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Species identification of archived fish bones collected from the Mediterranean Sea 100 years ago using molecular techniques.

Tesi di laurea in
Struttura e Connettività delle Popolazioni Marine

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Abstract

With the discovery that DNA can be successfully recovered from museum collections, a new source of genetic information has been provided to extend our comprehension of the evolutionary history of species. However, historical specimens are often mislabeled or report incorrect information of origin, thus accurate identification of specimens is essential. Due to the highly damaged nature of ancient DNA many pitfalls exist and particular precautions need to be considered in order to perform genetic analysis. In this study we analyze 208 historical remains of pelagic fishes collected in the beginning of the 20th century. Through the adaptation of existing protocols, usually applied to human remains, we manage to successfully retrieve valuable genetic material from almost all of the examined samples using a guanidine and silica column-based approach. The combined use of two mitochondrial markers cytochrome-oxidase-1(mtDNA COI) and Control Region (mtDNA CR), and the nuclear marker first internal transcriber space (ITS1) allowed us to identify the majority of the examined specimens using traditional PCR and Sanger sequencing techniques. The creation of primers capable of amplifying heavily degraded DNA have great potential for future uses, both in ancient and in modern investigation. The methodologies developed in this study can in fact be applied for other ancient fish specimens as well as cooked or canned samples.

1. Introduction

Prior to the last two decades the study of evolutionary genetics has largely depended on extrapolation of information from living organisms in an effort to reconstruct details of the past. However, modern DNA provides only indirect evidence of the historical processes that have occurred over evolutionary time. With the discovery that DNA can be successfully recovered from museum collections, such as archeological bones or dried tissues, a new source of genetic information is now available for extending our comprehension of the evolutionary history of many species. For example, the draft genome reconstruction of the pathogen bacterium *Yersinia pestis* (Kirsten et al. 2011) suggests how the analysis of ancient infectious diseases will provide contributions towards our understanding of host-pathogen coevolution. DNA extracted from ancient specimens can help identify the population dynamics for both humans (Yao et al 2003) and animals (Leonard et al. 2000; Riccioni et al. 2010). The research field of paleogenomics offers a wide array of solutions to present day problems and therefore the research of ancient remains and natural history collections is becoming increasingly important.

1.1 The Massimo Sella Archive

At the beginning of the twentieth century, Massimo Sella (an Italian scientist and professor) collected many bones of Atlantic bluefin tuna, *Thunnus thynnus*, while investigating their biology, population dynamics and habitat use. He collected many hooks attached to the giant tuna captured in traps throughout the waters of Italy and Libya, gleaning information concerning their migration routes (Sella M. 1928; 1929). Throughout fifteen years (from 1911 to 1926) and across the Mediterranean Sea, he collected thousands of individual skeletal specimens in the form of vertebrae, fins, teeth and whole skeletal remains of juvenile and adult fishes of Mediterranean large pelagic species, moving far beyond the study of only the Atlantic bluefin tuna.

The Massimo Sella Archive, now stored at the laboratories Marine Biology and Fisheries, Fano, and Genetics & Genomics of Marine Resources and Environment (GenoDREAM), Ravenna, of the University of Bologna is one of the biggest historical collections of fishes found in Italian waters. It is an invaluable resource for research into

historical genetic structuring and evolution of species in the past century (Riccioni et al. 2010).

Unfortunately, like many natural history collections arisen before the discovery of DNA, when professor Sella started to build up his archive, its ultimate role as a source of genetic material could not have been part of his plan and despite the remarkable preservation of some of the samples, most were not properly preserved. Moreover records of the specimens' origin and species identity are frequently incomplete, missing or incorrect. The use of molecular techniques can be used on this archive's specimens to confirm species identity and evaluate the presence of valuable DNA for further genetic purposes.

1.2 Problems of historical samples

The study of DNA from long-deceased organisms can offer many answers to questions raised when studying the genetic history of a certain species. However many technical problems and pitfalls need to be avoided to allow the molecular evolutionists access to the information locked away in the preserved genetic material.

Three major and persistent obstacles make DNA analysis of ancient skeletal remains a very challenging enterprise. First the total amount of DNA preserved in very old bones and teeth is greatly reduced, due to degradation over time and the genetic material that does remain is highly damaged (Pääbo et al. 2005). Secondly, historical DNA that has withstood the ravages of time is often contaminated with exogenous DNA from bacteria, fungi or other microbial organisms (Noonan et al. 2005). Finally regardless of the environmental conditions from which the sample is excavated or collected, contaminating organic and inorganic compounds such as humic acid and salts leaking from the surrounding soil can accumulate in the cavities over the years. These compounds can be carried over during the extraction and inhibit enzymatic reaction such as the commonly used polymerase chain reaction (Rohland 2012).

1.2.1 DNA damage

Within living cells, the integrity of DNA molecules is continually maintained by enzymatic repair processes (Lindahl 1993). After the death of an organism the cellular compartments that normally sequester catalytic enzymes are lost and as a consequence

DNA is rapidly degraded by enzymes such as lysosomial nucleases. In addition, DNA molecules are degraded by a host of bacteria, fungi, and insects (Englinton et al. 1991). When tissues become rapidly desiccated after death or DNA becomes adsorbed into mineral matrix, the DNA can be protected from enzymatic and microbial degradation (Pääbo et al. 2004). Some examples of this damage are oxidation as well as the direct and indirect effect of background radiation that will modify nucleic bases and the sugar-phosphate backbone of the DNA. Furthermore deamination and depurination and other hydrolytic processes will lead to destabilization and corruption of DNA molecules (Pääbo et al. 2004). The most common type of damage to DNA extracted from ancient remains is its fragmentation into short chains, generally between 100 to 500 base pairs (Hofreiter et al. 2001). This reduction in size is due to both enzymatic processes that occur slowly but steadily after death and enzymatic hydrolytic cleavage of phosphodiester bonds in the phosphate sugar back bone (Lindahl 1993; Shapiro 1982).
All these processes create problems for the retrieval of ancient DNA (henceforth aDNA) sequences (Table 1)

Table 1: Overview over different types of damage in ancient DNA (Pääbo et al. 2004)

Type of damage	Process	Effects on DNA
Strand breaks	Degradation by microorganisms Nucleases in the postmortem cell Other chemical processes	Reduction of overall DNA amounts Size reduction
Oxidative lesions	Damage to bases	Base fragmentation
	Damage to deoxyribose residues	Sugar fragmentation
		Nucleotide modification
DNA crosslinks	Reactions between DNAs as well as DNA and other biomolecules	e.g., Maillard products
Hydrolytic lesions	Loss of amino groups 1. adenine ⇒ hypoxanthine 2. cytosine ⇒ uracil 3. 5-methyl-cytosine ⇒ thymine 4. guanine ⇒ xanthine	Change of coding potential

1.2.2 Contamination

Higuchi et al. (1984) were the first to report the retrieval of informative DNA from an extinct animal, after they succeeded in cloning mitochondrial DNA sequences from a museum specimen of the Quagga (*Equus quagga quagga*), a member of the genus *Equus* that became extinct more than 100 years ago. This was followed by the cloning of repetitive nuclear DNA sequences from the skin of an ancient Egyptian 2400-year old mummy (Pääbo S. 1985). However for both of these studies only a very low proportion of the DNA was original, most of it was actually derived from extensive microbial contamination (Hagelberg & Clegg 1991). When conducting ancient DNA studies three kinds of contamination needs to be considered:

1. Contamination of sample surfaces through exposure to microorganisms, storage and collection contact and cross sample contamination during sample processing.
2. Reagents, labware and disposable laboratory supplies that could have been contaminated during manufacture, packaging or distribution.
3. PCR carryover that can inadvertently transfer between tubes during the course of the study.

A variety of methods are used by ancient DNA researchers and forensic scientists to remove or exclude sources of contamination before the DNA extraction actually begins: washing the surface of the sample with either ethanol, distilled water and bleach, mechanical removal of the surface layer, extracting material only from the interior of the sample, irradiating the surface with ultraviolet light (Kemp & Smith 2005).
Unfortunately, all of these methods offer only a partial solution to the contamination problem. For example, while the use of ethanol and its effectiveness to kill microbial organism is well established, it alone cannot physically remove the remains of nucleic acid from such dead organism. The use of bleach (sodium hypochlorite, NaClO) as a cleaning agent on ancient samples has been proven effective (Kemp & Smith 2005). However, since it produces chlorinated bases (Hayastu et al. 1971), prolonged exposure to bleach can lead to a progressive reduction of DNA in smaller and smaller pieces, reaching eventually individual bases (Whiteman et al. 2002).

The physical removal of a bone's surface layer, in order to reach the inner side of a sample cannot guarantee the elimination or the exclusion of contamination. Also the powder produced during the friction of the surface can produce cross sample contamination.
Finally, while the use of short wavelength (254nm) ultraviolet light has been recognized as capable of effectively destroying molecular bonds in DNA, the irregular surface shape of the samples can block the UV light from reaching and sterilizing the external surface of bones.

1.3 Developing a new protocol

Considering all the existing problems is crucial in the setting up of a aDNA extraction protocol that optimizes the DNA yield to be used for downstream tasks. Since aDNA is available only in a highly degraded state, the extraction methods that use overly aggressive chemical treatments, such as high temperatures or the use of strong detergents, need to be avoided to prevent further damage to the aDNA. To properly carry out this task two existing protocols (Riccioni et al. 2010; Dabney et al. 2013) were selected as starting points and through a collaboration with the department of Cultural Heritage at the University of Bologna, Ravenna, various modifications and tests were performed in order to optimize efforts.

Guidelines precautions

Paleogenomics has dealt with these problem through the publication of guidelines that are designed to ensure the quality of ancient DNA data, analysis and interpretation of results. Starting from a few relatively simple suggestions, these guidelines have evolved over time and have become a more exhaustive and extensive list of requirements resulting in the well-known nine key-criteria for authenticity defined by Cooper & Poinar (2000).

The nine criteria for authenticity:

1. Isolation of work areas: to separate samples and extracted DNA from PCR amplified products.
2. Negative control extractions and amplifications: to screen for contaminants entering the process at any stage.
3. Appropriate molecular behavior: owing to DNA degradation, the successful amplification of large DNA fragments in ancient DNA studies should be treated with caution.
4. Reproducibility: multiple PCR and extractions should yield consistent results.
5. Cloning of products: to assess for damage, contamination and jumping PCR.
6. Independent replication: the generation of consistent results by independent research groups.
7. Biochemical preservation: preservation of other biomolecules that correlate with DNA survival (e.g. collagen or amino-acid racemization) should indicate good sample preservation.
8. Quantification: by competitive PCR or Real-Time PCR to give an indication of the number of starting templates in the reaction.
9. Associated remains: are associated remains equally well preserved, and do they show evidence of contamination?

It needs to be underlined that these precautions should be followed especially when studying ancient human remains. For the analysis of non-primate historical specimens the use of these precaution is not strict.

1.4 Species identification

While the identity of most living adult fishes is essentially unambiguous, basing the identification on morphological attributes can be problematic in situations where physical characteristics are difficult to interpret, or are completely missing. The most abundant faunal remains are partial skeletons. Bones and teeth are the hardest tissues of vertebrates and while their preserved shape can often reveal the species to which they belong, distinguishing the actual species can prove to be quite problematic, if not impossible, when more members of the same family or genus share similar

morphological features. It has long been recognized that DNA sequence diversity, whether assessed directly or indirectly through protein analysis, can be used to discriminate species (Ward et al. 2005). Several protocols have been described for species identification of marine animals in recent years, based on different technologies, such as isoelectric focusing, high performance liquid chromatography, sodium dodecyl-sulfate polyacrylamide gel electrophoresis, enzyme-linked immune-absorbent assay, and starch gel electrophoresis (reviewed by Viñas & Tudela 2009). Among these, DNA-based methodologies are one of the most promising approaches since they provide very precise information, and due to their robustness, they can be applied to all of the different life stages of marine species and almost every kind of sample, including ancient ones.

1.4.1 Molecular markers

Diagnostic molecular markers can provide a means for positive identification when morphological identification is uncertain (Padial et al. 2010). Various molecular markers have been used to identify fish eggs and larvae including allozymes, polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) analysis, multiplex PCR and sequencing (reviewed by Paine et al. 2007). The genetic markers chosen for this study were mitochondrial DNA cytochrome-c-oxidase subunit I (COI), the mitochondrial DNA Control Region (mtDNA CR), and the ribosomal DNA first Internal Transcribed Spacer (ITS1) located in the nuclear genome.

1.4.2 Mitochondrial DNA

Mitochondrial DNA is more likely to survive in ancient specimens than nuclear DNA because they occur in much higher numbers per cell. Out of necessity there is an almost complete reliance on mitochondrial DNA sequences for phylogenetic studies of ancient faunal remains.

Primers

As already established the DNA that can be retrieved from ancient samples is short in length. This length of the DNA molecules compelled us to create new pairs of primers

capable of amplifying short fragments of diagnostic DNA. Primers were designed to attach to conserved regions with diagnostic bases located between both annealing sites

Cytochrome oxidase subunit 1

One of the most conserved protein coding genes in the mitochondrial genome is cytochrome oxidase subunit I (COI; Brown W.M. 1985). This gene is critical for cellular energy production and this functional importance restricts the frequency of mutations (Rawson & Burton 2002). The high level of conservation of COI allows for the design of a primer pair that successfully amplifies the same fragment across the diverse members of the Scombridae family. It is not surprising that the COI has broadly been used for species identification being the most commonly used gene region to be applied in DNA barcoding, thanks also to the creation of sequences databases such as the Barcode of Life Database System (Ratnasingham & Hebert 2007) and FISH-BOL (Ward et al. 2009).

1.4.3 The Introgression Problem

In the case of the genus *Thunnus* the validation of the genetic marker for species identification is critical due to the observed introgression between species (Bremer et al. 1997; Chow & Kishino 1995). This phenomenon, also known as introgressive hybridization, is defined as the movement of a gene from a species into the gene pool of another and has been described among several tuna species. This introgression can confound the results of genetic analysis and species misidentification can occur if the genetic marker is not appropriate. For instance, species identification based on nuclear genetic markers cannot distinguish between *T. thynnus* and *T. orientalis* (Chow et al. 2006). Also, the low genetic distance between *T. albacares, T. atlanticus, T. tonggol* can easily generate misleading results if a marker with low genetic variability is used (Alvarado Bremer et al. 2005). Another consideration is that some members of the *T. alalunga* species and *T. orientalis* are so close genetically (Alvarado Bremer et al. 1997, 2005; Chow et al. 2006) that depending on the methodology used the distinction between the two species may not be possible (Chow & Kishino 1995). About 2–3% of *T. thynnus* individuals show mtDNA sequence identical to *T. orientalis*. The same situation occurs vice versa, with about 2–3% of *T. orientalis* individuals having

mitochondrial DNA sequences equal to *T. thynnus*. Introgression can also occurs between *T. alalunga* and *T. thynnus*, with about 2–3% of the latter species' individuals having an identical sequence to *T. alalunga* (Viñas & Tudela 2009).

Mitochondrial Control Region and Internal Transcriber Spacer 1

The mtDNA Control Region (CR) was considered valid for species identification because previous studies based on this genetic marker had already analyzed *Thunnus* individuals and had detected introgression between some of them (Alvaradro Bremer et al. 2005). The nuclear Internal Transcriber Spacer 1 (ITS1) is a recombining, biparental marker, which can reveal recent gene flow and hybridization events (Mayer & Soltis 1999).Thus the combined use of both mtDNA CR and rDNA ITS1 can be considered ideal to look for introgression in the *Thunnus* genus and pairs of primers that amplified in these regions were also developed.

1.4.4 DNA Barcoding

DNA barcoding is a taxonomic method that uses a short genetic DNA marker of an organism to identify it as belonging to a particular species. The Barcode of the 650-bp COI sequence (Herbert et al 2003) is easily sequenced and usually provides greater than 97% likelihood of species identity for samples of birds (Herbert et al. 2004), mammals (Hajibabaei et al. 2007), fishes (Ward et al. 2005) and arthropods (Hajibabaei et al. 2006). Despite the fact that the mini-barcode system has dramatically broadened the application of DNA barcoding the accuracy provided when working with short DNA fragments, such as ancient DNA sequences, is greatly reduced (Figure 1; Meusnier et al. 2008).

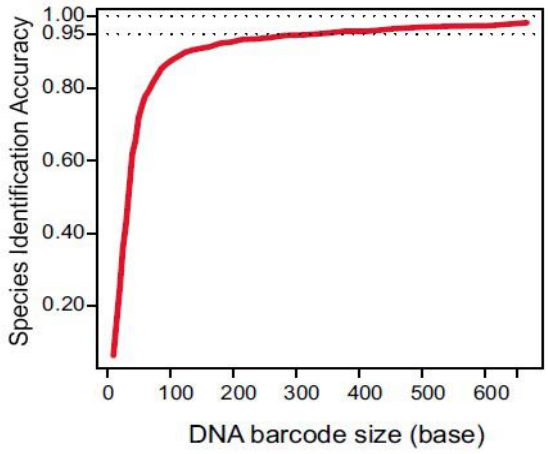


Figure 1: The identification accuracy is plotted vs. the length (in base pairs) of the submitted sequence (Meusnier et al. 2008).

The Barcode of Life Data Systems (also known as BOLD) is a sequence database specifically devoted to DNA barcoding of sequences in the COI region and is one of the most utilized system for the identification of species. At present, the algorithm that BOLD uses relies on distance-based identification despite the fact that such metrics have been rejected by the systematics community for almost two decades (Goldenstein et al. 2000). Previous barcoding of some scombrids has revealed the limitations of this distance-based approach (Lowenstein et al. 2009). For instance, recently separated taxa with large effective population sizes that are reasonably stable may constitute particular challenges for barcoding (Elias et al. 2007).

1.4.5 Phylogenetic trees

Almost every DNA barcoding study presents a phylogenetic tree as part of the standard analytical procedure (Ward et al. 2005; Chow et al. 2006; Viñas & Tudela 2009; Casiraghi et al. 2010). They are fast and easy to compute for large data sets but in the end they seem to fit only a graphic summarization capability (Collins& Cruickshank 2013). The main problem of phylogenetic trees is that the algorithms usually used will cluster the samples with the most likely similar taxa. This means that all the possible taxa need to be included in the tree, making the identification of an unknown species impossible (Collins et al. 2012). In situations of incomplete lineage sorting and species level paraphyly, tree based identification methods can only provide ambiguous or incorrect identifications (Lowenstein et al. 2009). Another drawback of phylogenetic trees is that they are incapable of properly allocating individuals when introgression is

involved. For example, Viñas & Tudela (2009) were able to generate trees for species of the *Thunnus* genus with different genetic markers (Figure 2). However, depending on the used marker these trees show different clustering of species (for example T. thynnus) and different bootstrap values. Also the variation among many different species can influence the bootstrap values, decreasing the validity of the tree. It is important to point out that this kind of problems are not resolved by using different tree inference methods such as neighbor-joining or maximum likelihoods (Collins & Cruickshank 2013)

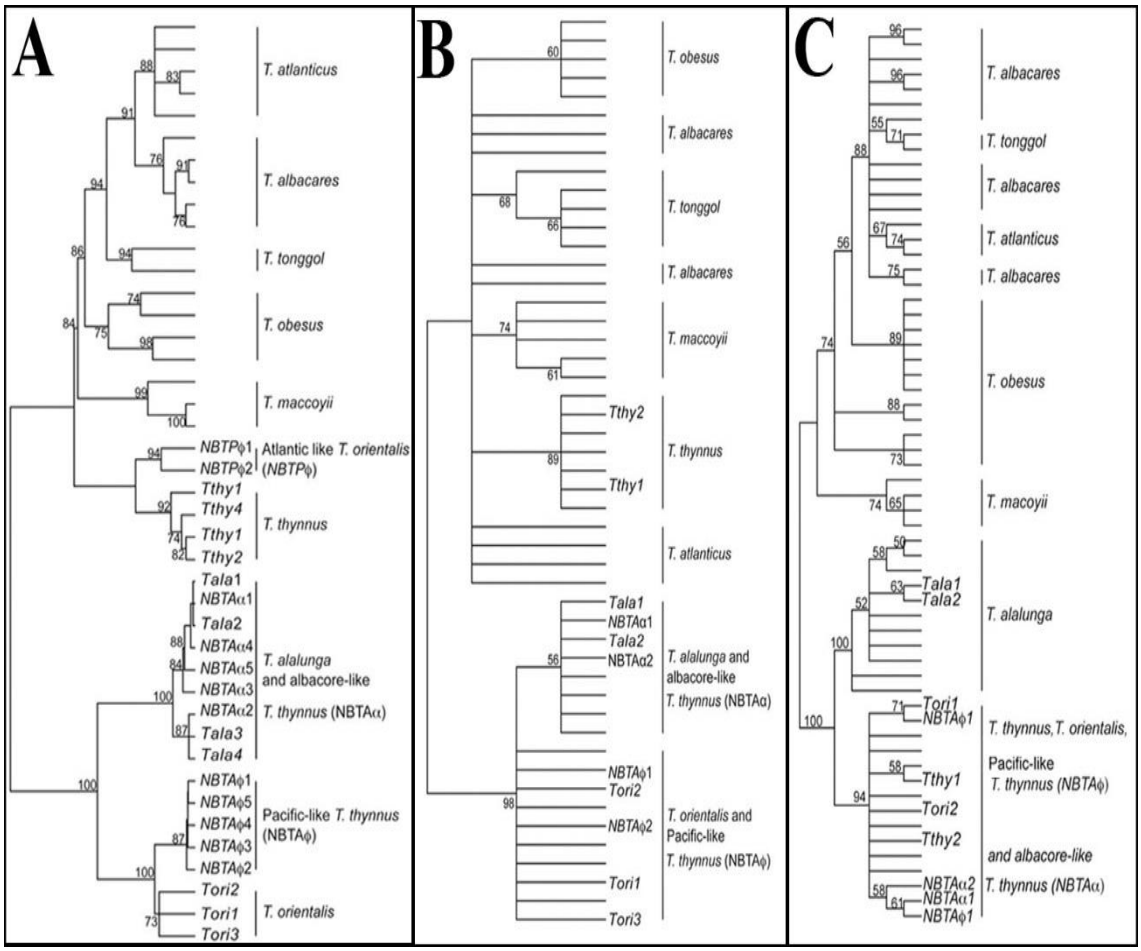


Figure 2: Phylogenetic trees of Thunnus sequences of mtDNA control region (A), mtDNA cytochrome oxidase 1 (B) and rDNA first internal transcriber spacer (C). The number on the nodes is the bootstrap value, not shown if below 60%.

1.4.6 Character Attributes

Even if DNA barcoding and phylogenetic trees have become standard methods for identifying organisms across all life stages, current phenetic (classification based

similarity) methods encounter trouble when working with short sequences. To overcome these issues species identification can be 'character based', whereby species are identified through the presence or absence of discrete nucleotide substitutions. Characteristic attributes are defined as diagnostic character states (genes, amino acids, base pairs or even morphological, ecological or behavioral attributes) that are found only in one clade but not in an alternate group that descends from the same node (Sarkar et al. 2002). Characteristic attributes are then divided into two major groups:

- Pure: when they are shared by all members of the clade and are absent from the other clades,
- Private: when they are shared only by some members of a clade but are absent from the other clades.

Both pure and private characteristic attributes can either be **simple** which are confined to a single nucleotide position, or **compound** which are combined states at multiple nucleotide positions.

After a particular characteristic attribute has been identified for a given taxonomic group, it can be used as diagnostic tool for species identification for this particular group (Rach et al. 2008)

2. Aim of the research

The study of ancient specimens, usually belonging to natural historical collections, has proved to be an invaluable source of genetic material. The information gathered analyzing old specimens can be used to investigate the evolutionary history of many species, including fishes. Thus it is essential to be able to find methods capable of successfully extracting and analyzing DNA from ancient samples. In this study we test DNA extraction protocols, usually suited for human analysis and inquire if this method can be applied, and eventually optimized, for historical fish samples. The final aim of this research was to evaluate the presence of valuable genetic material from dried specimens in the Massimo Sella archive, and to see if the genetic identification of the 100-years old samples is possible. Since standard identification methods fail when using heavily degraded genetic material, such as ancient DNA (aDNA), in this study we explore the possibility of using a characteristic attribute key to achieve species identification of historical fish specimens.

3. Materials and methods

3.1 Historical Samples

DNA was extracted from 207 vertebrae and one tooth, collected from the Massimo Sella archive in aDNA dedicated lab unit at the GenoDREAM. All of the specimens in the archive have been inspected and provisionally identified by researchers in the past. Whenever possible, data regarding the age, year of catch, weight and length, along with location of capture were collected (Appendix, Table S1).

3.2 Bone powder extraction

All the aDNA extractions were performed using an electrical drill, drillbits of different sizes and a hand-saw with a removable blade. To avoid cross-sample contamination all the bones were treated individually during both the cleaning and the powder extraction procedures.

3.2.1 Cleaning of the bones.

All bones were sprayed with bleach (3% sodium hypochlorite, eluted 1:1 with distilled water) and left to soak for up to ten minutes. After this time the sample was wiped clean with paper towel, rinsed with distilled water and air-dried. Once dried a thin surface layer was removed using sand paper, then the surface was rinsed with bleach and distilled water. All bones were left to dry for a minimum of 15 minutes under UV light (254nm wave length; Figure 3).

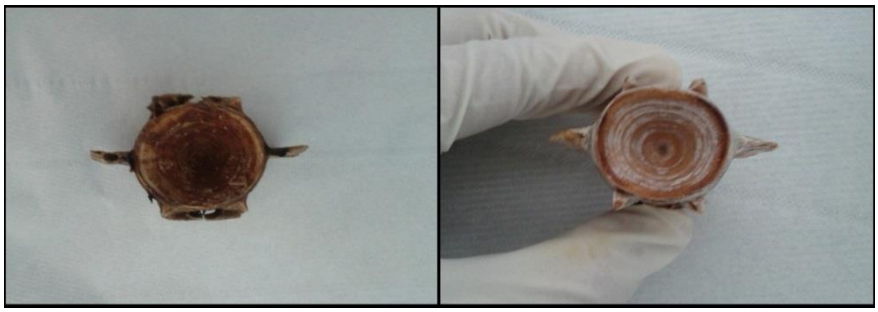


Figure 3*: Example of a vertebra before (left) and after (right) cleaning.*

3.2.2 Removal of bone powder

Depending on the size of the bone, the extraction procedure was adjusted to optimize the quantity of inner powder that could be extracted. In case of large vertebrae one or more holes were made with an electrical drill, paying particular attention not to pierce through the exterior surface of the bone (Figure 4A). If the bone wasn't large enough for drilling, then the powder was collected by cutting the whole vertebra in half with a saw and then removing a portion of the inner bone with a sterile scalpel (Figure 4B). In some cases vertebrae were so small that the entire bone was reduced to powder with a mortar and a pestle, after being carefully cleaned (Figure 4C). Since DNA from teeth is generally well preserved within the protective encasing of enamel, the dentine was simply removed from the tooth, in order to avoid unnecessary damage to the tooth (Figure 4D).

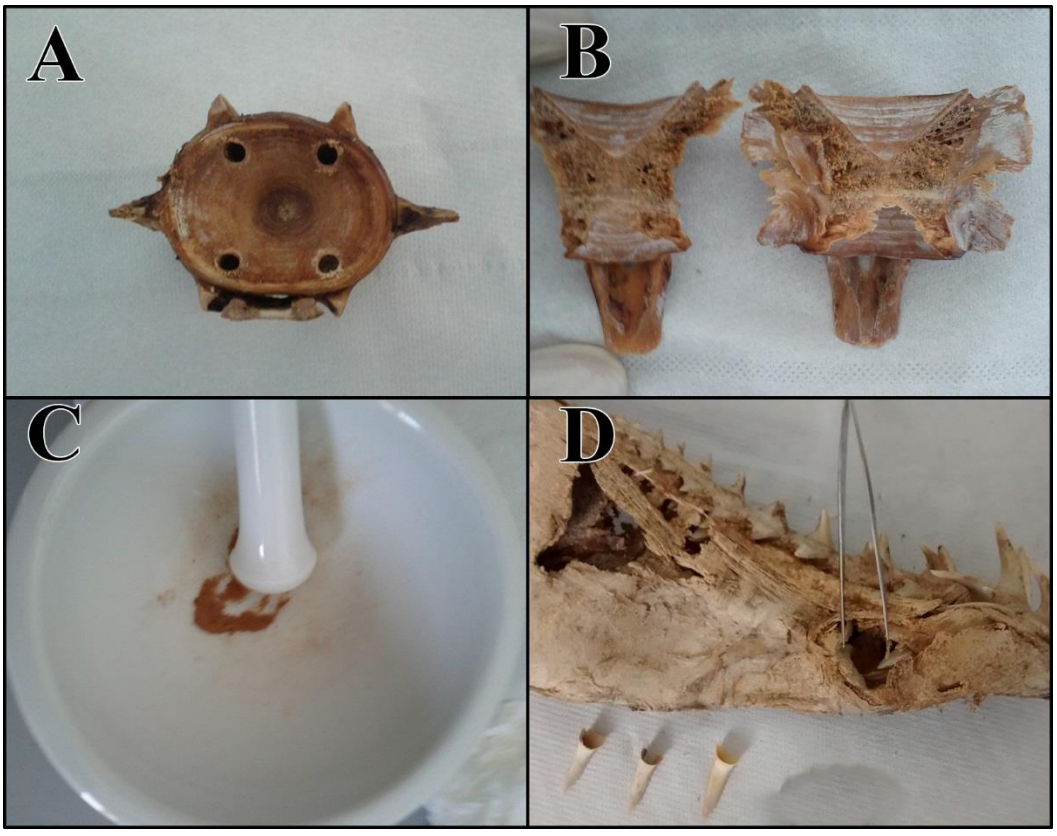


Figure 4: *Aftermath of the extraction performed with a drill (A), with a hand-saw (B) or by the reduction of the whole bone to powder (C). The dentine of the only dental sample was removed from teeth, the empty shell of enamel of the remaining teeth (D).*

3.3 aDNA extraction

All aDNA extractions were performed in a laboratory dedicated to ancient DNA analysis in which PCR products are forbidden. The work table was thoroughly decontaminated with ethanol and bleach after each extraction. All of the laboratory's supplies, with the exception of Qiagen's MiniElute™ Spin Column (guaranteed sterile by the manufacturer), were sterilized with UV light before every extraction. All of these precautions were followed in order to reduce contamination of the sample with exogenous modern DNA. A blank control (microtube containing distilled water) accompanied all the extraction sessions.

The two different protocols tested in this study were developed making changes to already existing protocols (Riccioni et al. 2010; Dabney et al. 2013). To understand which one of these protocols was the best suited to extract DNA from 100-year-old bones, 20 extractions were performed using each protocol (Box 1). To further understand if these protocols could be improved, 30 more extractions were performed with changes to the volume of the reagents (Box 2). The most important changes to the modified protocol of Dabney et al. (2013; heretofore referred as the "silica protocol") was the decision to double the volume of the extraction buffer and the binding buffer by splitting the bone powder of each sample into two different microtubes and then using a single silica column for the binding step. This modification allowed for complete digestion of the bone powder. The products of both protocols were then compared using quantification using Invitrogen's Qubit® 2.0 Fluorometer.

The final versions of the two protocols are featured below.

Dextran Blue Protocol (modified protocol of Riccioni et al. 2010)

All of the volumes (μL) for the Binding and Washing steps were doubled from the original version.

Extraction:

1. Incubate 200mg of bone powder overnight in a shaker at room temperature in 1.6 mL of EDTA buffer (0.5 M, pH 8.0).
2. Precipitate sample by centrifugation at 2500 rpm for 20 minutes.

3. Discard supernatant.
4. Add 1.6 mL of extraction buffer (0.1 M EDTA, 0.5% N-laurylsarcosine-Na salt) and 100 µL of 20 mg/mL Proteinase K to the decalcified bone precipitates.
5. Incubate overnight at 44°C in a shaker.
6. Centrifuge at 12,000 rpm for 10 minutes at room temperature.
7. Transfer 250 µL of extraction solution to another microtube.

Binding:

1. Add 3.5 µL of 1 µg/µL Dextran Blue, 500 µL of 4M NH4-acetate, and 1000 µL of 96% volume ethanol.
2. Precipitate DNA on ice for 10 min and then centrifuge at 14,000 rpm for 15 min at 4°C.
3. Discard supernatant.

Washing:

1. Add 500 µL of 70% volume ethanol and centrifuge at 14,000 rpm for 5 min at 4°C.
2. Discard supernatant.
3. Dry the sediment at 55°C on a block, or overnight on the bench.

Elution:

1. Add 50 µL of distilled sterile water.

Silica protocol (modified protocol of Dabney et al. 2013)

Before starting, split 100mg of bone powder from the same sample into two different eppendorfs, considering them as two different samples during the Extraction step. Then, during the Binding step, filter both aliquots using a single silica column.

Extraction:

1. Add 432 µL of EDTA (0.5M, pH 8.0) to each microtube.
2. Incubate for 16-24h, in a shaker at 37°C.
3. Add 70 µL of Proteinase K to each microtube.
4. Incubate for 16-24h, in a shaker at 37°C.
5. Centrifuge the eppendorfs for 3 minutes at 10,000 rpm.
6. Transfer supernatant into new microtubes.

Binding:

1. Add 1004 µL of Binding Buffer to each microtube.
2. Transfer 753 µL into a MiniElute™ Spin Column.
3. Centrifuge for 4 minutes at 14,000 rpm.
4. Add the remaining 753 µL to the respective column.
5. Centrifuge for 4 minutes at 14,000 rpm.
6. Repeat the steps 1-to-5 for the respective duplicate

Binding Buffer composition: Guanidine Thiocyanate (5M), Tween 20 (0.05%), Isopropyl alchol (40% vol/vol), Sodium acetate (90mM pH 5.2) and distilled water.

Washing:

1. For each MiniElute™ Spin Column add 750 µL of PE buffer.
2. Centrifuge for 2 minutes at 6,000 rpm.
3. Discard the flow-through.
4. Repeat once.
5. Dry-spin for 1 minute at 14,000 rpm.
6. Place the MiniElute™ Spin Column in a fresh 1.5-mL collection tube.

Washing Buffer composition: Qiagen's Buffer PE eluted with 96 % ethanol, by Qiagen's default instruction.

Elution:

1. Add 60 µL of pre-heated (60-70°C) distilled water.
2. Incubate room temperature for 10 minutes.
3. Centrifuge for 1 minute at 8,000 rpm.
4. Centrifuge for 2 minutes at 10,000 rpm.

3.4 Genetic analysis

3.4.1 Primer design

Primers capable of amplifying short sequences of the cytochrome oxidase subunit 1 (COI) gene, across the members of the Scombridae family were developed using Primer3 (Rozen & Skaletsky 1999). An alignment containing 74 reference sequences of different Scombridae species was used to identify sections of the gene that contained variable sites, diagnostic of species, with highly conserved flanking sequences appropriate for primer attachment. Primers where designed to amplify between 90-200 base pairs, because of the highly fragmented nature of historical DNA. The same strategy was used to design primers suited for *Xiphias gladius* (swordfish, the unique member of the Xiphiidae family in the Mediterranean) and two cartilaginous species of the Lamnidae family (shortfin mako, *Isurus oxyrinchus* and Great White Shark, *Carcharodon carcharias*). A similar approach was used for the design of *Thunnus* species-specific primers. Reference sequences are reported in the Appendix, Table S2.

All of the primers were designed with careful considerations given to the physical and structural properties of the oligos such as annealing temperature, guanine and cytosine content and tendency towards self-complementary binding. Finally all the primers were then tested using the PCR simulation software Amplifix (http://crn2m.univ-mrs.fr/pub/amplifx-dist).

A total of six pairs of primers where developed and can be seen in Table 2.

Table 2: List of the designed primers.

Name	Sequence	Targetted Species	Genetic Marker
FMSC1 Forward	5'-CGAGCTGAACTAAGCCAACC-3'	*Scombridae family*	mtDNA COI
FMSC1 Reverse	5'-GTCAGTTTCCAAACCCTCCA-3'	*Scombridae family*	mtDNA COI
TTCOX1 Forward	5'-CCCACGAATGAACAACATGA-3'	*Thunnus genus*	mtDNA COI
TTCOX1 Reverse	5'-CTCCAGCCTCAACTCCTGAA-3'	*Thunnus genus*	mtDNA COI
TTCR1 Forward	5'-AAATCGTCTAAGCCATACCAAGT-3'	*Thunnus genus*	mtDNA CR
TTCR1 Reverse	5'-TGGACTGGATGGTAGGCTCT-3'	*Thunnus genus*	mtDNA CR
TTITS1-II Forward	5'-GGGGGTTCAATGTCTCC-3'	*Thunnus genus*	rDNA ITS1
TTITS1-II Reverse	5'-TTTACACCGCACAGAGGTTG-3'	*Thunnus genus*	rDNA ITS1
FMXG1 Forward	5'-GCTGTCCTCCTTCTCCTCTC-3'	*Xiphias gladius*	mtDNA COI
FMXG1 Reverse	5'-GGTCAAAGAAGGCGGTGTTT-3'	*Xiphias gladius*	mtDNA COI
FMIO1 Forward	5'-GCCTTCCCCCGAATAAATAA-3'	*Lamnidae family*	mtDNA COI
FMIO1 Reverse	5'-TGCTAAGTTGCCAGCTAGGG-3'	*Lamnidae family*	mtDNA COI

3.4.2 Polymerase Chain Reaction

DNA amplifications were carried out using Promega's GoTaq® G2 Flexi DNA Polymerase kit and Biometra Tgradient96 thermocycler in 50µL reactions. A blank sample without aDNA was used in each run to test for contamination. The reaction buffer was created following the recipe listed in Table 3.

Table 3: Reaction buffer recipe used for a 50µL PCR.

Reagent	Stock concentration	Volume for each sample
H2O	-	26,75µl
GoTaq® Flexi Buffer	5X	5 µl
$MgCl_2$ Solution	25mM	6 µl
PCR Nucleotide Mix	10mM	2 µl
Forward Primer	10µmol/l	2,5 µl
Reverse Primer	10µmol/l	2,5 µl
GoTaq® G2 Flexi DNA polymerase template	5u/µl	0.26 µl
DNA	-	5 µl

Thermal cycles included an initial denaturation step for 3 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 30s, annealing between 58°C and 60°C (depending on the primers used) for 30s, extension at 72°C for 30s and a final elongation step for 3 minutes at 72°C.

Gel electrophoresis tests confirmed whether the amplifications were successful. We used a 1.5% resolution agarose gel, ideal for short length DNA.

3.4.3 Species identification

To construct a characteristic attributes key we visually inspected 5 mitochondrial COI reference sequences of each of the most common Scombridae family, downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), looking for particular variable sites that could serve as diagnostic sites for the species. All the sample's sequences, obtained via Macrogen's EZ-seq service, were analyzed and edited using FinchTV (http://www.geospiza.com/ftvdlinfo.html) software. Once verified these sequences were aligned, using ClustalW algorithm in MEGA 6.0 (Tamura et al. 2013), along with the reference sequences from NCBI. Finally, the diagnostic sites were checked to identify the species. To assess the identity of the five non-scombrid samples the use of species specific primers were considered sufficient.

A phenogram containing five reference sequences for each of the most common scombrids species was built using maximum likelihood inference methods and the Kimura-2-parameter (Kimura 1980) distance model. Statistical validation was tested performing bootstrap analysis (1000 repeats; Felseinstein 1985) on MEGA6.0. The tree was then analyzed, modified and scaled using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Barcoding analysis was performed querying the amplified sequences on public sequences databases BOLD (http://www.boldsystems.org/), for the mitochondrial COI sequences, and to NCBI's BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for mtDNA COI, CR and nuclear ITS1 sequences.

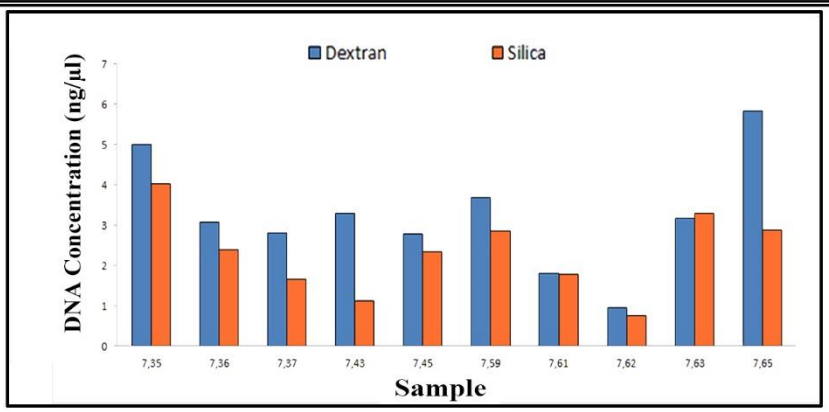
4. Results

4.1 aDNA extraction

Genomic aDNA from all 208 samples was successfully extracted and quantified (Appendix, Table S1) however not all of the extractions were successfully amplified (Table 4).

The comparison between non-modified versions of the two protocols revealed that the DNA yields of the two methods are not significantly different (p value >0.05; Box1).

Box1 - Comparison of DNA concentration in historical samples, generated using two different extraction protocols (Dextran Blue and Silica Column). Although the differences are quite obvious, the statistical differences between the two methods are not significant (p value > 0,05%)

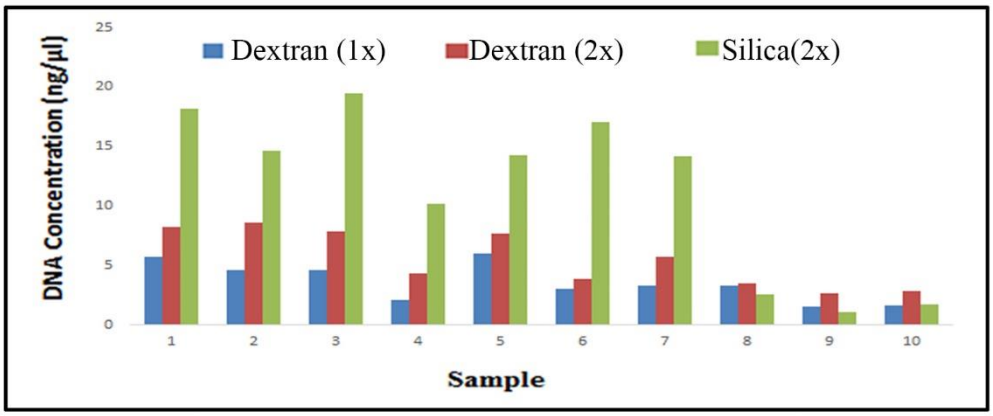


DNA Concentration (ng/µl)		
Sample	Dextran	Silica
7.35	5	4,04
7.36	3	2,41
7.37	2,81	1,67
7.43	3,29	1,14
7.45	2,8	2,34
7.59	3,68	2,86
7.61	1,82	1,78
7.62	0,968	0,768
7.63	3,19	3,3
7.65	5,84	2,89

T-Test: Two-Sample Assuming Unequal Variances	Dextran	Silica
Mean	3,2468	2,32
Variance	1,960169067	1,007
Observations	10	10
Hypothesized Mean Difference	0	
df	16	
t Stat	1,701927622	
P(T<=t) two-tail	**0,108115712**	

In contrast, the second comparison revealed that the genetic yields from the three extraction methods were significantly different (p value < 0.05; Box2).

Box2 - Comparison of DNA concentration in historical samples generated using three different extraction protocols. Dextran Blue(1x) original protocol, Dextran Blue(2x) doubled volumes and Silica(2x) two eppendorfs.The statistical differences between the three methods, tested with one another, are significant (p value < 0,05%)

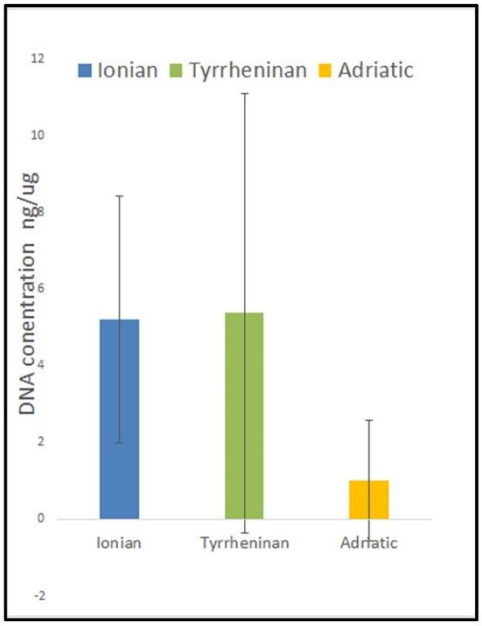


DNA Concentration(ng/µl)			
Sample	Dextran (1x)	Dextran (2x)	Silica (2x)
7.11	5,72	8,2	18,2
7.14	4,56	8,64	14,6
7.16	4,6	7,84	19,5
7.17	2,14	4,28	10,2
7.21	6,04	7,64	14,3
7.23	3,06	3,82	17,1
7.25	3,26	5,76	14,2
7.51	3,3	3,53	2,55
7.73	1,54	2,68	1,05
7.74	1,66	2,87	1,72

T-Test: Two-Sample Assuming Unequal Variances						
	Dextran (1x)	Dextran(2x)	Dextran(2x)	Silica(2x)	Dextran(1x)	Silica(2x)
Mean	3,588	5,526	5,526	11,342	3,588	11,342
Variance	2,5560178	5,59185	5,59185	50,154	2,55601778	50,15375
Observations	10	10	10	10	10	10
Hypothesized	0		0		0	
df	16		11		10	
t Stat	-2,146999		-2,4633		-3,3773824	
P(T<=t) two-t	**0,0474655**		**0,03149**		**0,00703351**	

A comparison of the concentration of the DNA extracted from all the *Thunnus* vertebrae, collected in three regions, is reported in Box 3. Statistical analysis revealed that while the averages are very different, due to the high variances this difference is not significant (p value> 0.05, F>Fcrit; Box 3)

Box 3. Comparison of DNA concentration averages of tuna samples collected from three regions. There is a not significant difference between the three regions (F> F crit, p value > 0.05)



Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*	*Standard Devation*
Ionian	50	260,11	5,2022	10,5099889	3,209328459
Tyrrheninan	50	268,717	5,37434	33,4324988	5,723971417
Adriatic	50	49,875	0,9975	2,52673356	1,573594258

ANOVA

Source of Variation	*SS*	*df*	*MS*	***F***	***P-value***	***F crit***
Between Groups	614,431044	2	307,21552	**19,8334842**	**2,36791E-08**	**3,057620652**
Within Groups	2276,99184	147	15,48974			
Total	2891,42289	149				

4.2 Amplifications

Some of the aDNA extracts showed different degrees of amplification while others did not amplify at all (Figure 6). Also, for several samples, a significant portion of the obtained sequences was too noisy to consider the sequence valid (Figure 7).

Table 4: List of primer pairs with their identified target taxa and amplification success rates.

Pair of primer	Target	Samples amplified	Clear sequences	Success rate(%)
FMSC1	*Scombridae family*	163	134	82,2
TTCOX1	*Thunnus genus*	51	41	80,4
TTCR1	*Thunnus genus*	36	30	83,3
TTITS1-II	*Thunnus genus*	37	24	66,7
FMXG1	*Xiphias gladius*	2	1	50
FMIO1	*Lamnidae family*	1	0	0,00

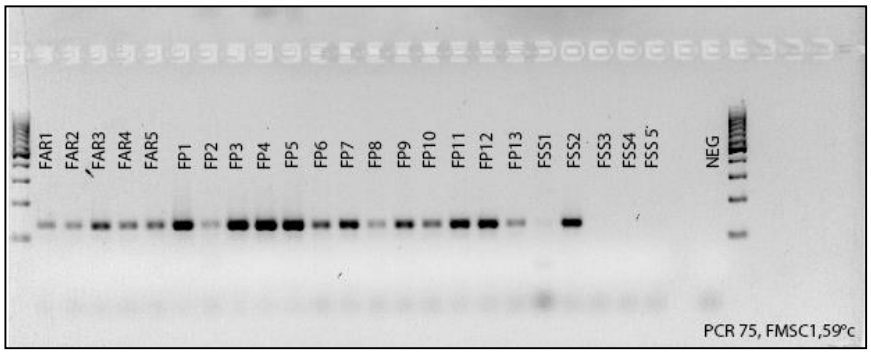


Figure 6: Example of a PCR electrophoresis gel showing differences in amplification efficiency. Highly effective amplifications are shown as dark bands, less effective amplifications as lighter bands and failed amplifications are columns with bands absent. Standard ladders are shown on the extremes of the gel as a measure of fragment length. In the present figure, the length of DNA amplified is approximately 100bp Sample codes are as follows: FAR = *Auxis* sp.; FP = *Sarda sarda*; FSS = *Scomber scombrus*; NEG = negative control.

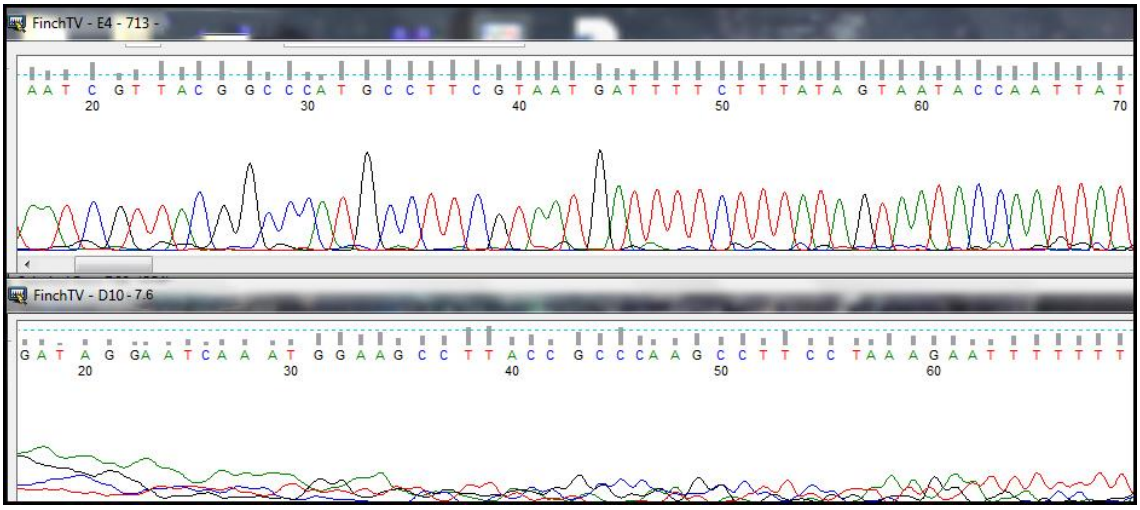


Figure 7: Example of two amplified sequences. Each letter represents the nucleotide base in that position. The gray rectangles above the letters represent the quality score of the base while the blue line represent a threshold of acceptance. The electropherogram on top is an example of successful amplification and sequencing, while the electropherogram on the bottom is exemplary of failure.

4.3 Species identification

The morphological identification of the samples (carried out by researchers in the past) provided a good starting point for the further genetic analysis. As expected the vast majority of the analyzed samples were identified as *T. thynnus;* however, due to the bad quality of some sequences the genetic identification was not possible for all of the amplified extractions (Table 5; for further detailed information see Appendix, Table S1).

Table 5: Overview of the samples identified by the original collectors using morphological methods and the genetic tools used in this study. All the morphological identifications refer to labels assigned by researcher in the past.

Putative species	Morphological ID	Genetic ID
Thunnus sp.	-	60
Thunnus thynnus	150	40
Scomber colias	17	10
Sarda sarda	13	13
Scomber scombrus	10	6
Auxis rochei	5	5
Xiphias gladius	3	1
Isurus oxyrinchus	2	0
Uknown Scombridae	8	0
Total	**208**	**135**

4.3.1 Characteristic attributes

Different characteristic attribute keys capable of differentiating species were found for each set of primers. However FMSC1 showed incapability to distinguish between the *Thunnus* species (Figure 8) while for the other pairs (TTCR1, TTCOX1 and TTITS1-II, specific for the *Thunnus* genus) these attributes were found (Figure 9).

Primer pair : FMSC1																		
	86	**94**	**97**	**106**	**107**	**109**	**112**	**121**	**130**	**133**	**140**	**142**	**145**	**148**	**155**	**163**	**167**	**169**
T. thynnus	G	T	G	G	A	C	C	C	C	T	G	A	G	T	A	A	A	T
T. alalunga	G	T	G	G	A	C	C	C	C	T	G	A	G	T	A	A	A	T
T. albacares	G	T	G	G	A	C	C	C	C	T	G	A	G	T	A	A	A	T
A. rochei	G	T	G	G	A	C	C	C	C	T	G	A	G	T	A	**G**	A	T
A. thazard	G	**C**	G	**A**	A	C	C	C	C	T	G	A	G	T	A	**G**	A	T
S. scombrus	**T**	T	**C**	**A**	**G**	C	C	**T**	C	T	**A**	**T**	**A**	**C**	**T**	A	**G**	**C**
S.colias	**T**	T	**C**	**A**	A	C	**T**	**T**	**T**	**C**	G	**T**	**A**	**C**	**T**	**G**	**G**	**C**
Sarda sarda	G	T	G	G	A	**T**	**T**	C	C	T	G	A	G	T	A	A	A	T
E. alletteratus	G	T	G	G	A	C	**T**	C	C	T	G	A	G	T	A	**C**	A	T

Figure 8: Character-based key for species of Scombrid derived from 49 reference sequences. Numbers in the first row represents the nucleotides positions, and are numbered following Ward et al 2005 sequences. The CA nucleotides are underlined.

Primer pair: TTCR1									
	260	**261**	**268**	**269**	**272**	**274**	**277**	**285**	**289**
T.thynnus	C	T	C	T	G	C	T	C	A
T. alalunga	C	**C**	C	T	**A**	**A**	T	**T**	**T**
T. albacares	**A**	T	**T**	**C**	**A**	**G**	**A**	**T**	A

Primer pair: TTCOX1			
	268	**271**	**260**
T.thynnus	C	T	C
T. alalunga	**T**	**C**	C
T. albacares	C	**C**	**A**

Primer pair: TTITS2				
260	**537**	**554**	**600**	**608**
C	A	T	C	T
C	A	T	**A**	T
A	**G**	**C**	C	**C**

Figure 9: Character-based key for the three species of *Thunnus*. The CA nucleotides are underlined and are numbered following Ward et al. 2005 (TTCOX1), Viñas et al. 2001 (TTCR1) and Viñas et al. 2009 (TTITS1-II).

5. Discussion

The species identification of historical samples from the Massimo Sella archive was overall a success. Through to the combined use of newly designed primers pairs we have developed a strategy that is able to identify historical fish remains Also, the combined use of both mitochondrial and nuclear markers proved to be a valid solution to account for introgression that can occur between some species the of *Thunnus* genus.

The resulting data of the comparison of the two original protocols tested show that the extraction from old specimens of the Massimo Sella archive is possible. Even if these two protocols displayed no significant differences in DNA concentrations, the yield of extracts for every single sample was considered unsatisfactory for the expected downstream applications (Next Generation Sequencing and SNP panel genotyping). After numerous trials and adjustments we managed to determine a variation of the protocols that allowed us to greatly increase the extraction's yield of both of the protocols. After evaluating the difference in yields between these various methods the optimized version of the Silica protocol was utilized to perform all of the 208 extractions, successfully completing the task.

However, it has to be pointed that for a variety of reasons, outlined below, the identification of all the examined specimens was not possible. Even if all of the 208 extracts were successfully quantified, spectrophotometry quantification (Invitrogen's Qubit) cannot reveal the origin of the analyzed DNA (exogenous vs. endogenous). Contamination remains the single most serious concern in the study of ancient DNA (Pääbo et al. 2004) as the presence of exogenous DNA usually exceed the amount of endogenous DNA by several fold (Noonan et al. 2005; Poinar et al. 2006; Green et al. 2006; Höss et al. 1996). While the use of species specific primer can help to eliminate the possibility of amplifying contaminant DNA, the use of more advanced (and expensive) quantification tools can characterize contaminations. For example, the use of a real-time PCR can help recognize the presence of a contamination through the use of melting curve analysis (Figure 10; Ririe et al. 1997).

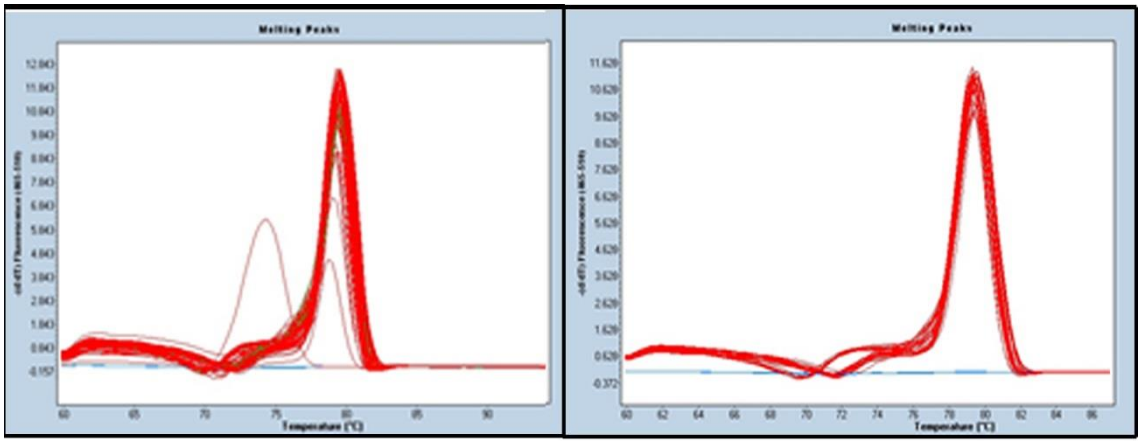


Figure 20: Example of a realtime-PCR performed on the same set of samples, amplified on different occasions, with TTCOX1 primer. On the left the small pink peak represents the presence of a contaminated sample (Puncher et al. 2015, unpublished data).

Another problem that needs to be taken into account is the presence of PCR inhibitors that could compromise amplification reactions. Several adjustments can be made to resolve this problem, however, some of these methods may be problematic due to the damaged nature of ancient DNA. For example, the dilution of the DNA extracts, in the hope that the inhibitory elements will be sufficiently diluted for successful amplification, can help eliminate this issue. However, samples with very low concentrations DNA can end up being diluted to a degree that it is no longer amplifiable (Kaestle & Horsburgh 2002). Despite the fact that the use of isopropyl alcohol (Hänni et al. 1995) and the employment of silica based protocol (Rohland & Hofreiter 2007) can eliminate some inhibitory problems, to be completely sure of PCR inhibitors presence the use of a quantitative approach (qPCR) should be applied (King et al. 2009).

Regardless of the remarkable conditions of the examined samples, some of the specimens were not properly preserved. As mentioned above, it is expected that 100 years of aging can produce serious damage to the DNA of the specimen, however some of the analyzed bones were particularly clean with some of them also displaying marks typically used for age assessments (Figure 11). It has to be underlined that almost all of the tuna's vertebrae collected from the Adriatic Sea had this appearance. It may be that many of these samples were cleaned using aggressive techniques. Thus the wide heterogeneity in the extracted DNA amounts from tuna bones could be attributed to these unknown cleaning methods. For example, some collectors routinely bleach or boil

bones in order to ensure long-term preservation. If this is the case for some of the bones contained in the Massimo Sella archive, then the integrity of DNA contained in those samples may be severely compromised.

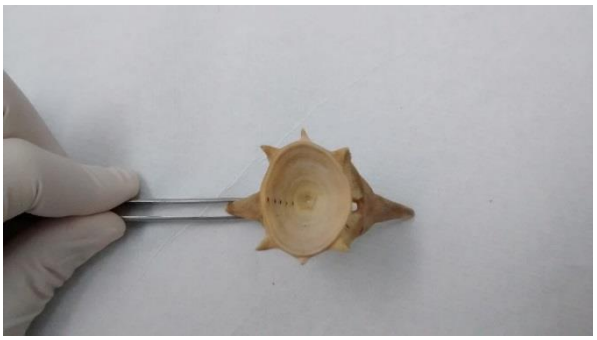


Figure 11: Vertebra of a tuna from the Adriatic Sea.

The design of primers capable of working with fragmented DNA strands allowed us to achieve, at least in part, the species identification. However, the standard methods of molecular identification didn't work well with our sequences. For both the direct interrogation of the BOLD System (figure 12) and the NCBI Basic Local Alignment Search Tool (BLAST; figure 13), the identification was not achieved.

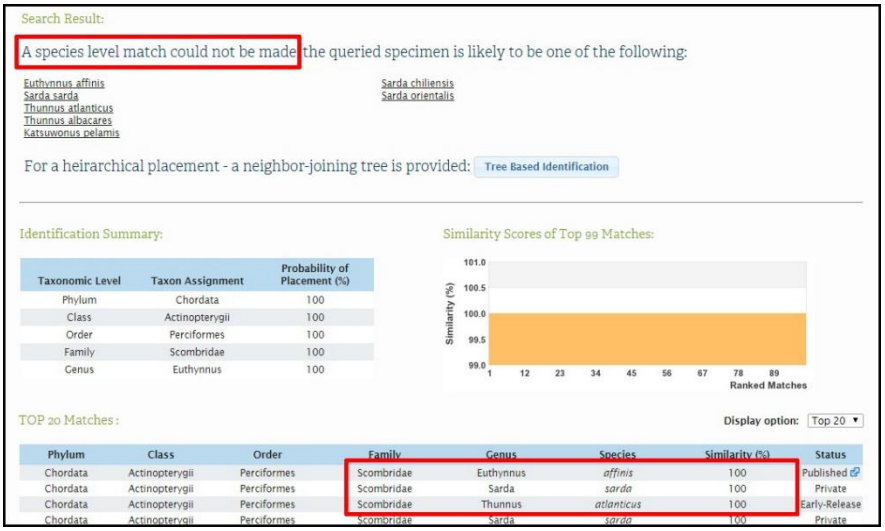


Figure 12: Submitted query of a FMSC1 amplicon, 90bp length to BoldSystem. Showed in the bottom red box are some of the many species that display a similarity score of 100% with the submitted sequence. The high number of compatible matches prevent the correct species identification of the sequence.

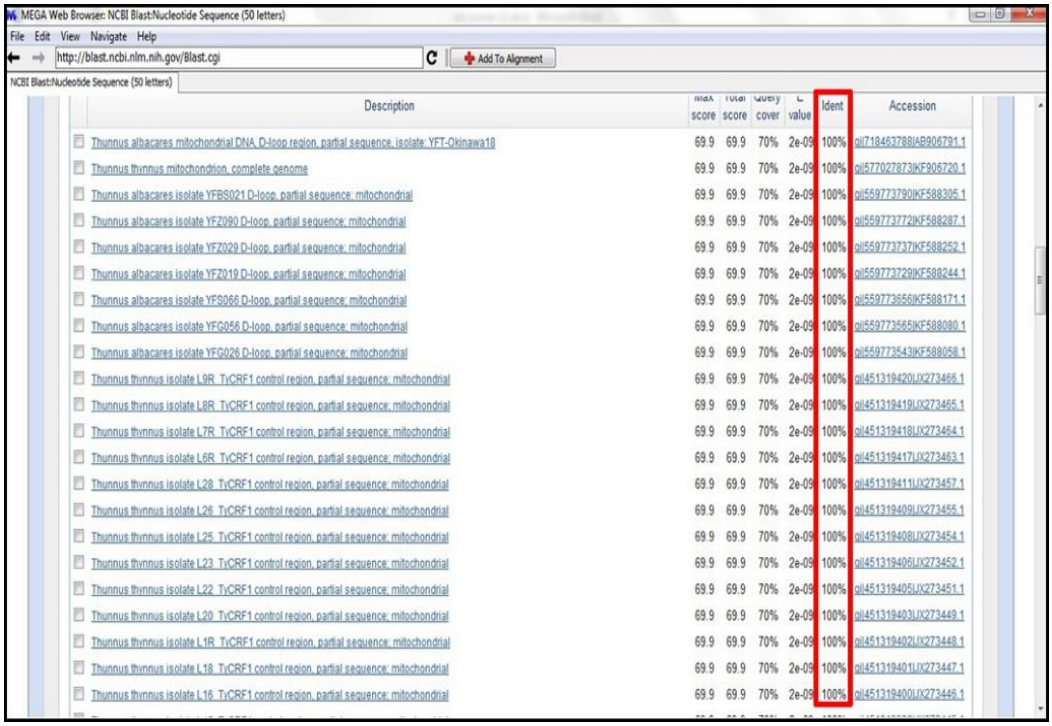


Figure 13: Submitted query of a TTITS1-II amplicon, 50bp length, to NCBI's BLAST. On the left column are displayed sequences of two different species of *Thunnus* that are compatible with the submitted sequence. In the red box the similarity score is highlighted. The 100% compatibility of both the two species prevents the correct identification of the sequence.

As already mentioned, the use of a phylogenetic trees is rarely efficient when conducting species identification (Collins & Cruickshank 2013); sequences can fail to cluster correctly and the identification of sequences is often ambiguous or incorrect (Lowenstein et al. 2010). The maximum likelihood tree constructed in this study proved, as expected, to be a non-effective way to cluster species and unknown specimens, due to the high similarity between the short sequences (Figure 14).

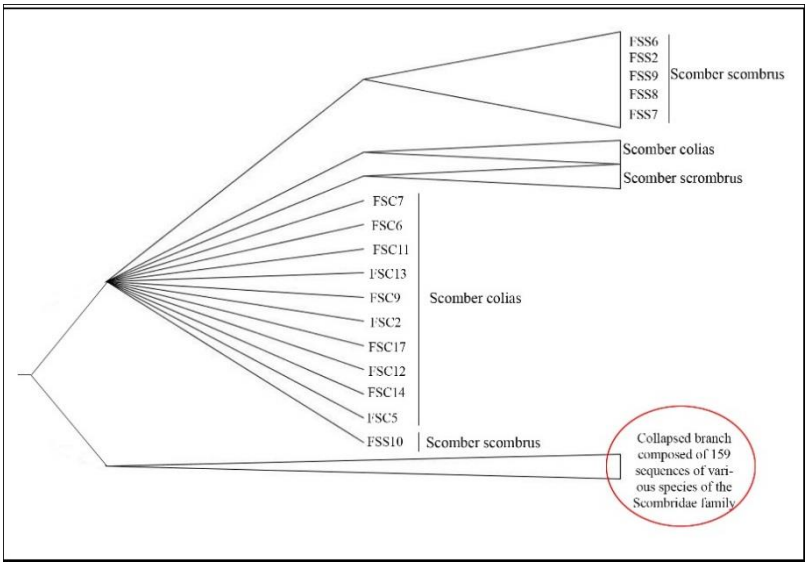


Figure 14: Maximum likelihood phylogenetic tree of 135 mtCOI amplified sequences and 70 mtCOI reference sequences. The bottom branch represents a collapsed tree of 159 sequences of various different species, clustered together.

The identification was therefore obtained with the use of a Characteristic Attribute (CA) key (Sarkar et al. 2002). The first step of this identification was done with the use of the FMSC1 primer pair. This set of primers was able to amplify across all the Scombridae family members, and provided us DNA sequences long enough to look for CA diagnostic for Scombridae. The joined use of both "simple" (only one nucleotide) and "compound" (more than one nucleotide) CAs gave us an identification that was unambiguous for almost all of the specimens.

For example, the nucleotide #121 was a cytosine (C) for all of the examined member of the Scombridae family. However, when in this location a thymine (T) was found, the #121 nucleotide identify that sequence as either belonging only to *Scomber colias* or *Scomber scrombrus*. The nucleotide site #130 displayed a cytosine for all the scombrids with the exception of *Scomber colias* that showed a thymine in that location (see Figure 8). Therefore the use of the simple CA (#130) tell us that the species is *Scomber colias*, the use of the compound CA (both #121 and #130) identify the specimen as *Scomber scrombrus* (Figure 15).

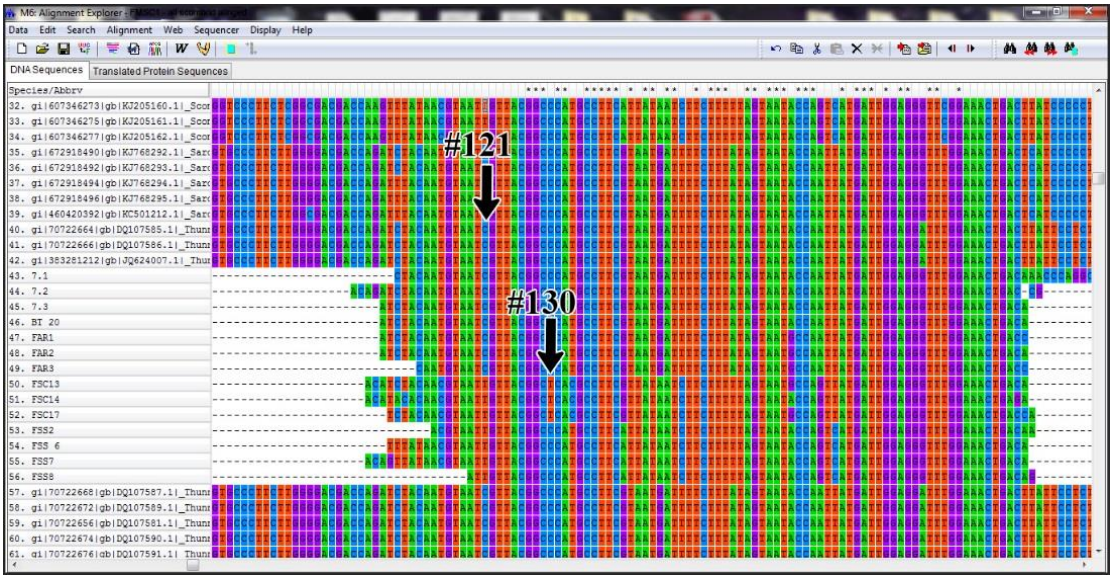


Figure 15: Example of an alignment file with some of our sample FMSC1 sequences (from 7.1 to FSS8), aligned with reference sequences. Pointed by the black arrows are two of the nucleotides sites diagnostic for *Scomber colias* and *Scomber scrombrus*.

Unfortunately, the identification to species level was not possible for all individuals. While it is theoretically possible to distinguish between *Auxis rochei* and *Auxis thazard* using the CA at the nucleotide position #94, the length of the obtained sequences was too short for that nucleotide to be sequenced. A similar problem was encountered with *Sarda sarda* individuals (nucleotide positions #109 and #112) which, due to the short length of the amplified sequences, were not possible to be distinguished from *Thunnus spp*. Another drawback of the FMSC1 primer pair is that a distinction between *Thunnus* species was not possible, since no CAs were present in the amplified sequence (Figure 8).

However the combined use of the other pairs of genes and primers (specifically designed for the *Thunnus* genus) allowed us to confirm, at least in part, that the tested *Thunnus* samples are indeed *Thunnus thynnus*. In fact, CAs were found in all of the other primer sets utilized. The analysis of 37 *Thunnus sp.* nuclear ITS1 sequences amplified with the primer pair TTITS1-II successfully identified 22 of them as *T. thynnus*. While CAs capable of discriminating between tuna species were present in the amplified ITS1 sequences, most of those sequences are unreadable or lacking nucleotides due to sequencing errors, making the identification for all the 37 amplicons impossible (Figure 16).

Figure 16: Example of an alignment file with some of our sample TTITS1-II sequences (from H10 to G6) aligned with reference sequences. Indicated by the black arrows are two of the nucleotide sites diagnostic for *Thunnus* spp. The white spaces indicate missing nucleotides in the sequences.

The presence of diagnostic CAs was also found in the mitochondrial Control Region sequence marker amplified by the primer pair TTCR1, which proved to be capable of differentiating between tuna species, confirming that 30 of them are *Thunnus thynnus*. Similar to the TTITS1-II sequences, however, some of them were not considered for the identification because of poor quality reads. Despite the high amount of sequences obtained with the mitochondrial COI marker (TTCOX1) none of them were long enough to include the identified CAs, making these sequences unusable (Figure 17).

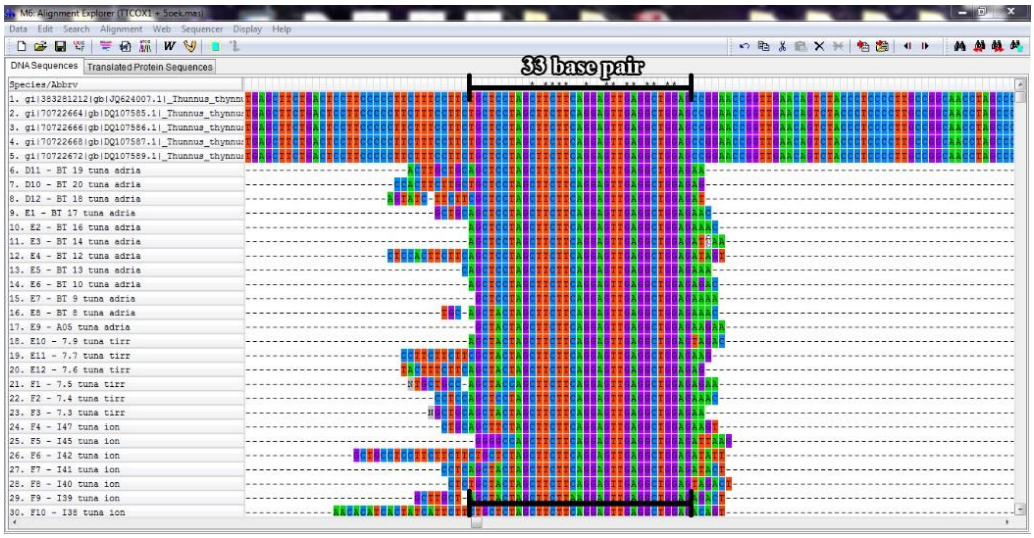


Figure 17: Example of an alignment file with some of TTCOX sequences (from D11 to F10) aligned with reference sequences.

Finally the identification of only one of the five non scombrid specimens was possible due to the bad condition of the historical samples. The three putative swordfish's vertebrae were not enclosed by a bone matrix and despite the successful amplification of two samples, only one of them was positively identified as *Xiphias gladius*. None of shark's samples could be genetically identified.

The consecutive use of a primers capable of amplify for all scombrids, followed by the use of tuna specific primers proved to be an effective strategy for sample identification. The combined use of both mitochondrial and nuclear markers also proved to be a valid method for identification of introgression, as 11 of 51 specimens addressed as Atlantic bluefin tuna are confirmed to be *T. thynnus* by both TTCR1 and TTITS1-II (Appendix, Table S1). Even if the genetic recognition was not possible for all the specimens, the overall accordance of all the different molecular markers used, paired with the morphological identification confirms that the specimen identification was performed correctly.

The genetic techniques and markers developed in this study offer a wide array of uses for the near future. These methods can be suited for temporal analysis of population dynamics in fishes. Also further research with these tools can provide invaluable genetic information that can be applied for next-generation sequencing. The methods developed in this work can be also used for modern day problems, such as food forensics. For example, the identification of cooked and canned tunas is problematic due to damage caused by the cooking of the flesh (Makie et al. 1999). In this situation the use of our primers (able to amplify short DNA sequences) can be used for the identification of incorrectly labelled food items.

6. Conclusions

This study deal with the species identification of historical fish bone specimens of 100-year old feasible. Despite the high number of problems encountered, the procedures optimized in this work prove that the goal was actually reached, at least resolving some remaining problems. However, these results also show that more work needs to be done in order to be able move towards further goals. As methods to isolate and sequence endogenous ancient DNA continues to improve, ancient DNA studies will remain limited by problems typical of the field. However as the number and accuracy of such studies is increasing, paleogenomics is poised to play an increasingly important role in improving our understanding of evolutionary processes over short and medium term, hence the study of natural history collections will play a major role in the near future.

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8. Appendix

Table S1: List of all the gathered data

Sample	DNA ng/ug	Powder mass(mg)	Putative Species	Identifcation with FMSC1	Identifcation with TTITS1-II	Identifcation with TTCR1	Catch location	Year of catch	Years (rings on vertebra)	Weight when cought (Kg)
I 1	6,48	100	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 2	0,37	99	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 3	2,25	110	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 4	4,72	98	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 5	6,28	99	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 6	1,1	92	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 7	3,42	102	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 8	3,13	105	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 9	2,9	120	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 10	3,49	105	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 11	4,28	180	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 12	5,2	190	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 13	4,4	200	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 14	10	223	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 15	13,6	257	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 16	7	234	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 17	4,32	171	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 18	5,56	189	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 19	9,2	190	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 20	15,7	260	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 21	0,85	296	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 22	3,44	195	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 23	5,04	276	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 24	7,72	268	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 25	7,52	218	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 26	3,9	153	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 27	4,16	220	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 28	7,32	227	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 29	9,48	214	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 30	5,16	274	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 31	11,2	105	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 32	2,54	95	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 33	5,88	90	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 34	4,8	101	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 35	5,4	96	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 36	6,36	98	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 37	11	108	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 38	3,34	109	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 39	1,4	104	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 40	5,04	108	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 41	7,28	114	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Ionian Sea	unknown.1911-1926	unknown	unknown
I 42	6,68	103	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 43	2,13	95	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 44	3,56	100	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 45	4,56	99	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 46	1,35	103	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown
I 47	2,51	104	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 48	2,5	104	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
I 49	1,71	107	*Thunnus sp.*				Ionian Sea	unknown.1911-1926	unknown	unknown

Table S1 – continue: List of all the gathered data

Sample	DNA ng/ug	Powder mass(mg)	Putative Species	Identifcation with FMSC1	Identifcation with TTITS1-II	Identifcation with TTCR1	Catch location	Year of catch	Years (rings on vertebra)	Weight when cought (Kg)
I 50	2,88	103	*Thunnus sp.*	*Thunnus sp.*			Ionian Sea	unknown.1911-1926	unknown	unknown
7.1	1,14	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	28,7
7.2	0,764	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	17,3
7.3	2,56	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	18
7.4	2,38	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	16,4
7.5	3,96	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.6	2,14	100	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	12,5
7.7	3,5	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.8	1,96	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.9	2,96	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	16,7
7.10	5,9	100	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	14
7.11	18,2	123	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.12	1,13	133	*Thunnus sp.*		*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	20
7.13	5,72	141	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	19,4
7.14	14,6	85	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Tyrrhenian Sea	1911	unknown	21,4
7.15	1,74	140	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	16,4
7.16	19,5	75	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	24
7.17	10,2	118	*Thunnus sp.*		*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	18,9
7.18	5,68	38	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	20
7.19	2,46	20	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.20	5,16	159	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Tyrrhenian Sea	1911	unknown	14
7.21	14,3	113	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.22	6,68	48	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.23	17,1	86	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	19,6
7.24	3,36	132	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.25	14,2	106	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	17,2
7.26	11,4	80	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	18,7
7.27	18,4	106	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.28	0,908	152	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.29	10	70	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.30	20,8	162	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.31	0,416	80	*Thunnus sp.*				Tyrrhenian Sea	unknown.1911-1926	unknown	unknown
7.35	4,04	101	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*	*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	17
7.36	2,41	153	*Thunnus sp.*			*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	20
7.37	1,67	110	*Thunnus sp.*			*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	27
7.43	1,14	118	*Thunnus sp.*			*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	27
7.45	2,34	125	*Thunnus sp.*			*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	
7.51	2,55	59	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	22
7.58	0,936	66	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	21
7.59	2,86	111	*Thunnus sp.*			*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	20
7.61	1,78	100	*Thunnus sp.*	*Thunnus sp.*		*Thunnus thynnus*	Tyrrhenian Sea	1911	unknown	22
7.62	0,768	112	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	18
7.63	3,3	93	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	25
7.65	2,89	115	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	29
7.67	1,78	135	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	31
7.70	3,96	122	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	unknown
7.73	1,05	86	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	16
7.74	1,72	100	*Thunnus sp.*				Tyrrhenian Sea	1911	unknown	25,5
7n.1	2,42	126	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	20

Table S1 – continue: List of all the gathered data

Sample	DNA ng/ug	Powder mass(mg)	Putative Species	Identifcation with FMSC1	Identifcation with TTITS1-II	Identifcation with TTCR1	Catch location	Year of catch	Years (rings on vertebra)	Weight when cought (Kg)
7n.2	1,32	137	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	18
7n.3	0,565	152	*Thunnus sp.*	*Thunnus sp.*			Tyrrhenian Sea	1911	unknown	21,5
A02	1,38	51	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A05	1,44	55	*Thunnus sp.*		*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A22	0,548	130	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
A26	0,235	20	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A28	0,13	20	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A31	0,688	110	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A34	0,182	70	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A35	0,316	114	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A37	0,192	70	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A49	0,079	90	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A51	0,049	100	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A53	0,428	100	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
A54	1,04	150	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
A58	0,187	110	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A59	0,62	30	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A64	0,082	90	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
A66	0,31	127	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
A69	0,334	80	*Thunnus sp.*	*Thunnus.sp*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	5	unknown
A74	0,194	20	*Thunnus sp.*	*Thunnus.sp*	*Thunnus sp.*		Adriatic Sea	unknown.1911-1926	5	unknown
A79	0,251	140	*Thunnus sp.*	*Thunnus.sp*			Adriatic Sea	unknown.1911-1926	5	unknown
BT1	0,6	90	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT2	0,83	100	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT3	1,26	90	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT4	0,54	100	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT5	0,61	70	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	5	unknown
BT6	0,376	110	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT7	0,306	90	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT8	2,77	100	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	8	unknown
BT9	3,8	120	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT10	3,88	180	*Thunnus sp.*	*Thunnus sp.*	*Thunnus thynnus*		Adriatic Sea	unknown.1911-1926	3	unknown
BT11	1,08	140	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	4	unknown
BT12	0,288	130	*Thunnus sp.*	*Thunnus sp.*	*Thunnus sp.*		Adriatic Sea	unknown.1911-1926	6	unknown
BT13	1,68	210	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	8	unknown
BT14	0,216	120	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	6	unknown
BT15	1,31	100	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT16	0,628	80	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT17	0,104	140	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT18	0,64	90	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT19	0,42	90	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	4	unknown
BT20	10,3	110	*Thunnus sp.*	*Thunnus sp.*			Adriatic Sea	unknown.1911-1926	6	unknown
BT21	0,548	130	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	6	unknown
BT22	0,412	70	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	8	unknown
BT23	0,452	120	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	8	unknown
BT24	0,286	80	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	8	unknown
BT25	1,06	130	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT26	2,13	120	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT27	0,752	120	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	4	unknown

Table S1 – continue: List of all the gathered data

Sample	DNA ng/ug	Powder mass(mg)	Putative Species	Identifcation with FMSC1	Identifcation with TTITS1-II	Identifcation with TTCR1	Catch location	Year of catch	Years (rings on vertebra)	Weight when cought (Kg)
BT28	1,88	170	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	5	unknown
BT29	1,44	100	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	4	unknown
BT30	0,592	150	*Thunnus sp.*				Adriatic Sea	unknown.1911-1926	4	unknown
FSC1	0,136	78	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC2	0,304	88	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC3	0,796	111	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC4	0,162	99	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC5	0,378	88	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC6	0,376	60	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC7	0,596	50	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC8	0,66	70	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC9	0,213	50	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC10	2,1	70	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC11	0,566	100	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC12	0,248	60	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC13	0,368	40	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC14	0,259	50	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FSC15	0,311	70	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC16	0,548	100	*Scomber colias*				Not clear	unknown.1911-1926	unknown	unknown
FSC17	0,0166	100	*Scomber colias*	*Scomber colias*			Not clear	unknown.1911-1926	unknown	unknown
FAR1	0,178	105	*Auxis Rochei*	*Auxis sp.*			Not clear	unknown.1911-1926	unknown	unknown
FAR2	0,304	110	*Auxis Rochei*	*Auxis sp.*			Not clear	unknown.1911-1926	unknown	unknown
FAR3	0,568	110	*Auxis Rochei*	*Auxis sp.*			Not clear	unknown.1911-1926	unknown	unknown
FAR4	0,152	100	*Auxis Rochei*	*Auxis sp.*			Not clear	unknown.1911-1926	unknown	unknown
FAR5	0,698	110	*Auxis Rochei*	*Auxis sp.*			Not clear	unknown.1911-1926	unknown	unknown
FP 1	8	100	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 2	1,08	120	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 3	3,03	85	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 4	8,64	120	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 5	8	120	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 6	1,14	104	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 7	0,512	102	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 8	1,16	112	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 9	1,51	115	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 10	0,334	96	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 11	0,237	107	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 12	0,309	100	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FP 13	0,848	130	*Sarda sarda*	*Sarda sarda*			Not clear	unknown.1911-1926	unknown	unknown
FSS 1	0,608	60	*Scomber scombrus*				Not clear	unknown.1911-1926	unknown	unknown
FSS 2	0,296	50	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FSS 3	0,178	40	*Scomber scombrus*				Not clear	unknown.1911-1926	unknown	unknown
FSS 4	0,52	70	*Scomber scombrus*				Not clear	unknown.1911-1926	unknown	unknown
FSS 5	0,568	110	*Scomber scombrus*				Not clear	unknown.1911-1926	unknown	unknown
FSS 6	0,277	40	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FSS 7	0,282	50	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FSS 8	0,27	40	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FSS 9	0,24	70	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FSS 10	0,7	70	*Scomber scombrus*	*Scomber scombrus*			Not clear	unknown.1911-1926	unknown	unknown
FI 1	1,23	110	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 2	0,436	90	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 3	0,284	100	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 4	4,08	110	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 5	1,22	70	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 6	4,22	110	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 7	2,8	100	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
FI 8	2,48	100	*Scombridae family*				Not clear	unknown.1911-1926	unknown	unknown
F3	0,268	100	*Xiphias Gladius*				Not clear	unknown.1911-1926	unknown	unknown
F13	0,305	100	*Xiphias Gladius*				Not clear	unknown.1911-1926	unknown	unknown
F28	0,508	100	*Xiphias Gladius*				Not clear	unknown.1911-1926	unknown	unknown
FM1	0,144	120	*Isurus oxyrinchus*				Not clear	unknown.1911-1926	unknown	unknown
FM2	0,208	100	*Isurus oxyrinchus*				Not clear	unknown.1911-1926	unknown	unknown

Table S2: List of all the reference sequences

Cytochrome oxydase c sub-unit 1									
Sequence	**Species**	**Sequence**	**Species**	**Sequence**	**Species**	**Sequence**	**Species**	**Sequence**	**Species**
FJ605793	*Thunnus thinnus*	FJ605787	*Thunnus obesus*	FJ605782	Thunnus obesus	FJ605808	*Thunnus alalunga*	EU392206	*Thunnus albacares*
DQ835873	*Thunnus thinnus*	FJ605765	*Thunnus obesus*	FJ605783	Thunnus obesus	GQ414565	*Thunnus alalunga*	DQ35945	*Thunnus albacares*
DQ835874	*Thunnus thinnus*	FJ605766	*Thunnus obesus*	FJ605784	Thunnus obesus	GQ414571	*Thunnus alalunga*	DQ35946	*Thunnus albacares*
DQ835879	*Thunnus thinnus*	FJ605762	*Thunnus obesus*	FJ605802	Thunnus obesus	HQ167713	*Thunnus alalunga*	DQ35948	*Thunnus albacares*
DQ835880	*Thunnus thinnus*	DQ835861	*Thunnus obesus*	FJ605803	Thunnus obesus	HM007772	*Thunnus alalunga*	DQ35949	*Thunnus albacares*
FJ605746	*Thunnus thynnus*	DQ835862	*Thunnus obesus*	FJ605807	Thunnus obesus	HM007773	*Thunnus alalunga*	DQ35951	*Thunnus albacares*
FJ605750	*Thunnus thinnus*	DQ835863	*Thunnus obesus*	HQ611138	Thunnus obesus	HM007774	*Thunnus alalunga*	DQ35952	*Thunnus albacares*
FJ605752	*Thunnus thinnus*	DQ835864	*Thunnus obesus*	HQ611139	Thunnus obesus	JN007752	*Thunnus alalunga*	DQ35953	*Thunnus albacares*
FJ605757	*Thunnus thinnus*	DQ835865	*Thunnus obesus*	HQ611140	Thunnus obesus	JN007753	*Thunnus alalunga*	DQ35954	*Thunnus albacares*
FJ605758	*Thunnus thinnus*	DQ835867	*Thunnus obesus*	JN644297	Thunnus obesus	JN007754	*Thunnus alalunga*	DQ35955	*Thunnus albacares*
FJ605759	*Thunnus thinnus*	DQ835868	*Thunnus obesus*	JN644299	Thunnus obesus	JN007755	*Thunnus alalunga*	DQ35956	*Thunnus albacares*
FJ605760	*Thunnus thinnus*	DQ835869	*Thunnus obesus*	JN644304	Thunnus obesus	JN007756	*Thunnus alalunga*	DQ35957	*Thunnus albacares*
FJ605761	*Thunnus thinnus*	DQ835870	*Thunnus obesus*	JN644305	Thunnus obesus	JN007757	*Thunnus alalunga*	DQ835947	*Thunnus albacares*
FJ605764	*Thunnus thinnus*	DQ835871	*Thunnus obesus*	DQ107629	Thunnus obesus	JN007758	*Thunnus alalunga*	DQ835950	*Thunnus albacares*
FJ605769	*Thunnus thinnus*	DQ35866	*Thunnus obesus*	DQ107630	Thunnus obesus	JN007759	*Thunnus alalunga*	DQ885058	*Thunnus albacares*
FJ605806	*Thunnus thinnus*	FJ605743	*Thunnus obesus*	DQ107642	Thunnus obesus	JN007760	*Thunnus alalunga*	DQ885059	*Thunnus albacares*
GQ414566	*Thunnus thynnus*	FJ605744	*Thunnus obesus*	DQ107643	Thunnus obesus	JN007761	*Thunnus alalunga*	DQ885060	*Thunnus albacares*
GQ414567	*Thunnus thynnus*	FJ605747	*Thunnus obesus*	DQ107644	Thunnus obesus	JQ624006	*Thunnus alalunga*	DQ885061	*Thunnus albacares*
GQ414568	*Thunnus thynnus*	FJ605749	*Thunnus obesus*	EU752221	Thunnus alalunga	DQ107645	*Thunnus alalunga*	DQ885062	*Thunnus albacares*
GQ414569	*Thunnus thynnus*	FJ605751	*Thunnus obesus*	EU752222	Thunnus alalunga	DQ107646	*Thunnus alalunga*	EF609627	*Thunnus albacares*
GQ414572	*Thunnus thynnus*	FJ605756	*Thunnus obesus*	EU752223	Thunnus alalunga	DQ107647	*Thunnus alalunga*	EF609628	*Thunnus albacares*
JQ624007	*Thunnus thynnus*	FJ605770	*Thunnus obesus*	DQ835818	Thunnus alalunga	DQ107658	*Thunnus alalunga*	FJ605778	*Thunnus albacares*
DQ107585	*Thunnus thynnus*	FJ605771	*Thunnus obesus*	DQ835819	Thunnus alalunga	DQ107659	*Thunnus alalunga*	FJ605785	*Thunnus albacares*
DQ107586	*Thunnus thynnus*	FJ605772	*Thunnus obesus*	DQ835820	Thunnus alalunga	FJ605791	*Thunnus albacares*	GU324193	*Thunnus albacares*
DQ107587	*Thunnus thynnus*	FJ605773	*Thunnus obesus*	DQ835821	Thunnus alalunga	FJ605788	*Thunnus albacares*	GU324194	*Thunnus albacares*
DQ107589	*Thunnus thynnus*	FJ605774	*Thunnus obesus*	DQ835822	Thunnus alalunga	FJ605768	*Thunnus albacares*	GU324198	*Thunnus albacares*
FJ605796	*Thunnus obesus*	FJ605775	*Thunnus obesus*	DQ835823	Thunnus alalunga	FJ605763	*Thunnus albacares*	GU324199	*Thunnus albacares*
FJ605792	*Thunnus obesus*	FJ605776	*Thunnus obesus*	DQ835824	Thunnus alalunga	FJ605754	*Thunnus albacares*	GU440556	*Thunnus albacares*
FJ605789	*Thunnus obesus*	FJ605779	*Thunnus obesus*	FJ605767	Thunnus alalunga	EU752224	*Thunnus albacares*	HM452165	*Thunnus albacares*
FJ605790	*Thunnus obesus*	FJ605780	*Thunnus obesus*	FJ605798	Thunnus alalunga	EU752225	*Thunnus albacares*	HM452166	*Thunnus albacares*
FJ605786	*Thunnus obesus*	FJ605781	*Thunnus obesus*	FJ605804	Thunnus alalunga	EF609629	*Thunnus albacares*	HM007768	*Thunnus albacares*

Table S2 – continue: List of all the reference sequences

cytochrome oxydase c sub-unit 1									
Sequence	**Species**	**Sequence**	**Species**	**Sequence**	**Species**	**Sequence**	**Species**	**Sequence**	**Species**
HM007769	*Thunnus albacares*	DQ107583	*Thunnus atlanticus*	DQ107640	Thunnus maccoyii	KJ968132	*Katsuwonus pelamis*	JF492935	*Auxis rochei*
HM007770	*Thunnus albacares*	DQ107584	*Thunnus atlanticus*	DQ107641	Thunnus maccoyii	KC500273	*Auxis thazard*	JF492936	*Auxis rochei*
HM007771	*Thunnus albacares*	DQ107588	*Thunnus atlanticus*	FJ605799	Thunnus orientalis	KC500274	*Auxis thazard*	JN644283	*Auxis rochei*
JN644300	*Thunnus albacares*	FJ226521	*Thunnus tonggol*	FJ605800	Thunnus orientalis	KC500275	*Auxis thazard*	JN644296	*Auxis rochei*
JN644293	*Thunnus albacares*	FJ226522	*Thunnus tonggol*	FJ605801	Thunnus orientalis	KC500276	*Auxis thazard*	JN644298	*Auxis rochei*
JN644308	*Thunnus albacares*	FJ226523	*Thunnus tonggol*	GQ414564	Thunnus orientalis	KC500277	*Auxis thazard*	JN644301	*Auxis rochei*
DQ107648	*Thunnus albacares*	FJ226524	*Thunnus tonggol*	GQ414570	Thunnus orientalis	KC500278	*Auxis thazard*	JQ178238	*Auxis rochei*
DQ107649	*Thunnus albacares*	FJ237957	*Thunnus tonggol*	DQ107581	Thunnus orientalis	KC500279	*Auxis thazard*	KF528384	*Auxis rocheis*
DQ107650	*Thunnus albacares*	FJ237958	*Thunnus tonggol*	DQ107590	Thunnus orientalis	KC500280	*Auxis thazard*	KF528385	*Auxis rocheis*
DQ107651	*Thunnus albacares*	FJ237959	*Thunnus tonggol*	DQ107591	Thunnus orientalis	KC500281	*Auxis thazard*	KF528386	*Auxis rocheis*
DQ107652	*Thunnus albacares*	FJ237960	*Thunnus tonggol*	DQ107592	Thunnus orientalis	KC500282	*Auxis thazard*	KC501208	*Sarda sarda*
DQ35881	*Thunnus atlanticus*	JN644286	*Thunnus tonggol*	DQ107631	Thunnus orientalis	KC500283	*Auxis thazard*	KC501209	*Sarda sarda*
DQ35882	*Thunnus atlanticus*	JN644288	*Thunnus tonggol*	DQ35925	Katsuwonus pelamis	KC500284	*Auxis thazard*	KC501210	*Sarda sarda*
DQ835883	*Thunnus atlanticus*	JN644291	*Thunnus tonggol*	DQ35926	Katsuwonus pelamis	KC500285	*Auxis thazard*	KC501211	*Sarda sarda*
DQ835884	*Thunnus atlanticus*	JN644294	*Thunnus tonggol*	DQ35927	Katsuwonus pelamis	KC500286	*Auxis thazard*	KJ768292	*Sarda sarda*
DQ835885	*Thunnus atlanticus*	JN644306	*Thunnus tonggol*	DQ35928	Katsuwonus pelamis	KC500287	*Auxis thazard*	KJ768293	*Sarda sarda*
DQ835886	*Thunnus atlanticus*	DQ107632	*Thunnus tonggol*	DQ35929	Katsuwonus pelamis	KC500288	*Auxis thazard*	KJ768294	*Sarda sarda*
GU225687	*Thunnus atlanticus*	DQ107633	*Thunnus tonggol*	DQ35930	Katsuwonus pelamis	KC500289	*Auxis thazard*	KJ768295	*Sarda sarda*
GU225688	*Thunnus atlanticus*	DQ107634	*Thunnus tonggol*	DQ35931	Katsuwonus pelamis	KC500290	*Auxis thazard*	KJ09601	*Sarda sarda*
GU224626	*Thunnus atlanticus*	DQ107635	*Thunnus tonggol*	DQ35932	Katsuwonus pelamis	KC500291	*Auxis thazard*	KC501198	*Sarda sarda*
GU224627	*Thunnus atlanticus*	DQ107636	*Thunnus tonggol*	DQ835924	Katsuwonus pelamis	KC500292	*Auxis thazard*	KC501199	*Sarda sarda*
GU224628	*Thunnus atlanticus*	FJ605741	*Thunnus maccoyii*	GU225629	Katsuwonus pelamis	DQ835850	*Auxis rochei*	KC501200	*Sarda sarda*
GU224629	*Thunnus atlanticus*	FJ605742	*Thunnus maccoyii*	GU225630	Katsuwonus pelamis	DQ835851	*Auxis rochei*	KC501201	*Sarda sarda*
GU224630	*Thunnus atlanticus*	FJ605753	*Thunnus maccoyii*	HQ945893	Katsuwonus pelamis	DQ835852	*Auxis rochei*	KC501202	*Sarda sarda*
GU224631	*Thunnus atlanticus*	FJ605755	*Thunnus maccoyii*	HQ945894	Katsuwonus pelamis	DQ835853	*Auxis rochei*	KC501203	*Sarda sarda*
GU224632	*Thunnus atlanticus*	FJ605777	*Thunnus maccoyii*	JF49705	Katsuwonus pelamis	DQ835854	*Auxis rochei*	KC501204	*Sarda sarda*
GU224633	*Thunnus atlanticus*	FJ605794	*Thunnus maccoyii*	JF49706	Katsuwonus pelamis	FJ226516	*Auxis rochei*	KC501205	*Sarda sarda*
GU224634	*Thunnus atlanticus*	FJ605795	*Thunnus maccoyii*	JF49707	Katsuwonus pelamis	FJ226517	*Auxis rochei*	KC501206	*Sarda sarda*
GU224635	*Thunnus atlanticus*	DQ107637	*Thunnus maccoyii*	JN653476	Katsuwonus pelamis	FJ226518	*Auxis rochei*	KC501207	*Sarda sarda*
JQ843089	*Thunnus atlanticus*	DQ107638	*Thunnus maccoyii*	JN653477	Katsuwonus pelamis	FJ226519	*Auxis rochei*	JQ774716	*Scomber colias*
DQ107582	*Thunnus atlanticus*	DQ107639	*Thunnus maccoyii*	KJ968131	Katsuwonus pelamis	FJ226520	*Auxis rochei*	JQ774717	*Scomber colias*

Table S2 – continue: List of all the reference sequences

Cytochrome oxydase c sub-unit 1					
Sequence	**Species**	**Sequence**	**Species**	**Sequence**	**Species**
JQ774718	*Scomber colias*	KJ205161	*Scomber scrombrus*	KC501891	Xiphias gladius
JQ774719	*Scomber colias*	KJ205162	*Scomber scrombrus*	KC501892	Xiphias gladius
JQ775108	*Scomber colias*	KJ205163	*Scomber scrombrus*	KJ709526	Euthynnus alletteratus
JQ775109	*Scomber colias*	KJ768300	*Scomber scrombrus*	KF461181	Euthynnus alletteratus
JQ775110	*Scomber colias*	KJ768301	*Scomber scrombrus*	KF461180	Euthynnus alletteratus
JQ775111	*Scomber colias*	KJ09609	*Scomber scrombrus*	KC00752	Euthynnus alletteratus
JQ775112	*Scomber colias*	DQ88495	*Carcharodon cacharias*	KC00751	Euthynnus alletteratus
JQ775113	*Scomber colias*	EU388646	*Carcharodon cacharias*	GU225629	Euthynnus alletteratus
JQ775114	*Scomber colias*	FJ518941	*Carcharodon cacharias*	GU225630	Euthynnus alletteratus
KJ709873	*Scomber colias*	FJ518942	*Carcharodon cacharias*	HQ945893	Euthynnus alletteratus
KJ709874	*Scomber colias*	FJ518943	*Carcharodon cacharias*		
KJ709875	*Scomber colias*	FJ518944	*Carcharodon cacharias*		
KJ709876	*Scomber colias*	GU440260	*Carcharodon cacharias*		
KJ709877	*Scomber colias*	JF493076	*Isurus oxyrinchus*		
KJ768298	*Scomber colias*	JF493696	*Isurus oxyrinchus*		
KJ09606	*Scomber colias*	JF493697	*Isurus oxyrinchus*		
KJ09607	*Scomber colias*	JF493698	*Isurus oxyrinchus*		
KJ09608	*Scomber colias*	JX034003	*Isurus oxyrinchus*		
KC501363	*Scomber scrombrus*	JX034004	*Isurus oxyrinchus*		
KC501364	*Scomber scrombrus*	JX034005	*Isurus oxyrinchus*		
KC501365	*Scomber scrombrus*	JX034006	*Isurus oxyrinchus*		
KC501366	*Scomber scrombrus*	JX124792	*Isurus oxyrinchus*		
KC501367	*Scomber scrombrus*	KC015502	*Isurus oxyrinchus*		
KC501368	*Scomber scrombrus*	KJ709676	*Xiphias gladius*		
KC501369	*Scomber scrombrus*	KJ709951	*Xiphias gladius*		
KC501370	*Scomber scrombrus*	KC501885	*Xiphias gladius*		
KC501371	*Scomber scrombrus*	KC501886	*Xiphias gladius*		
KC501372	*Scomber scrombrus*	KC501887	*Xiphias gladius*		
KF930384	*Scomber scrombrus*	KC501888	*Xiphias gladius*		
KJ205159	*Scomber scrombrus*	KC501889	*Xiphias gladius*		
KJ205160	*Scomber scrombrus*	KC501890	*Xiphias gladius*		

Table S2 – continue: List of all the reference sequences

Mitochondrial Control Region				First internal transcriber space			
Sequence	**Species**	**Sequence**	**Species**	**Sequence**	**Species**	**Sequence**	**Species**
AF390345	*Thunnus alalunga*	JN988641	*Thunnus albacares*	GQ414554	*Thunnus thynnus*	AB212005	*Thunnus alalunga*
AF390346	*Thunnus alalunga*	JN988642	*Thunnus albacares*	GQ414555	*Thunnus thynnus*	AB212006	*Thunnus alalunga*
AF390347	*Thunnus alalunga*	JN988643	*Thunnus albacares*	GQ414560	*Thunnus thynnus*	AB212007	*Thunnus alalunga*
AF390348	*Thunnus alalunga*	JN988644	*Thunnus albacares*	GQ414561	*Thunnus thynnus*	AB212008	*Thunnus alalunga*
AF390349	*Thunnus alalunga*	JN988645	*Thunnus albacares*	GQ414562	*Thunnus thynnus*	AB127402	*Thunnus alalunga*
AF390350	*Thunnus alalunga*	AF390430	*Thunnus thynnus*	GQ414563	*Thunnus thynnus*	KM055358	*Thunnus alalunga*
AF390351	*Thunnus alalunga*	AF390431	*Thunnus thynnus*	AB212012	*Thunnus thynnus*	AB212029	*Thunnus albacares*
AF390352	*Thunnus alalunga*	AF390432	*Thunnus thynnus*	AB212011	*Thunnus thynnus*	AB212030	*Thunnus albacares*
AF390353	*Thunnus alalunga*	AF390433	*Thunnus thynnus*	AB212010	*Thunnus thynnus*	AB212031	*Thunnus albacares*
JN988635	*Thunnus albacares*	AF390434	*Thunnus thynnus*	AB21209	*Thunnus thynnus*	AB212032	*Thunnus albacares*
JN988636	*Thunnus albacares*	AF390435	*Thunnus thynnus*	AB211999	*Thunnus alalunga*	AB212033	*Thunnus albacares*
JN988637	*Thunnus albacares*	AF390436	*Thunnus thynnus*	AB212000	*Thunnus alalunga*	AB212034	*Thunnus albacares*
JN988638	*Thunnus albacares*	AF390437	*Thunnus thynnus*	AB212001	*Thunnus alalunga*	AB212035	*Thunnus albacares*
JN988639	*Thunnus albacares*	AF390438	*Thunnus thynnus*	AB212002	*Thunnus alalunga*	AB212036	*Thunnus albacares*
JN988640	*Thunnus albacares*	AF390439	*Thunnus thynnus*	AB212003	*Thunnus alalunga*	AB212037	*Thunnus albacares*
				AB212004	*Thunnus alalunga*		

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