workers, showed sufficient level of reliability in the identification between the two *Mugilidae* species. These results are in accordance with the findings of previous authors (Fischer *et al.*, 1981; Harrison, 2016; Thomson, 1997; Trewavas and Ingham, 1972). In particular, the more specific characters are those related to head length development of labial split (both in profile and ventral vision) and lastly to number of soft rays of the anal fin (Figure 2). The examination of mouth shape and length (Figure 3) in relation to eye margin, together with development of pectoral fin (if conveniently tilt forward) as regards to rear-ocular space and, in the event of doubt, the count of soft rays of the anal fin (Figure 4), represent confident criteria for effectiveness of a practical diagnosis, or at least a screening, in mono or polyspecific fishings of *Mugil* spp. on the Eastern Central Atlantic coasts by local fishermen. At present, the systematic control of the species identity upstream of the supply chain can be supported by the examination (on a sampling basis) through molecular analysis aimed to determine the presence of genetic markers specific for each species. Our results, related to a blind trial (the samples were delivered to the laboratory not reporting the species) on three female specimens for each of the two species *Mugil cephalus* and *Mugil capurrii*, confirmed the correct species identification in all the sampling units of muscle and ovary. The molecular method showed the discriminatory capability in terms of verification of species identification and compliance to the requirements for the labelling of fishery products. Taking into account the different importance of the two approaches in forensic sciences, they should be considered as complementary in the routinely practice, both by fisheries operators and by competent authorities, as it is happening for example, in the field of *integrative taxonomy* of fishes as the *Mugilidae* family. This integrate approach combines morphological and meristic analysis with newer disciplines, such as molecular genetics (González-Castro and Ghasemzadeh, 2016).

Conclusions

According to other authors (Durand *et al.*, 2017), DNA barcoding may be an effective aid to traditional taxonomy, designed to facilitate fast and accurate species identification, especially among the *Mugilidae*. Although these results should be considered preliminary, we have confirmed that DNA barcoding discriminates against the two mullet species (*Mugil cephalus* and *Mugil capurrii*), and can identify individually isolated fish ovary and muscle portions from these species. However, in order to better control the identity of species upstream of the supply chain and in particular the whole fish from which the ovaries are extracted, practical and up-to-date criteria of external morphology and meristic characters (Figure 2) are still necessary. This will help in the systematic and immediate use in the fishing and selection stages of *Mugilidae* batches or, as reported by previous authors (Ward *et al.*, 2009), barcoding and morphological analysis should go *hand-in-hand*.

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