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ASSESSMENT OF THE POPULATION STRUCTURE AND TEMPORAL CHANGES IN SPATIAL DYNAMICS AND GENETIC CHARACTERISTICS OF THE ATLANTIC BLUEFIN TUNA UNDER A FISHERY INDEPENDENT FRAMEWORK

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Preface

Due to rampant over-exploitation, the biomass of top predator species has declined by as much as 90% since the 1950s (Myers and Worm 2003). When these stocks have reached commercial exhaustion, fisheries have focused on the next lower trophic level, serially depleting marine resources in what has become known as “Fishing down food webs” (Pauly et al. 1998). Among commercially exploited fish, all three species of bluefin tuna (Pacific, Southern and Atlantic) have undergone some of the most significant declines in abundance and geographic range contractions (Worm and Tittensor 2011). As each of these species are large, long-lived, and have a high economic value, restricted spawning areas and short spawning periods, they are particularly susceptible to over-exploitation (Collette et al. 2011). Although the Atlantic bluefin tuna (BFT; *Thunnus thynnus*) has been targeted by fisheries in the Mediterranean Sea for thousands of years, it has only been in these last decades that the exploitation rate has reached far beyond sustainable levels; introducing a high risk of fisheries’ decline and stock collapse (MacKenzie et al. 2009).

An understanding of the population dynamics, exploitation rates and the influence of environmental variables that affect large pelagic fish (e.g. tunas, billfishes and sharks) is crucial for stock management and conservation of entire marine ecosystems. In 2006, the International Commission for the Conservation of Atlantic Tunas (ICCAT) adopted a 15-year recovery plan for bluefin tuna in the eastern Atlantic and Mediterranean (EC N. 644/2007). In addition to the new restrictions placed on fisheries (restricted seasons, size limits, banning of search planes) a multidisciplinary monitoring programme was introduced in order to increase our understanding of the species' biological traits. Using an array of technologies (satellites tags, genetic markers and microchemistry of otoliths) researchers determined that the population structure of BFT is likely to be more complex than the current two population management model. A pattern indicative of genetic heterogeneity has been detected within the Mediterranean (Carlsson et al. 2004, 2007; Riccioni et al. 2010); rising suspicions that the basin may contain numerous distinct populations. Population structuring studies using of molecular tools should make use of samples with identified origins (ie. young tuna that haven't developed the capacity to travel great distances). Several genetic studies that analyzed adult specimens have been unable to detect significant differentiation of populations. Monitoring of early life history stages can also inform decision makers about the health of the species based upon recruitment and survival rates.

PhD objectives

In an effort to contribute to the conservation of the species and the development of a more informed stock assessment, this PhD project was developed to elucidate a more accurate understanding of BFT population structuring and spatial dynamics. The aims of the project were 1) to assess the accuracy of larval identification methods, 2) determine the genetic structure of modern BFT populations, 3) assess the self-recruitment rate in the Gulf of Mexico and Mediterranean spawning areas, 4) estimate the immigration rate of BFT to feeding aggregations from the various spawning areas, and 5) develop tools capable of investigating the temporal stability of population structuring in the Mediterranean Sea. Funding for the research contained in this thesis was by and large provided by ICCAT's Atlantic-wide Research Programme for Bluefin Tuna (GBYP) through the Biological and Genetic sampling and analyses consortium and the Data Recovery project conducted between 2012 and 2015.

In order to establish a baseline of understanding concerning the basic biological traits of BFT, its morphological characteristics, geographic distribution and the fisheries that target them are detailed in **Chapter 1**. Current efforts to study BFT spatial dynamics and the tools used for estimating the remaining biomass are reviewed. Several inhibitory elements that challenge the development of effective conservation and management are also reviewed at length.

Misidentification of larvae can lead to uncertainty about the spatial distribution of a species, confusion over life history traits and population dynamics, and potentially disguise the collapse or recovery of localized spawning areas. **Chapter 2** details several weaknesses in modern morphology-based taxonomy including demographic decline of expert taxonomists, flawed identification keys, reluctance of the taxonomic community to embrace advances in digital communications and a general scarcity of modern user-friendly materials. Recent advances in molecular techniques useful for specimen identification and population studies are discussed at length. In this same vein, **Chapter 3** contains a description of the molecular identification of larvae (n=188) collected from three spawning areas in the Mediterranean Sea by different institutions. Several techniques were used to analyze the genetic sequences and an extensive comparison of the results is presented. The results revealed important differences in the accuracy of the taxonomic identifications carried

out by different ichthyoplanktologists following morphology-based methods.

Molecular techniques used for wildlife conservation are evolving rapidly and their use by fisheries researchers is steadily on the increase. Using a Genotyping-by-Sequencing (a Next Generation Sequencing technique) approach a large consortium operating within the GBYP developed a panel of high performance SNPs capable of distinguishing populations of BFT. The process by which the 95 Single Nucleotide Polymorphisms (SNPs) were selected and technically validated is described in **Chapter 4**. This tool could be used to improve Atlantic BFT stock assessments and management as well as in seafood traceability studies and investigations. **Chapter 5** details how the SNP panel was used to genotype 752 individuals captured between 2007-2013 from fourteen locations throughout the species' range, including several previously uncharacterized sites. Given the scale of sampling, this study is the most comprehensive assessment of modern bluefin tuna population structuring to date. These efforts were made with the aims to further validate the newly developed SNP panel as well as contribute to our understanding of the species' populations structuring. The results confirm much of what has already been learned from tagging and otolith studies with the exception of evidence suggestive of a small amount of mixing between eastern and western populations.

Effective fishery management benefits from the existence of baseline information concerning population size and demographics before intense commercial exploitation. Given that BFT have been intensively targeted by fisheries for thousands of years, analysis of DNA extracted from historical samples recovered from archives, museums and archaeological excavations can help to reveal elements of the past necessary for establishing something of a pseudo-baseline (Nielsen et al. 1999). **Chapter 6** provides a historical description of the relationship between humans and bluefin tuna, the state-of-the-art of historical DNA studies, and a description of new protocols developed for ancient DNA analysis. Using novel molecular techniques, DNA was extracted from bluefin tuna vertebrae excavated from late iron age and ancient roman settlements in coastal Iberia (Portual and Spain, 4th-2nd century BC; n=23) and Byzantine-era Constantinople (4th-15th century AD; n=6), as well as vertebrae from the Massimo Sella archive located at the University of Bologna (Ionian, Tyrrhenian and Adriatic Seas, early 20th century; n=150). In **Chapter 7**, I have described how a 96 SNP panel was developed to genotype historical and modern samples in order to elucidate changes

in population structuring and allele frequencies of loci associated with selective traits. The panel is composed of 48 SNPs discovered during the GBS research that was described in the previous chapter (i.e. funded by ICCAT GBYP Phase 4). The remaining 48 SNPs were derived from transcriptomic work conducted in 2012 by the GenoDREAM laboratory (Pintus 2013). In total 269 modern samples and 190 historical samples will be genotyped using this panel and unfortunately the results are not available to be reported here (expected in July 2015); however, the process by which the project was developed and executed is explained in detail.

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Table of Contents


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Chapter 1

Queen of Tunas

In a tiny, humble and impeccably tidy kitchen located down a small side street in Tokyo, Japan, Jiro methodically unpackages large chunks of fish that he ordered at the Tsukiji market earlier that morning. The smell of steaming rice perfumes the air and the sounds of the beginning day creep through the backdoor that stands ajar. An old well-fed cat can be seen lying in a pool of sunlight on the pavement outside. Jiro pauses and sensuously runs his fingertips over the soft deep red flesh of the fish to whom he owes his livelihood. Certainly his reputation as one of Tokyo's preeminent sushi chefs is largely due to five great qualities by which he strives to live his life: 1) A chef must be serious and consistent in his work; 2) A chef must always strive to improve their work; 3) Cleanliness. If a restaurant isn't clean, then the food isn't going to taste good; 4) Impatience. A sushi chef is an independent composer that shrugs collaboration and insists on having things done their way, and; 5) Passion. A sushi chef must be a perfectionist. His eyes breathe in the colour and contrast of the pearly striations and magenta almost translucent muscle. "Without fish", he thinks to himself, "we can't do business. However that doesn't mean they should catch all the fish to the brink of extinction." And with that, Jiro hastily places all of the fresh fish in the display case and continues with his preparations for the lunch rush. As always, the restaurant will be full.

1.1 The Atlantic bluefin tuna

Ranked among both the fastest and largest bony fishes in the world, the Atlantic bluefin tuna (BFT; *Thunnus thynnus*) has captivated coastal civilizations in the Mediterranean since time immemorial (see Chapter 6). Recognized for their immensity, the largest of the species can grow to 700kg and nearly 4m in length (Fig. 1). Seen in their natural environmental, most humans have observed them from above, as immense dark shadows herding their prey into tight bait balls, spraying plumes of silver spray into the air as they cut through the water at unfathomable velocities. Once removed from the sea, one is able to observe a rainbow of iridescent blues, greens, silver and white that fade from midnight navy blue on the dorsal surface to a mottled silver on the ventral surface. This colouration allows the tuna to blend with the dark deep waters when seen from above and the bright sunlight when viewed from below. They are fusiform in shape, meaning that they are wide in the middle and tapered at both ends. Like all scombrids, BFTs have a spiny depressible dorsal fin that can be tucked away into slot on their backs to reduce drag in the water during fast burst swimming. Behind this is a second dorsal fin followed by a series of tiny finlets that lead to a large, rigid and lunate caudal fin capable of producing powerful thrust. On their ventral side another row of finlets run anteriorly to their spiny anal fin. It is believed that the scombrid upper and lower finlets function to channel water flow into the vortex created by the caudal fin, thereby increasing the efficiency of both directional control and swimming velocity (Nauen and Lauder 2000). Aligned with the deepest section of their body are the BFT's pelvic and pectoral fins. BFT are negatively buoyant and require their pectoral fins to provide lift while they swim, otherwise they would sink. As BFT approach their prey they pull their pectoral fins into the grooves along their sides and blast through the water like torpedoes. They are a perfection of streamlining and even their eyes are completely flush with their bodyline. It is a truly formidable hunter with many prey items and only a handful of predators (squids, sharks, orcas).

Fig.1: K. Fraser with a 679kg BFT caught in Nova Scotia, 1979 (Photo courtesy of IGFA).

Stomach content analysis of BFT caught in the central Mediterranean revealed an array of 91 taxa

of prey items including 54 teleosts, 20 cephalopods and 13 crustaceans. Among the preferred food items were deepwater species like lanternfish (*Hygophum benoiti*), dragonfish (*Chauliodus sloani*) and shortfin squid (*Illex coindetti*), which accounted for approximately half of the diet (Battaglia et al. 2012). The authors of that study concluded that BFT focus on fauna that migrate vertically throughout the water column during the night and larger prey during the day. A similar study in the Gulf of St. Lawrence, Canada, revealed a much less diverse diet dominated by teleosts (mostly herring, *Clupea harengus*, and mackerel, *Scomber scombrus*) as well as several species of crustaceans, two species of bivalve, cephalopods, brown algae and a starfish (Pleizier et al. 2012). BFT captured in the Ligurian Sea appear to feed on small pelagic fishes and Ligurian krill, *Meganyctiphanes norvegica.* In fact, one individual of 117 cm had a stomach felled almost exclusively with krill (Orsi Relini et al. 2010). Finally, stable isotope analysis of BFT captured in the Balearic Sea suggests that gelatinous zooplankton constitute a significant fraction of their diet, especially young BFT (Cardona et al. 2012). Overall, it appears that BFT are generalist predators, changing prey items based on their location, focusing on abundant prey items and feeding in both benthic and pelagic habitats.

Tunas have a highly specialized vascular system that ensures efficient elimination of wastes and circulation of oxygen. A very powerful and large heart pumps blood packed with high concentrations of red blood cells, comparable to the hematocrit of diving mammals, creating a blood pressure three times higher than most other fishes. They are obligate ram ventilators, meaning that they must constantly swim with their mouths open in order to ventilate their gills. The surface area of their gills is extremely high relative to other teleosts, allowing them to extract upwards of 50% of the oxygen in the water passing over their gills (Joseph et al. 1988). Countercurrent heat exchange networks (or *rete mirabilis*) located throughout their circulatory system allows for the retention of heat in their core which gives their strong muscles the ability of rapid bursts of activity that can send them through the water at velocities higher than 100 km/h. A high mass of anaerobic white muscle provides an additional burst of energy when the tuna is already swimming at high speeds for extended periods of time. Their eyes are else fed with the same warm blood allowing them the same advanced hunting ability in deep cold waters as surface waters, as oxygen is transferred from the blood to the muscles more efficiently at higher temperatures (Bushnell and Holland 1989). Without eyelids, they spend a life time surveying the sea. These adaptations come at a cost

however; large BFT must avoid remaining in warmer waters for extended periods of time as they run the risk of overheating (Block et al. 2001; Teo et al. 2007). BFT with surgically implanted archival tags have been observed frequenting environments containing a wide range of temperatures (2.8 - 30.6°C) while maintaining constant internal temperatures of 25°C through both behavioural and physiological thermoregulation mechanisms (Block et al. 2001).

1.2 Distribution, ecology and movements

Burdened with massive oxygen demands, BFT spend their entire lives (up to 32 years) wandering the oceans, swimming endlessly without rest. These wanderings can take them far and wide. There are in fact three species of bluefin tuna that occupy all of the world's oceans (Fig. 2): the Atlantic (*T. thynnus*), Pacific (*Thunnus orientalis*) and southern bluefin (*Thunnus maccoyii*). This work focuses exclusively on the Atlantic bluefin tuna whose habitat presently spans the entire North and Central Atlantic Ocean, North Sea, Mediterranean, North and Black Seas and the Gulfs of Mexico and St. Lawrence. Fishery, tagging and survey data agree that the majority of adults feed in the Atlantic Ocean, Bay of Biscay, North Sea and Gulf of St Lawrence during the summer, autumn and winter months. Their movements appear to be dictated by the location of their prey, leading some to mid-Atlantic upwelling areas, some to the cold coastal waters of the North Sea, Canada, Iceland and Greenland, and others south to warmer waters of the Caribbean, Canary Islands and western Africa. In the early spring, these migratory groups of adults follow ancient migratory routes that lead them to spawning areas in the Mediterranean Sea and Gulf of Mexico (GOM).

Interestingly, the Atlantic species is the only bluefin tuna with more than one spawning area. Recent studies using an array of technologies including satellite tagging, genetic markers and microchemical analyses suggest that the population structure of BFT is much more complex than previously believed (Rooker et al. 2008, 2014; Galuardi et al. 2010; Cermeño et al. 2012; Aranda et al. 2013; Riccioni et al. 2010, 2013; Fraile et al. 2014). It's long been recognized by fishermen, poets, philosophers and historians that a group of BFT are resident in the Eastern Mediterranean Sea. Historical accounts suggest that this resident group have a north-south migratory route that stretches from the Black Sea, through the Sea of Marmara, into the northern Aegean, south to the Ionian Sea and eastwards into the Levantine Sea. During the 1960s, the Japanese fishing fleet captured large quantities of BFT off the coast

of Brazil and there have long been suspicions that they can be found in the southern Atlantic Ocean, particularly in the Gulf of Guinea and as far south as South Africa from January to March (Richards 1976; Takeuchi et al. 1999).

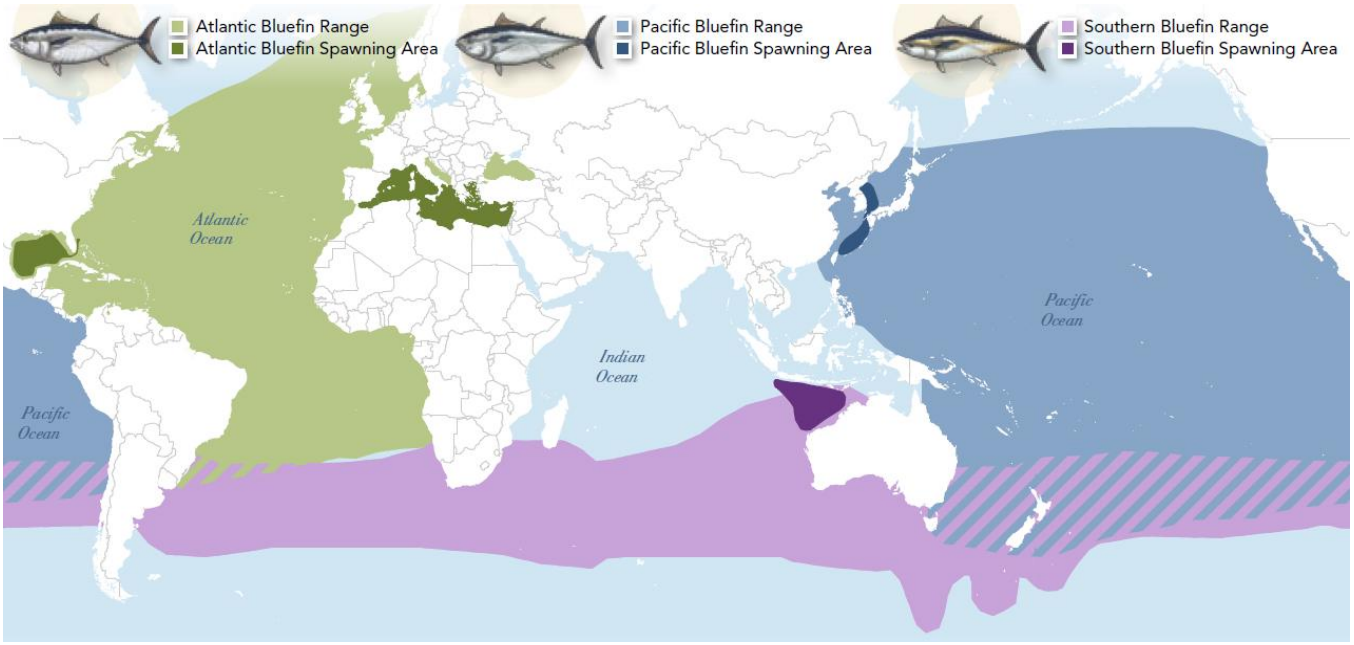

Fig. 2: Geographic distribution of three species of bluefin tuna (source: GreenInfo Network in Boustany 2011)

After decades of heavy exploitation, the species began showing signs of decline and stocks began to rapidly contract. Catches of BFT waned and disappeared from the North Sea in the 1960s, coastal waters of Brazil in the 1970s and Black Sea in the early 1980s (Mather et al. 1995; Fromentin and Powers 2005 MacKenzie and Myers 2007; MacKenzie and Mariani 2012). Worm and Tittensor (2011) estimated that in the 50 years following the 1960s, the abundance of BFT in the ocean reduced by more than 70% and their geographic range contracted by 53%. A renewed effort to learn more about the species, its spawning behaviour, migratory patterns, demographics and reproductive potential began in earnest.

Researchers have made use of an array of techniques to deduce the movements of BFT after they have been captured. At the beginning of the 20th century, Prof. Massimo Sella, the Director of the Marine Biological Station in Rovinj (Croatia), amassed a collection of fishing hooks that had been removed from BFT captured in tuna traps in the waters of Italy, Croatia and North Africa (Sella 1930). The materials and knots used to make the hooks could be traced to origins in Norway and the Strait of Gibraltar. This was likely the first tagging study to give information about the migratory behaviour of BFT after leaving the Mediterranean. Similarly, various studies have documented the presence of scars and wounds consistent

with cookie cutter shark, *Isistius brasiliensis*, bites on BFT captured in the Bahamas and the Canary Islands as well as Norway and the Mediterranean Sea (Di Natale 2010). Since this species of shark is only found in tropical waters south of Cape Hatteras and the Strait of Gibraltar, this information started giving biologists an indication of the latitudinal movements of BFT outside of the Mediterranean and GOM in the 1970s and 1980s. Archeologists and historians have also provided fisheries scientists with new insights into historical BFT distributions and fisheries. Archeologists have uncovered bones belonging to BFT in the northern Black Sea, as far afield as the Sea of Azov (Pantikapaion), a region in which BFT haven't been observed for centuries (Morales et al. 2007). Ancient Greek and Roman naturalists like Strabo, Aristotle and Appian wrote about angry herds of female tunas spawning in the Maiotis swamps, which are now known as the Sea of Azov (Tichii 1917). This comes as no surprise, since the region was once rich in shads, anchovies, sprats sardines and mackerels, all of which are favourite prey items of BFT (Morales et al. 2007). Much information can be gathered from Aristotle's History of Animals. In that seminal text, Aristotle speaks of tuna that spend the summer in the Black Sea, winter in the Aegean and migrate through the Sea of Marmara, Dardanelles and Bosporus Strait during the spring. According to Aristotle, it was widely believed at the time that BFT could see better out of their right eye and as such migrated along the southern shore of the Bosporus when entering the Black Sea and exited by the north shore, on the steps of Byzantium. During the 1970s and early 1980s, information concerning the distribution of BFT in the Black Sea is confusing and sometimes contradictory. Some authors suggest that the species was limited to the western half of the Black Sea (Muus and Dählstrøm 1975), while others proposed that they could only be found along the sea's southern shores (Whitehead et al. 1984). This archeological and historical information suggests that the retreat from the Black Sea may have begun long before their disappearance in the 1980s.

Modern tagging technologies employed by wildlife biologists to track the movements of marine animals range in capacity for data collection and cost (passive tags, acoustic tags and various electronic archival tags). Passive ID tag studies have shown that young and adult BFT display distinct geographical and seasonal movement patterns (Mather 1980). Dozens of young BFT (3-44kg) have been documented making trans-Atlantic migrations between the mid-Atlantic Bight and the Bay of Biscay (in both directions). One exceptional case from 1962 is a single tuna released in the Bahamas and recaptured in

Norway less than 2 months later (Rodewald 1967). Records of fish that migrated from the west to the east suggest that this migration is intermittent and that the frequency and number of fish that make the journey might be influenced by environmental elements, such as westerly winds, which differ on an annual basis (Rodewald 1967). According to a review of ID-tagging data, Mather (1980) proposed the following generalizations to be applied to BFT populations during the 1950s-1970s: 1) BFT spawned in the western Atlantic concentrate in the Mid-Atlantic Bight during their first year. 2) Small fish (3-44kg) feed in coastal waters of the Mid-Atlantic Bight during warm months and move offshore during the winter. In years when westerly winds are strong, large contingents of BFT cross the Atlantic to the Bay of Biscay. 3) Medium-sized BFTs (45-130kg) have similar movements as the smaller fish but exaggerated in scale, covering the Gulf of Maine and the Grand Banks. These animals spawn in or close to the Gulf Stream in the spring. 4) Giant BFT (>130kg) have even more extensive movements, expanding northward in the summer and fall into the Gulf of St Lawrence and Newfoundland, and south to Bermuda. In the springtime, giant BFT spawn in the GOM, and adjacent parts of the Caribbean and the Atlantic. During the summer they either feed off the coast of New England and Canada or cross the Atlantic to Norwegian waters. Mather (1980) also offered the following description of the movement of eastern BFT, albeit characterized by the author as conjectural and over-simplified: 1) Young-of-the-year (YOY) concentrate off the Atlantic coast of Morocco in the fall and approach the coastline in the spring. 2) In their second year, small fish move further afield to the Bay of Biscay and southern coast of Morocco and the Canary Islands. 3) Medium BFT enter the Mediterranean in May-June for spawning and return to the Atlantic in July-August. Others probably spawn sporadically somewhere in the Atlantic. Summers are spent in the Bay of Biscay or off the coast of Morocco, while winters are likely spent in the vicinity of the Canary Islands. Giant BFT have much the same migration patterns during the spawning season; however, large contingents likely pass west of the British Isles and summer off the coast of Norway. The giants that do make this journey arrive lean and hungry after traversing the near barren expanse of the open ocean. All available tag and recapture data suggest that migrating tuna follow favourable currents across the Atlantic; however, their exact routes were unknown until the advent of archival tags.

Many studies have used pop-up satellite and archival tags in which BFT were tagged in the north western Atlantic (Lutcavage et al. 2012; Block et al. 2001, 2005; Galuardi *et al.* 2010: Wilson et al. 2011)

and the north eastern Atlantic and Mediterranean (De Metrio et al. 2001, 2004, 2005; Block et al. 2005; Tudela et al. 2011; Cermeño et al. 2012, 2015; Aranda et al. 2013). All of these studies have confirmed that BFT have wide migratory routes that span the Atlantic Ocean. To date none of these tagging studies have shown a single individual entering both the GOM and the Mediterranean Sea within its lifetime, providing support for the natal homing theory (Rooker et al. 2008a). Individuals that have been observed in the GOM forage along the east coast of North America, including the Gulf of St Lawrence, and travel as far east as a few hundred miles short of the north western tip of the Iberian peninsula before returning to the west. These individuals haven't been observed travelling into the waters of Iceland, Greenland or the North Sea. Individuals that have been observed in the western and central Mediterranean can remain within the basin for years or enter the Mediterranean from Atlantic foraging areas for the spawning season only. BFT that migrate between the Mediterranean and the Atlantic have been observed as far south as the Canary Islands and as far north as Iceland, Norway and Greenland. They too forage along the east coast of the USA and Canada, entering into the Gulf of St Lawrence and skirting a trajectory tracing a diagonal between northern Florida and the Bahamas. Interestingly, no tagging study has ever shown individuals from the western and central Mediterranean migrating into the Levantine Sea (east of Greece and the Gulf of Sidra). Nor have any studies shown BFT from the Levantine Sea exiting the Mediterranean or travelling much further west than Sicily, for that matter. Multiple studies have shown mature individuals avoiding both of the known reproductive areas during the spawning season, suggesting that some of the populations skip spawning events or additional spawning takes place in the Atlantic Ocean (McGowan and Richards 1989; Lutcavage et al. 1999; Block et al. 2001; Goldstein et al. 2007; Galuardi et al. 2010; Wilson et al. 2011). A summary of the discussed tagging studies which amount to the lion's share of studies using electronic tags is provided in the Appendix.

Over the past three decades researchers have been using the unique properties of a component of the inner ear of fish, or otoliths, to extract information about their movements and life-history patterns (Campana 2005). Analysis of otolith chemistry can help to define life history variations within populations, provide information about the movements and life history traits of single groups of fish, retrace an individual's movements through different environments, assess connectivity and mixing of groups, and determine parentage and natal origins of fish (Elsdon et al. 2008). The chemical signature of

an individual's environment is preserved in the layers of biogenic calcium carbonate (typically aragonite) deposited on the protein matrix that make up otoliths. Unlike bone, they are metabolically inert, meaning that the annual layers of growth are neither reabsorbed nor reworked as the fish ages (Campana and Neilson 1985). Concentrations of several isotopes (carbon, oxygen, calcium, strontium, sulfur and lead) and elements (strontium, barium, manganese, iron and lead) can vary between each layer and can be considered as a natural chemical tag or chemical chronology of an individual's entire life (Campana 2005). By analyzing each of these layers separately, researchers can trace the movements of fish and determine the natal origin of individuals based on the chemical content of the otolith core.

Researchers have been applying these methods to the study of otoliths of BFT, determining that these structures confirm many of the lessons learned from tagging studies (Rooker et al. 2002; Rooker et al. 2003; Rooker et al. 2008a; Rooker et al. 2008B; Rooker et al. 2014; Fraile et al. 2014). An early attempt to use otolith microchemistry as a tool for population assignment of BFTs focused on differences in the concentration of several elements (lithium, magnesium, calcium, manganese, strontium and barium) between various nursery areas in the Mediterranean Sea (Alboran, Ligurian, and Tyrrhenian Sea) and Western Atlantic (New Jersey and Rhode Island; Rooker et al. 2003). Using multivariate analysis, Rooker et al. (2003) found significant differences in the otolith microconstituents of second year BFT juveniles (fork lengths between 66-70 cm) captured in the Western Atlantic (n = 12) and the Ligurian Sea (n = 8). Discriminant analysis, using all six elements, indicated that 71% of these individuals were correctly assigned to the nursery sites that were captured in. When each element was analyzed separately, only lithium showed significant differences between the two areas. A comparison between second year and young-of-the-year (25-42 cm fork length) BFT captured at three nursery areas in the Mediterranean (Alboran, Ligurian and Tyrrhenian Seas) showed differentiation between sites; however, two out of three sites also showed interannual differentiation between age classes. Classification accuracy for all tests ranged between 62% and 85%, suggesting that the technique required additional optimization or both nursery areas contain individuals of mixed origin. Due to the low number of individuals analyzed (between nine and 15 per site) and interannual variation within sampling sites, this study could hardly be considered conclusive. In comparison, an earlier study by some of the same authors investigating the population structure of Pacific bluefin tuna, *T. orientalis*, uncovered much higher levels of distinction

(assignment accuracy of 100%) between major nursery areas in the Pacific Ocean and marginal seas using three elements (Rooker et al. 2001). This difference between resolution of nursery areas for the two species is likely due to the fact that Atlantic BFT have at least two major spawning areas and the Pacific bluefin tuna has one.

A later study analyzing differences in carbon and oxygen isotopes (δ13C or δ18O) and many more samples (38-154 individuals per sampling location) was able to achieve much higher levels of confidence (Rooker et al. 2008a). For example, the vast majority of adults captured in the Mediterranean Sea and GOM showed evidence of natal homing (95.8% and 99.3%, respectively). Smaller fish captured in the Mid-Atlantic Bight area were shown to be of mixed origin (44.3% - 57.4% eastern origin). Large fish captured in the same area and northwards into the Gulf of Maine and Gulf of St. Lawrence contained an increasing proportion of western origin fish (94.8% and 100%, respectively). As such, Rooker et al. (2008a) concluded that trans-Atlantic east-west migrations are performed for the most part by young BFT.

On the whole, the tagging studies described above indicated that giant BFT exiting the Mediterranean after spawning are likely to be heading into the cold waters of the northeast Atlantic, Norwegian Sea and North Sea. Since large BFT are more thermally resistant and better suited for foraging in colder waters, it may eventually be determined that selection of foraging waters is dependent on both water temperature and size/age of BFT.

Similar studies using the same technique and many of the same sampling areas showed variable influence of δ13C concentrations in determining natal origin of BFT from a variety of age classes (Rooker et al. 2008b, 2014; Fraile et al 2014). In one study, δ13C failed to distinguish between individuals caught in the Mediterranean and the Western Atlantic all together (Rooker et al. 2008b). Several studies showed significant inter-annual variability in otolith chemistry at single sample site that was, for the most part, less than the differences found between sites (Rooker et al. 2008b, 2014; Fraile et al 2014). Collectively, these studies provide evidence that the feeding aggregations found in the 1) Mid-Atlantic Bight are composed of young BFT from both sides of the Atlantic (Rooker et al. 2008b), 2) central north Atlantic, on either side of the 45° W management boundary, contain a mix of adults from the east and west (Rooker et al. 2014), and 3) Bay of Biscay are juveniles and adults of eastern origin (Fraile et al. 2014). Moreover, giant BFT found in the waters of New Brunswick, Prince Edward Island and Nova Scotia within the Gulf

of St Lawrence as well as the Atlantic coast of Nova Scotia are almost exclusively of western origin (Schloesser et al. 2010). Combined, this data tells a tale of juveniles with more limited migratory movements, highly migratory adolescents that remain in relatively warm waters and adults that display increased latitudinal movements, entering into colder regions, yet remain closer to spawning areas than the younger immature BFT.

In 2009, the feeding aggregate in the Bay of Biscay contained a small number of individuals (2.7%) of western origin; however, this representation decreased to zero in 2010 and 2011, suggesting that the magnitude of transatlantic migrations differs from year to year, confirming the aforementioned hypothesis (Rodewald 1967). Close to the Strait of Gibraltar, western immigrants have been captured in Moroccan traps but appear to be absent from traps on the north side of the Strait in Spanish and Portuguese waters (Rooker et al. 2014). BFT caught in the waters around the Balearic Islands, Sardinia and Malta all seem to be of eastern origin, while several individuals appearing to be of western origin have been captured near Cyprus (Rooker et al. 2014). According to Rooker et al. (2004), otolith $\delta^{18}O$ values were significantly higher for YOY captured in the eastern nursery areas (Mediterranean and Bay of Biscay) in which the water is both cooler and more saline than the GOM and east coast of the USA. Additional sampling in the northern Aegean, Marmara and Black Sea should be conducted in order to determine if these western-type BFT caught near Cyprus are not a third group with distinct otolith chemistry influenced by the unique physico-chemical characteristics of those water masses where spawning and nursery areas were once suspected in the past.

Finally, PCBs and organochlorine pesticides have also been used to confirm the general conclusions of natal homing and mixing of both stocks in the western Atlantic (Dickhut et al. 2009). Since this study used YOY (23-38 cm FL) captured offshore from the mid-Atlantic Bight as a baseline for western origin fish, they may have sacrificed some assignment power as this region is known to contain a mix of young fish from both spawning areas. Additional calculations are required to compare fish of different age and size using this method as these chemicals accumulate in tissues as BFT feed, grow and age. The authors suggest that young fish captured in the Mediterranean Sea with western-like concentrations of pollutants in their tissues are representative of young BFT that migrate to the western Atlantic within their first year, taking on characteristics of those waters and return to the Mediterranean

years later for spawning. In this way, this methodology can provide information about BFT movements as well as origin.

The development of genetic techniques as a means of determining genetic population structuring can be viewed as a means to overcome the interannual variation endemic to otolith chemistry studies which is subject to environmental variation as well as the prohibitively expensive satellite tagging methods and inability to attach large tags to young fish. The use of molecular techniques to achieve these ends will be discussed at length in Chapters 5 and 6.

1.3 Reproductive biology

When sexually active, BFT are broadcast spawners that aggregate in favourable waters during the spring spawning season (conditions and locations are described in Chapter 2). According to some authors, BFT from the western Atlantic reach maturity after 6-10 years while those in the east mature at 4-6 years (Baglin 1976, 1982; Corriero et al. 2005; Díaz and Turner 2007). Some confusion persists as to the actual definition of maturity; whether it pertains to individuals of a specific size (>110cm), those that are capable of producing gametes (vitellogenic oocytes) or individuals that are actively participating in spawning events and contributing to recruitment (Corriero et al. 2003). Díaz and Turner (2007) estimated the age of maturity for BFT in the western Atlantic using extrapolations from the average length of fish caught by Japanese longliners in the GOM between January and June. Using this method loaded with assumptions (only mature BFT are present in the GOM during these months, all mature BFT from the west enter the GOM for spawning, Japanese longliners catch an unbiased representation of all age classes), they estimated that the age of 50% maturity is around 12 years and concluded that the number of BFT reaching maturity before the age of 9 is negligible. Not only is this estimate scientifically questionable but it is also rather unrealistic to consider that BFT in the west mature a full 5-8 years later than eastern fish. Considering that many juvenile and adolescent BFT of both eastern and western origin spend their first years feeding together in the western Atlantic, it is unlikely that a difference in maturation rates is a result of diet or environmental conditions. This leaves genetic differences as the sole potential cause of life history differences; however, genetic differences between the two populations are simply not great enough to cause such heterogeneity (see Chapter 5).

Gauging maturity based on a gonadosomatic index (GSI), which is the ovary weight as a

percentage of total body weight, offers a much more direct analysis of sexual development. Mature females should have a GSI ranging between 2.0 and 5.3 and a high proportion of yellow-orange ova that are 0.85-1.2 mm in diameter (Baglin 1982). Using both the GSI and histological analysis of ovaries, Baglin (1976, 1982) estimated that western BFT begin to mature between the ages of 5 and 6 years. Using the same methods, Medina et al. (2002) estimated that BFT begin maturing at 3 years of age. Despite reports of regional differences in BFTs, such as higher GSIs in Eastern Atlantic medium sized fish, larger ovaries in giant fish from the west (Baglin 1976, 1982) and slight differences in second dorsal fin length and number of pectoral fin rays (Rivas and Mather (1976), eastern and western BFT are probably more similar than much of the literature suggests. These differences are likely a result of sampling bias more than anything else. In fact, a 2014 study using a novel method for assessing sexual maturity, by characterization of intra-pituitary gonadotopins, determined that the current paradigm of different maturity schedules on either side of the Atlantic is false (Heinisch et al. 2014).

As with many other teleost species, female BFT have higher fecundity (number of mature eggs that can be released within a single spawning season) at larger sizes (Baglin 1976). For example, BFT measuring 205 cm and 156 kg are capable of releasing 13.6 million eggs, while a female 269 cm in length (fork length) and 284 kg can carry as many as 40.6 million eggs (Baglin 1982). Mature eggs measuring around 0.47 mm in diameter (dependent on water conditions like salinity and health of female) begin to develop when mature BFT enter into spawning areas where sea surface temperatures exceed 20.5°C (Richards 1976; Medina et al. 2002; Alemany et al. 2010). The ambient sea surface temperature in the Gulf of Mexico during the spawning season is normally >25°C, much higher than large BFT are accustomed to in the Atlantic Ocean. As a result, the mean body temperature of BFT in the Gulf of Mexico during the spawning period (26.7 ± 1.6°C) is roughly 2°C higher than when the fish are feeding in the Atlantic Ocean (24.7 ± 2.3°C; Teo et al. 2007). Similar temperatures (22.5 - 24.9°C) are reached in the Levantine Sea during spawning events (Karakulak et al. 2004). Tagging of mature adults entering the Gulf of Mexico during the spawning period has revealed a unique spawning behaviour described as shallow oscillatory diving during the night (Block et al. 2001; Teo et al. 2007). This behaviour may be an attempt at thermoregulation, wherein diving into cold deep water (203 ± 76 m) provides the necessary cool down from surface waters, while frequently returning to the surface maintains their presence in the spawning

aggregation for as long as physiologically possible. Alternatively, rapid and repeated movements through depths of varying temperatures while maintaining a higher than average body temperature may have a role in stimulating the release of gametes. Given the correct cues, spawning of BFT appears automatic and has even been observed in fattening cages in the Mediterranean (Gordoa et al. 2009). Held captive in warm surface waters and prevented from diving into deeper waters, these captive BFT are likely spawning in response to increased body temperatures. This temperature-dependent impulse to release gametes may account for the occurrence of eggs and larvae outside of known spawning areas (Baglin 1976; Richards 1976; McGowan and Richards 1989). Given higher than average sea temperatures, BFT may be stimulated to release gametes in less than favourable bodies of water in the absence of conspecifics. Immediately following repeated spawning behaviour, which persists for 2-3 weeks, BFT swim to deep waters, as if in an effort to cool down as quickly as possible (Block et al. 2001; Teo et al. 2007). Given these observations, rising sea temperatures resulting from climate change will almost certainly have an impact on the spawning behaviour and location of BFT.

There are seven oocyte developmental stages in the ovaries of BFT: perinucleolar, lipid, early vitellogenic, late vitellogenic, migratory nucleus, pre-hydrated and hydrated (Corriero et al. 2003). Within spawning areas, several of these developmental stages can be found among the fish captured there, indicating that BFT are asynchronous multiple spawners (Medina et al. 2002). In the Gulf of Mexico, BFT spawn during April, May and June with a peak occurring in late May and early June, while in the Mediterranean it occurs during May, June and July with a peak in the eastern Mediterranean in late May to early June followed by a peak in the western Mediterranean in late June to early July (Baglin 1976, 1982; Richards 1976; Karakulak et al. 2004; Heinisch et al. 2008; Knapp et al. 2014).

Once fertilized, eggs passively float in the water currents and hatch after approximately 30 hours (De Metrio et al. 2010). The waters that larvae are born into are usually oligotrophic and after exhausting their yolk sac they begin feeding on small plankton after 3 days. By day 25 post-hatching, BFT larvae become aggressive predators, consuming large amounts of pelagic larvae of other fish species as well as conspecific eggs and larvae (De Metrio et al. 2010). By mid-November, young BFT can grow to be about 40cm in length, at which point they begin to aggregate, school and move *en masse* to nursery areas (Mather et al. 1995). After one year, BFT grow to be more than 50 cm in length, by their third year they'll

have grown to approximately 1 m in length and by eight years they weigh over 100 kg (Restrepo et al. 2010). Given the opportunity, BFT can live over 30 years of ago and can reach over 3 m in length (Mather et al. 1995; Restrepo et al. 2010).

1.4 Fisheries

Exploitation of BFT stocks by pre-industrial civilizations certainly had an impact on our collective development; influencing trade routes, organization of labour, politics and the way we regard ownership of the sea and coastal lands (see Chapter 6 for further details). It wasn't until after the 1950s that the pendulum swung the other way and humans started to have an impact on populations of bluefin tuna. Following World War II, rich fishery grounds reopened and the American industrial complex was at a state of historical capacity. Tuna canneries were the third largest employers in San Francisco and new tuna super trawlers were opening up new areas of exploitation. Devastated by the ravages of war, Japan refocused on the sea as source of protein for their impoverished masses and the Pacific bluefin tuna was to play a major role. Following market scares associated with bluefin tunas tainted by both mercury and radiation (a result of the USA's testing of the H-bomb at Bikini Atoll), Japan's fishing fleets expanded beyond the Pacific Ocean and began capturing bluefin tunas in the Atlantic in the 1960s. The expansion of the fleet and popularity for fatty tuna meat was the result of the introduction of mechanical refrigeration that was then available in homes, restaurants and factory trawlers. In Richard Ellis' *Tuna: A love story* (2008), he credits the moment that the BFT market exploded to the arrival of two Japanese purse seiners off eastern Canada and New England in 1971-1972. The Japanese were willing to pay fishermen amounts of money for whole bluefin that were, until that time, unheard of. Witnessing the profits to be made, European countries started to abandon the traditional, seasonal and fixed method of fishing by tuna traps and began modernizing the bluefin fleet in order to exploit offshore stocks year round. Armed with spotter planes, gillnets, longlines and purse seiners, a reckless assault on the Mediterranean commenced. In the north eastern Atlantic, the Norwegian purse seining fleet expanded rapidly and yielded profits for little more than a decade (Miyake et al. 2004). Under pressure from both legitimate and clandestine fisheries, historical stocks of ABFT vanished from the North Sea (1960s), coastal waters of Brazil (1970s) and the Black Sea (1983) (Mather et al. 1995; Miyake et al. 2004; Fromentin and Powers 2005). In the west, a similar pattern of dramatic increases in both fishing capacity and catches was followed by a crash in yields

some two decades after the birth of the fishery. The demand for BFT in the sashimi market of Japan has greatly increased its value and as a result the rate of exploitation (Ellis 2008; Fonteneau 2009; Fromentin 2009; Longo 2011). This unyielding appetite for bluefin tunas and the profits to be had from their exploitation has led Dr. Barbara Block, one of the world's leading experts in the species, to famously crown the species as the “cocaine of the sea”. In 2001, Carl Safina wrote, “On the world markets the bluefins (and to some extent bigeye tuna) are “boutique species” commanding excessive prices among a few people with excessive appetites for luxury and status-enhancing displays of wealth. For all the difficulty with management politics, in no sense can these fisheries be considered as producers of Food for the Masses.”

1.5 Conservation and management challenges

1.5.1 The International Commission for the Conservation of Atlantic Tuna

In 1965, the Food and Agriculture Organization of the United Nations called for a conference of plenipotentiaries to prepare and adopt a convention for the purpose of establishing a commission for the conservation of tuna and tuna-like fishes in the Atlantic Ocean. At that meeting, which took place in Rio de Janeiro during May of 1966, the International Commission for the Conservation of Atlantic Tuna (ICCAT) was established by representatives from 17 countries. The commission was ratified in 1966 and charged with the responsibility of managing thirty species of large pelagic fishes, including all tunas, swordfish, billfish, mackerels, and the shortfin mako and blue shark in the Atlantic Ocean, Gulf of Mexico and Mediterranean. ICCAT now consists of 50 contracting parties (CPs), of which the EU is a single party. BFT are managed as two stocks separated by the 45ºW meridian: a western stock which consists of individuals supposedly spawning in the Gulf of Mexico and foraging in the north western Atlantic and an eastern stock with spawning areas in the Mediterranean Sea and foraging waters in the Mediterranean and north eastern Atlantic (Block et al. 2005; ICCAT 2009). If population structuring does occur within the Mediterranean Sea, as some experts have suggested (Carlsson et al. 2004, 2007; Fromentin 2009; Riccioni et al. 2010, 2013), then replenishment of exhausted sub-populations will not follow a scenario consistent with the existing stock assessment model.

ICCAT's scientific committee, the Standing Committee on Research and Statistics (SCRS), is made up of scientists from various countries who compile catch data, produce statistical reports, model

population trends and offer advice for management policies. The Commission's managers are then expected to use this scientific advice to draft fishing management policies which take the form of recommendations to the member nations. Recommendations are passed by a simple majority vote consisting of a quorum of 2/3 of the fifty Cps, who each have a single vote. Each CP is responsible for implementing, monitoring and enforcing recommendations.

In 1981, ICCAT adopted strict catch quota regulations in the Western Atlantic where the fleet consists for the most part of vessels from Japan, the USA and Canada. Quota regulations did not appear in the eastern Atlantic until 1995. Due to the economic interests at stake and the overcapacity of the European fleet it is unsurprising that misreporting and fraud are causing management efforts to fail. In 2006, ICCAT introduced a multi-annual recovery plan which has contributed to a decreased overall harvest of both adult and juvenile fish (ICCAT 2006b). Between 2007 and 2008, the EU implemented a short term (6 month) emergency conservation regulation in response to new evidence of damaging fishing practices by six EU countries. Stocks are finally showing signs of improvement and WWF has declared the BFT's recovery a resounding success (García et al. 2013; ICCAT 2015b). Although, the rate and nature of this recovery is still very much uncertain, as the SCRS admits that the quality of data currently provided by fishery dependent sources are inadequate to formulate an accurate stock assessment (ICCAT 2013; Fromentin et al. 2014). The SCRS is advocating caution until the speed and magnitude of the recovery can be confirmed (ICCAT 2015). As such the SCRS was unable to agree upon an upper limit for the Total Allowable Catches (TACs) that would not jeopardize the recovery of the stock in their 2015 report to the commission. The SCRS did however, suggest a gradual increase in the TACs to a level equal to the most precautionary maximum sustainable yield (MSY) but were unable to agree upon a time frame and rate of increase. In response, the commission has decided to increase the quota by 20% per year over the next 3 years, starting in 2015. In essence, this means a 71.5% increase in the eastern Atlantic and Mediterranean quota between 2015 and 2017 (Table 1).

1.5.2 Poor implementation of management plans

Despite the presence of ICCAT, BFT stocks continued to plummet throughout the 1980s and 1990s. Carl Safina (2001) wrote, “The bluefin tuna is emblematic for failure to find the political resolve to act toward recovery of severely depleted fish despite having generally reliable scientific information.”

Safina is referring to the fact that between 1970 and 1989, ICCAT and its member parties oversaw the disappearance of 90% of BFT giants (>8 years) and 50% of medium adults (6-7 years) in the western stock (ICCAT 1991). Although the commission was advised by the SCRS at the time to reduce catches to as close to zero as possible, they continued to allow an annual quota of 1160-2660 tonnes for scientific monitoring during the 1980s, while catches of adult BFT continued to plummet (Fig. 3; Safina 1993).

Table 1: Science-based TAC recommendations provided by ICCAT SCRS, catch quotas set by ICCAT, yields reported by contracting parties, and SCRS estimates of actual yield in metric tonnes by year (ICCAT 1995-2015).

Year	Science-based TAC recommended (t)	Quota set by ICCAT (t)	Reported yield (t)	SCRS estimate (t)
1996	N/A	N/A	46,033	>50,000
1997	N/A	N/A	46,983	>50,000
1998	20,000	N/A	44,700	>50,000
1999	<25,000	32,000	32,454	>50,000
2000	<25,000	29,500	33,754	>50,000
2001	<25,000	32,143	34,557	>50,000
2003	<26,000	32,000	28,205	>50,000
2004	<26,000	32,000	32,567	>50,000
2005	<26,000	32,000	35,845	>50,000
2006	N/A	32,000	30,647	>50,000
2007	15,000	29,500	32,398	61,000
2008	15,000	28,500	23,849	25,760-34,120
2009	8,500-15,000	19,950	19,751	18,308
2010	13,500	13,500	11,328	N/A
2011	12,900-13,500	12,900	9,779	N/A
2012	12,900-13,500	12,900	11,474	N/A
2013	12,900-13,500	13,500	13,133	N/A
2014	12,900-13,500	13,500	N/A	N/A
2015	*23,256 in steps over 2-3 years	16,142	N/A	N/A
2016	*see above	19,296	N/A	N/A
2017	*see above	23,155	N/A	N/A

*SCRS scientists were not able to reach a consensus on the number of steps to complete the rebuilding plan, or on the management strategies.

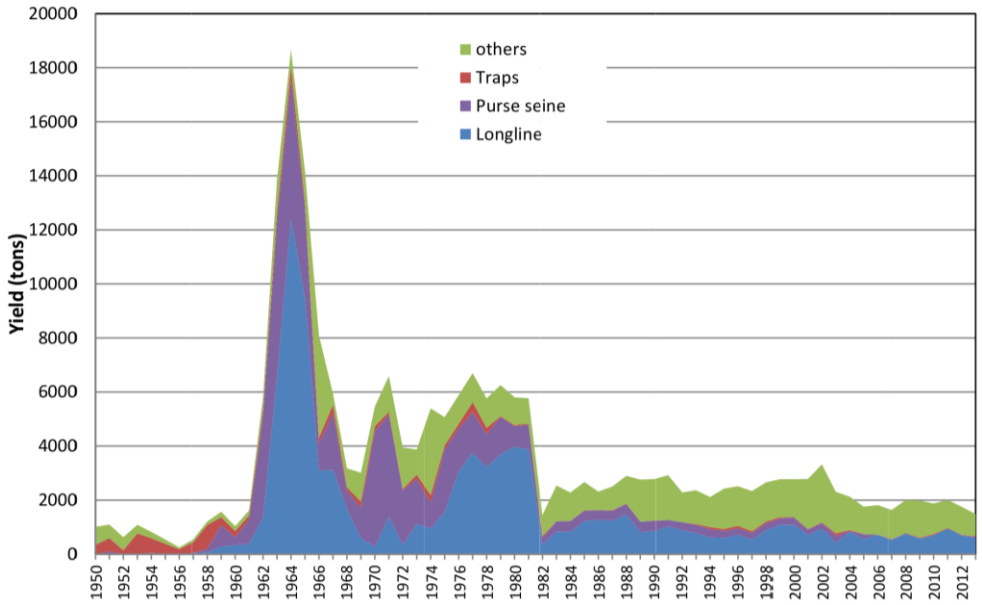

Fig. 3: Reported catch of BFT in the Western Atlantic from 1950 to 2013 by fishing gear (ICCAT 2015).

In the eastern Atlantic and Mediterranean, the Commission set catch quotas well above the levels recommended by the SCRS every year between 1999 and 2009 (Table 1). The catches that were reported to the commission by the contracting parties during that period were consistently higher than quotas, except for 2003 and 2008, which revealed ICCATs lack of enforcement capacity. The SCRS estimated that the actual catches, including IUU fishing, were consistently 2 to 3 times higher than the advisable limit. The most exaggerated difference between scientifically advised limits (15,000 t) and actual yields (~61,000 t) was in 2007, one year after ICCAT published their Recovery Plan (Fig. 4). An independent review funded by WWF revealed that EU vessels from France, Spain and Italy (France in particular), in collusion with Libya, were largely responsible for the IUU fishing during the early 2000s that led to such high catches above and beyond the quotas (WWF 2006). The years of frustrated negotiations and political intrigue that paved the way towards reform are detailed in Safina (1993). Many of the negotiations were held behind closed doors and involved the influence of both industry and conservation lobbyists. Advice from the SCRS certainly showed the way forward and many of the contracting parties were in favour of reform; however, several countries consistently sabotaged the process and placed the species on a path towards collapse. Some conservationists blamed ICCAT as a whole and crowned the commission with the moniker of the International Conspiracy to Catch All Tuna. The way in which many of the member states of ICCAT have failed to uphold their responsibilities, while others have actively withheld data and done their best to sabotage the conservation process is widely regarded as an “international disgrace”.

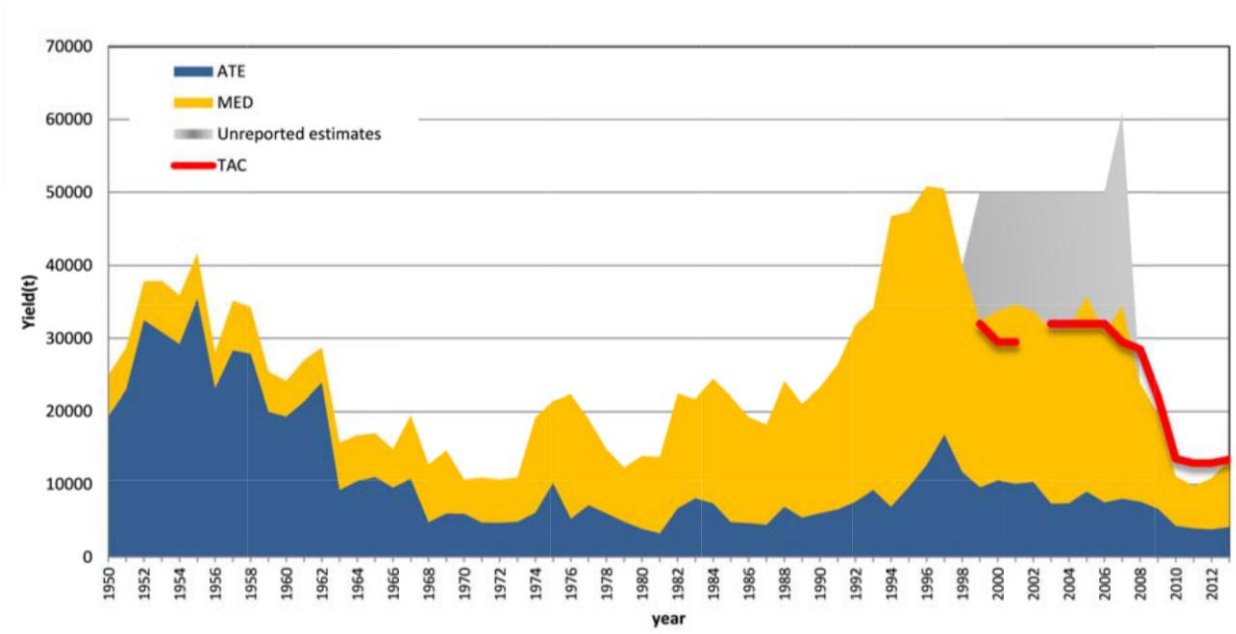

Fig. 4: Reported catch of BFT in the Eastern Atlantic (ATE) and Mediterranean (MED) from 1950 to 2013 together with an estimate of the unreported catch (calculated by the SCRS using fishing capacity and mean catch rates over the previous decade; ICCAT 2015).

An independent performance review commissioned by ICCAT in 2008 determined that BFT attracts 85% of the media coverage and criticism from the international community, despite the fact the species accounts for only 7% of the overall tuna caught within the concerned management area (Hurry et al. 2008). The authors remarked that the challenges faced by ICCAT are shared by all regional fisheries management organizations (RFMOs); however, ICCAT's exceptionally high membership consisting of 50 contracting parties (over twice as many as all other RFMOs, aside from the WCPFC which has 26) exacerbate the issues. When too many politicians gather around a table there is a tendency for them to focus more on one another and the process of negotiating for higher allocations than on the science leading towards sustainability (O'Leary et al. 2011). The authors concluded that the failure of ICCAT to reach its objectives is in a large part due to the lack of compliance from many of the CPs and that many of the problems plaguing the commission could be fixed if the CPs fulfilled their responsibilities and adhered to the recommendations provided by the commission. According to the report, “ICCAT has developed reasonably sound conservation and fisheries management practices, which, if fully implemented and complied with by Contracting Parties, Cooperating non-Contracting Parties, Entities and Fishing Entities (CPCs), would have been expected to be effective in managing the fisheries under ICCAT’s purview.” Moreover, the SCRS appears capable of providing sound advice to the commission, despite the significant challenge presented when CPs routinely fail to deliver data, which is often inaccurate, on schedule. This

review was performed in 2008 and many improvements have been made since. Quotas have been drastically reduced since then, IUU fishing has been addressed and the stock is finally showing signs of improvement (García et al. 2013; ICCAT 2015b).

The BFT fishery isn't unique in regard to inflation of quotas by CP representatives and fisheries ministers in particular. A 2011 study estimated that between 1987 and 2011 politicians chose to increase scientifically recommended TACs for 68% of eleven commercial fish species across nine management zones (O'Leary et al. 2011). Politicians set TACs lower than scientifically advised in only 14% of cases reviewed. On average, politicians set TACs 33% higher than scientifically advised and in one case in the Bay of Bay Biscay management area politicians set TACs 1100% higher than advised for hake. There wasn't a single case in the 14-year period in which a moratorium advised by scientists was enacted into law. The authors concluded that "productive and sustainable fisheries will not be achieved if Fisheries Ministers' cavalier disregard for scientific advice continues".

1.5.3 Illegal, Unreported and Unregulated fisheries and Flags of Convenience

As previously mentioned, ICCAT has a membership of 50 contracting parties, including several countries that do not border on the Atlantic Ocean but obviously have invested interests in the fish that live there (The Republic of South Korea, China, Philippines, Japan and Vanuatu). All contracting parties have a single vote in the commission, including the EU which is considered as one single contracting party. One interesting and concerning case is that of Vanuatu, a small Pacific island nation of 228,000 residents which also has a vote on the commission. According to the list of vessels longer than 20m in length that are registered with ICCAT and authorized to operate in the convention area, there are 20 vessels that fly the flag of Vanuatu. Upon closer inspection, 14 of these vessels are operated by Japanese corporations. In fact only one long lining vessel is owned and operated by a company from Vanuatu. Other countries operating vessels with the flag of Vanuatu are Taiwan (3), China (1) and Namibia (1). The actual owners of these vessels have addresses in Panama (8), Singapore (3), Seychelles (1), Liberia (2), Cook Islands (1), Taiwan (3) and China (1). Moreover, sixteen of these 20 vessels are carriers with authorization to receive transhipments from vessels registered in Japan, Taiwan, South Korea, China, Belize and the Philippines. The same applies for the 18 vessels flying the flag of Belize of which only two are owned and operated by companies based in Belize. Although none of the Belizean vessels are owned

or operated by Japan, three carrier vessels are owned and operated by Chinese corporations and the rest are under the yoke of companies in the USA, Estonia, Greece, Spain, Ghana, South Africa, Panama, Germany and the Ivory Coast. Seventeen of the 25 vessels flying the flag of Panama are named “Chung Kuo No. X” and are owned and operated by companies based in Panama, all of whom have headquarters in Taiwan. Registering a vessel in a sovereign state different from the ship's owners in an effort to avoid regulations and give the appearance of capacity reduction is a practice known as purchasing Flags of Convenience (FOC). If the state issuing the FOC decides to remove a vessel from their registry and revoke the privileges of their flag, vessels can obtain a new FOC from another state within 24 hours (Gianni 2004). In fact many vessels change both the name of their vessel and FOC on a regular basis (known as “flag-hopping”) in order to avoid traceability.

Japan and Taiwan have long been a challenge in the management of all species of bluefin tuna, in Pacific, Atlantic, Indian and Southern Oceans. Mengerink et al. (2010) explains the politics, aggressions and tribulations shared by these two tuna nations in entertaining detail. During the early 2000s, Japan and Taiwan were both encouraged to reduce their fishing capacity and instead of doing so, they simply decorated their vessels with FOCs and moved their fleet abroad. When pressured further, Japan went on the offensive and attacked Taiwan's overcapacity and overuse of FOCs. By 2004, 50 out of 51 of Taiwan's long-lining tuna vessels with freezer capacity were flying FOCs from countries like Vanuatu, Panama, Belize, Cambodia, Bolivia and Georgia. Between 2000 and 2004, Japan publicly circulated reports condemning Taiwan for Illegal, Unreported and Unregulated (IUU) fishing practices at several meetings of the Western and Central Pacific Fisheries Commission (WCPFC). Several years after Japan's campaign to expose Taiwan's illegal operations, it surfaced that Japan was hunting southern bluefin tuna at a rate 3 times higher than their quota allowed and were actively concealing their catch data from inspectors at the same time. The managing director of the Australian Fisheries Management Authority, Richard McLoughlin had this to say about this revelation in 2006, "Essentially the Japanese have stolen $2 billion worth of fish from the international community [by dint of their own under-reported fishing and their import of bluefin from IUU vessels], and have been sitting in meetings for fifteen years saying they are as pure as the driven snow” (Scheiber et al. 2007).

IUU fishing activities are also occurring within the Atlantic, in recent years and by vessels that

have permits to operate there, issued to them by ICCAT. Cross referencing of all vessels contained in the ICCAT Record of Vessels with the Greenpeace International Blacklist, a database of fishing vessels and companies engaged in Illegal, Unregulated and Unreported (IUU) fishing, produces a list of 8 vessels with questionable pasts. One of these is a Taiwanese owned freezer cargo ship (Lung Yuin) using a flag from Vanuatu which was inspected in Japan in 2004. The Japanese authorities discovered that the Lung Yuin had received trans-shipments of frozen bigeye tuna 25 Taiwanese vessels and three Vanuatu flagged fishing boats owned by Taiwanese companies which all gave false information about where the fish had been caught. The Lung Yuin also had two log books, one of which was true and the other false. Among the 25 Taiwanese longliners that provided false information to the authorities are the Yuh Yeou 236 and Shin Yeou 6, both of which are on the list of vessels currently operating with ICCAT permits in the Atlantic. Also on the list is a Spanish vessel (Albacora Uno) accused of shark finning in the Marshall Islands and illegally discarding a ton of skipjack in 2012, an Italian longliner (Biagio Anna) accused of illegally using driftnets in the Tyrrhenian and Ionian Seas in 2006, a trawler from the Russian federation (Kapitan Bogomolev) illegally fishing in the waters of Senegal-Guinea Bissau in 2013, a south Korean longliner (Oryong 316) accused of not reporting its catches during 2006 and a purse seiner owned by a French company and operating out of the Ivory Coast (Solevant) illegally fishing in Liberia and forgery of a fishing license in 2012. The fraudulent commercial practice of purchasing FOCs for vessels and registering shell companies abroad with the full intent of importing all yields to a corporation's sovereign state is known as fish, seafood or food laundering.

1.5.4 Sea ranching

Much of the BFT fishery is now focused on sea ranching, a fishing practice by which schools of bluefin tunas are encircled by large purse-like nets and towed to cages to be fattened for several months to years before slaughter. Sea ranching operations can now be found along the coasts of nearly all countries in the Mediterranean and the Atlantic coasts of Portugal and Morocco. This practice has greatly confused stock assessments that rely on accurate estimates of length, weight and age at capture. Thousands of fish are moved from purse seine nets to towing or transport cages and then into fattening cages and catches are inevitably mixed, combined and confused. The increased popularity of purse seining and greatly reduced number of BFT has heralded the decline of the traditional trap fisheries in the Mediterranean, which had

maintained a consistent 500 year record of standardized fishing effort. Since tuna ranching activities involve live, fast swimming BFT underwater, it is very difficult to estimate the biomass of fish removed from the wild and placed into captivity each year. From 1997 to 2004, while reported purse seine catches were being reduced, exports of BFT from Mediterranean ranches to Japan increased rapidly until exports exceeded catches (Miyake 2005). While a portion of this unaccounted for difference between export and catch can be credited to the fattening of fish, it's also suggestive of IUU fish being laundered through the growing number of farms (De Stefano and Van der Heijden 2007). It's been estimated that between 2005-2011, approximately 60% of the eastern BFT catch ended up in tuna fattening farms (Ortiz et al. 2014). As such, ICCAT has now requested that a percentage of every transfer from purse seine nets to towing cages is sacrificed in order to estimate sizes and weights of the fish captured via extrapolation from a random sampling (ICCAT 2010b, 2012b). This of course, would require an estimate of the total number of fish contained in the nets. Developing accurate stock assessments in the past were difficult enough but now the SCRS is forced to make estimates from random samples of random samples [repetition intended] and the complexity of the issues have multiplied. The task has certainly become more challenging for observers who are incapable of counting fish in the water, unless they join them. WWF has expressed concern for the ease with which the hunters, ranchers and farm stewards will be able to launder IUU fish through the burgeoning tuna farm industry (ICCAT 2015b). For several years, ICCAT and members of the SCRS have been working on developing a underwater camera monitoring system capable of counting fish and estimating their biomass as they pass through the doors connecting cages. Unfortunately a few technical issues (imaging system has problems with multiple fish entering the cage at once, accuracy of estimates reduces with non-perpendicular orientation of fish in front of camera, reduced water visibility influences accuracy of estimates) have slowed the introduction of this technology. Ranching has also provided new opportunities fishermen to profit from the capture of smaller BFT because they will eventually be fattened to an acceptable market size within the cages (De Stefano and Van der Heijden 2007). Fromentin and Ravier (2005) suggested that if the fishery avoided the capture of juveniles (>35kg) than the fishery could sustain higher levels of exploitation and the stock could be more productive.

Not only does tuna ranching threaten the welfare of BFT stocks but it's also an incredibly inefficient food production practice from an ecological perspective. Some 20 kg of bait fish are required to

produce 1 kg of BFT, which is the highest food conversion ratio (FCR) of any farmed fish in the world (Volpe 2005; Longo 2011). Since the rapid expansion of the ranching industry, bait fish stocks have also been placed under additional pressure and EU ranches are now forced to import bait fish to feed their captive BFT. Europe is injecting millions into subsidies for an industry that is literally converting their wild stocks of herring, mackerel, sardines, anchovy and squid into sashimi to feed the Japanese.

1.5.5 Subsidies

The rapid growth of the tuna ranching industry (vessels and fattening cages) has been well-subsidized by European taxpayers at both the federal and EU level as well as investment companies and multi-national super corporations from around the world; Japan in particular (WWF 2004; De Stefano and Van der Heijden 2007; Longo 2011). The hypocrisy of EU subsidies, which are intended to reduce fishing capacity, made the headlines in 2009 when a European Commissioner admitted that €33.5 million had been spent on new purse seiners and modernization of existing vessels between 2000 and 2008 (Pope 2009). The industry has also been effective in taking advantage of a legal loophole allowing it to garner a windfall in subsidies intended for the development of aquaculture in the EU. In essence, tuna ranching is a capture fishery masquerading as aquaculture. WWF estimates that between 2000 and 2003, the tuna ranching industry has obtained in excess of €19-20 million in public funds for operations in the Mediterranean Sea (WWF 2004). Savvy operators can acquire as much as 75% of initial investment costs from national and regional government bodies for new ranching operations WWF 2004). These financial transfers from the public to the private sector have become a serious threat to sustainable BFT fisheries because they distort investment decisions for modernization of fishing fleets and the expansion of ranching operations (Sumaila and Huang 2012).

1.5.6 Selective fishing practices

Each fishing gear used to capture BFT is subject to both time and area restrictions. For example, in 2015 large-scale pelagic longline vessels over 24 m are permitted to operate in the eastern Atlantic and Mediterranean from 1 January to 31 May with the exception of the area delimited by West of 10°W and North of 42°N, as well as in the Norwegian Economic Zone, where fishing is permitted from 1 August to 31 January. Purse seining has a much more restricted season which runs from 26 May to 24 June in the eastern Atlantic and Mediterranean and from 25 June to 31 October in the Norwegian Economic Zone. While longlining targets various age classes (as well as numerous species of bycatch,

including turtles, sharks and seabirds) over a five month period there are concerns that purse seining, which takes the lion's share of the annual quota (Fig. 5), targets a more restricted range of age classes. Historical accounts and observations by modern fisheries scientists suggest that age classes don't mix completely and that the older larger BFT enter the spawning areas and are followed later by the smaller less fecund age classes (Badham 1854). If this is the case, then the quota given to the Spanish purse seiners operating in the Mediterranean, which was filled in 23 hours in 2014, is likely to consist of a limited variety of fish as they pass through the narrow Strait of Gibraltar. Furthermore, this restricted age group is targeted by both purse seiners and longliners during the week of 26 May to 31 May when both gear types are in use in the same area. This targeted selection of a restricted age class can have negative impacts on the demographics of the species, reduce recruitment rates because of the selective removal of large more fecund individuals and confuse stock assessments that rely on accurate age composition estimates.

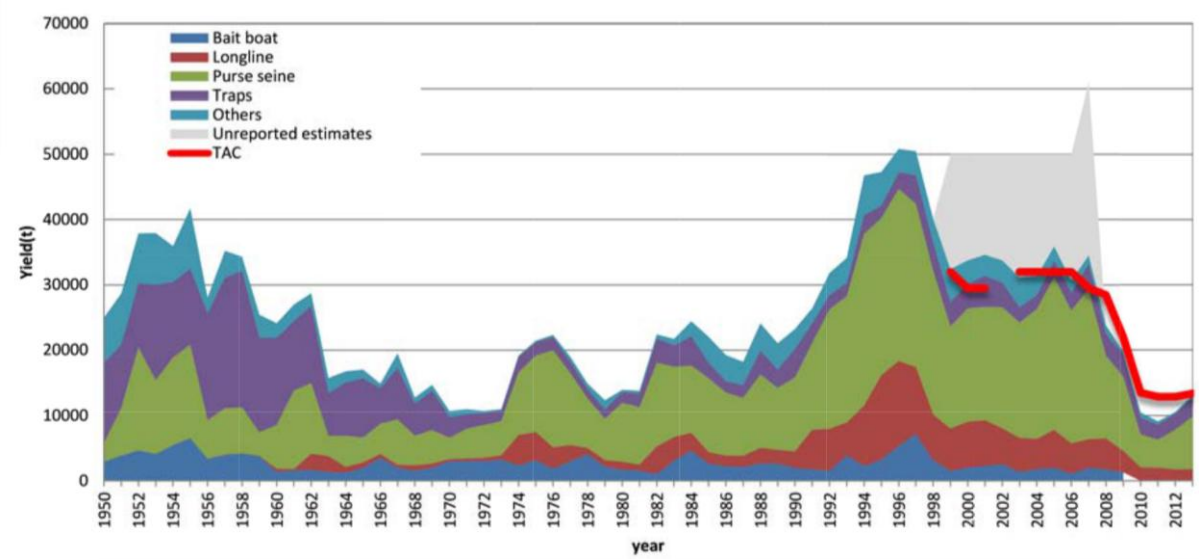

Fig. 5: Reported catch of BFT in the Eastern Atlantic and Mediterranean from 1950 to 2013 by gear type together with an estimate of the unreported catch (ICCAT 2015).

1.5.7 The Atlantic-wide research programme for bluefin tuna (GBYP)

In 2008 the SCRS proposed to the Commission that a programme be developed in which research into data mining, aerial surveys, tagging design studies and programme coordination is undertaken. Several Contracting Parties agreed to the value of such an endeavour and expressed an interest in providing additional funds for its initiation. A Steering Committee containing the SCRS Chair, the ICCAT Executive Secretary or his/her Assistant, bluefin tuna rapporteurs, and an outside expert in tuna research

was appointed soon thereafter. The adopted name for the programme was the GBYP, or Grande Bluefin Tuna Year Programme, which has since become known as "The Atlantic-wide research programme for Atlantic tuna". The programme was initiated during March 2010 with the following three priorities:

1. Improve basic data collection through data mining (including information from traps, observers, and VMS), developing methods to estimate sizes of fish caged, elaborating accurate CPUE indices for Mediterranean purse seine fleets, development of fisheries-independent information surveys and implementing a large scale scientific conventional tagging programme;

2. Improve understanding of key biological and ecological processes through electronic tagging experiments to determine habitat and migration routes, broad scale biological sampling of live fish and dead fish landed (e.g. gonads, liver, otoliths, spines, etc.), histological analyses to determine bluefin tuna reproductive state, biological and genetics analyses to investigate mixing and population structure; and ecological processes, including predator-prey relationships;

3. Improve assessment models and provision of scientific advice on stock status through improved modelling of key biological processes (including growth and stock-recruitment), further developing stock assessment models including mixing among areas, and developing and use of biologically realistic operating models for more rigorous management option testing.

In order to assist the GBYP participants, ICCAT recommended that the Contracting Parties, Cooperating non-Contracting Parties, Entities or Fishing Entities should provide maximum assistance for the procurement of permits to operate in relevant maritime areas and the airspace over these areas. The GBYP was granted a 20 t research mortality allowance and exempted from size limits, fishing gear restrictions and seasonal closures (ICCAT 2011b). During the first year of operations, or Phase 1, efforts were focused on aerial surveys, data mining and tagging. In its second year, the GBYP expanded to include the "Biological and Genetic sampling and analyses" consortium, which is composed of 13 entities and 7 sub-contracting parties from 13 countries and specializes in research focused on otolith microchemistry and shape, genetics and growth calibration. Between 2012 and 2014, the consortium sampled 8,842 BFT, acquiring 4,165 otoliths, 626 gonads, 6,107 muscle and fin tissue samples for research purposes.

Overall, the scientific and economic conditions have been suitable for an ambitious re-analysis of the population structure of BFT. The conservation demand, willingness of the industry to embrace new scientific endeavours and political support for research all made it possible for the most comprehensive genetic survey of BFT populations to date. The following chapters detail three years of research in pursuit of novel insights into the population structuring and spatial dynamics of BFT in both space and time.

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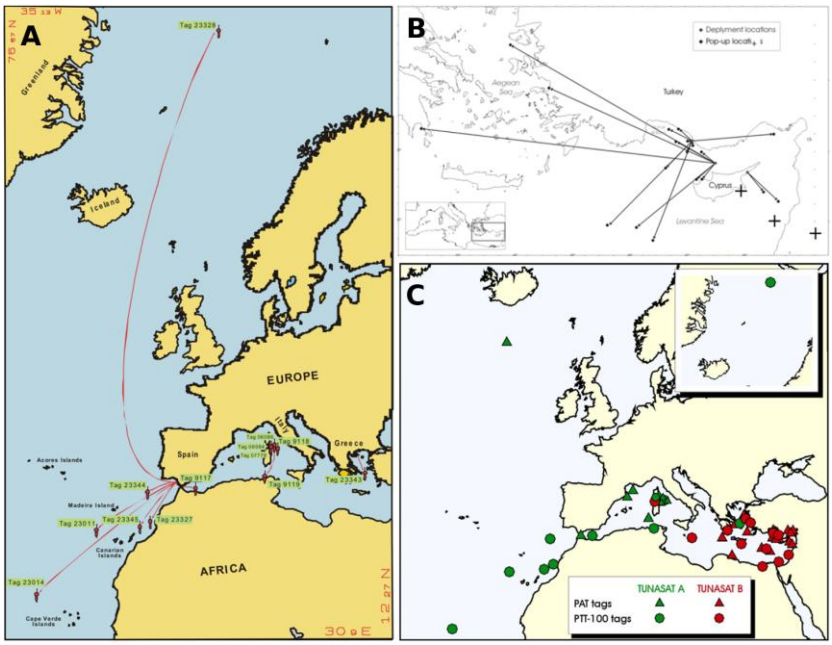
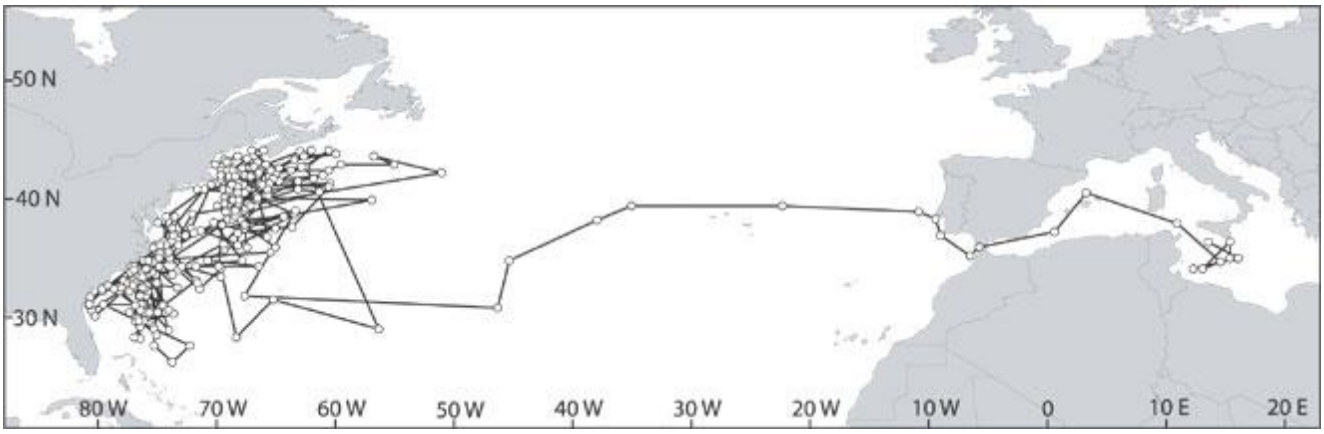
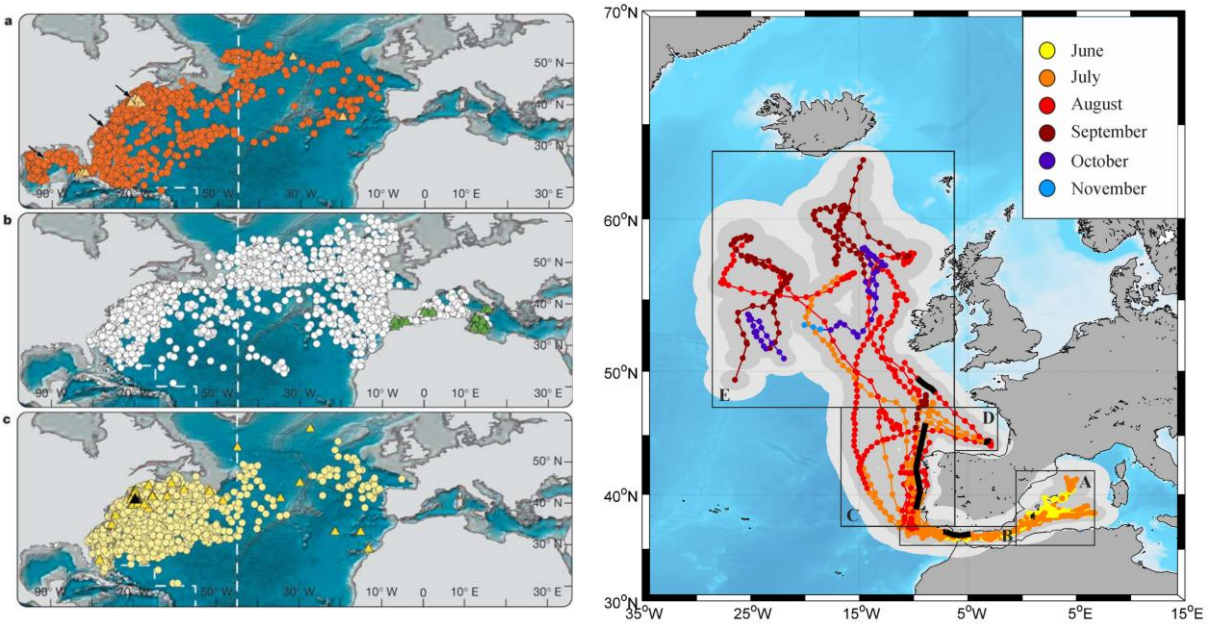
Appendices



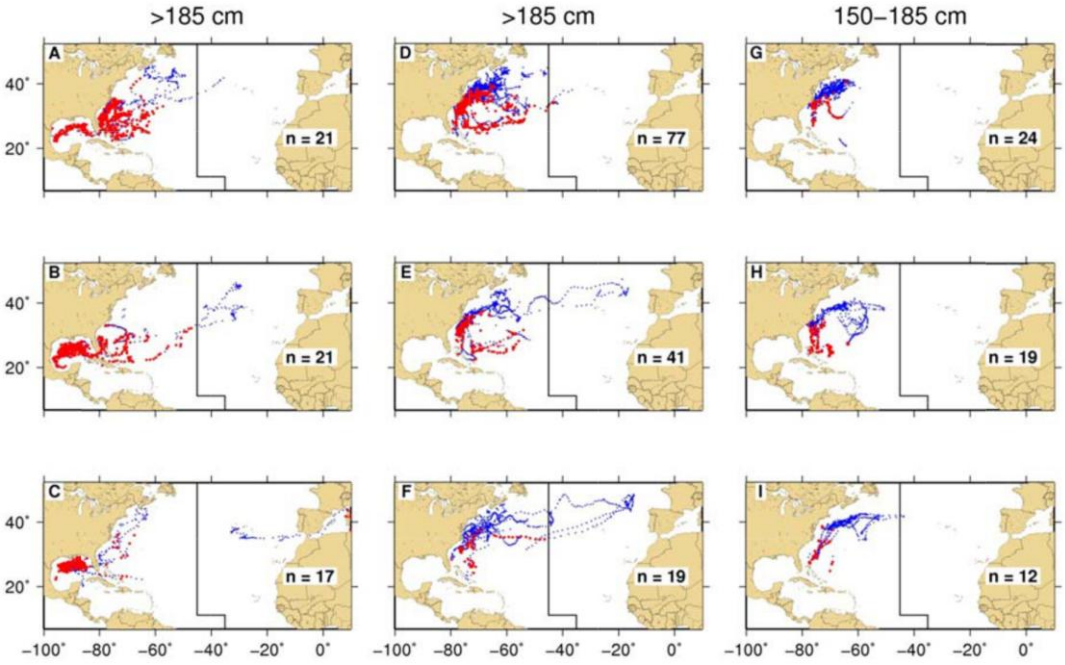
Appendix 1. PSAT trajectories of A) 13 BFT tagged in Barbate (Spain, n=7), Sardinia (Italy, n=2), Corsica (France, n=4) and Chalkidiki (Greece, n=1) between 1998 and 1999 during April-November. Days at liberty range between 5 and 246 (from De Metrio et al. 2001) and **B)** 19 BFT tagged in northern Cyprus and the Bay of Antalya in the Levantine Sea. Deployment (+) and pop-up (•) locations of the satellite tags released 3-67 days at liberty (from De Metrio et al. 2004). C) PSAT pop-up locations of 52 BFT tagged in Cyprus, the Levantine Sea, the Aegean, Corsica and Sardinia after 5-300 days. The report does not specify date of tagging nor does it provide trajectories between tagging and pop-up locations (from De Metrio et al. 2005). All images have been modified using GIMP 2.8.14.



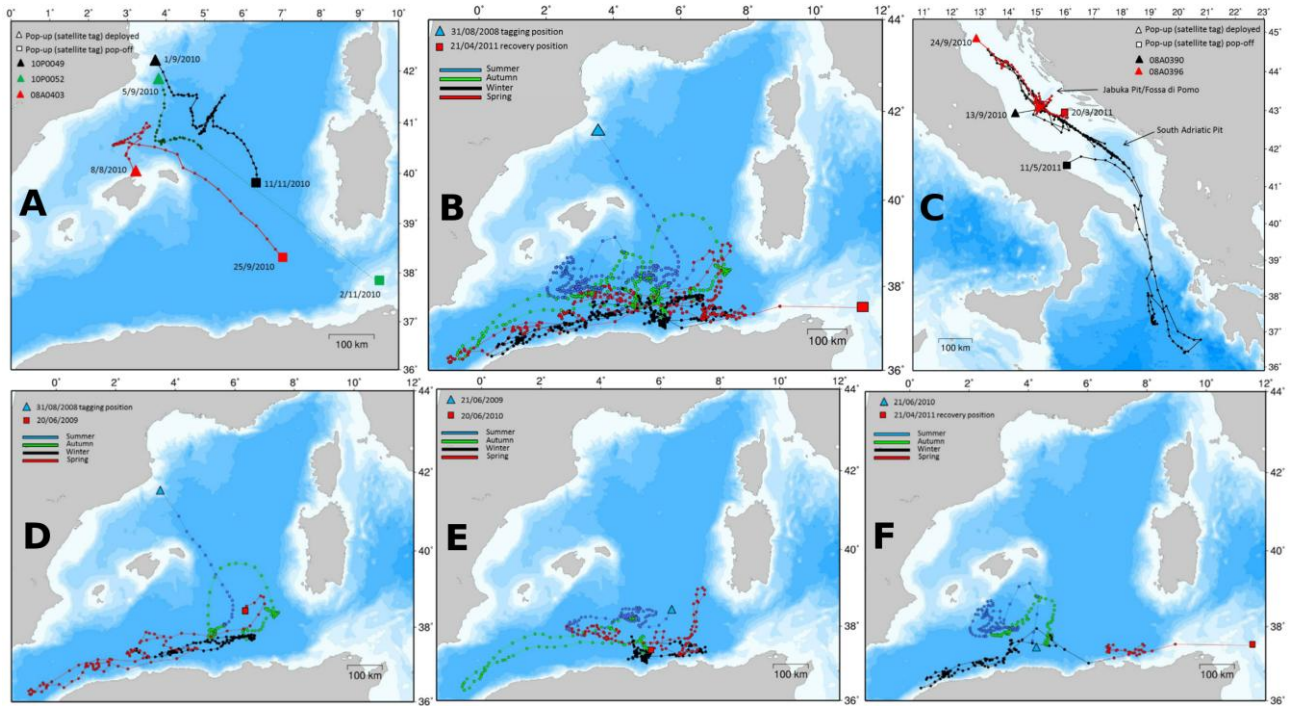
Appendix 2. PSAT trajectory of a single BFT (203 cm CFL at release), tagged in North Carolina, that spent three consecutive years in the western Atlantic (1997-2000) prior to a trans-Atlantic crossing into the Mediterranean Sea (Block et al. 2001, modified with GIMP 2.8.14).



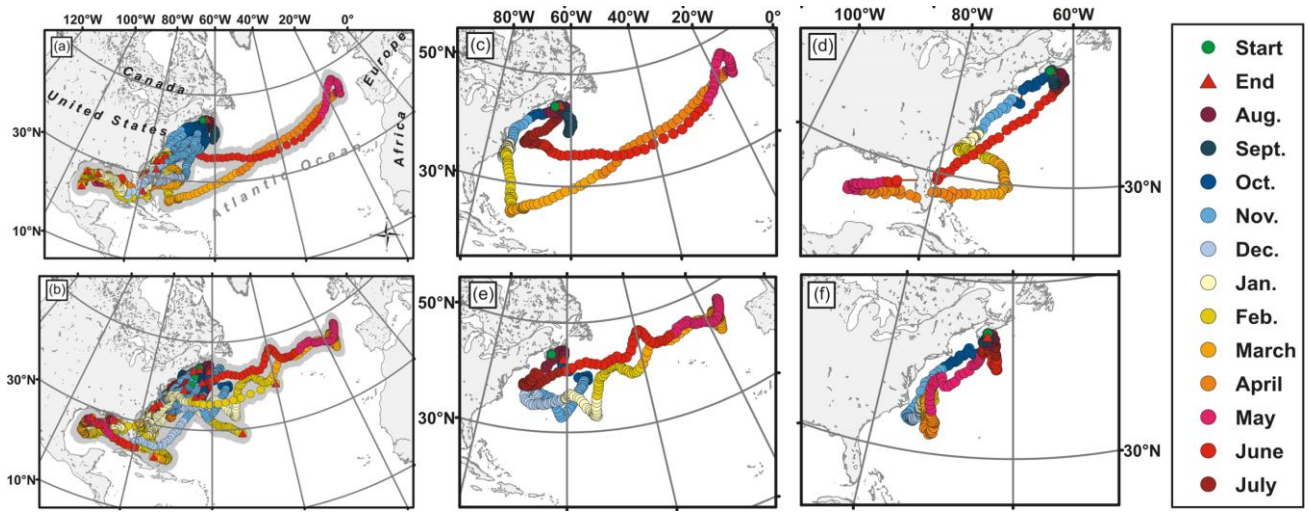
Appendix 3. Left) Positions of PSAT tagged BFT released from 3 locations (arrows) in the USA between 1996 and 2004. **a)** 36 BFT classified as western breeders (219 ± 27 cm CFL at release, median time at large 579 days), **b)** 26 BFT classified as eastern breeders (207 ± 17 cm CFL at release, median time at large 926 days), **c)** 268 BFT that did not visit the eastern or western spawning areas (202 ± 16 cm CFL at release, median time at large 141days). Triangles in all figures represent tag pop-up location (from Block et al. 2005, modified with GIMP 2.8.14). **Right)** PSAT trajectories of 13 BFT tagged in the Western Mediterranean during early June (2009-2011) after more than 45 days at liberty (from Aranda et al. 2013, modified with GIMP 2.8.14).



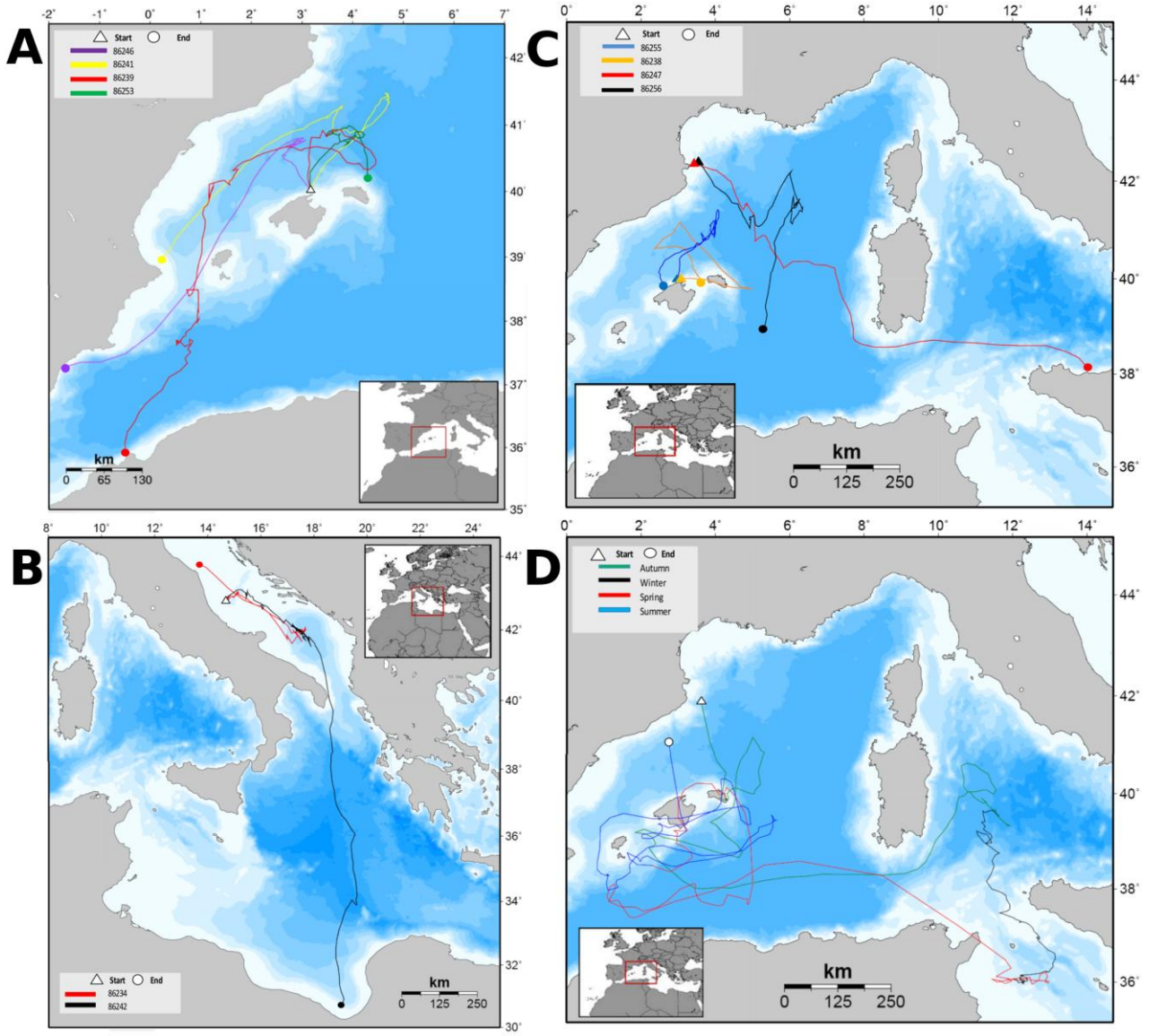
Appendix 4. PSAT trajectories of 126 adult BFTs tagged in the NW Atlantic during three different time periods: December-January (A,D,G), February-March (B,E,H) and April-June (C,F,I). Red points indicate ambient maximum temperatures (SST) between 22-26°C. All images are from Lutcavage et al. (2012) and have been modified with GIMP 2.8.14.



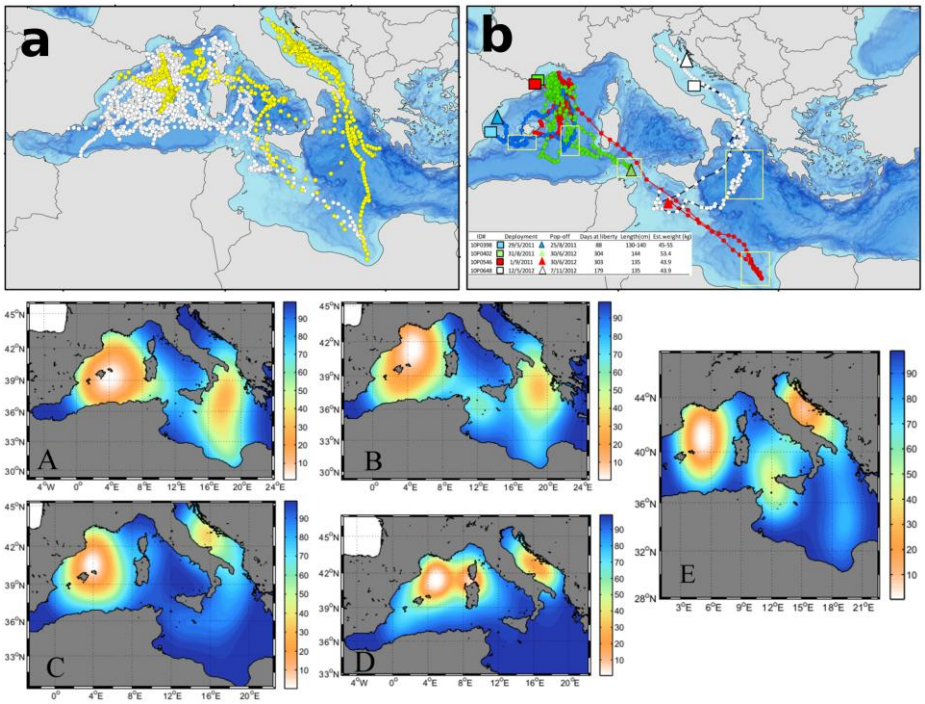
Appendix 5. A) PSAT trajectories of 3 BFT tagged in the Balearic Sea during August and September. BFT were at liberty for >30 days (dates of tagging and recovery are featured in figure). **B, D, E, F)** Trajectories of individual juvenile BFTs tuna tagged with archival tags during August 2008 in NE Spain and recovered after 32, 10, 12, and 10 months at liberty. **C)** PSAT trajectories of 2 BFTs tagged in the Adriatic Sea after 6-8 months at liberty. All images are from Cermeño et al. (2012) and have been modified with GIMP 2.8.14.



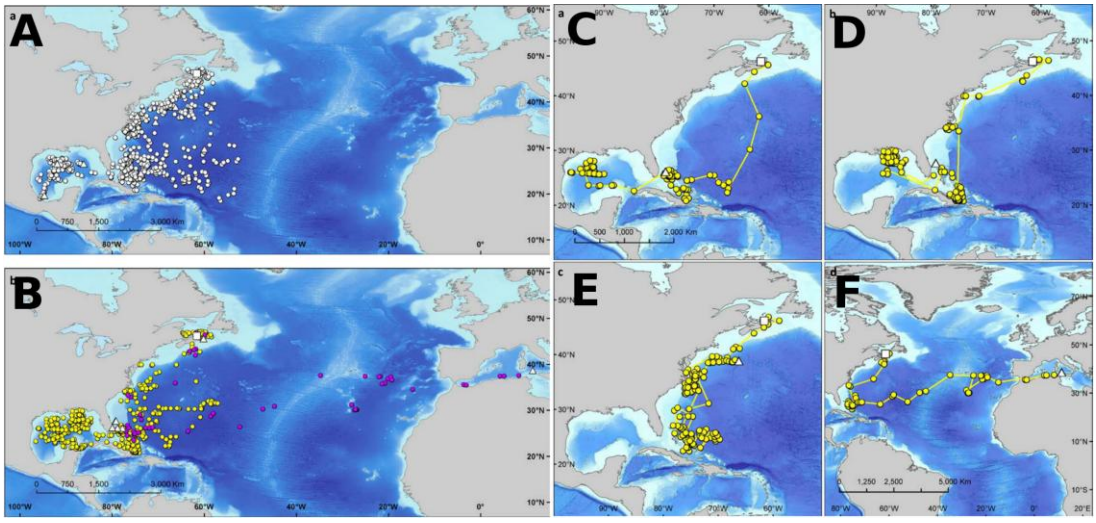
Appendix 6. PSAT trajectories of 32 adult BFT tagged offshore from Nova Scotia in 2005 (**a**, 268±56kg) and 2006 (**b**, 227±58kg). Tags remained on fish for 145±28 days in 2005 and 150±76 days in 2006. **c, d, e, f**) PSAT trajectories of individual BFT. All images are from Galuardi et al. (2010) and have been modified with GIMP 2.8.14.



Appendix 7. PSAT trajectories of **A**) four BFT tagged in mid-August 2008 off Mallorca Island, **B**) four tunas tagged during 2009 (Blue and Yellow: two adult BFT tagged in mid-August off Mallorca; Red and Black: two adult BFT tagged in early September in the Gulf of Lions), **C**) two BFT tagged in the Adriatic Sea during 2009, and **D**) a juvenile tuna tagged with an archival tag during August 2008 in the Gulf of Lions and recovered close to the initial position after 391 days at liberty. All images are from Tudela et al. (2011) and have been modified with GIMP 2.8.14.



Appendix 8. a) Daily positions of 38 BFT tagged with PSAT and archival tags. White circles represent positions of BFT tagged in the western Mediterranean (n = 28 pop-up tags + 1 internal tag), yellow circles represent positions of BFT tagged in the Adriatic (n = 9 pop-up tags). **b)** Daily positions of four tagged BFT displaying signs of spawning behaviour in areas highlighted in yellow squares. Seasonal Utilization Distributions (UDs) of BFT during the (**A**) spawning period (i.e. May 15 to July 15), (**B**) spring, (**C**) summer, (**D**) autumn and (**E**) winter. All images are from Cermeño et al. (2015) and have been modified with GIMP 2.8.14.



Appendix 9. Pooled geo-locations of BFT tagged with satellite pop up tags off Cape Breton Island (Canada) in **A**) 2007 and **B**) 2008 (squares and triangles represent the tagging and pop up locations, respectively). PSAT trajectories of individual BFT that entered the Gulf of Mexico (**C, D**), the Mediterranean Sea (**F**), ir did not visit either one of the two known major spawning areas (**E**). All images are from Wilson et al. (2011) and have been modified with GIMP 2.8.14.

CHAPTER 2

TROUBLED TAXONOMY

Squeezing through a narrow crack in a thin layer of partly digested outer egg membrane, or chorion, a small larva enters into the big blue expanse. Large predatory eyes take in the vast body of water that spins away into eternity on three sides with crystalline shards of light beaming through the ocean's surface that form the rooftop of their new universe overhead. The waters are warm but the black depths below promise dangers and radiate cold. Menacing shadows dart about the lonesome tiny figure suspended in the blue. Instantly the tiny fish recognizes one of its newborn brethren surveying its new world only a few fin thrusts away. Midnight black pupils become fixed and a deep longing penetrates the bluefin's fragile awareness. Fraternal love and hunger mix as one in the ebb and flow of turbulent surface waters. In the next moment, a surge of water and gaping jaws lined with tiny razor sharp teeth shock the bluefin back into the reality of predator and prey and one sibling disappears into the mouth of another. Alone and free, the baby bluefin tuna quickly swims into the void.

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Title Misidentification of bluefin tuna larvae: A call for caution and taxonomic reform.

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2.1 Introduction

Once properly identified, fish eggs and larvae can provide vital information for troubled fisheries, like that of BFT, concerning population structuring, spawning seasons and locations, as well as spawning stock biomass (SSB) or recruitment trends. Significant relationships between SSB and the abundance of eggs and larvae have been observed in several species including small pelagics (anchovy, Pacific and Atlantic mackerel; Lockwood et al. 1981; Lasker 1985; Stratoudakis et al. 2006; Lo et al. 2010) and demersal species (rockfish, cod, plaice, sole; Moser et al. 2000; Armstrong et al. 2001). As such, egg and larval abundance indices are commonly used to calibrate, improve and validate stock assessment models, which are often subject to inaccurate fisheries data (Hsieh et al. 2005). For decades, scientists from ICCAT member nations have been using BFT larval indices generated from surveys conducted in the Gulf of Mexico to calibrate Virtual Population Analyses for the western stock (Scott et al. 1993; Scott and Turner 2003; Ingram et al. 2010). In 2013, the first standardized BFT larval indices for a Mediterranean spawning site were published based on larval surveys conducted by the Spanish Institute of Oceanography around the Balearic Islands in the western Mediterranean (Ingram et al. 2013). Temporal shifts in BFT larvae abundance and condition can provide important information about recruitment success relative to short and long term environmental changes (Alemany et al. 2010; Lindo-Atichati et al. 2012; García et al. 2013a). Larval surveys can also help to determine community assemblage dynamics in order to develop additional data for the development of ecosystem-based management approaches (Richardson et al. 2010). Surveys of this nature have revealed that highly exploited species exhibit more variability in abundance in response to climate change than under-utilized species (Hsieh et al. 2006). In the context of a rapidly changing environment and a swollen fishing fleet, our ability to accurately identify and monitor BFT throughout its life history is critical for their effective management.

Clearly, the distribution and quantity of early life stage fishes can provide a wealth of information but what happens when eggs and larvae are classified and counted incorrectly? *Surveys monitoring the abundance* of fish eggs collected in the Irish Sea in 2001 were used by ICES to estimate the SSB of cod (*Gadus morhua*), plaice (*Pleuronectes platessa*) and sole (*Solea solea*). The resulting estimates far exceeded those given by Virtual Population Analysis (Armstrong et al. 2001). Subsequent genetic analysis revealed that many of the eggs had been misidentified, leading to large over-estimations of cod SSB (Fox

et al. 2005). Elsewhere, inaccurate estimates of egg diameters used to identify sciaenids may have led to over-estimations of up to 50% of the SSB of black drums, *Pogonias cromis,* in Chesapeake Bay (Daniel and Graves 1994). A recent study focusing on the ability of researchers from five different laboratories in Taiwan to identify larvae determined that the average accuracy of identification was 80.1%, 41.1% and 13.5% at family, genus and species levels, respectively (Ko et al. 2013). Families containing the most misidentified larvae in that study were Sparidae, Scorpaenidae, Scombridae, Serranidae and Malacanthidae. Recently, Puncher et al. (2015: C**hapter 3**) revealed that Atlantic bluefin tuna have been misidentified in the Mediterranean Sea. In that study, they showed that more than half of larvae submitted by three Mediterranean institutions to an ICCAT funded BFT research project were the wrong species.

In this review, we explore the events leading up to the misidentification of BFT larvae in the Mediterranean and present the argument that additional mistakes are likely to have occurred in the past and have a high likelihood of happening again in the future unless corrective measures are taken. In order to establish context for our concerns, we first provide a small review of problematic assumptions that have been made based upon what we have learned from young fish that have been identified as BFT. This is followed by a review of the problems associated with morphology-based taxonomy and its efficacy as a primary tool within the framework of large-scale tuna larvae surveys. We critically examine the resources available to survey teams and make several suggestions for improvements. Finally, we review the merits of morphology-based taxonomy and molecular techniques and highlight the need for a harmonization of both methods in order to minimize costs and optimize wildlife conservation and fishery management efforts.

2.2 A Context for Concern

Larval surveys have confirmed that BFT spawn during May and June in the eastern Mediterranean, June and July in the western Mediterranean (Duclerc et al. 1973; Alemany et al. 2006; Heinisch et al. 2008), and April to June in the Gulf of Mexico and Florida Straits (Baglin 1976; Richards 1976; Fig. 1). BFT larvae have also been observed in the Gulf of Guinea during February, March and August (Richards 1976), as well as the Black Sea during summer months, until they disappeared from that body of water in the 1980s (Vodianitskii and Kazanova 1954; Akyuz and Artuz 1957). Significant numbers of BFT larvae have also been identified off the coasts of Turkey (Bay of Mersin) and Tunisia

(Oray and Karakulak 2005; Giovanardi and Romanelli 2010; Zarrad et al. 2013; Koched et al. 2013). In 1983, Piccinetti and Piccinetti Manfrin (1993) identified BFT larvae off the northern coast of Egypt; however, their presence in those waters has not been verified since. Some researchers have suggested that spawning may also occur in the mid-Atlantic, after tagged adults were found there during spawning months (Lutcavage et al. 1999). Of course, this might also be evidence that BFT do not necessarily spawn annually (Galuardi et al. 2010). The much restricted BFT fishing season in most parts of the Mediterranean Sea is scheduled to take place during these spawning periods (currently 26 May to 24 June; ICCAT 2014). Many other fisheries choose to safeguard mature fish from capture until spawning has completed (Grüss et al. 2014). Of course, many (not all) of the BFT captured in the Mediterranean Sea are there for a few weeks alone, for the sole purpose of reproduction (Aranda et al. 2013) and a great deal of revenue would be lost if this season were to be closed. Future BFT conservation efforts may focus on protection of spawning areas and seasons, as they have been previously identified by the presence of their eggs and larvae.

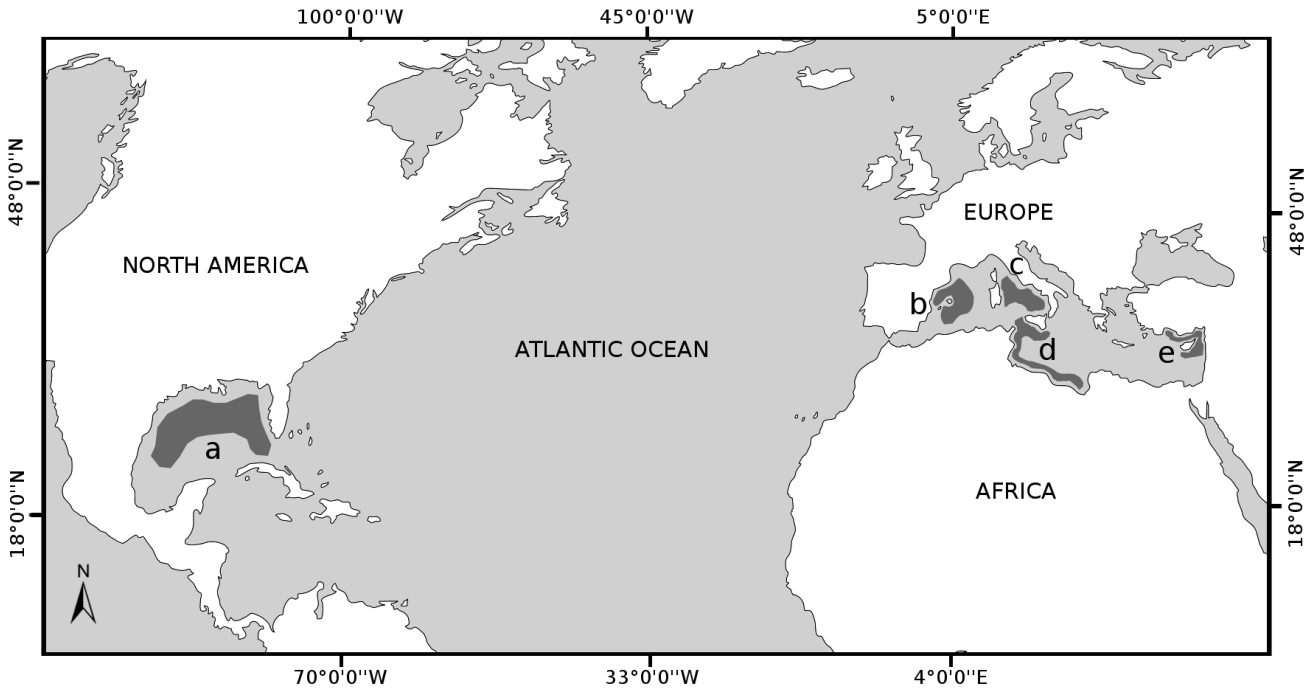

Fig. 1 Map showing known spawning areas (shaded polygons) of the Atlantic bluefin tuna in the Gulf of Mexico (a), Balearic Sea (b), Tyrrhenian Sea (c), Ionian Sea (d) and Levantine Sea (e). Figure was generated using gimp 2.8 (GNU Image Manipulation Program 2.8.14).

Much of our understanding concerning favourable environmental conditions for spawning of BFT comes from research based in the western Mediterranean Sea. Larval surveys in this area have shown that changes in relative abundances of different species are directly influenced by hydrodynamics (Alemany et al. 2010; Reglero et al. 2012). BFT larvae are most abundant in surface waters ranging between 23-28°C, where two water masses collide and create complex hydrodynamic conditions lacking strong, directional flows (García et al. 2005a; Reglero et al. 2012; Muhling et al. 2013). As Mediterranean waters are generally oligotrophic and do not provide ample food for the high bio-energetic demands of quickly developing BFT larvae, spawning typically occurs at the formation of frontal structures, the boundaries of anti-cyclonic gyres, and upwelling zones typical of oceanic islands, wherein both food particles and larvae are entrained (García et al. 2005a; Aguilar et al. 2009; Mariani et al. 2010; Lindo-Atichati et al. 2012). The Balearic Islands in the western Mediterranean are arguably the most productive of BFT spawning areas (Alemany 2008; García et al. 2013b). Within these waters, BFT larvae tend to be more abundant in areas where incoming Atlantic water masses and resident surface Atlantic waters, already modified by a longer stay in the Mediterranean, converge (García et al. 2005a; Alemany et al. 2006, 2010; Reglero et al. 2012). The discovery of low nutrient concentrations and reduced primary production in some areas with high concentrations of larvae have led some researchers to suggest that predation on non-BFT fish larvae and cannibalism plays an important role in the survival and development of BFT recruits. The continual spawning of BFT seen over a period of weeks produces sub-cohort overlap, exposing smaller larvae to predation by older recruits; a behaviour that appears to be favoured by higher sea temperatures, when growth rates increase and size classes become more distinct (Reglero et al. 2011; García et al. 2013a). The Tyrrhenian and Ionian Seas also produce high quantities of BFT larvae, particularly off the south eastern coast of Sicily (Cape Passero), where the confluence of the Atlantic Ionian Stream and upwellings result in a retention area rich in potential prey items (Lafuente et al. 2002; García et al. 2005b). Predictions concerning the impacts that climate change and future ocean conditions will have on BFT populations will be based on these larval abundance studies. Unfortunately, this research is taking place on a limited geographic scale and until additional resources (monetary and human expertise) are allocated elsewhere, this will continue to be the case.

Our current understanding of BFT spawning in the Mediterranean Sea suggests that the western

basin is the most productive source of young bluefin tuna; however, spawning also takes place in the central and eastern Mediterranean Sea. Discrepancies in larval survey results from this region have cast some doubt on the location, consistency, timing and success of spawning events. For example, a 1994 larval survey of the entire Mediterranean collected 1160 tuna larvae, including 183 BFT, none of which were caught in the Levantine Sea (Piccinetti et al. 1997). These results are not surprisingly, since the survey was conducted between 21 June and 7 July, several weeks after the established spawning season (Karakulak et al. 2004) and south of Cyprus, instead of in the Bays of Antalya and Mersin, north of the island, where BFT larvae are often found (Oray and Karakulak 2005). Japanese surveys have reported low densities of BFT larvae in hauls taken in the mid-Levantine Sea around the Herodotus Basin (Tsuji et al. 1997, Nishida et al. 1998). These larvae may have originated at the periphery of the Rhodes and Léraptra anti-cyclonic gyres, which are composed of inflowing Atlantic waters south of the islands of Rhodes and Crete, respectively; a hypothesis that fulfils both the hydrodynamic and island proximity requirements for BFT spawning (Robinson et al. 1992). Waters exiting these gyres to the south are carried eastward by the Mid-Mediterranean Jet Stream and later bifurcate south to the waters of the Herodotus Basin and north to the Bay of Antalya. Alternatively, spawning may also be occurring between the island of Cyprus and the coastlines of Syria and Lebanon, where a number of smaller seasonal anti-cyclonic gyres form. These water masses are carried northward by the Cilician Current along the southern coast of Asia Minor, where they can be retained in eddies formed in the Bays of Mersin and Antalya (Robinson et al. 1992, Özsoy et al. 1993).

BFT larvae are commonly transported over significant distances by both jet stream waters and meandering gyres throughout their distribution. Larvae appear to accumulate in retention areas with high concentrations of food particles (including conspecific larvae), wherein they develop into juveniles and migrate en masse to nursery areas (McGowan and Richards 1989). In the Western Atlantic, seven-day-old BFT larvae (>4.0 mm) have been captured off the coast of North Carolina using a mesh size of 0.33m, well outside of known spawning areas (McGowan and Richards 1989). Although ocean currents in that region are capable of transporting larvae from the Gulf of Mexico and Straits of Florida, approximately 970 km, the origin of larvae around Cape Hatteras is unknown. The majority of BFT larvae captured in that area have been large (4.6 - 6.5 mm), while smaller larvae (<3.5 mm) are noticeably absent among

samples. Similarly, the majority (65%) of larvae captured in the northern Levantine Sea have been large (5-7 mm, approximately 10 days post hatch), opening up the possibility that their origin may lie in a different body of water; although, some smaller larvae (3.1 mm) have also been captured (Oray and Karakulak 2005). Moreover, most larval surveys conducted in the Levantine Sea have used nets with a 1 mm mesh size which are unlikely to retain the entire smaller size fraction of larvae (Oray and Karakulak 2005). Therefore, it is currently inadvisable to compare the results of larval surveys taking place in the eastern Mediterranean with other locations, since they cannot be considered as quantitative hauls. Clearly, the execution of additional standardized larval surveys, supported with genetic identification of samples and hydrographic modelling, are required in the Levantine Sea to the extent that they have been performed elsewhere in the Mediterranean Sea.

2.3 Potential for errors in BFT larvae identification

In general, identification of tuna eggs and the larvae of some tuna species using morphological characteristics alone is incredibly difficult, requiring an in depth knowledge of taxonomy as well as patience and experience. Two of the world's former leading tuna taxonomists warned that "the young stages of fishes of the family Scombridae are among the most difficult to identify to generic levels and particularly to the species level" (Richards and Pothoff 1974). Kohno et al. (1982) cautioned that an "extensive knowledge of individual, growth-associated, and geographic variations" in patterns of melanophores is required to make an accurate identification of *Thunnus* species. Unique patterns of red pigmentation have also been used to distinguish species; however, this type of pigmentation is most prominent in larvae caught during the night (Matsumoto et al. 1972) and is lost during formalin fixation and ethanol preservation (Richards et al. 1990). Due to the difficulties associated with correctly identifying larger larvae (>5 mm) by pigmentation patterns alone, some authors advise using osteological characteristics; however the process of clearing, staining and examining is time consuming, unfeasible for large surveys and often results in additional loss of samples (Richards et al. 1990). Identification based upon morphometrics alone is unfeasible due to body distortions occurring during fixation or rapid growth spurts that distend and stretch the body during rapid early development (Matsumoto et al. 1972). Making matters worse, fish larvae are often so damaged during sampling that identification to species level using morphological features becomes impossible (Paine et al. 2007). Due to the scarcity of expertise in larval

taxonomy, species identification is commonly outsourced to distant laboratories that specialize in the sorting of plankton (Scott et al. 1993; Ingram et al. 2010; Matarese et al. 2011; Lindo-Atichati et al. 2012). Some researchers have resorted to limiting identification of their captured larvae to lower taxonomic levels (Hernandez et al. 2010; Lindo-Atichati et al. 2012), while others have turned to genetics to identify to species level (Chow et al. 2003, 2006).

The ability of field technicians to identify larvae to species level is limited by the quality of materials and tools that they are given to accomplish this challenging task. In some fields of study, misidentification of species can reach as high as 90%, which can lead to serious errors concerning community composition and population inferences (Vecchione et al. 2000). Decades ago, before recent clarification of tuna systematics, Richards (1976), a reputed ichthyoplanktologist warned, "For a group like the tunas I am sceptical of reports based on eggs and early larvae unless these young stages have been raised to identifiable sizes." Unfortunately, culturing unknown eggs and larvae until they can be accurately identified has proven extremely difficult for some long-range pelagic species, including BFT (Hyde et al. 2005). Rearing of BFT from eggs through to their juvenile stages has been completed only recently (de la Gandara et al. 2010). As a result, most larval surveys rely on a small handful of taxonomic keys, illustrations and journal publications for species identification (unless one of the world's few tuna taxonomists or a well-trained technician is onboard to assist with the painstaking task of identifying thousands of larvae). When sourcing texts that specialize in BFT systematics, confusion quickly ensues as most texts are quite old and use several of 15 synonyms, seven of which were generated by Linnaeus alone (Froese and Pauly 2015). A few keys are easily obtained on the internet, while most require a personal contact with someone in the industry willing to share digital copies. Obviously since most texts are several decades old, there is no standardized formatting or consistency in quality. Several texts that are routinely referenced by current larval studies are written in Japanese (Ueyanagi 1966, Yabe 1966), Italian (Sanzo 1932a; Scaccini 1975), French (Duclerc et al. 1973) and Spanish (Dicenta 1975). Some of these keys are very old and after several generations of scanning and copying of illustrations, morphological features contained therein have become difficult to distinguish.

Early taxonomic descriptions of scombrids were often based on misidentified larvae or eggs and are thus riddled with errors and confusion (Alemany 2008). The unreliable nature of these guides was

identified as early as 1974 by Richards and Pothoff (1974), who called them contradictory and complained that they made use of too many different criteria for comprehensive identifications. The authors of some of these early works have admitted that errors were made when identifying the larvae that were later used to build identification keys (Matsumoto et al. 1972; Richards and Pothoff 1974). Unfortunately, efforts to replace these early works with accurate descriptions have yet to take place. As a result, successive publications based on these works have carried the errors forward. The origin of these mistakes are the pioneer works on tuna larvae in the Mediterranean in which BFT larvae and those of other tuna species were misidentified, namely Ehrenbaum (1924) and Sanzo (1932). For example, the larvae identified by Ehrenbaum (1924) as *T. thynnus* were in all likelihood *T. alalunga*, according to the pigmentation pattern of the specimens illustrated in their publication. In short, the illustrated larvae all but lack diagnostic black pigmentation in the trunk, except for a few dorsal and/or ventral melanophores on the urostile, a pattern that is maintained from hatching to advanced postlarval stages (Alemany 1997 and references therein) and a characteristic that is not shared by any other tuna species inhabiting the Mediterranean Sea. Using fertilized eggs captured at sea, Sanzo (1932, 1933) described eggs and early hatched larvae of *T. thynnus* and *T. alalunga* (at that time *Orcynus thynnus* and *Orcynus germo*, respectively), following a comparison with mature eggs extracted from the ovaries of captured adult females. However, the continuous row of ventral melanophores, preanal ventral pigmentation and few dorsal melanophores in the caudal area featured in the resulting drawings and descriptions suggest that Sanzo (1932, 1933) was unintentionally working with *A. rochei* specimens (Alemany 1997 and references therein) and not *T. thynnus* and *T. alalunga* as intended. Later, Scaccini et al. (1973) confirmed these errors when eggs identified as BFT, according to Sanzo's descriptions, released larvae belonging to other species. In fact, larvae identified by Scaccini et al. (1973) as BFT were probably *A. rochei,* based on a photograph featured in the publication of a recently hatched larva. These two erroneous works by Ehrenbaum (1924) and Sanzo (1932) were the only references taken into account by Padoa (1956) for describing BFT larvae, in what has become one of the most extensively used fish larvae identification guides for the Mediterranean Sea. From these dubious origins, drawings and descriptions of early life stage BFT and albacore by Sanzo (1932, 1933) continue to resurface in fish larvae identification guides of species inhabiting in the Atlantic Ocean, including Fritzsche (1978), Fahay (2007) and Richards (2005).

Confusion between illustrations and descriptions of *T. thynnus* and *T. orientalis* has also persisted for decades as many of the most relied upon guides predate the separation of the two species (Collette 1999). For example, Yabe et al. (1966) published an in depth description of *T. orientalis* and *T. maccoyii* and their distribution in the Pacific Ocean, containing eight detailed illustrations of *T. orientalis* larvae. Jones et al. (1978) then used the illustrations by Yabe et al. (1966) as well as several other drawings of *T. orientalis* by Ueyanagi and Watanabe (1964) and Ueyanagi (1966) for their in depth description of *T. thynnus*. Fahay continued this practice of recycling illustrations in two publications in 1983 and 2007 in which they use the same drawings by Yabe et al. (1966). In their 1983 publication, they indicate in a footnote that the *T. thynnus* larvae were "Pacific material"; however, this important detail was omitted from their later publication in 2007 (Fahay 1983, 2007). It is unfortunate that this occurred, considering that this key was published years after the *Thunnus orientalis* species designation in 1999. Certainly, these mistakes in the pioneer works have confounded many scientists for decades and are undoubtedly the root of the issue surrounding BFT misidentifications.

During the 1970s several attempts were made to clarify the confusion surrounding the taxonomy of early life stage scombrids. In a thorough review, Richards and Pothoff (1974) warned that established osteological diagnostic features sometimes contradict conclusions based on pigmentation. Later, Dicenta (1975) noted encountering a great amount of difficulty when attempting to differentiate between the eggs and larvae of *T. thynnus* and *A. thazard* (the authors likely meant *A. rochei*). Following the examples provided by Sanzo (1932, 1933), Scaccini (1975) claimed that there are no differences to be found in the pigmentation of *T. thynnus* and *T. alalunga* at sizes smaller than 5 mm. Soon after Kohno et al. (1982) identified 191 larvae as *T. thynnus* and *T. alalunga* using guides by Scaccini et al. (1975) and Fritzche (1978), among others, and determined that the patterns of melanophore distribution were inconsistent within each species, concluding that the use of pigments as diagnostic features was limited. Since all of these studies were working from the same erroneous guides, it comes as no surprise that new sources of confusion, such as intraspecific variation (resulting from *A. rochei* specimens analyzed as *T. thynnus*), were discovered and propagated.

It has now been over two decades since this literature-based discussion fell silent and although early life stage scombrid taxonomy has since been resolved, a comprehensive review clarifying the

confusion and identifying errors contained in existing identification guides has yet to be published. Modern internet-based standards are disappointing in terms of illustrations used, or lack thereof. FishBase (Froese and Pauly 2015) features an illustration of a 25-day-old juvenile *T. orientalis* incorrectly labelled as *T. thynnus* (Miyashita et al. 2001), while ICCAT's species identification sheets contain a single illustration of a juvenile *T. thynnus*. As Godfray (2002) pointed out, the quantity of "taxonomic information available on the web is pitiful, and what is present (typically simple lists) is of little use to non-taxonomists". Tunas are obviously no exception to this generalization and new efforts should be made to definitively identify and describe each species different larval morphotypes with the support of genetic analyses and/or conclusive rearing experiments.

As a first contribution to this effort, we offer a clarification of some of the more contentious elements affecting the correct identification of BFT larvae in the Mediterranean. Currently, there are no correct and complete descriptions of BFT larval development published, including illustrations of the different developmental stages from eggs and yolk sac larvae to advanced post-larvae, based on Mediterranean material. Despite the scarcity of descriptive materials, several incomplete yet useful references do exist. For example, Alemany (1997) contains an original illustration of Mediterranean BFT flexion larvae and the larval stages following yolk sac reabsorption have already been correctly described by Dicenta (1975), who pointed out that the most distinctive species specific feature of these larvae is the presence of dorsal melanophores located mid-trunk. Despite some differences that may exist between the pigmentation pattern of Pacific and Atlantic BFT (Jones et al. 1978; Kohno et al. 1982), the flexion and postflexion stages of *T. thynnus* can be identified using the descriptions of *T. orientalis* larvae in Ueyanagi and Watanabe (1964), Ueyanagi (1966) and Yabe et al. (1966). Moreover, recent rearing experiments carried out in the Mediterranean (de la Gándara et al. 2010) have corroborated the observations provided by Kaji et al. (1996), confirming that *T. orientalis* and *T. thynnus* yolk sac larvae have the same pigmentation pattern; namely a very large dorsal melanophore in the middle of the trunk, which extends to the primordial fin (Fig. 2a). After the yolk sac has been absorbed, this melanophore becomes smaller and migrates to the trunk, giving rise to additional small dorsal melanophores and the typical dorsal melanophore pattern of BFT larvae (Fig. 2b,c). This unmistakable pigmentation pattern can also be observed in larvae that have been preserved in formalin (Fig. 3).

Fig. 2. Photographs of Altlantic bluefin (*Thunnus thynnus*) larvae reared from eggs collected from spawning induction cages at El Gorguel (Cartagena, Spain) and cultivated at the larval rearing plant at the Spanish Insitute of Oceanography in Mazarrón (de la Gándara et al. 2010). Larval developmental stages represented are: yolk sac (a), 8 days post hatching (b), and 14 days ays post hatching (c). Photographs taken by F. de la Gándara and adapted for publication using GNU Image Manipulation Program 2.8.14.

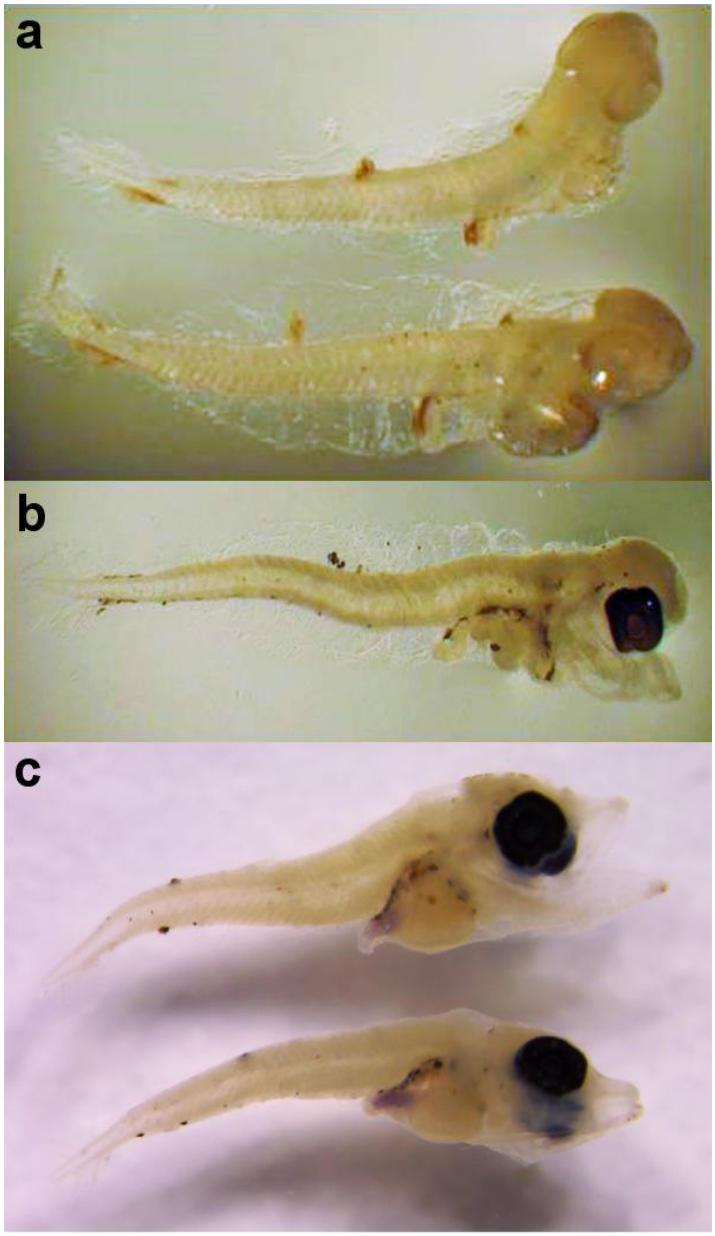

Fig. 3 Photographs of Altlantic bluefin (*Thunnus thynnus*) larvae captured in the Balearic Sea and preserved in formalin. Approximate age of larvae are: <24h post hatching yolk sac larvae (a), 24h post hatching (b), 48h post hatching (c). All photographs were taken by F. Alemany and adapted for publication using GNU Image Manipulation Program 2.8.14.

Bullet tuna, *A. rochei*, are also found in the Mediterranean Sea and have similar conspicuous dorsal pigmentation at flexion and preflexion stages; however, this is restricted to a few melanophores in the caudal peduncle area which extend anteriorly as the larvae develop (Alemany 1997). Other characteristics, such as ventral pigmentation (digestive organs and cleithrum) in *Auxis spp.* and a much more intense black pigmentation of the first dorsal fin in BFT, allow for the discrimination of the two species at similar developmental stages. In summary, it is possible to identify scombrid larvae found in the Mediterranean Sea based on morphological characters alone, unfortunately overcoming the confusion

cultivated in the past is an obstacle that will be difficult to overcome until accurate and complete descriptions are published.

2.4 Required modernization of larval taxonomy

BFT larval surveying efforts would greatly benefit from a modern revision of outdated taxonomic keys and focus should be given to providing new keys and high quality photographs and illustrations online in a digital format. Over a decade ago, Godfray (2002) suggested that a revitalization of taxonomy is required, advocating that it is ideally suited for Information and Communication Technologies because of its information rich nature and large number of meticulously crafted illustrations. Still, for the most part, taxonomy simply hasn't made the technological leap to modern digital communciation tools. One may argue that taxonomy, as a science, is being left behind in an age of information that demands a digital interface for nearly all tasks performed in modern research. Guerra-García et al. (2008) declared that taxonomy is in a crisis, warning that funding for taxonomy is generally inadequate and that expert taxonomists are few and in demographic decline. Over a decade ago, Boero (2001) commented on the state of decline of taxonomy, remarking that since publication records of researchers are used as a performance measure, the dismal if not non-existent impact factors of taxonomic journals are not attractive for young researchers trying to establish themselves in their careers. As such, the number of professional and amateur taxonomists has undergone a long and persistent decline since the 1950s (Hopkins and Freckleton 2002). In 2003, Wilson (2003) estimated that as few as 6,000 biologists were working in taxonomy worldwide. The culmination of these numerous pitfalls has slowed the progress of species identification and the assessment of global biodiversity; an ethereal obstacle now described as the "Taxonomic Impediment". The disadvantages associated with morphological taxonomy weighed against the increasing efficiency of molecular techniques are driving more researchers towards DNA barcoding for routine species identification.

A heated debate has been raging between advocates of morphological and molecular taxonomy since the inception of DNA barcoding. Some opponents of molecular taxonomy have argued that DNA barcoding explores a very restricted portion of the genome and this may lead to systematic errors in classification. They argue that the use of a single standard sequence is equivalent to classifying organisms based upon a single morphological feature (Tautz et al. 2002). Seberg et al. (2003) expressed concerns

over a monopolization of information by wealthier nations, warning that an expensive and centralized DNA-based taxonomy would add to an existing North-South divide in taxonomy. It could be argued that the open-access nature of online sequence databases is acting to decentralize knowledge that was once the sole possession of aged taxonomists with years of education unavailable to many. It is for this reason that some have claimed that "DNA barcoding promises to entirely democratize the taxonomic process" (Packer et al. 2009).

Others have suggested that the merits of morphological taxonomy and barcoding can combine to improve systematics overall (Stevens et al. 2011; White and Last 2012). DNA barcoding has the potential to accelerate species identifications and diversity assessments by increasing the synergy between field biologists, reference collections and sequencing facilities (Swartz et al. 2008). A key directive of centralized DNA barcoding efforts, such as the Barcode of Life Initiative, is matching known species with archived voucher specimens to DNA sequences (Ratnasingham and Hebert 2007). Likewise, morphological taxonomists are needed to recognize and describe new species as well as confront the daunting task of matching pre-existing descriptions with ever-expanding sequence libraries (Tautz et al. 2002). Similar efforts must also focus on the taxonomic description of early life stage fishes. Kendall and Matarese (1994) estimated that only 10% of the described marine fish species had known larvae and as few as 4% had published descriptions of their eggs. Certainly barcoding has a role to play in these efforts.

2.5 Advances in molecular analysis

Several genetic techniques have been used by researchers over the past two decades to identify early life stages of fishes and resolve previously unexplored challenges in fisheries science (Daniel and Graves 1994; Vandersea et al. 2008; Boley and Heist 2011). The most commonly used molecular technique for identification of fish species is the PCR amplification and sequencing of a ~650bp fragment of the cytochrome oxidase subunit 1 (*CO1*) gene located in the mitochondrial genome. The *CO1* gene has been proposed as the basis for a global bio-identification system for all animals (or Barcode of Life Project) for the following reasons: 1) variability of sequences between individuals of the same species is negligible when compared to the variation between species, 2) moderate mutation rate, and 3) characteristic flanking regions that require a limited number of "universal" primers (Hebert et al. 2003). An extension of the Barcode of Life project is the Fish Barcode of Life Initiative (FISH-BOL), which

seeks to barcode all of the estimated 32,257 recognized species of fish using the same gene (http://www.fishbol.org; Ward et al. 2009). Barcoding by *CO1* sequences has been used to identify the diversity of reef fish larvae in the Pacific Society Islands (Hubert et al. 2010), Great Barrier Reef (Pegg et al. 2006) and Caribbean Sea (Victor et al. 2009; Valdez-Moreno et al. 2010; Baldwin et al. 2011). *CO1* barcodes have also been used to verify the species of difficult to identify larvae of snapper in the Straits of Florida (D'Alessandro et al. 2010), mackerel off of south-eastern Australia (Neira and Keane 2008), sculpin in the Bering Sea (Matarese et al. 2011), scombrids in the western Atlantic and mid-Pacific (Paine et al. 2007, 2008), sandlance in the Yellow, East and Bering Seas (Kim et al. 2010), and entire communities of medium-sized pelagic larvae in the Straits of Florida (Richardson et al. 2010). Other genes neighbouring the *CO1* gene, such as the ATCO region and 16S rRNA gene, have been used to identify *Thunnus* eggs and larvae offshore from the Mariana Islands and Yucatán Peninsula, respectively (Kawakami et al. 2010; Muhling et al. 2011); however, molecular barcoding techniques have yet to be applied to large-scale larval surveys focusing on *Thunnus thynnus*. The application of these techniques has provided many solutions to long unanswered mysteries; however, with every advance new challenges are inevitably encountered.

As with many other aspects of working with young tunas, identification of species by *CO1* sequences is not a straightforward enterprise. Due to the similarity between species in the *Thunnus* genus, some researchers have experienced difficulty when identifying unknown samples using genetic distance based approaches, like phylogenetic tree construction (Alvarado Bremer et al. 1997; Richards et al. 2006; Paine et al. 2007). Viñas and Tudela (2009) were unable to resolve *Thunnus orientalis* and *T. thynnus* clades using a handful of sequences (n=4-8) and as a result advocated for the use of the mitochondrial control region as an alternative marker for the barcoding of tunas. Since the geographic distribution of *T. orientalis* does not overlap with the spawning areas of *T. thynnus*, the *CO1* marker can be used as a barcoding marker for tuna larvae collected in the Gulf of Mexico and Mediterranean Sea. By using a different analytical approach, Lowenstein et al. (2009) identified seven nucleotides in the *CO1* sequence that distinguish *T. thynnus* for *T. orientalis* and one simple pure characteristic attribute that differentiates *T. thynnus* from all other tunas. As such, the use of individual diagnostic loci to identify species, or characteristic attribute keys, has proven effective for differentiation of scombrid species (Lowenstein et al.

2009). Barcoding of BFT mitochondrial DNA has also revealed that 2-3.3% of Atlantic BFT share nearly identical mtDNA with albacore and Pacific bluefin tunas, due to past hybridization events and subsequent introgression of the mitochondrial genome (Viñas et al. 2011; Alvarado Bremer et al. 2005). As such, nuclear markers, like the rDNA first internal transcribed spacer (*ITS1*) region, has been advocated by some as a means by which albacore and BFT can be differentiated (Chow and Kishino 1995; Chow et al. 2006; Paine et al. 2007; Viñas and Tudela 2009). Using the sequencing approach and tools described above, identification of unknown scombrid larvae in the Mediterranean Sea is straightforward and unambiguous.

Once a species' DNA barcode is published, researchers are able to develop a suite of genetic tools capable of identifying that species without direct sequencing of DNA. A 33-probe suspension bead array has been used to identify 23 different species of California marine fish eggs, including commercially relevant species such as Pacific mackerel (*S. japonicus*), hake (*Merluccius productus*), Pacific barracuda (*Sphyraena argentea*), white seabass (*Atractoscion nobilis*), California halibut (*Paralichthys californicus*), and diamond turbot (*Hypsopsetta guttulata*) (Gleason and Burton 2012). The developers of this method have suggested that their DNA probes could be scaled up to simultaneously identify the eggs of as many as 100 species. More traditional and economic techniques such as multiplexing of haplotype-specific PCR products and electrophoresis have been used to rapidly identify collections of clupeiform larvae captured in Senegal (Durand et al. 2010). Similarly, researchers in Hawaii have developed a species-specific set of primers that, when combined with electrophoresis, were capable of distinguishing the larvae of six species of billfish, two species of dolphinfish and wahoo (*Acanthocybium solandri*) onboard a research vessel in only 3 hours (Hyde et al. 2005). Advances have also been made to simplify the process of distinguishing scombrid larvae. For example, Paine et al. (2008) developed a quick molecular diagnostic tool capable of differentiating albacore and bluefin tuna using a restriction enzyme (*Eagl*) to digest amplified fragments of *ITS1*.

Next Generation Sequencing (NGS) is a rapidly evolving array of technologies now being widely employed for fisheries management tasks. SNP panels generated through NGS have been developed in order to discriminate species and populations of albacore tuna, Pacific lamprey and blue catfish (Albaina et al. 2013; Hess et al. 2014; Li et al. 2014). In Europe and America, SNP panels have been used to

distinguish between species and populations of salmon as well as farm raised and wild fish (Amish et al. 2012; Drywa et al. 2014; Houston et al. 2014; Larson et al. 2014). The expectation is that these panels will be used for hatchery and marketplace traceability, improvement of breeding programs and wildlife conservation efforts. For example, ICCAT, through the Atlantic-wide Research Programme for Bluefin Tuna (GBYP), is currently funding research to develop a high performance array capable of distinguishing populations of bluefin tuna. NGS technologies also provide the opportunity to quickly ascertain the species composition of entire communities of plankton. Researchers at the Plymouth Marine Laboratory in England recently compared the results of a plankton survey in which species were identified via both a metagenetic and morphological approach (Lindeque et al. 2013). Using 454 pyrosequencing they identified 135 operational taxonomic units (OTUs), over twice the number (58) identified using a morphological approach, including rare species and parasites. This approach may not be capable of giving an exact number of larvae by species collected in a given sample but it can give a relative abundance of each. Fisheries organizations and multi-institutional larval surveys, plagued by sampling and species identification bias, have much to gain from this new technology. Although NGS techniques have been very expensive in the past, its cost is declining rapidly and new markets are opening up in the developing world (Willette et al. 2014).

NGS can produce millions of barcodes per day, the cost of which is decreasing at a much higher rate than predicted by Moore's Law, according to the statistics kept by the National Human Genome Research Institute. Stein et al. (2014) conducted an in depth analysis of the costs associated with the sorting and identification of freshwater macroinvertebrates, fish and diatoms using morphology-based taxonomy, sequencing and the metagenetic analysis of bulk samples of organisms using the IonTorrent PGM™ platform. The cost and time estimates required to obtain results using these separate approaches were based on data provided by commercial laboratories in the USA between 2010 and 2012. Their estimates did not include the cost of sample collection (common for all approaches) and are comparable to what would be paid by a large fishery-based larval survey. They concluded that Sanger sequencing costs 1.7 - 3.4 times more than a morphology-based taxonomic approach (1200-1700USD per 1000 fish). Although the bulk sample NGS approach is incapable of identifying individual fish and provides relative abundances of each taxa instead, its cost were comparable or slightly less than the morphology-based

approach. The time and costs required for Sanger sequencing includes the same steps as traditional methods (sorting of samples, removal of debris, voucher specimen analysis) with additional DNA extraction, PCR amplification and sequencing, adding another 5 USD in cost per individual analyzed. Despite these additional steps, the Sanger sequencing approach is able to provide results within days or weeks of sampling (depending on access to sequencing services), compared to the months required for traditional morphology-based identifications. Modern NGS platforms can provide results in 3 - 5 days, as it eliminates the time required for sorting of the specimens, and costs between 0.50 – 2 USD per individual. However, Stein et al. (2014) believe that Sanger sequencing is more accurate than NGS analysis due to shorter sequence lengths and amplification biases. Researchers are now beginning to use aspects of each approach that optimize their resource costs: use of voucher specimens identified by a morphology-based approach, NGS techniques to discover new barcoding sites or diagnostic/informative loci, followed by Sanger sequencing of low numbers of samples or genotyping of high numbers of individuals with microarrays (Shokralla et al. 2014).

2.6 Guide of good practice in BFT larval studies

Due to the unreliable, inconsistent and incomplete nature of catch data provided to tuna fishery scientists, managers are increasingly looking to alternative sources of information. If larval and egg abundances are to be more widely used by tuna fishery scientists, it is imperative that problems associated with sample identification are resolved. Considering that tuna taxonomy has suffered from confusion in the past and is in need of modernization it comes as no surprise that an understanding of BFT larval dynamics remains incomplete. We recommend an elaboration of high quality drawings of the complete developmental series of BFT, accompanied by descriptions based on a high number of individuals from throughout the species range in order to account for intraspecies variability. High definition photographs should accompany each drawing, to help technicians recognize diagnostic features in more realistic and familiar representations of each larval stage. Equipped with these new tools, efforts should be made to *c*onfirm that spawning is taking place in areas where BFT larvae have been documented only once or have been identified by only one group of researchers or technicians. If natal homing is indeed a reproductive strategy of BFT, conservation efforts should target remote locations where only a few larvae have been captured, so that rare genotypes can be catalogued. BFT originating in these areas are likely to possess

specialized adaptations (egg buoyancy, sperm motility and larval growth rates) to unique local environmental conditions (temperature, salinity, density, current velocity, nutrient availability etc.) (Mackenzie and Mariani 2012), which may prove important for the species' survival in future oceans modified by climate change (Hobday et al. 2015). Regardless of the sampling location, nets with 0.33-0.50 mm mesh size should be employed with a tow velocity of two nautical kilometres per hour in order to ensure collection of smaller size fractions of larvae. This would allow a comparison of yields across all geographic areas.

Advances in NGS and historical DNA analysis are allowing researchers new access to archived samples (larvae, scales, otoliths, spines and vertebrae), revealing important ecological and evolutionary changes in fish stocks and populations over time (Riccioni et al. 2010, 2013; Cuveliers et al. 2011; Poulsen et al. 2011; Seeb et al. 2011). Although molecular sampling techniques have been making great advances in reducing impact on voucher specimens in the recent past (Nielsen and Hansen 2008; Gibbon et al. 2009), the same cannot be said for the majority of morphological taxonomists that still preserve their samples in formalin; an outdated practice that renders genetic analysis very difficult if not impossible (Ward et al. 2009). Many BFT larval studies have used formalin as a preservative, which severely limits future genetic verification of samples (Piccinetti et al. 1997; García et al. 2002; Koutrakis et al. 2004; Alemany et al. 2006; Isari et al. 2008; Alemany et al. 2010; Catalán et al. 2011; Koched et al. 2013). After discovering the way in which formalin corrupts the body shape and pigmentation of larvae, Ueyanagi (1966; 1969) began preserving their larvae in 70% ethanol. Researchers with the California Cooperative Oceanic Fisheries Investigations use a conversion factor of 1.098 to convert from formalin fixed length to live length because of formalin-induced shrinkage of larvae (Lo et al. 2010). Admittedly, ethanol also distorts diagnostic morphological features; however, because it is non-toxic ethanol is less hazardous for field surveys. Therefore, we strongly suggest that future surveys store all collected samples in >70% ethanol at -20°C. Ethanol that is either denatured or purified to concentrations >96% should be avoided, due to the presence of additional chemicals that hinder DNA preservation and interfere with downstream genetic applications. Alternatively, replicate samples collected during survey tows by means of a bongo net, or Folsom plankton splitter post-collection, can be preserved in both ethanol and formalin. Researchers working with nematodes have found that DESS, a solution containing a few common

laboratory chemicals, is capable of preserving morphological features and genetic integrity for extended periods of time at room temperature (Yoder et al. 2006).

Finally, we suggest that all routine fisheries work involving larvae should make use of taxonomists and geneticists in order to ensure both accuracy of results and efficient use of financial resources. Genetic barcoding and NGS techniques are legitimate tools that can support species identification and play a crucial role in fisheries management efforts.

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Chapter 3

A Fish by Another Name

Again the parent's pointed jaws comprest

By force expell them from their pleasing rest.

But void of all remorse the Tunnies feed

On their own spawn, and gulp th' enliven'd seeds

With strange repast the cruel parents blest

Devour their eggs, and praise the monstrous feast

-Oppian, 2nd century

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The following chapter has been accepted for publication in PloS ONE. G.N.Puncher composed the entire work but received a great amount of valuable input from his advisors at the University of Bologna: A. Cariani and F. Tinti. Additional co-authors provided advice on the manuscript's content and revisions.

Title Molecular identification of Atlantic bluefin tuna (*Thunnus thynnus*, Scombridae) larvae and development of a DNA character-based identification key for Mediterranean scombrids.

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3.1 Introduction

Atlantic bluefin tuna (BFT, *Thunnus thynnus*) are the largest of tunas roaming the world's oceans and have fed and fascinated humankind for millennia. Vast herds of BFT ply the cold waters of the North Sea and North Atlantic Ocean for prey in the winter, returning to spawning grounds in the Mediterranean Sea and Gulf of Mexico in the spring and early summer. Fisheries have long taken advantage of these migratory patterns, slaughtering multitudes of tuna by hook, gaff, harpoon and net as they migrated through coastal waters to feed Roman legions, burgeoning principalities, fishery empires and modern multinational corporations. This unrelenting appetite has recently brought stocks to the brink of collapse. Gone are the large BFT that spawned in the Black Sea and swelled the market places of Istanbul up until the mid 1980s (Mather et al. 1995; Karakulak et al. 2004). BFT stocks that inspired a revolution in fishing gear in the North Sea, sustaining a French, Norwegian, German, Dutch and Danish fleet for several decades crashed in 1963 (Mather et al. 1995). In the 1960s, a massive shoal of BFT that appeared off the coast of Brazil, was quickly targeted by Japanese long-liners and faded into the annals of history in as little as 7 years (Fromentin and Powers 2005; Fromentin et al. 2014a). After many years of decline, the fisheries organization responsible for managing BFT stocks, the International Commission for the Conservation of Atlantic Tunas (ICCAT), introduced a recovery plan and stocks have started showing signs of recovery, although the rate of recovery remains uncertain (Fromentin et al. 2014b; ICCAT 2014).

Currently, ICCAT manages BFT stocks as two populations: one which spawns in the Gulf of Mexico and forages in the North Western Atlantic (NWA) and a second that spawns in the Mediterranean Sea and forages in the Mediterranean and North Eastern Atlantic (NEA) (Block et al. 2005). Despite evidence suggesting that these two populations mix, ICCAT divides their ranges along the 45º longitude and manages them separately. Novel insights developed from an array of technologies including satellites tags, genetics and microchemistry suggest that the population structure of BFT is more complex (Rooker et al. 2008, 2014; Galuardi et al. 2010; Cermeño et al. 2012; Aranda et al. 2013; Riccioni et al. 2010, 2013). If regional population structuring exists, it is paramount for the welfare of the species that it be maintained, in order to conserve genetic biodiversity and evolutionary potential. An accurate and confident model of the population structure of BFT and the factors that affect their distribution is key to their continued viability.

In the past 15 years, several molecular techniques have been used in an effort to develop a more accurate vision of BFT population structure and dynamics in line with the results developed by electronic tagging campaigns and traditional ecological knowledge (summary and references in ICCAT 2013). Unfortunately, the results of these studies have been inconclusive and often contradictory. Due to the highly migratory nature of BFT, some research groups investigating the species' genetic population structure are now using only young tuna for their research as it is widely assumed that eggs, larvae and tuna of less than a few months age do not disperse far from their point of origin.

The location and abundance of early life stage fish is also used to improve stock assessments and our understanding of BFT spatial dynamics. Indices developed from the abundance of eggs and larvae collected from spawning sites have been used over 120 times to adjust and substantiate stock assessments of 18 different species within five teleost families throughout the world (Stratoudakis *et al.* 2006). For decades, scientists from ICCAT member nations have been using larval indices generated from surveys conducted in the Gulf of Mexico to calibrate Virtual Population Analyses (VPAs) of western Atlantic BFT (Scott et al. 1993; Scott and Turner 2003; Ingram et al. 2010). In 2013, the first standardized BFT larval indices for a Mediterranean spawning site were published based on larval surveys conducted by the Oceanographic Institute of Spain (IEO) around the Balearic Islands in the western Mediterranean (Ingram et al. 2013). Temporal shifts in BFT larvae abundance and condition can also provide important information about recruitment success, relative to short and long term environmental changes (Alemany et al. 2010; Lindo-Atichati et al. 2012; García et al. 2013). Surveys that have monitored the distribution of tuna larvae have shown that changes in relative abundances of different species are directly influenced by hydrodynamics (García et al. 2005a; Alemany et al. 2006; Aguilar et al. 2009; Alemany et al. 2010; Mariani et al. 2010; Reglero et al. 2012; Muhling et al. 2013). In the context of a rapidly changing environment, our ability to properly identify and monitor fish species throughout their life history is critical for effective wildlife management and conservation efforts (Costa and Carvalho 2007; D'Alessandro et al. 2010).

Unfortunately, early life stage fishes are often inaccurately identified by inexperienced technicians (Vecchione et al. 2000, Ko et al. 2013). These errors can lead to a misunderstanding of the spatial distribution of species, confusion over life history traits and population dynamics, inaccurate estimations

of recruitment rates, survivorship and stock biomass, and potentially disguise the collapse or recovery of localized spawning sites (Armstrong et al. 2001; Fox et al. 2005). The potential causes for misidentifications of tuna larvae are several: 1) Identification of tuna eggs and the larvae of some tuna species, using morphological characteristics alone, is very challenging, requiring an in depth knowledge of taxonomy, patience and experience (Richards and Pothoff 1974; Kohno et al. 1982; Richards 2006), 2) samples are often badly damaged during collection or as a result of preservation (Matsumoto et al. 1972; Richards et al. 1990; Paine et al. 2007), 3) some tuna identification keys are inaccurate and require updating (Puncher et al. unpublished), and 4) expert taxonomists in general are few and in demographic decline (Boero 2001; Hopkins and Freckleton 2002; Wilson 2003).

Due to the difficulties of identifying tuna larvae researchers occasionally outsource the task to distant laboratories such as the Sea Fisheries Institute, Plankton Sorting and Identification Center in Poland (Scott et al. 1993; Ingram et al. 2010; Matarese et al. 2011; Lindo-Atichati et al. 2012); however, misidentification of larvae has occurred at that facility in the past as well (F. Alemany personal communication). Others have simply resorted to assigning scombrids to lower taxonomic levels (Boehlert and Mundy 1994; Lindo-Atichati et al. 2012). Advances in molecular techniques and genetic barcoding now offer another solution to this problem.

There are a variety of ways in which researchers using genetic barcodes analyze their data in order to identify their specimens. The most commonly used tools for sequence association in barcoding studies are: 1) alignment with voucher sequences from online databases using Basic Local Alignment Search Tool (BLAST) tools provided by National Center for Biotechnology Information or the ID system provided by the Barcode of Life Database (BOLD), 2) Neighbour-Joining trees, and 3) classification using a molecular key of characteristic attributes. The BLAST program uses a heuristic algorithm to identify the sequences contained in GenBank that are most similar to the query sequence provided by the user (Altschul et al. 2009). The ID System by BOLD employs a Hidden Markov Model (HMM) for alignment construction and returns only sequence matches that are less than 1% divergent from the query sequence (Ratnasingham and Hebert 2007). Neighbour-Joining (NJ) trees are ubiquitous among barcoding publications. They use a hierarchical clustering method to construct a phenogram based on a distance matrix of similarity between reference and query sequences. These distance matrices can be constructed

by various methods which can impact species identification accuracy and measures of confidence. The Kimura 2-parameter model (K2P) for NJ trees has become the default model for most fish species identification studies; however, researchers have recently been challenging this assumption (Little and Stevenson 2007; Zhang et al. 2011; Collins et al. 2012; Srivathsan and Meier 2012; Collins and Cruickshank 2013).

This study describes how larvae collected from the Strait of Sicily, Western Ionian Sea and Levantine Sea were acquired from three different institutions for genomic analysis within ICCAT's Atlantic wide research programme for bluefin tuna (GBYP). All larvae had been provisionally identified as *Thunnus thynnus* by technicians using morphology-based methods. Each larva was barcoded using a 650bp fragment of the *CO1* gene and identified to species in an effort to assess the accuracy of identification. We have also compared the effectiveness of various methods used for associating sample sequences to reference or voucher sequences. We review the overall effectiveness of the Neighbour-Joining tree approach and compare two methods used for distance matrix construction (p-distance vs. K2P). Finally, we develop and assess a character-based key which uses unique genetic characteristics in much the same way as taxonomic keys that identify organisms based on diagnostic morphological features.

3.2 Materials and Methods

3.2.1 Sample collection

Strait of Sicily – Larval tows were performed by Istituto per l'Ambiente Marino Costero of the National Research Council of Italy (IAMC-CNR) in the waters off Sicily's southern coast (35°30'N-38°90'N, 12°38'E-15°10'E; Fig. 1), on board the R/V "Urania", during 17-21 July 2011 and 5-19 July 2012 using two Bongo nets with 40 and 90cm diameters equipped with 1 mm black mesh. Bongo nets were towed obliquely from the surface to 100 m and back to the surface at two knots. All larvae were preserved in 96% ethanol and transported to the laboratory where they were identified to family, genus or species level when possible, using various taxonomic keys (Pothoff and Richards 1970; Pothoff 1974; Fahay 1983, 2007; Kawamura et al. 2003). A total of 88 larvae with a mean length of 4.9 ± 1.5 mm and provisionally identified as tunas were sent to the GenoDREAM laboratory at the University of Bologna for genetic barcoding. An additional 4 non-scombrid larvae were also provided as outliers.

Fig. 1: Map of the Mediterranean Sea and surrounding area with three larvae sampling sites: A) Strait of Sicily, B) Ionian Sea and C) Levantine Sea.

Capo Passero, Ionian Sea – OCEANA's 2008 larval survey aboard the R/V Marviva Med was the first BFT larval survey undertaken by an NGO team in the Mediterranean (Aguilar et al. 2009). Larval tows were conducted 15 July - 11 August 2008 using a bongo 90 net with a quadrangular mouth opening equipped with 500 µm mesh for horizontal surface plankton tows east of Sicily (36°30'N-37°33'N, 15°35'E-15°59'E; Fig. 1). The net was towed at 2-2.5 knots at the surface for a constant duration of 10 minutes. Upon removal from the sea, all larvae were immediately preserved in ethanol. All larvae were then dispatched to experts at IEO facilities in Palma de Mallorca where they were identified to species by experts. A total of 58 larvae identified as *T. thynnus* were dispatched to the GenoDREAM laboratory in November 2013 for genetic analysis.

Levantine Sea - A larval cruise was conducted by Istanbul University during 20-24 June 2012 along the southern coast of Turkey (36°07'N-36°10'N, 33°33'E-33°47'E; Fig. 1). Larvae were collected from surface waters using a Bongo net of 90 cm diameter and 1 mm mesh size, towed at 2-2.5 knots for 10 minutes. All captured larvae were immediately preserved in 96% ethanol and identified to species level on-board using microscopes and the taxonomic key by Richards (2005). A total of 38 larvae with a mean length of 6.9 ± 1.5 mm were provisionally identified as *T. thynnus*. These larvae were then bisected and the caudal sections were dispatched to GenoDREAM lab for genetic verification.

Ethics Statement

All larvae used in this study were collected from the Mediterranean Sea using plankton nets and sacrificed via immersion in 96% ethanol, according to standard larval survey practices. Permission to conduct the larval surveys and all legal permits were granted by the International Commission for the Conservation of Atlantic Tuna (ICCAT) and the General Fisheries Commission for the Mediterranean. ICCAT is the intergovernmental organization responsible for the management of bluefin tuna in the Atlantic Ocean and Mediterranean Sea and all research was coordinated through their "Atlantic-wide research programme for bluefin tuna" (GBYP). ICCAT issued a recommendation (Rec. 11-06) allowing the parties involved in this research to collect and sacrifice larvae for the purposes of genetic research as well as ship samples from one country to another (Certificate No. ICCAT RMA12-049).

3.2.2 DNA extraction

All 188 scombrid and non-scombrid larvae were digested overnight in a Proteinase K solution and genomic DNA was extracted from each and purified using Promega's Wizard®SV96 Genomic DNA Purification kit and vacuum manifold. Fragments of the *CO1* gene (~ 650 bp) were amplified (PCR) using FishF2 (5'-TCGACTAATCATAAAGATATCGGC- AC-3') and FishR2 (5'-ACTTCAGGGTGACCGAA GAATCAGAA-3') primers first published by Ward et al. (2005). PCR reactions were performed in 50 µL volume consisting of 1x PCR Buffer, 1.0 µM of each primer, 160 µg/mL of BSA, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$, 2.5 U/mL of Invitrogen Taq polymerase and ~100 ng of template DNA. PCR conditions consisted of 94°C for 3 min, 35 cycles of 30 sec at 94°C, 30 sec at 52°C, and 30 sec at 72°C, with a final extension at 72°C for 3 min. Due to the introgression of the mitochondrial genome of *T. alalunga* into the *T. thynnus* gene pool (Alvarado Bremer 1997; Chow et al. 2006; Paine et al. 2007; Ward et al. 2009; Viñas and Tudela 2009), all larvae that were identified as *T. alalunga* using the *CO1* gene were then barcoded using sequences from the *ITS1* region. DNA extractions from archived tissue samples of adult *T. thynnus* (2) and *T. alalunga* (2) were used as reference standards for all further analyses. Fragments of the *ITS1* gene (~680bp) were amplified using the *ITS1*F (5'-TCCGTAGGTGAACCTGCGG-3') and *ITS1*R (5'-CGCTGCGTTCTTCATCG-3') primers designed by Chow et al. [60]. All other PCR reagents were the same as above and conditions were as follows: 94°C for 3 min, 35 cycles of 30 sec at 94°C, 30 sec at 50°C, and 30 sec at 72°C, with a final extension at 72°C for 3 min.

After receiving all *CO1* sequences from Macrogen Europe (Amsterdam, Netherlands), they were aligned in MEGA v.6 (Tamura et al. 2013) using the ClustalW algorithm and trimmed to 612bp.

3.2.3 BLAST and BOLD search engines

All larval sequences were converted to FASTA format and submitted to a nucleotide BLAST through the NCBI website (http://www.ncbi.nlm.nih.gov/). The first five sequence matches with highest similarity to database references (max ident) were analyzed for species consistency. Similarly, all sequences were submitted to BOLD's Identification System (http://www.boldsystems.org/) for comparison with all species level barcode records of animals (148,815 sequences as of 09 Nov 2014). All matches were analyzed using the percent similarity score.

3.2.4 Neighbour joining tree analysis

Ten reference sequences from each of thirteen scombrid species were downloaded from BOLD (n=122) and GenBank (n=8) (Appendix 1). Sequences were mined from GenBank only when the number of sequences on BOLD were insufficient. Aside from the BFT reference sequences, eight of the species occur in the Mediterranean Sea and may have larvae associated with those of BFT (*Auxis rochei, Auxis thazard, Scomber colias, Scomber scombrus, Euthynnus alletteratus, Katsuwonus pelamis, Sarda sarda, T. alalunga,*). The remaining four species are closely related to the other species and were included in order to rule out the occurrence of genetic introgression (*Scomber japonicus, Thunnus albacares, Thunnus maccoyii, Thunnus atlanticus*). Reference sequences were sourced from throughout each species' geographic distribution in order to have ample representation of genetic variation. Various phenograms (all sequences, only reference sequences, all larvae and reference sequences belonging to Mediterranean species) were built using the Neighbour Joining method (Saitou and Nei 1987) with both the Kimura-2-parameter (K2P) distance model (Kimura 1980) and p-distances (Nei and Kumar 2000)] for distance matrix construction. The statistical support of each node was tested using the bootstrap (Felsenstein 1985) analysis with 1000 replications in MEGA6 (Tamura et al. 2013). Selected trees were modified using FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

3.2.5 Assignment by characteristic attribute key

All variable sites of an alignment containing all reference and larvae sequences were highlighted using MEGA6. This simplified alignment was inspected for loci containing unique nucleotides that were diagnostic of particular species. The character-based key constructed here differs from those constructed by Lowenstein et al. (2009) in that it requires first assignment to genus and then species. In this way we have used nucleotides that may not be unique to single species or genera. Rather we are providing a multi-step assignment tool which uses multiple characteristics, much like a taxonomic key. According to the terminology used by Lowenstein et al. (2009), we have selected "pure characteristic attributes" (PCAs), or specific nucleotides located at variable sites that are unique to single clades and thus diagnostic for single clades. Some of these characteristic attributes stand alone and called "simple CAs" whereas other loci should be used in combination and are thus called "compound CAs". Once the key was constructed all larval sequences were assigned to species accordingly.

3.3 Results

DNA extractions from all larve were successfully amplified, sequenced and analyzed. Sequences from 84 larvae with photograohic records were uploaded to the Barcode of Life Database (Project: MLRV; Accession numbers: MLRV001-15 to MLRV084-15). All other sequences were uploaded directly to the GenBank database (KT003822 to KT003924). A few unexpected challenges were encountered concerning the reference sequences downloaded from both BOLD and GenBank. One *T. thynnus* reference sequence (BOLD: GBGCA443-10; GenBank: GQ414572) affiliated with the work of Viñas and Tudela (2009) corresponds with *CO1* sequences of *T. alalunga*. Within that work, the authors clearly state that this sequence belongs to a BFT with introgressed mtDNA from albacore; however, no mention of this is associated with the sequences itself in either database. They also describe how two sequences belonging to Pacific-like *T. thynnus* (Atlantic bluefin with introgressed *T. orientalis* mitochondria) were used and published in GenBank (GQ414570 and GQ414573). Both of these sequences were later incorporated into the BOLD database (GBGCA445-10 and GBGCA442-10, respectively); however, in both databases GQ414570 is identified as *T. thynnus*, while QQ414573 is identified as *T. orientalis*. Another sequence featured in [61], which they referred to as *T. orientalis,* has since been uploaded to GenBank (GQ414566) and BOLD (GBGCA449-10) under the title of *T. thynnus*. BOLD and BLAST queries, as well as our CA

key, clearly identify this as a *T. orientalis* sequence. It is likely that the authors have confused sequences and their IDs at some point (confirmed by J. Viñas). Finally, a single *T. orientalis* sequence (GenBank: JN097817; BOLD: GBGCA1390-13) uploaded by several researchers from the South Korean National Fisheries Research & Development Institute matches those of *T. thynnus.*

3.3.1 BLAST assignment

For all but two sequences, BLAST provided a species match with an identity similarity higher than 99% (Table 1). The median value for maximum similarity scores across the type five selected matches for each larva was 100%. The remaining two larvae from the Strait of Sicily received identity similarity scores of 93% and 95% for sequences belonging to bullet tuna (*Auxis rochei*, Scombridae) and *T. thynnus*, respectively. Overall, only 42% of larvae were identified as *T. thynnus.* In fact, nearly as many (39%) were identified as bullet tuna.

Table 1: Species and origin of larvae identified with *CO1* and *ITS1* markers, neighbour-joining reconstruction and BLAST.

Species	Strait of Sicily	Capo Passero	Levantine Sea
Auxis rochei	53	0	21
Eythynnus alleteratus	2	0	12
Scomber japonicus	1	0	0
Thunnus alalunga	11	0	0
Thunnus thynnus	21	58	0
Non-scombrid larvae	4	0	5
Total	92	58	38

The larvae collected in the Strait of Sicily were composed of five different scombrid species with only 21 identified as *T. thynnus*. Although the samples from the Levantine Sea contained fewer taxa, none of the larvae provided were identified as *T. thynnus*. All larvae collected from the Ionian Sea, offshore from Capo Passero were identified as *T. thynnus* and received identity similarity scores of 100%. The four non-scombrid larvae from Sicily were identified as *Chromis sp.*, picarel (*Spicara smaris*, Centracanthidae), greater weever (*Trachinus draco*, Trachinidae), and pygmy lanternfish (*Lampanyctus pusillus*, Myctophidae). Five larvae from the Levantine Sea were assigned to two additional outlier taxa: brown comber (*Serranus hepatus*, Serranidae) (2) and common pandora (*Pagellus erythrinus*, Sparidae) (3). All larvae provisionally identified as albacore using *CO1* sequences and later barcoded with the *ITS1* gene clustered with *T. alalunga* standards (data not shown), thereby ruling out the possibility of false species identification due to hybridization/mtDNA introgression.

3.3.2 BOLD assignment

All larvae were identified by BOLD with a confidence score of 99-100%, aside from the two individuals discussed above for which a "no match" was returned. No match was given by BOLD because of the program's 1% divergence threshold. All other specimen identifications were equal to those provided by BLAST.

3.3.3 Neighbour-joining tree analysis

Neighbour joining tree analysis using *CO1* sequences produced well-defined clusters of candidate larvae with reference sequences (Fig. 2). All larvae clustered with reference taxa in the same manner as the BLAST and BOLD results. Phenograms containing only the larvae and reference sequences for species found in Mediterranean Sea showed lowest bootstrap probability (BP) for branch nodes separating the true tunas (BFT and albacore) from the other scombrids (BP = 33 - 35) and a clade containing the true tunas and *E. alletteratus* from the other species (BP = 30 – 34). The node containing the *A. rochei* reference sequences and 74 larvae was the least stable with BPs of 53 – 59. All other nodes had BPs > 80.

Phenograms based on distance matrices using p-distances (Fig. 3) had consistently higher BP values than those built with K2P distances (Fig. 2). P-distance based phenograms also tended to exclude larval sequences from clusters, as is the case with one larva in the Auxis spp. cluster in Fig. 2. When the larval sequences were removed from the alignments, all BP values increased (Fig. 3). Predictably, BP values for the A. rochei (BP = 39) and T. thynnus (BP = 17) nodes increased most dramatically. When *T. atlanticus*, *T. maccoyii*, *T. obesus* and *T. albacares* reference sequences were included in the alignments, the neighbour-joining tree based on p-distances failed to differentiate clusters for each and combined all within the same cluster, alongside the *T. thynnus* and related larvae sequences (BP = 61; Fig. 4). The topology of this same clade in the corresponding K2P-based phenogram changed somewhat but the composition remained the same, albeit the BP value was much lower (BP = 35).

The *ITS1* NJ tree constructed from sequences of voucher specimens and larvae identified as *T. alalunga* did not reveal any introgressed BFT.

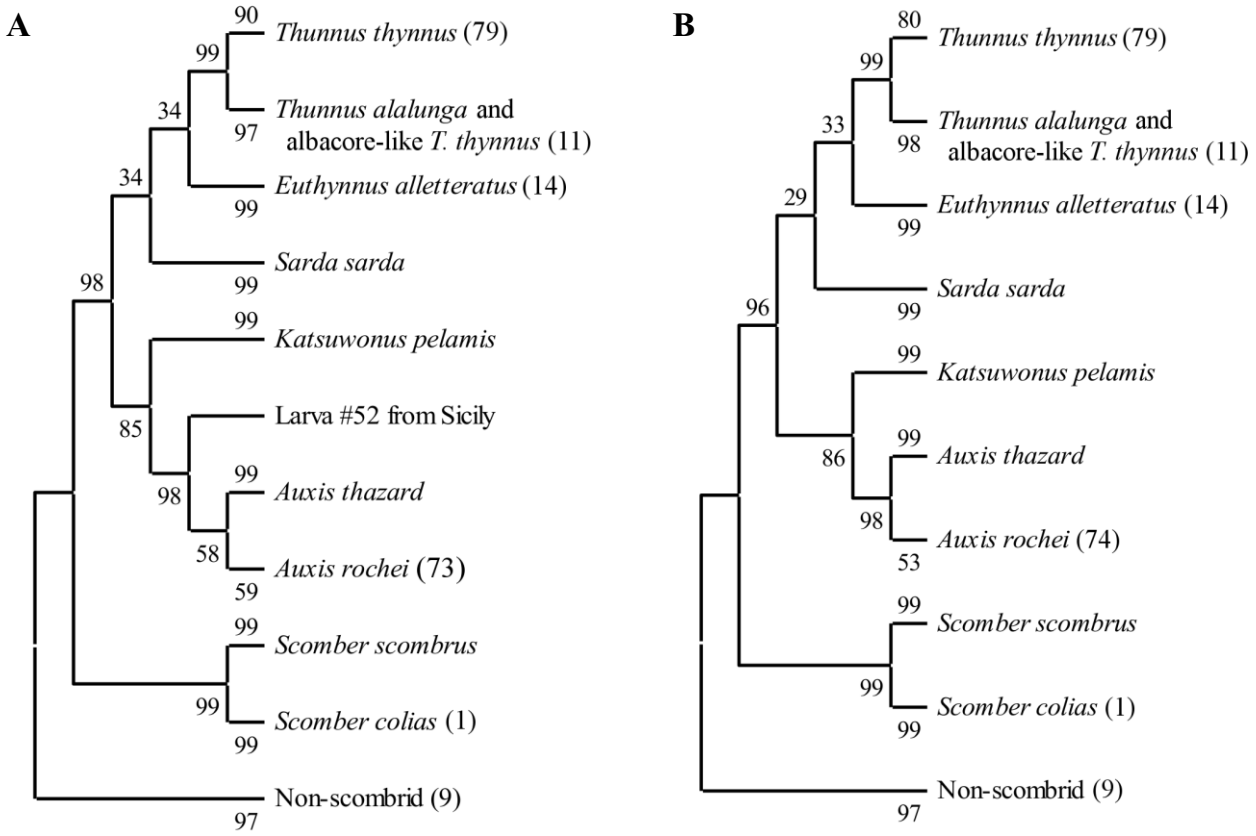

Fig. 2: Neighbour-joining phenograms of Mediterranean scombrid reference sequences clustered with number of unknown larvae in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to each branch (Felsenstein 1985). All ambiguous positions were removed for each sequence pair. There were a total of 612 nucleotide positions in the final dataset. (A) The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 280 nucleotide sequences. (B) The evolutionary distances were computed using the Kimura 2-parameter model [64] and are in the units of the number of base differences per site. The analysis involved 280 nucleotide sequences.

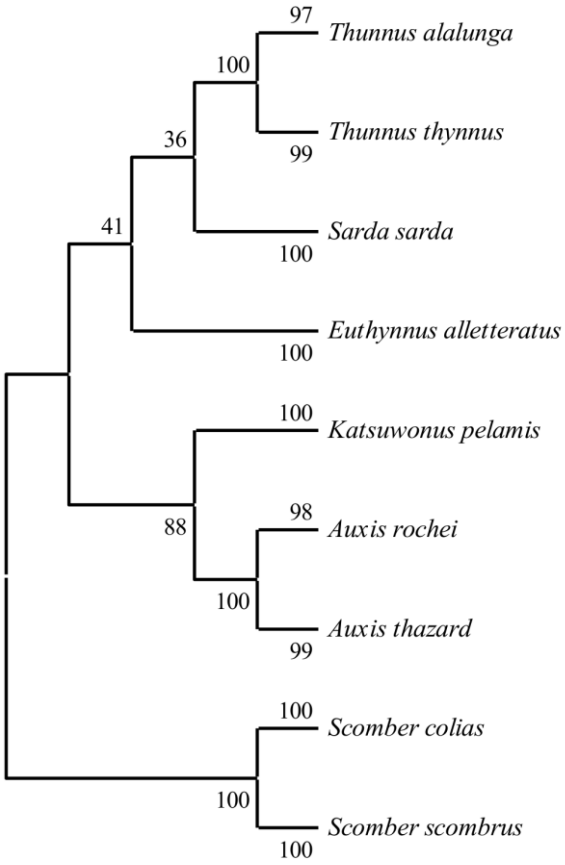

Fig. 3: Neighbour-joining phenogram of Mediterranean scombrid reference sequences only. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 91 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 612 nucleotide positions in the final dataset.

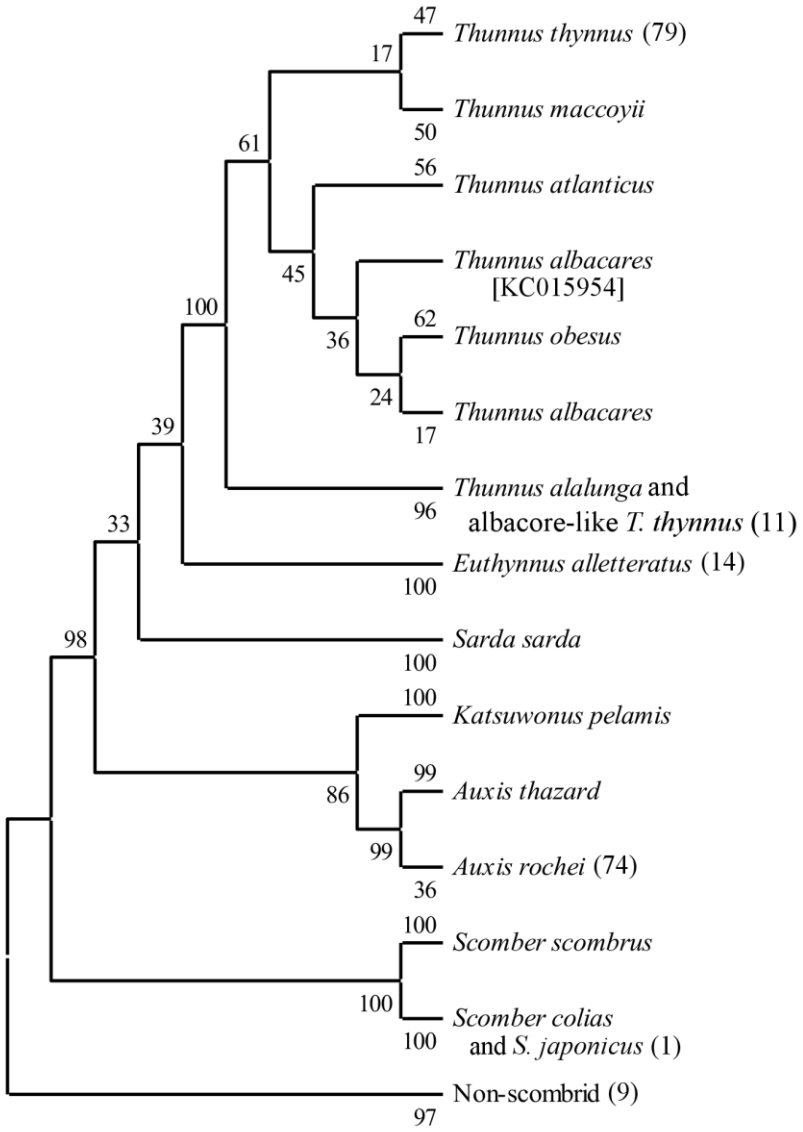

Fig. 4: Neighbour-joining phenogram of reference sequences (including non-Mediterranean *Thunnus* species) clustered with number of unknown larvae in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 330 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 612 nucleotide positions in the final dataset.

3.3.4 Character-based assignment

Construction of a character-based key uncovered 68 nucleotides capable of distinguishing all reference taxa. As the primary purpose of our research was the isolation of *T. thynnus* larvae from the rest of the catch, *Thunnus spp.* sequences were the first to be isolated using four diagnostic nucleotides (Table 2). Using these nucleotides a total of 90 larvae were identified as members of the *Thunnus* genus, representing less than half of all larvae. Six bases separating *T. alalunga* reference sequences from the other five *Thunnus* species identified eleven albacore tuna among the larvae. A single thymine located at position 231 of our alignment was capable of isolating all *T. thynnus* references from the other species of the same genus. When these criteria were applied to the larvae, a total of seventy-eight larvae were identified as bluefin tuna. One outstanding individual was identified as *T. maccoyii*, *T. atlanticus*, *T. albacares or T. obesus* using these criteria. Since none of these species can be found in the Mediterranean Sea, this is likely an example of genetic introgression. Three bases, found to be discriminatory for the *Auxis* species of the Mediterranean, identified seventy-four larvae among the remaining samples. All of these *Auxis* candidate larvae were identified as *A. rochei* using eight bases that were found to discriminate between *A. rochei* and *A. thazard* reference sequences. Using six diagnostic loci, fourteen *Euthynnus alletteratus* larvae were identified. Eleven characteristic loci set the *Scomber spp.* apart from the other species and ten loci were used to identify a single larva. Six diagnostic bases were found for *Katsuwonus pelamis* and *Sarda sarda* but none of the larvae analyzed were identified as members of either species. Since the character-based identification key was developed for the identification of scombrids only, the remaining nine larvae could not be identified.

3.4 Discussion

3.4.1 (Mis)Identification of larvae

The 11 species identified among the samples are typical of plankton surveys conducted in the region and during this sampling season. BFT larvae are commonly associated with larvae of other scombrids, having been previously captured with dense concentrations of bullet tuna off the coasts of Tunisia (Giovanardi and Romanelli 2010; Koched et al. 2012), Sicily (Aguilar et al. 2009) and the Balearic Islands (Alemany et al. 2010). They have also been found alongside albacore tuna (Aguilar et al. 2009; Alemany et al. 2010) and Atlantic black skipjack (Euthynnus alletteratus, Scombridae) (Alemany et

al. 2010). Among these species, BFT are found in proportionally higher concentrations in deep offshore waters beyond shelf breaks (Alemany et al. 2006; Koched et al. 2012). Among these species, BFT are found in proportionally higher concentrations in deep offshore waters beyond shelf breaks [25,70]. Researchers in Italy (Cuttitta et al. 2004) captured *L. pusillus* larvae in the Strait of Sicily in June-July 2000. Larvae of *S. hepatus, Chromis chromis* (Pomacentridae)*, P. erythrinus* and *T. draco* have been captured in the Aegean during June of 2003-2006 (Isari et al. 2008). Alemany et al. (2006) have encountered all the species that we have identified in the Balearic Islands, aside from *P. erythrinus* and Atlantic chub mackerel (*Scomber colias,* Scombridae). Accurate identification of these samples to species level is a testament to the versatility of the genetic marker used and the potential of the ever-expanding resources of the Barcode of Life Database.

Table 2: Characteristic attributes capable of distinguishing scombrids in the Mediterranean Sea. The position of each nucleotide is given in relation to 612bp alignment of all sequences. Diagnostic nucleotides at each locus are given in parentheses.

Taxa	**Diagnostic nucleotides**
Thunnus spp.	327[A], 372[G], 525[T], 540[T]
Thunnus alalunga	228[T], 273[G], 438[C], 495[T], 606[G], 609[T]
Thunnus thynnus	231[T]
T. thynnus, *T. albacares*, *T. maccoyii*, *T. obsesus, T. atlanticus*	228[C], 273[A], 495[C], 606[A], 609[C]
Auxis spp.	393[T], 453[T], 456[C] (all 3 must be part of the package).
Auxis rochei	225[T], 247[T], 315[C], 336[T], 348[C], 465[A], 468[A], 486[T]
Auxis thazard	225[C], 247[C], 315[T], 336[C], 348[T], 465[G], 468[G], 486[C]
Euthynnus alletterratus	303[G], 312[A], 408[G], 426[G], 498[G], 553[T]
Scomber spp.	81[T], 127[G], 210[A], 235[C], 249[G], 258[G], 260[C], 351[C], 393[C], 434[G], 519[T]
Scomber colias/japonicus	93[C], 192[T], 225[G], 240[G], 306[C], 312[G], 321[A], 342[T], 414[C], 423[A], 436[G], 438[A], 561[T], 612[C]
Scomber scombrus	67[G], 72[T], 129[C], 240[A], 303[A], 306[A], 321[G], 507[G], 543[T], 546[T]
Sarda sarda	216[T], 258[T], 264[C], 279[G], 543[G], 567[T]
Katsuwonus pelamis	366[T], 378[T], 390[A], 501 [G], 555[G], 582[T]

The high number of correctly identified BFT larvae within the Sicilian samples was expected, since large quantities *of T. thynnus* are typical of the Sicilian Channel, western Ionian and southern Tyrrhenian Seas (Sanzo 1932; Piccinetti et al. 1997). To date, BFT larvae have been found in highest concentration in Mediterranean waters around Sicily, the Balearic Islands and the southern coast of Turkey (Tsuji et al. 1997; García et al. 2005a, b, 2013). It comes as no surprise that all 58 larvae provided by the IEO and OCEANA-Marviva project were correctly identified, since their staff are leading experts in the field of ichthyoplanktology. However, the fact that no BFT larvae were provided amongst the 38 larvae received from the Levantine Sea calls into question all previous publications on the subject of BFT reproduction in that area. For example, Oray and Karakulak (2005) captured 121 bluefin tuna (*T. thynnus*), 94 bullet tuna (*A. rochei*) and 22 Atlantic black skipjack (*E. alletteratus*) larvae in the Northern Levantine basin during larval surveys conducted in 5-18 June 2004. Their publication established a benchmark in the literature by which many assumptions of BFT movements, reproductive behaviour and population structuring has been made. However, during that survey, the researchers, capture protocols and larvae identification methods were the very same that procured the misidentified larvae discussed herein. The possibility that larvae from the Levantine Sea have been misidentified in the past, demands a review of the timing, location and extent to which BFT are spawning in the eastern Mediterranean. If BFT spawning areas are indeed limited in number, then their accurate identification and subsequent conservation from over-exploitation, habitat alteration and pollution is critically important (Hyde et al. 2005).

3.4.2 DNA extractions and use of molecular markers

It is noteworthy that we were able to extract high quality DNA, and identify to species level, larvae that had been archived in ethanol at room temperature for over 5 years. This possibility should be taken into consideration for all collections containing similarly preserved wildlife specimens. Additionally, the molecular markers used were effective at identifying all larvae to species level. The use of *CO1* for sample identification has been criticized, as it lacks the capacity of discrimination among the Neothunnus tribe and the Pacific and Atlantic BFT (Ward et al. 2005; Viñas and Tudela 2009). Since none of the members of the Neothunnus tribe or *T. orientalis* are present in the Mediterranean Sea, this was not a concern for our study. Our reconstruction of NJ trees including the additional non-Mediterranean *Thunnus* spp. sequences were also unable to reliably distinguish the members of that genera, aside from *T.*

alalunga which consistently clustered independently from the others. Some of the earliest molecular work using a suite of allozymes also found albacore to be most divergent and uncovered very little divergence between *T. albacares*, *T. maccoyii* and *T. orientalis* (Elliott and Ward 1995). DNA sequences from the mitochondrial control region suggest that BFT is a sister taxa of the southern bluefin tuna and that the albacore and Pacific bluefin tuna (PBFT) form a divergent monophyletic clade (Alvarado and Bremmer 1997). The high number of diagnostic loci contained in our *CO1* sequences which discriminate between the albacore and the other Thunnus species support this claim. Clearly, the level of divergence between species is dependent upon the character used for comparison. For example, the high level of divergence between the Pacific and Atlantic bluefin tuna shown when analyzing the mitochondrial control region (Alvarado Bremer et al. 1997) vanishes when comparing ITS1 gene sequences (Chow et al. 2006). This could be a result of historical hybridization of the PBFT with albacore which resulted in the transfer of the albacore mtDNA genome into the PBFT line (Elliott and Ward 1995).

The presence of erroneous or misleading reference sequences in both GenBank and BOLD is both troublesome and concerning. Surely, it was not the intention of the founders of these databases that users should have to check the origin and associated publications of every sequence. The presence of sequences belonging to hybrid organisms in genetic reference databases is confusing and requires additional traceability and documentation.

3.4.3 BLAST and BOLD assignment

The results generated by BOLD's global alignments and BLAST's local alignments were consistent with one another. The identity similarity threshold used by BOLD prevented the identification of two larvae from the Strait of Sicily, whose identity were later confirmed by BLAST, NJ trees and our character-based identification key. Lowenstein et al. (2009) complained that BOLD performed poorly during their attempts to identify species used in sushi but the database was still in its infancy then and it has come a long way since and now contains over 4 million collected from 146 countries. During this period of rapid growth, BOLD began featuring sequences mined from GenBank in their Public Data Portal. We have found several sequences that are in clear violation of the data standards that were established when BOLD was first introduced. The founders of BOLD have clearly stated that sequences do not undergo any kind of centralized review and that the quality of data featured in the database is

ultimately dependent upon the individuals that have uploaded the data [48]. At the moment, more than half of the *Thunnus thynnus* sequences featured in BOLD have been mined from GenBank. This hybridization of these two databases, without BOLD's once lauded traceability standards, threatens to undermine the reputation and usefulness of BOLD.

3.4.4 Neighbour-joining trees

The NJ trees accurately identified larvae to species level for taxa with reference sequences included in the alignments. Clearly, for this approach to work, query larvae must cluster with voucher sequences; an obvious disadvantage when compared to the BLAST and BOLD approaches. The use of NJ trees for species identification has recently come under fire from various sources (Little and Stevenson 2007; Collins and Cruickshank 2013). An entire section focused on NJ trees has been featured in a recent publication entitled "The seven deadly sins of DNA barcoding" (Collins and Cruickshank 2013). A major disadvantage associated with NJ trees surfaces when individual sequences are assigned to the space between two reference clusters. On these occasions researchers are forced to retreat to lower taxonomic levels (Paine et al. 2008). By increasing the number of sequences used for each reference taxa, one can decrease the frequency of these ambiguous outcomes (Ross et al. 2008). Our results suggest that the models used for the assembly of distance matrices are also important for the reduction of ambiguous results. Although the K2P model is the most widely used model for NJ tree construction in fish barcoding studies, we have found that distance matrices based on p-distance provide higher BP values. Recent critical reviews of NJ tree construction agree that identification of species using NJ trees based on K2P distances can be inappropriate to the task and more suitable, less complex models can prove more effective (Collins et al. 2012; Srivathsan and Meier 2012). In fact, Collins et al. (2012), wrote that K2P "was without exception a poorly approximating model at the species level". Why the K2P model is so widely used in fish barcoding studies is a mystery. Srivathsan and Meier (2012) suggest that the widespread use of K2P is a result of its use by early barcoding proponents who wanted to highlight the extreme differences between species. Perhaps researchers are simply following the examples of their peers or that of BOLD which uses the K2P model for taxa identification trees (Ratnasingham and Hebert 2007).

3.4.5 Character-based assignment

Realizing the faults inherent in the NJ tree approach, some researchers have found that a character-

based method of specimen identification has proven more appropriate to the task (Richardson et al. 2007; Lowenstein et al. 2009). Paine et al. (2007) constructed a character-based key for identification of degraded tissue samples using reference sequences from 17 species of the Scombridae common to the Western Atlantic Ocean. Our molecular key differs significantly, since the molecular key of Paine et al. (2007) begins with position 575 of our alignment, thereby providing only 37 bases for comparison. The molecular key of Lowenstein et al. (2009) was generated from alignments shifted only 40 base pair positions towards the 3' end of the *CO1* sequence. The molecular key of Lowenstein et al. (2009) includes *Thunnus* species only, as they were making efforts to develop a tool for seafood traceability. Interestingly, they did not include in their study the skipjack tuna, *Katsuwonus pelamis*, a globally cosmopolitan fish and by far the world's most important tuna fishery in the globe. They, too, discovered the same individual nucleotide that distinguishes *T. thynnus* from all other tunas. Many of the diagnostic loci discovered by Lowenstein et al. (2009) no longer function after the inclusion of the *Scombrus* spp. and *Auxis* spp. sequences. Character-based keys for scombrid species have also been developed for the mitochondrial control region and *ITS1* (Alvarado-Bremer et al. 1997; Chow et al. 2006).

3.5 Conclusion

Misidentification of early life stage fishes has already occurred in various commercial species, leading to inaccurate estimations of spawning stock biomass (Daniel and Graves 1994; Fox et al. 2005). We have shown here, for the first time that tuna larvae collected from the Mediterranean Sea have been misidentified by larval survey crews. If the larvae and eggs of BFT are to be used to improve stock assessments in the future, it is imperative that problems associated with species identification are resolved. Fishery independent data, such as larval abundance, are certainly welcome for the betterment of stock assessments where traditional fishery data has lost its credibility; however, scientific rigour and quality control must also accompany this data or we run the risk of repeating the mistakes of the past. Genetic barcoding is a legitimate technique that can support species identification and play a crucial role in fisheries management efforts. We suggest that all routine fisheries work involving larvae should make use of both morphological taxonomists and geneticists in order to ensure both accuracy of results and efficient use of financial resources. We also call upon the few taxonomic world experts to update the identification keys associated with fish species of economic and conservation concern, embrace the digital community

and pass their knowledge onto new generations through training courses, either *in situ* or with the various Information and Communications Technology platforms now available. Most barcoding efforts are dependent on online databases for voucher sequences; therefore, it is crucial that quality standards are upheld if the barcoding effort is to retain its legitimacy. Enough doubt has been cast on the inappropriate use of NJ trees for specimen identification purposes that they are now regarded by many simply as an attractive communication tool; however, they are still being widely used. Conversely and despite nearly two decades of use in scombrid identification, character-based keys are still not used as a reliable and recognized tool. Perhaps it is the user-friendly automated style of NJ trees that have kept phenograms popular, despite their fallibility. Several efforts are being made to automate the generation of character-based keys and their use as specimen identification tools. The BOLD System now features a Diagnostic Characters analysis suite and R has a package (Spider) dedicated to their use (Brown et al. 2012). Character-based keys also have potential for translation into microarrays and high throughput NGS genotyping platforms. Certainly, standardized high throughput genotyping tests will have profound impacts on the future of larvae identification and fisheries surveys.

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Appendix 1. Reference sequences used for alignments, phenogram analysis and CA development. Unless stated otherwise, all sequences were recovered from BOLD.

Auxis rochei (DSFSF123-09, AB105165.1 [GenBank], AB103468.1 [GenBank], GBGC0053-06, GBGC1666-06, GBGC3356-07, GBGC3358-07, GBGC3359-07, GBGC3360-07, GBGC7423-09)

Auxis thazard (TOBA316-09, FOA810-04, GBGC1669-06, GBGC3350-07, GBGC3351-07, GBGC3354-07, GBGC3355-07, GBGC7414-09, GBGC7415-09, GBGC7416-09)

Scomber colias (FCFPS032-06, AB488406.1 [GenBank], FCFPS117-06, FCFPS118-06, GBGC3320-07, GBGC3321-07, GBGC3322-07, GBGC3323-07, GBGC3324-07, DSFSF201-09)

Scomber japonicus (HQ611117.1 [GenBank], DSFSF202-09, DNATR081-12, MFC235-08, FARG481-08, FARG482-08, FARG486-08, FMV165-08, RFE268-05, RFE269-05)

Scomber scombrus (FCFP095-05, FCFPS040-06, FCFPS041-06, FCFPS152-06, FOA796-04, FOA800-04, SCAFB741-07, SCFAC837-06, SCFAD497-09, GLF063-14

Euthynnus alletteratus (AB099716.1 [GenBank], BZLWE263-08, CSFOM035-10, DNATR775-13, DNATR775-13, DNATR776-13, DNATR777-13, LIDM537-07, MFSP619-10, MFSP620-10, MXII103-07)

Katsuwonus pelamis (MXII150-07, DSFSF147-09, ANGBF6825-12, ANGBF6832-12, ANGBF6837-12, GBGC1667-06, GBGC4959-08, GBGC4960-08, GBGC4961-08, GBGC4962-08)

Sarda sarda (DNATR075-12, DNATR1220-13, DNATR1221-13, DNATR1223-13, DNATR1239-13, CSFOM065-10, MLFPI110, MLFPI227, MLFPI99, MLFP100)

Thunnus alalunga (FOA868-04, GU256526.1 [GenBank], JN086151.1 [GenBank], CSFOM079-10, GBGCA665-10, GBGCA675-10, GBGCA706-10, RFE231-05, RFE233-05, RFE404-05)

Thunnus albacares (ANGBF6823-12, FOA869-04, FOA870-04, MFC185-08, RFE248-05, RFE250-05, SAFC038-11, SCFAC184-05, TZMSC142-05, WLIND461-07)

Albacore -like Thunnus thynnus (GBGCA443-10)

Thunnus thynnus (FOA947-05, FOA948-05, FOA945-05, DNATR1723-13, DNATR1724-13, GBGC0049-06, GBGC0803-06, GBGCA446-10, SCFAC660-06, SCFAC661-06)

Thunnus maccoyii (FJ605741.1 [GenBank], FOA874-04, FOA875-04, FOA876-04, FOA877-04, GBGCA176-10, GBGCA678-10, GBGCA679-10, GBGCA720-10, GBGCA731-10)

Thunnus obesus (SAFC026-11, ANGBF6817-12, ANGBF6820-12, ANGBF6821-12, FCHIL181-06, FCHIL182-06, FOA881-04, FOA882-04, GBGCA722-10, GBGCA726-10)

Thunnus atlanticus (FOA952-05, MEFM1002-06, MEFM1003-06, MEFM1004-06, MEFM1030-06, MFSP1883-11, MFSP1884-11, MXII115-07, MXII119-07, TOBA079-09)

Chapter 4

Discovery of single nucleotide polymorphisms in the bluefin tuna genome using a genotyping-by-sequencing approach.

May 27, 2014 – Inigo Rodriquez entered into the classroom with his head hanging low, avoiding the inquisitive stares of his classmates. He silently dropped his bag to the floor beside his desk, nudged his chair aside, sat down with a heavy sigh and laid his head down on the desktop. The other students knew it wasn't wise to press the obviously morose Inigo and turned their gaze forward. The sunshine from the classroom window blinded the little boys sleepy eyes and he was once again sailing across a sea of blue by his father's side. After years of anticipation he had finally been invited to set sail with his father and uncle on the family's tuna purse seiner. He had worked into the night while his friends played in the waning spring sunsets. His father had managed to clear three weeks from his school calendar and the whole town had known that Inigo was setting sail to hunt the mighty bluefin with his father, as the Rodriguez boys had done for generations. But it hadn't lasted three weeks. Not even 24 hours. After only 23 chaotic hours of madness, his uncle had received the call over the radio. The quota was caught.
The sound of his teacher's voice barely registered as thoughts swam in circles in his mind. He blinked away a tear of frustration and balled his fists. His teacher carried on, knowing it unwise to interrupt the boy's meditation. Inigo didn't care to listen to his teacher as he was occupied with greater matters than algebra. Why had the government stopped them from fishing on the first day at sea, when there were obviously so many tuna still in the ocean? It just wasn't fair!

Publication Note:

The following two chapters decribe the accomplishments of a consortium of partners funded by the International Commission for the Conservation of Atlantic Tuna. The majority of the analysis described below has been executed by the G.N. Puncher along with the written content of the chapters. Samples were provided by the various groups associated with the author affiliations and DNA extractions were conducted at AZTI Tecnalia, the University of the Basque Country and by G.N. Puncher at the University of Bologna. All Next Generation Sequencing library preparation, sequencing and data preparation occurred at KULeuven in Belgium with direct consultation from G.N. Puncher and A. Cariani at the University of Bologna. The contents of Chapters 4 and 5 are currently being drafted for publication.

Title Population structure and genetic management unit delineation in the bluefin tuna using a genotyping-by-sequencing approach.

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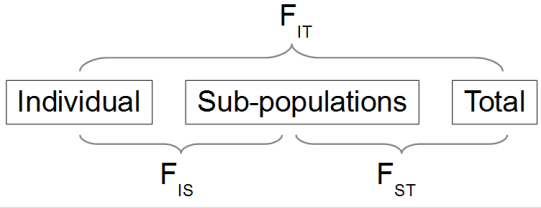
4.1 Introduction

Molecular tools are increasingly used by fisheries researchers to inform management decisions. Over the past three decades, various genetic techniques have been used to identify the species of adult fish and larvae (fragment length polymorphisms, barcoding, metagenetics) in order to understand species distribution, movement and habitat use. These techniques are now being used to investigate various forms of seafood fraud such as mislabeling, laundering and falsification of documentation (Lowenstein et al. 2009; Miller et al. 2012; Nielsen et al. 2012). Genetic population structure studies are able to infer the spatial dynamics, migratory movements and kinship of commercially targeted species in order to delineate management and conservation units. This kind of work is essential for an accurate understanding of mixing rates so that contributions from each stock to annual catches can be accurately estimated for stock assessment purposes (Habicht et al. 2010). Determining the origin of individual fish captured at feeding aggregations can help to identify habitat use and thus areas and times appropriate for conservation actions. Information concerning genetic adaptations and shifts in a species' spatial distribution in response to changing environmental parameters can help predict the impacts of climate change (Hoffman and Willi 2008; Nielsen et al. 2009). Molecular tools are often used for phylogeographic studies that aim to identify barriers to the movements of populations and species, providing information about their preferred environmental conditions and sensitivity to natural and anthropogenic pressures (Dudgeon et al. 2012). Knowledge concerning the distribution of past populations as inferred by these studies can help to predict future distributions in response to changing environmental conditions. Genetic diversity can inform fisheries managers about a population's genetic effective size, or an estimate of the number of fish in the spawning stock that contributed gametes to the sampled cohort. As the number of adults descreases in a population, genetic drift and inbreeding increase, resulting in loss of adaptive variation and accumulation of detrimental genes (Hutchinson et al. 2003; Poulsen et al. 2006; Dudgeon et al. 2012). This information is vital for the assessment of a population's wellbeing and can be used to help maintain a species' adaptive potential. Molecular tools are also being used in aquaculture to manage inbreeding, identify stocks, and monitor impacts on natural populations (Bekkevold et al. 2006). The accidental release of highly inbred aquaculture stock into the wild has reduced the genetic diversity of natural stocks and transferred deleterious traits to wild fish (Coughlan et al. 2006). Finally, genetic research has identified that

industrialized fishing has had numerous genetic, physical and demographic impacts on commercially targeted species (Allendorf et al. 2008).

In the past 15 years, several molecular techniques have been exploited in an effort to elucidate a more accurate depiction of *Thunnus thynnus* population structure and spatial dynamics in line with the results from electronic tagging campaigns and traditional ecological knowledge. The sophistication and resolution of molecular techniques has been constantly evolving and recent results are showing great potential for adding clarity to this issue that has interfered with the optimal management of the species (see Appendix 1 for a summary of all molecular analyses of BFT to date). PCR amplified sequences from the mitochondrial DNA control region (mtDNA CR) were the first to show evidence of differentiation between the western Atlantic and Mediterranean populations (Alvarado Bremer et al. 1999); although, the effectiveness of this marker was called into question when a later study was unable to detect differences between populations over multiple years (Ely et al. 2002). A third study that followed soon thereafter contained an analysis of allele frequencies from 37 allozyme loci and found a single locus that differed significantly between the western and eastern groups (Pujolar 2003). As if to validate their previous results, Alvarado Bremer et al. (2005) analyzed 607 samples from the western and eastern Atlantic and Mediterranean Sea using a 450bp sequence of mtDNA CR and found no significant differences between the eastern and western stocks of BFT. Two studies have since shown significant divergence of Gulf of Mexico and Mediterranean populations using mtDNA CR sequences and microsatellites (Carlsson et al. 2007; Boustany et al. 2008). Population geneticists use fixation indices to describe the distribution of genetic diversity within a species (Box 1). For each of these studies the fixation index used to differentiate populations (F_{ST}) was estimated at between 0.005 and 0.0129. A review of population genetic parameters for 57 marine species estimated mean and median F_{ST} values of 0.062 and 0.02, respectively, for all species combined (Waples 1998). If BFT populations are differentiated, then the F_{ST} values used to define these differences are lower than the average for marine fish. Of course, this is to be expected for a species with ocean-wide migratory patterns, long lifespans,

Box 1. Illustration representing fixation indices, or measures of heterozygosity, used to estimate the differentiation of populations (F_{ST}), inbreeding within sub-populations (F_{IS}), and genetic diversity of an entire species (F_{IT}).



broadcasting of gametes and repeat spawning isolated to the marine environment.

The same pattern of inconsistent results can be seen among the studies that analyzed the genetic differences between sampling locations in the Mediterranean. Evidence of genetic structuring within the Mediterranean was first published in 2004, when mtDNA CR sequences and microsatellites showed genetic divergence of young-of-the-year captured in the Ionian and Tyrrhenian Seas (Carlsson et al. 2004). Three years later, this study were repackaged to include additional samples from the Gulf of Mexico and the Western Mediterranean and 25 samples from the Ionian Sea were re-assigned to the "eastern Mediterranean" (Carlsson et al. 2007). Unsurprisingly, weak signals of population divergence were once again found between the eastern and western Mediterranean. In 2010, a study comparing samples collected from six locations in the western and central Mediterranean (Adriatic, Tyrrhenian, Ligurian, Alboran, Algeria and Sardinia) using eight microsatellites, calculated significant F_{ST} values for 25 of the 28 pairwise comparisons between populations (Riccioni et al. 2010). This analysis included two groups of historical samples from 1911 (Tyrrhenian) and 1926 (Adriatic), which were significantly different from all other samples, including modern samples from the same location. A similar study in 2013, reanalyzed the same data with the addition of 60 individuals from the Levantine Sea (Riccioni et al. 2013). The authors found evidence of at least three sub-populations within the Mediterranean that appear to be structured along an environmental gradient (salinity and temperature). Conversely, other studies using both microsatellites and mtDNA CR sequences have been unable to detect divergence among Mediterranean sampling locations (Boustany et al. 2008; Viñas et al. 2011). The first effort to characterize BFT populations in the western and eastern Atlantic using single nucleotide polymorphisms (SNPs) produced the most significant evidence for differentiation of populations to date (Albaina et al. 2013). In that study, 17 SNPs revealed significant differentiation of BFT captured in the north west Atlantic and groups in the Bay of Biscay (F_{ST}= 0.120±0.091, $p < 0.01$) and the western Mediterranean (F_{ST} =0.116±0.078, $p < 0.01$). These F_{ST} estimates are an order of magnitude higher than previous estimates using the other approaches. Due to the limited number of Western Atlantic samples used in their study (n=15), the results should be verified with an increased number of samples.

Several important factors can influence the outcome of investigations into genetic structuring of BFT populations. As discussed in Chapter 1, several locations from which some of the aforementioned

studies collected samples, are feeding aggregations that are visited by BFT from both the eastern and western stocks. Moreover, genetic divergence has been observed between collections of fish captured within months of each other at the same feeding location (Carlsson et al. 2006). Therefore, it is wholly inappropriate to make pairwise comparisons using samples from these locations for genetic structure analyses, since they cannot be considered populations. Furthermore, considering the highly migratory nature of the species, it is also unwise to analyze groups of adult BFT captured outside of spawning locations, since they are likely to be of mixed origin. The only studies that can be awarded any credit for providing valid information concerning divergence of populations are those that have used larvae and/or young-of-the-year. Comparisons should also include samples from multiple years in order to avoid false positives for divergence due to sampling bias, temporal variation in the survival of larvae, and sweepstake effects (Larson and Julian 1999). Of course, the choice of molecular marker used also appears to have an influence on the outcome of population investigations.

4.1.1 SNP Discovery and the role of Next Generation Sequencing in fisheries research

It has been nearly a decade since the first protocol for NGS, or high throughput sequencing, was published (Margulies et al., 2005) and its impact on biological studies has been profound. The technological advancement that led to a veritable revolution in genetics was a massive scaling up of capillary electrophoresis, allowing for millions of simultaneous reactions occurring in parallel. The reactions happen rapidly and hundreds of Gigabases can be produced in a single run of short stretches of DNA that have been fragmented and assembled into libraries. NGS refers to a number of different technologies that rely on either emulsion PCRs (semiconductor sequencing, pyrosequencing and sequencing by ligation) or slide-based PCRs (reversible terminator sequencing and ligation sequencing). Of course, NGS is well known for the advances that it has allowed in the sequencing of entire genomes; however, its capacity to rapidly identify SNPs throughout the genome at both coding and non-coding regions has had a major influence on conservation genetics. These tools have allowed for significant advances in fisheries research, including fine-scale population structuring, population assignment, kinship analysis, local adaptations and estimations of effective populations size (Seeb et al. 2011).

SNPs can be discovered by a number of different methods, including Sanger sequencing of targeted genes products and expressed tag sequences, shotgun sequencing, and Next Generation

Sequencing. At least 80 SNPs are recommended for the detection of low levels of differentiation among highly mobile species (Ryman et al. 2011) so they are often assembled into panels containing at least 96 loci. SNP panels have been used to identify species and populations of albacore tuna, Pacific lamprey, salmon, and blue catfish, and distinguish between wild and natural populations of salmon (Habicht et al. 2010; Albaina et al. 2013; Amish et al. 2012; Drywa et al. 2014; Hess et al. 2014; Houston et al. 2014; Larson et al. 2014; Li et al. 2014). In 2011, Molecular Ecology Resources dedicated in entire issue (22 publications) to the use of SNPs in conservation genetics (mostly fish species). One of the main directives of FishPopTrace (Stokstad 2010), an EU funded consortium of 15 research groups, was the use of SNPs in research related to fish population analysis, seafood traceability and monitoring, control, and enforcement in the fisheries sector.

4.1.2 Aim of the study

The goal of this study was to develop a SNP panel capable of distinguishing groups of young BFT within spawning and nursery areas in the western Atlantic and Mediterranean Sea. Since mtDNA CR sequences and microsatellites have both provided mixed results in this regard, we hope to add more clarity to BFT population structuring using the increased resolution and genome coverage of SNPs. A genotyping-by-sequencing approach using the Illumina HiSeq platform will be used for SNP discovery. In fisheries related research, these methods have been applied to studies focused on species with relatively simple population structuring (limited range, anadromous, single spawning events). This study may present particularly unique challenges in that BFT is a highly migratory and long-lived species, with large effective population sizes, high genetic diversity and at least two large spawning areas.

4.2 Materials and Methods

4.2.1 Tissue sampling and DNA extractions

A total of 105 larvae and 450 young-of-the-year (YOY) individuals were captured at various spawning areas and adjacent waters in the Mediterranean Sea, Gulf of Mexico and Cape Hatteras from 2007 to 2012 during the late spring and early summer months (Table 1, Figure 1). All larvae used in this study were collected using plankton nets and sacrificed via immersion in 96% ethanol, according to standard larval survey practices. In addition to morphological identification, all larvae were barcoded to ensure proper species assignment (Chapter 3). All YOY were captured with hand lines and trolling lines.

Permission to conduct sampling surveys and all legal permits were granted by the International Commission for the Conservation of Atlantic Tuna (ICCAT). ICCAT is the intergovernmental organization responsible for the management of bluefin tuna in the Atlantic Ocean and Mediterranean Sea and all research was coordinated through their "Atlantic-wide research programme for bluefin tuna" (GBYP). ICCAT issued a recommendation (Rec. 11-06) allowing the parties involved in this research to collect and sacrifice young BFT for the purposes of genetic research as well as ship samples from one country to another (Cert. No. ICCAT RMA12-049).

Table 1: Samples of Atlantic bluefin tuna used for development of a 384 SNP genotyping panel using data generated by from Genotyping-by-Sequencing.

Sampling location	Sample code	Age class	Year	N. individuals
Levantine Sea (LEV)	EM-LS11-V	Larvae	2011	10
	EM-LS11-0	Young-of-the-Year	2011	29
	EM-LS12-0	Young-of-the-Year	2012	40
	EM-LS12b-0	Young-of-the-Year	2012	21
Strait of Sicily (STS)	CM-SI11-0	Young-of-the-Year	2012	21
	CM-SI12-V	Larvae	2012	21
	CM-SI12-0	Young-of-the-Year	2012	40
Balearic Islands (BAL)	WM-BA09-0	Young-of-the-Year	2009	41
	WM-BA10-0	Young-of-the-Year	2010	45
	WM-BA11-0	Young-of-the-Year	2011	40
	WM-BA12-0	Young-of-the-Year	2012	40
Tyrrhenian Sea (TYR)	WM-TY11-0	Young-of-the-Year	2011	37
	WM-TY12-0	Young-of-the-Year	2012	40
	WM-TY12b-0	Young-of-the-Year	2012	40
Gulf of Mexico (GOM)	WA-GM07-V	Larvae	2007	11
	WA-GM08-V	Larvae	2008	14
	WA-GM09b-V	Larvae	2009	25
	WA-GM09-V	Larvae	2009	24
Cape Hatteras (CAP)	WA-GM08-0	Young-of-the-Year	2008	16
Total				555

Approximately 20mg of muscle tissue/finclip from each of the 450 YOY and 105 complete or partial (caudal fin or eyeball) larvae were digested overnight in a Proteinase K solution. Genomic DNA was extracted from all samples and purified using Promega's Wizard®SV96 Genomic DNA Purification kit and vacuum manifold following the manufacturer's protocols. DNA was eluted with 60µl of distilled water heated to 60°C. DNA concentration and purity was measured with a NanoDrop.

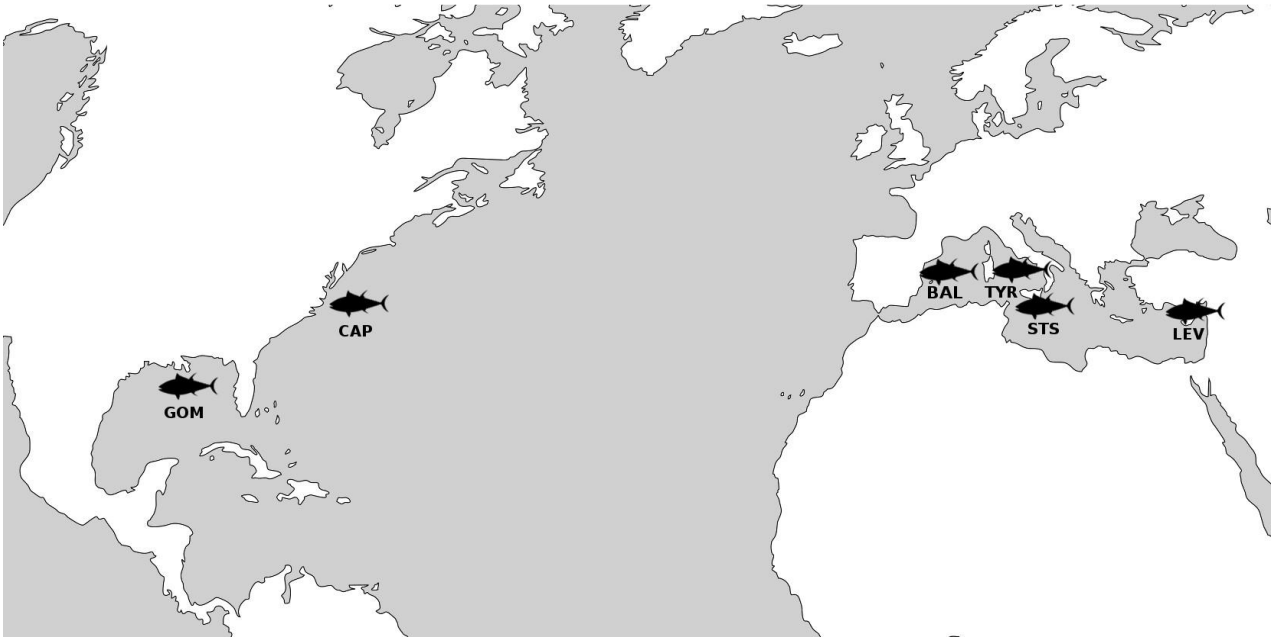


Fig. 1: Sampling locations (GOM = Gulf of Mexico; CAP = Cape Hatteras; BAL = Balearic Islands; TYR = Tyrrhenian Sea; STS = Strait of Sicily; LEV = Levantine Sea).

4.2.2 Genotyping-By-Sequencing

Extracted genomic DNA was digested with the *Ape*KI, a restriction enzyme which has a 5bp recognition site (5'GCWGC - 3'CGWCG). As in Elshire et al. (2011), the following standard Illumina sequencing adaptors were ligated to all fragments:

5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxx

5'-CWGyyyyAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

where, ''xxxx'' and ''yyyy'' denote the barcode and barcode complement and sequences, respectively. The second, or ''common'', adapter has only an ApeKI-compatible sticky end:

5'-CWGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG

5'-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

The GBS libraries were sequenced on HiSeq2500 (Illumina) 100 bp paired-end module, multiplexing 188 individuals on each sequencing lane with barcodes developed within the project, at the Genomics Core Facility (KULeuven) in collaboration with BioGenomics. Due to the very large number of fragments sequenced and discovered polymorphisms, individuals with less than 500k reads were resequenced to achieve a sufficient depth of sequencing for reliable genotype calling.

4.2.3 De novo SNP discovery

As recommended in Elshire et al. (2011), barcodes and low-quality edges were removed from the raw Illumina DNA sequence data using the Universal Network Enabled Analysis Kit pipeline (UNEAK, Lu et al. 2013), which is an extension of the TASSEL v3.0 program. UNEAK identifies SNPs in the absence of reference genomes by clustering similar 64 bp sequences (tags) that differ by a single base pair (tag pairs or reciprocal tags). The pipeline also employs a network filter that removes any cluster that includes more than one SNP. In this manner, 324,433 SNPs were discovered and among these an average of ~50% of SNPs were called per individual from each sampling location. SNPs were filtered according to the representation of a minimum number of individuals per sample. All SNPs with less than 70% representation among all individuals within each sample were removed, resulting in a dataset containing 27,316 SNPs.

The dataset was then converted to a Genepop file format using PGD Spider (Lischer and Excoffier 2012), and a basic quality check analysis was performed using the Adegenet package in R (Jombart and Ahmed 2011) and GENETIX (Belkhir et al. 1996-2004). An excess of heterozygosity was observed for the majority of loci which warranted additional tests and analysis that revealed an insufficient and unequal distribution of read coverage. As such, the *de novo* SNP discovery pipeline was abandoned for reasons discussed in the Results and Discussion sections. After recognizing this issue, the data for all individuals were pooled within five geographic sampling areas or strata (Gulf of Mexico and Cape Hatteras were merged into a western Atlantic stratum) and reanalyzed using pairwise comparisons of the total number of reads per allele per locus.

4.2.4 Genotyping-by-Sequencing allele frequency analysis

4.2.4.1 Reference genome assembly

A genome reference was assembled using data generated by genome sequencing of a single individual captured offshore from the Balearic Islands (unpublished data). The genomic library was sequenced in 2 × 75-bp paired-end mode on a HiSeq 2000 (Illumina). A total of 94,000,207 paired-end reads were produced and these were trimmed to 71bp due to bad quality endings. ABySS (1.3.7; Simpson et al. 2009) was used to create a *de novo* assembly of the genome. ABySS was run with standard parameters, and a k-mer size of 31. This resulted in an assembly of 6,309,067 contigs and a genome size of 944 Mb (additional details provided by AbySS are provided in Box 2). As quality control for the assembled genome, the original data was mapped using Bowtie2 (version 2.2.0; Langmead and Salzberg 2012) in the local modus.

Box 2: Summary statistics from the *de novo* assemble genome using AbySS.

No. of contigs	6,309,067
Total length	944,001,291
No. of contigs >= 500 bp	278,295
Largest contig size	55660
N50	3185
N75	1721
L50	56,015
L75	122,741
GC percentage	39.5%

4.2.4.2 Reference-based SNP discovery

The aforementioned raw paired-end sequence data (Section 2.2) was de-multiplexed using GBSX (Herten et al. 2015). GBSX trims barcodes and adaptors, allowing for one sequencing error in the barcodes and enzyme restriction site, thereby greatly improving paired end read joining and mapping. Reads from re-sequenced individuals that were distributed across multiple lanes were recombined and overlapping read pairs were merged using FLASH (v. 1.2.7; Magoč and Salzberg 2011). Details concerning the reads that were retained after this filtration step are featured in Box 3. Merged read pairs were then pooled according to strata and mapped against the genome reference ad mapped to the reference genome

Box 3: Percentage of reads that were retained after FLASH tool demultiplex filtration.

Strata	% of data retained	Number of reads
Western Atlantic	86.87	146,942,658
Balearic Islands	90.09	225,310,444
Ligurian Sea	90.36	36,418,929
Tyrrhenian Sea	86.88	282,901,496
Strait of Sicily	87.36	135,885,940
Levantine Sea	91.58	124,845,382

(from section 2.4.1) using Bowtie2 (version 2.2.0; Langmead and Salzberg 2012) using the end-to-end modus. Merged reads were filtered based on a mapping quality equal to or greater than 20 (or mapped to a particular location with 99% certainty). The genomic coordinates and corresponding coverage statistics for the targeted regions were retrieved using BEDtools (version 2.17.0; Quinlan and Hall 2010). Depth of coverage for each location in the genome was normalized for each stratum using the following formula:

$$\text{Normalized depth location} = \text{Location depth} \times \frac{\text{Highest total read number over all strata}}{\text{Total read number of this stratum}}$$

Locations with a normalized depth of 50 or more, over all strata, were selected for the identification of representative SNPs for all strata.

SNP calling was performed for all strata, based on the quality filtered mapping, in one run using FreeBayes (version 9.9.10; Garrison and Marth 2012). Similar settings to the human genome project SNP calling were used: mapping quality >20, and base quality >15. By performing SNP calling simultaneously on the mapped data of all strata, SNP information for all candidate positions in all strata were obtained, including positions that were homozygous for certain strata.

4.2.4.3. SNP selection

Given that the read data for each locus was pooled by strata it wasn't possible to calculate individual allele frequencies. As such, a proxy for allele frequency was developed using the available read counts. SNPs were selected for a 384 SNP genotyping panel based upon a delta value (DV), which was calculated for each locus using the following formula:

$$DV = \text{Pop.1}\left(\frac{\text{Number of reads for reference allele}}{\text{Number of reads for both alleles}}\right) - \text{Pop.2}\left(\frac{\text{Number of reads for reference allele}}{\text{Number of reads for both alleles}}\right)$$

A minimum of 18 read counts for each locus per stratum was set as a threshold in order to maintain confidence in SNP validity. Preference was given for SNPs that had delta values between 1.0 and 0.7 (high), 0.7 and 0.6 (mid) and 0.6 and 0.5 (low) (Table 2). Additional SNPs with exceptional read coverage were selected to complete the 384 SNP panel. Finally, all SNPs were filtered to ensure compliance with the criteria of Fluidigm genotyping design (minimum flanking sequence length of 60 bp and <65% GC content).

Table 2: Number of SNPs selected for the 384 trial SNP panel in order to differentiate between five strata.

	WATL vs LEV	WATL vs BAL	WATL vs TYR	WATL vs STS	LEV vs BAL	LEV vs STS	LEV vs TYR	BAL vs STS	TYR vs STS	TYR vs BAL
High	8	4	0	2	1	14	9	9	1	5
Mid	7	9	0	3	1	19	8	19	1	12
Low	23	31	3	28	3	29	29	38	14	23
Total	38	44	3	33	5	62	46	66	16	40

Using the SNP flanking regions derived from the aforementioned incomplete reference genome, all sequences were then compared with cod (*Gadus morhua*), sea bass (*Dicentrarchus labrax*) and general teleost sequences available through GenBank using the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1997).

4.2.5 384 SNP panel validation with 188 individuals

In an effort to validate the SNPs selected *in silico,* 188 of the 555 individuals used for the SNP discovery process (Table 3) were genotyped using a 384 SNP-typing array. Genotyping was carried out with the BiomarkTM HD platform (Fluidigm) and run with ROX as the passive reference dye and FAM and HEX SNPtype™ Assays for specific allele calling.

Table 3: Sampling details of young BFT samples selected for SNP panel validation.

Sampling location	Sample code	Age class	Year	Stratum	N. individuals
Levantine Sea	EM-LS11-0	Young-of-the-Year	2011	LEV	27
	EM-LS12-0	Young-of-the-Year	2012	LEV	21
Strait of Sicily	CM-SI11-0	Young-of-the-Year	2011	STS	21
	CM-SI12-0	Young-of-the-Year	2012	STS	27
Balearic Islands	WM-BA11-0	Young-of-the-Year	2011	BAL	24
Tyrrhenian Sea	WM-TY12-0	Young-of-the-Year	2012	TYR	24
Gulf of Mexico	WA-GM07-V	Larvae	2007	WATL	2
	WA-GM08-V	Larvae	2008	WATL	8
	WA-GM09b-V	Larvae	2009	WATL	10
	WA-GM09-V	Larvae	2009	WATL	8
Cape Hatteras	WA-GM08-0	Young-of-the-Year	2008	WATL	16
Total					188

A quality check was performed in order to remove SNPs that were non-polymorphic, of low quality or failed to amplify. The hierarchical Bayesian method described in Beaumont and Balding (2004), implemented in Bayescan software (Foll and Gaggiotti 2008) was used to detect loci with strong support

for selection (-log10PO > 1). The Beaumont and Nichols (1996) FDIST approach, implemented in Lositan (Antao et al. 2008) using a 0.995 confidence interval was used to identify SNPs showing evidence of balancing selection, which were then removed. Using Genepop on the Web v4.2 (Raymond and Rousset 1995; Rousset 2008), SNPs were analyzed for heterozygosity excess/deficiency using a global test with the following Markov chain parameters: 1,000 dememorization steps, 100 batches and 1,000 iterations per batch. STRUCTURE software (v2.3.4; Pritchard et al. 2000; Hubisz et al. 2009), a Bayesian model-based clustering algorithm, was used to visualize the population structure and estimate the extent of admixture by individual and population. For each analysis, a burn-in period of 10k with 50k MCMC reps was employed, using the admixture ancestry model, correlated allele frequencies and sampling locations used for the LOCPRIOR model. Lambda values were inferred and the number of potential ancestral clusters analyzed was equal to the total number of strata. For each value of k the analysis was run 10 times. The results showed that the samples collected from the Levantine Sea in 2012 behaved much differently than all other populations and were thus removed from further analyses. An analysis of the final SNP dataset using BELS software (backward-elimination locus-selection) selected the optimal SNPs for discrimination of population structuring (Bromaghin 2008). A total of 59 SNPs were selected for optimization of discrimination between the samples collected from the Gulf of Mexico and the Mediterranean Sea. Another 48 SNPs were selected for their discriminatory power between sampling locations within the Mediterranean, 11 of which had already been selected for trans-Atlantic discrimination.

Genotypes for all 188 individuals from the remaining 96 SNPs were then re-analyzed *in silico*. Hardy-Weinberg tests were performed using Genepop on the Web v4.2 (Raymond and Rousset 1995; Rousset 2008) fixed with the following default Markov chain parameters: 1000 dememorization, 100 batches and 1000 iterations per batch. Alpha values were adjusted using the Bonferroni correction. As above, outlier loci were identified using Lositan (Antao et al. 2008). Various population structuring statistics (F_{IS}, F_{ST}, F_{IT}) were calculated with Arlequin v3.5.1.2 (Excoffier et al. 2009). Genetic differentiation between samples was estimated by pairwise F_{ST} using FSTAT v.2.9.4 (Goudet 1995), testing significance with the adjusted nominal level (α=0.05) for multiple comparisons (Bonferroni correction). STRUCTURE software (v2.3.4; Pritchard et al. 2000) was used to elucidate patterns of population structuring. Throughout all analyses, the admixture model and LOCPRIOR option were

selected. Preliminary analysis for the optimal number of ancestral clusters (k) made use of 10k burnins and 50k MCMC reps with 20 replicates at each of value of k ranging from 1 to the total number of groups analyzed. These results were then analyzed with STRUCTURE harvester and a final range of 3 k values was re-analyzed with 500k burnins and 750k MCMC reps. Clustering of groups using the 59 and 96 SNP panel was further analyzed using the Adegenet package v1.4-2 in R, which provides Discriminant Analysis (DA, defining groups), based upon a dataset of Principal Components (PC, reducing data to several dimensions) constructed from multi-locus genotypes (Jombart 2008; Jombart and Ahmed 2011).

4.3 Results

4.3.1 De novo SNP discovery using the UNEAK pipeline

Comparisons between read coverage by individual and measures of DNA quality and quantity (nucleic acid purity, DNA purity and DNA concentration) revealed no significant correlation (Fig. 2). Therefore, the DNA extractions used for analysis were satisfactory for the required purpose. However, preliminary analyses of allele frequencies continually showed an excessive number of heterozygotes as thresholds of read count were increased.

Additional analysis of a subset of 22 individuals with more than 2 million reads revealed the same results. More detailed analysis of datasets with minimum thresholds of 3, 4 and 5 read counts per SNP locus revealed a trend of increasing heterozygosity uncharacteristic of wild populations. The majority of loci within the dataset requiring a minimum read count of 3 had observed heterozygosity values around 0.4 (Fig.3A). When the minimum read count threshold was increased to 4 and 5, values of heterozygosity increased at an unexpected rate (Fig.3B,C). Within the 5 minimum read count dataset, the difference between expected and observed heterozygosity was overwhelmingly negative for the majority of all loci (Fig.3D).

Overall observed heterozygosity within each dataset was then plotted alongside the expected heterozygosity and the difference between the two (Fig. 4). The data trend shows a deficit of heterozygotes at 2 read counts and a steadily increasing frequency of heterozygotes after 4 read counts. These uncharacteristic trends that appear to be independent of DNA quality and quantity were suggestive of errors in the work pipeline. For this reason, the analysis of individual genotypes was abandoned and an alternative approach was developed.

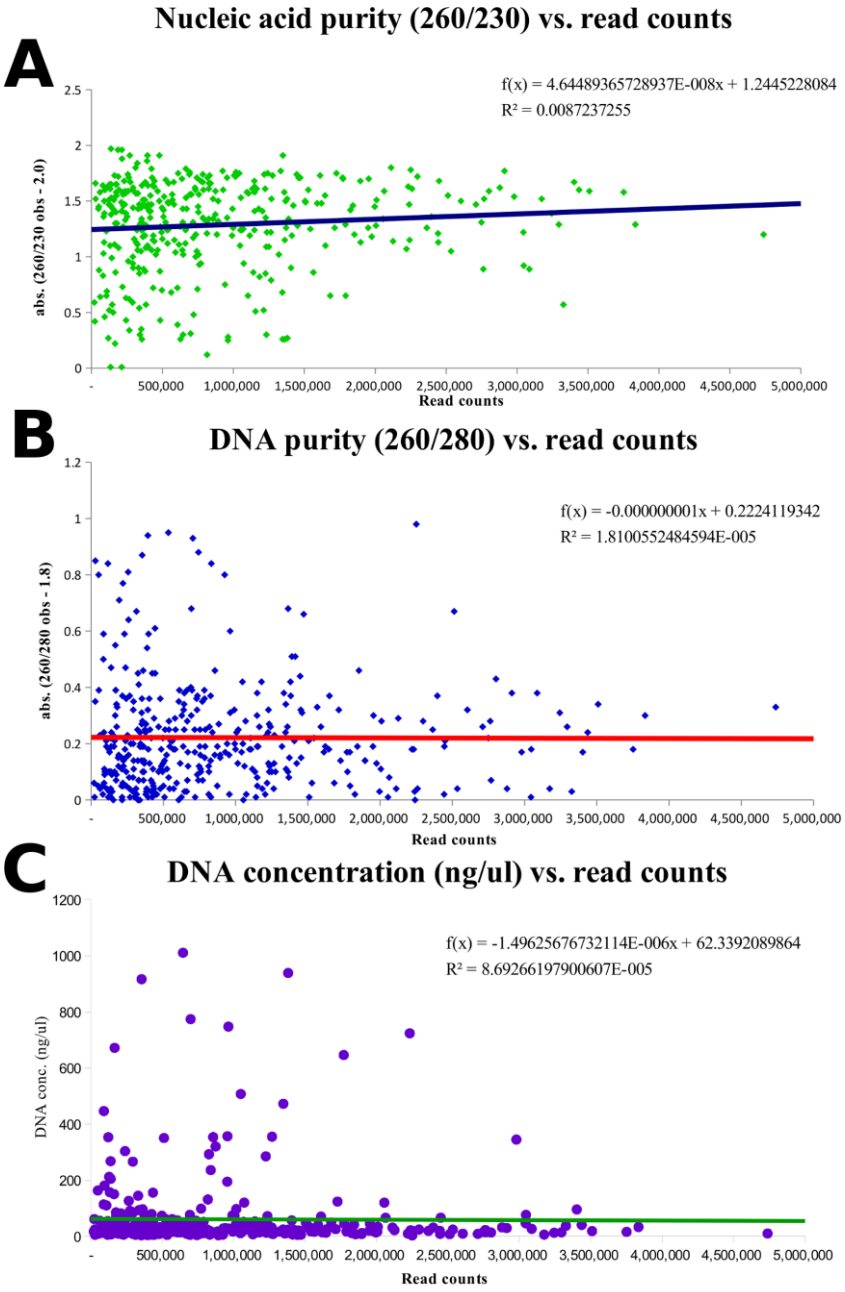


Fig. 2: Total read coverage for 384 randomly selected individuals compared to nucleic acid purity (260/230 ratio; **A**), DNA purity (260/280 ratio; **B**) and DNA concentration (ng/µL; **C**).

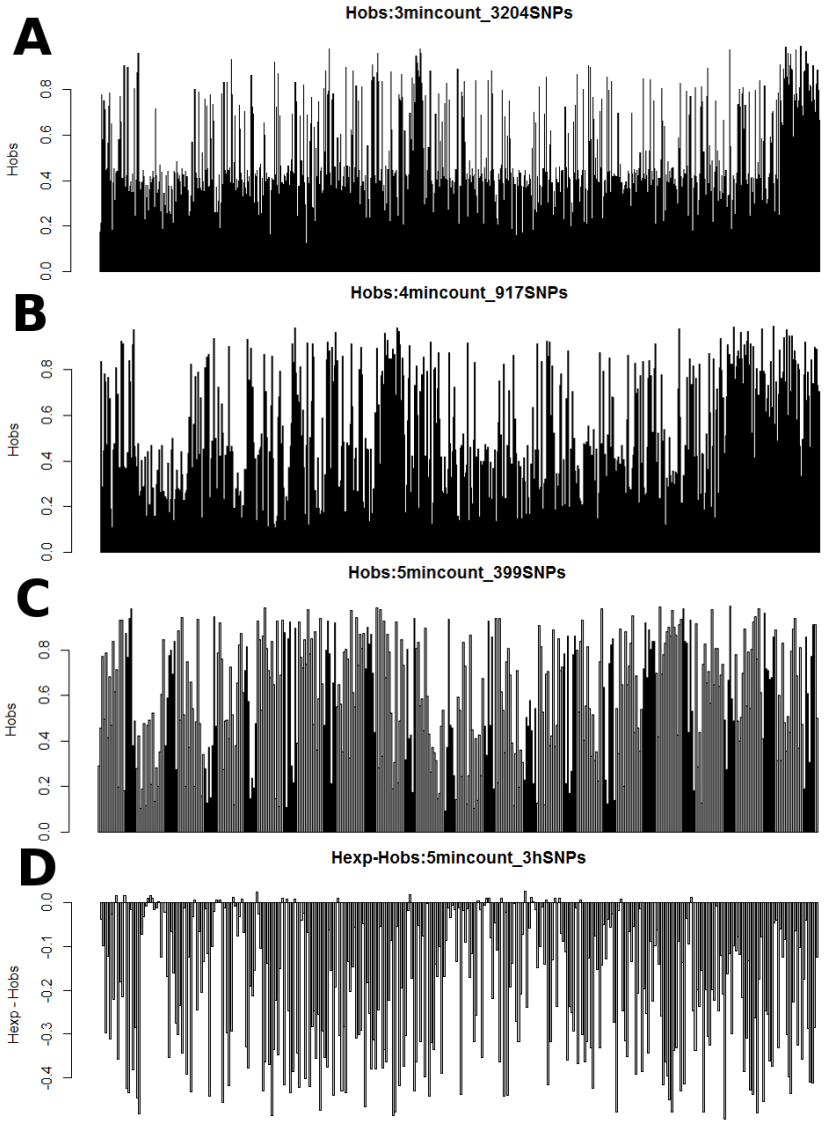


Fig. 3: Observed heterozygosity of ~1300 individuals after implementation of minimum read counts/loci/individual filters: minimum read counts of 3 (**A**), 4 (**B**), 5 (**C**). SNP loci were eliminated from the analysis if read count thresholds were not achieved. Analysis of minimum read count >=3 dataset consited of 1311 individuals, 3204 loci and 39.4% missing information; >=4 dataset consisted of 1308 individuals, 917 loci and 37.2% missing information; >=5 dataset consisted of 1305 individuals, 399 loci and 32.4% missing information. Calculated differences between observed and expected heterozygosity for all loci in the 5 minimum read count dataset (**D**).

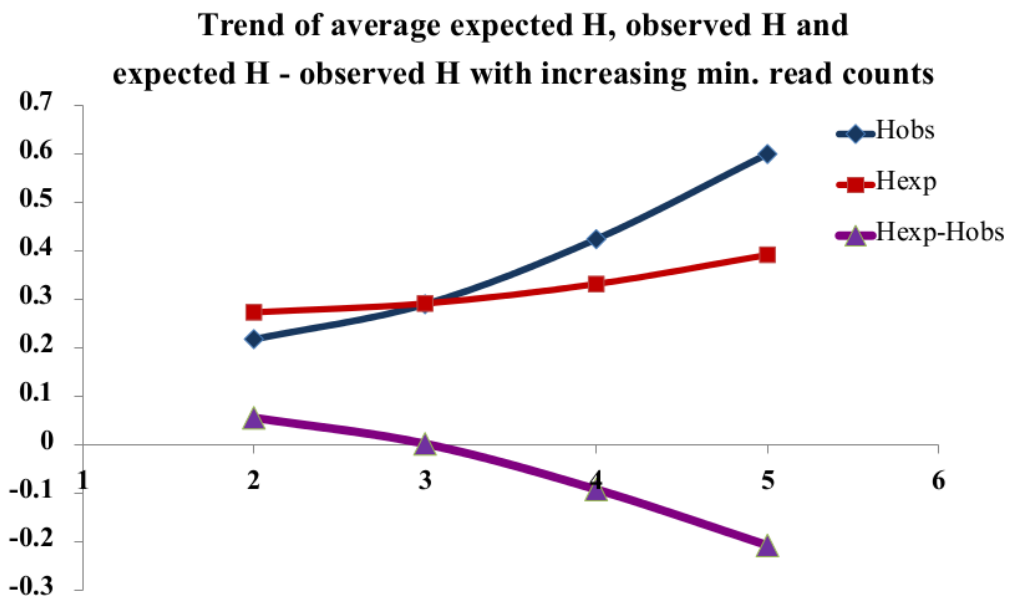


Fig. 4: Overall observed (Hobs; blue) and expected (Hexp; red) heterozygosity from four datasets containing individual genotypes with 2-5 minimum read counts per loci. The difference between expected and observed heterozygosity (Hexp-Hobs; purple) is plotted for each dataset.

4.3.2 Genome assembly

GBSX processing resulted in the retention of a much higher percentage of reads (88.9% overall) after FLASH tool filtration (Table 4). Merging of read pairs produced longer sequences (up to 180bp) which, when mapped against the genome reference, resulted in much improved mapping quality and confidence (**Table 5**). In total 866,121 locations in the dataset passed the mapping and normalized depth filtration criteria. SNP calling by FreeBayes resulted in 2,467,103 SNP positions, of which 236,332 intersected with the >50 coverage mapped locations. After filtering for only biallelic SNPs (among all strata), a total of 184,895 SNPs were found to be sufficiently represented among all strata.

Table 4: Results from the analysis of original data mapped to the assembled genome reference.

Description	% paired-end reads	% reads	No. of paired-end reads	No. of reads
Pair Aligned concordantly exactly 1 time	68.07	68.07	63,984,850	127,969,700
Pair Aligned concordantly >1 times	6.09	6.09	5,720,552	11,441,104
Pair Aligned discordantly 1 time	2.34	2.34	2,203,070	4,406,140
Not aligned as pair, aligned exactly 1 time	-	3.82	-	7,189,521
Not aligned as pair, aligned >1 times	-	18.18	-	34,176,081
Overall alignment	-	98.50	-	185,182,546
Unique alignment	-	74.24	-	139,565,361

Table 5: Mapping statistics of reads per strata after combining read pairs using FLASH tools.

	Total reads	Aligned 0 times	Aligned exactly once	Aligned more than once	Alignment rate	Reads with Phred score > 20
Western Atlantic (WATL)	146,942,658	4.69%	57.95%	37.36%	95.31%	72.84% 107,036,577
Balearic Islands (BAL)	225,310,444	2.19%	53.67%	44.14%	97.81%	66.29% 149,362,671
Tyrrhenian Sea (TYR)	186,901,496	17.94%	46.17%	35.89%	82.06%	57.00% 106,528,635
Strait of Sicily (STS)	135,885,940	13.44%	51.79%	34.77%	86.56%	64.02% 86,989,987
Levantine Sea (LEV)	124,845,382	3.81%	57.99%	38.20%	96.19%	71.2% 88,948,045

4.3.4 SNP genotyping

All 188 individuals were sufficiently genotyped for population structure analysis. From the pool of 384 SNPs, 77 were non-polymorphic, unrecognized or provided low quality data. Thirteen SNPs had minor allele frequencies of less than 10% across all populations. An additional 53 SNPs were removed, 44 of which showed evidence of balancing selection and nine consistently failed to provide enough data for statistical analysis. In total 34% of the *in silico* discovered SNPs failed the technically validation. Finally, 33 SNPs were removed as they were found to be outside of Hardy-Weinberg Equilibrium. This left a pool of 221 SNPs for which average percentage of missing data per SNP locus was 2.57 ± 3.16% with median value of 2.4%. The average percentage of missing data by individual was 2 ± 6% with a median value of 0%. YOY captured in the Levantine Sea in 2012 were removed from the dataset, as their data proved so divergent from all other populations that their inclusion interfered with the analysis.

4.3.5 Genetic diversity and Hardy-Weinberg Equilibrium

The mean heterozygosity across all loci was 0.344 ± 0.155 with the lowest value (0.322 ± 0.200) assigned to larvae from the Gulf of Mexico 2008 and the highest value (0.347 ± 0.161) assigned to the Levantine Sea 2012 samples. Of the final 221 SNP candidates, all behaved according to the HWE, following Bonferroni correction.

4.3.6 Detection of outlier loci

The hierarchical Bayesian method described in Beaumont and Balding (2004), implemented in Bayescan software (Foll and Gaggiotti 2008) detected only one SNP showing support for selection (-

log10PO > 1). Outlier detection using Lositan software revealed a total of 8 candidate loci for positive selection and 12 candidate loci for balancing selection (Fig. 5A, B). After selection of the final 96 SNP panel, the analysis was run again with the same settings, revealing 2 candidate loci for positive selection and 3 candidate loci for balancing selection had been retained (Fig. 5B).

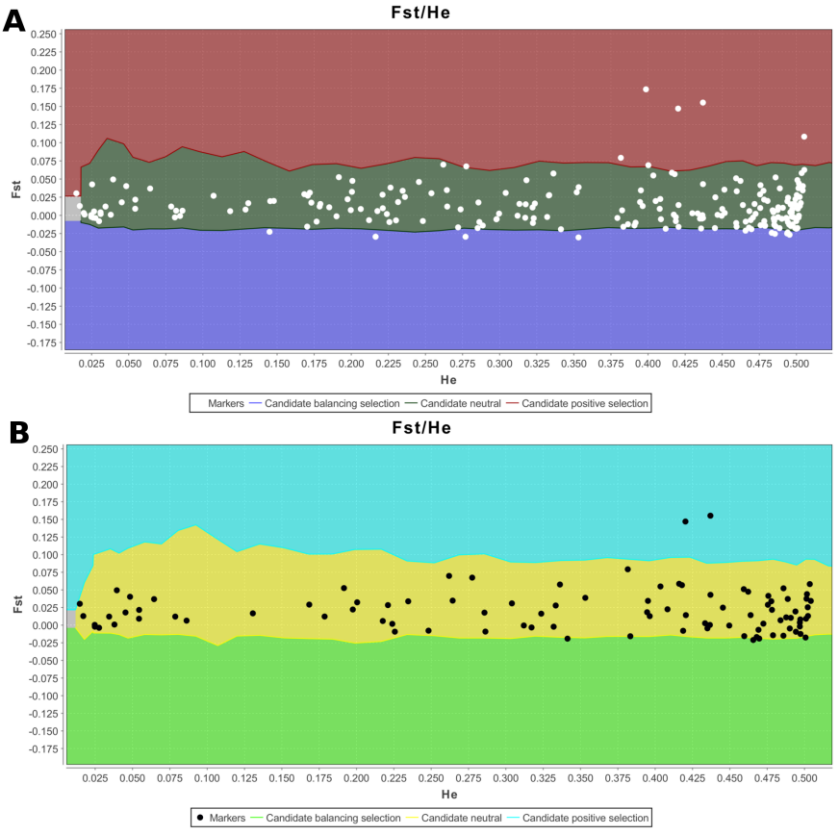


Fig. 5: Lositan analysis results for datasets containing 167 individuals and 221 SNPs (**A**) or 96 SNPs (**B**). In both figures, the lower and upper coloured band represents a threshold inwhich loci are candidates for balancing selection and positive selection, respectively. The confidence interval was set at 0.995.

4.3.7 Populations Genetics

Using genotypes from the final 95 SNP panel, the genetic differentiation between all samples was significant, with a global F_{ST} value of 0.0155 (p-value<0.05). The global F_{IS} was calculated to be 0.0429, demonstrating that only 25% of the variation in the dataset is due to between population differences. Analysis of the entire dataset with genotypes from all 95 SNPs revealed significant differences between the Western Atlantic samples and 3 strata (Balearic, Strait of Sicily 2011, Levantine Sea). The Tyrrhenian Sea samples also differed significantly from fish captured in the Balearic Sea and the Strait of Sicily 2011 (Table 6). Analysis of the dataset with just 48 SNPs that were selected because of discriminatory power within the Mediterranean showed that only one significant pairwise difference persisted (Strait of Sicily

2011 and the western Atlantic; Table 7). However, when all 167 samples were analyzed with the 59 SNP panel designed to differentiate between western Atlantic and Medditerranean populations, every pairwise comparison with the western Atlantic was significant (Table 8). F_{ST} values ranged between 0.0165 (Strait of Sicily 2012) and 0.0369 (Levantine Sea).

Table 6: Matrix of F_{ST} values calculated using 96 SNP genotypes for 167 individuals from 6 samples. FST values are provided under the diagonal and indicators of significance (*) and non-significance (NS) are shown above the diagonal. Significant F_{ST} values are highlighted in yellow.

	W. Atlantic	Balearic Sea	Tyrrhenian Sea	S. Sicily 2011	S. Sicily 2012	Levantine Sea
W. Atlantic		*	NS	*	NS	*
Balearic Sea	0.0162		*	NS	NS	NS
Tyrrhenian Sea	0.0161	0.0165		*	NS	NS
S. Sicily 2011	0.0218	0.0077	0.0152		NS	NS
S. Sicily 2012	0.009	0.0062	0.0104	0.0091		NS
Levantine Sea	0.0274	0.0083	0.0216	0.0106	0.0081	

Table 7: Matrix of F_{ST} values for 167 individuals from 6 samples calculated using a 48 SNP panel designed to discriminate between groups of BFT in the Mediterranean Sea. FST values are provided under the diagonal and indicators of significance (*) and non-significance (NS) are shown above the diagonal. Significant F_{ST} values are highlighted in yellow.

	W. Atlantic	Balearic Sea	Tyrrhenian Sea	S. Sicily 2011	S. Sicily 2012	Levantine Sea
W. Atlantic		NS	NS	*	NS	NS
Balearic Sea	0.0152		NS	NS	NS	NS
Tyrrhenian Sea	0.0044	0.0232		NS	NS	NS
S. Sicily 2011	0.0192	0.0154	0.0268		NS	NS
S. Sicily 2012	0.0042	0.0155	0.0169	0.0173		NS
Levantine Sea	0.0284	0.0157	0.0355	0.0177	0.0188	

Table 8: Matrix of F_{ST} values for 167 individuals from 6 samples calculated using a 59 SNP panel designed to discriminate between BFT from the Western Atlantic and Mediterranean Sea. FST values are provided under the diagonal and indicators of significance (*) and non-significance (NS) are shown above the diagonal. Significant F_{ST} values are highlighted in yellow.

	W. Atlantic	Balearic Sea	Tyrrhenian Sea	S. Sicily 2011	S. Sicily 2012	Levantine Sea
W. Atlantic		*	*	*	*	*
Balearic Sea	0.0229		NS	NS	NS	NS
Tyrrhenian Sea	0.0256	0.0033		NS	NS	NS
S. Sicily 2011	0.028	-0.0022	0.0016		NS	NS
S. Sicily 2012	0.0165	-0.0024	0.001	-0.0033		NS
Levantine Sea	0.0369	0.0015	0.0105	0.0017	0.0011	

4.3.8 Detection of genetic clusters

Cluster analysis based on the Bayesian algorithm performed in STRUCTURE revealed strong differentiation of western Atlantic and Mediterranean samples as well as genetic structuring within the Mediterranean Sea (Fig. 6). The 48 SNP subset showed pronounced population structuring within the Mediterranean at 3 different levels of assumed ancestral populations. The 59 SNP subset showed significant differentiation of the western Atlantic from all samples in the Mediterranean. Results from the DAPC analysis were similar, with samples from the western Atlantic differentiated from the Mediterranean samples and some amount of structuring between Mediterranean groups (Fig.7).

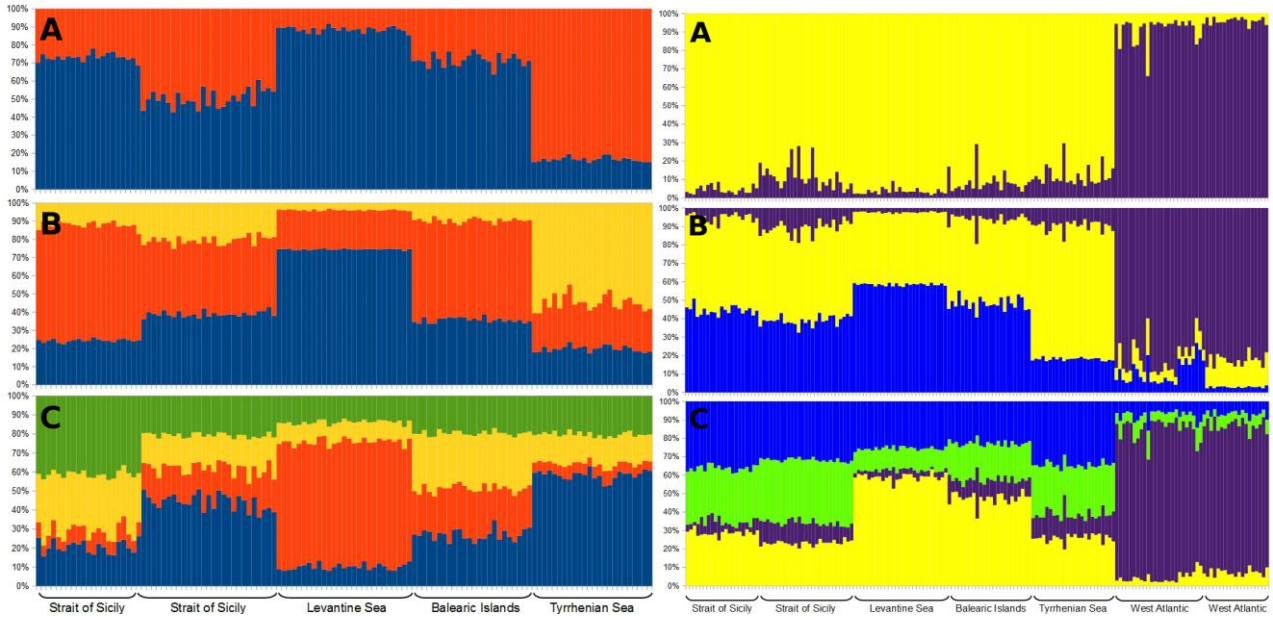


Fig. 6: STRUCTURE analysis of 123 BFT collected from the Mediterranean Sea using a subset of 48 SNPs from the validated 96 SNP panel. The model used assumes 2 (A), 3 (B) and 4 (C) ancestral populations (Left). STRUCTURE analysis of 167 BFT from the Mediterranean and Western Atlantic using a subset of 59 SNPs from the 96 SNP panel developed by the BGSA Genetics Consortium. The model used assumes 2 (A), 3 (B) and 4 (C) ancestral populations (Right).

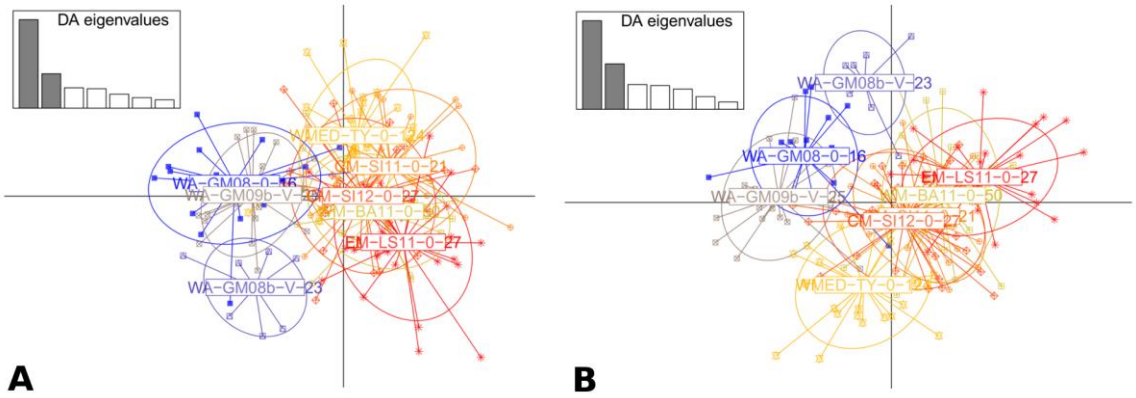


Fig. 7: DAPC analysis of 167 BFT collected from the Mediterranean Sea and western Atlantic using 96 SNPs (A) and a subset of 59 SNPs (**B**). Circles and dots in shades of blue are samples from the western Atlantic and warmer colours are associated with the Mediterranean Sea samples.

4.4 Discussion

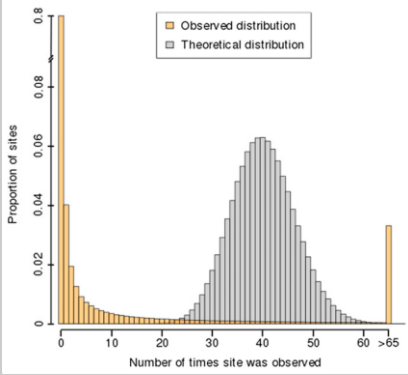
Several errors were made in the development and analysis of the original GBS dataset. These errors were detected when increases in minimum read count thresholds resulted in a constant increase in heterozygosity throughout the dataset. By increasing the number of read counts required to define an individual genotype, one expects to eliminate heterozygotes that have been incorrectly called as homozygotes due to the low detection rate of alternative alleles. In our dataset the average heterozygosity by locus increased to 60%, while many loci had heterozgosity values between 90-100% at minimum read counts as low as five. Some GBS protocols have advised that loci with mean observed heterozygosity >0.75 should be discarded (Nielsen et al. 2011; White et al. 2013). If this advice had been followed, then we wouldn't have had a dataset to analyze.

We initially suspected that the dataset suffered as a consequence of low sequencing coverage due to low quality DNA; however quality control analysis of sequencing coverage and several measures of DNA quality and quantity suggested that the two were uncorrrelated. Errors likely began with the use of *Ape*KI as the restriction enzyme (RE) during the preparation of sequencing libraries. In the years that have passed since the begnining of this project, several studies have shown that *Ape*KI library preparations couple with Illumina sequencing results in low, biased error prone coverage of genome sequences. Shortly after encountering issues in our dataset, a study was published claiming that, “The GBS protocol generally provides low coverage if the enzyme *ApeKI* is used” (Lu et al. 2013). That same year another study published results from an analysis of the performance of three restriction enzymes (*Ape*KI, *Pst*I and

*Eco*T22I) which determined that *Ape*KI was the worst performing RE relative to the others (De Donato et al. 2013). The fundamental difference was credited to *Ape*KI's five base pair cut site (GC[A/T]GC), compared to the six base pairs targeted by the other REs. Not only is *Ape*KI shorter than the other REs (enabling it to attach to the genome more frequently) but it also contains an ambiguous base which can increase the number of candidate attachment sites. Digestion using *Ape*KI results in a high number of missing data, irrespective of the quality of data that one begins with (De Donato et al. 2013). Even for samples with high depth coverage, researchers can expect to have ~30% missing data for any given individual in a dataset (Beissenger et al. 2013).

A great deal of bias can be introduced to the dataset during the sequencing of GBS libraries. Since the *ApeKI* cut site targets sections of DNA heavy in GC content, genomic regions rich in the nucleotides are heavily fragmented resulting in some genomic regions being over- or underrepresented (Minoche et al. 2011). A 2013 study found that GBS had a non-uniform distribution of sequence reads in that cut site adjacent sequence fragments are left virtually unsequenced while other portions have read depth >1000x the mean (Beissenger et al. 2013). Box 4 shows the results of a study comparing the theoretical distribution of reads per sequence versus the realized distribution of reads when using *Ape*KI combined with the Illumina platform (Beissenger et al. 2013). The authors of that study determined that the median number of reads per sequence fragment was zero, substantially less than expected (~40).

Box 4: Observed and theoretical frequency distribution of the number of times that optimally sized *Ape*KI fragments were sequenced (from Beissinger et al. 2013).



Using *Ape*KI for DNA digeston results in the production of many short and repetitive fragments of DNA (De Donato et al. 2013), which can easily be incorrectly aligned, resulting in paralogs that appear as heterozygous loci. Heterozygotes can be incorrectly identified as homozygotes even among regions with high read coverage (Lu et al. 2013). Minoche et al. (2011) has analyzed the origin of sequencing errors generated by the Illumina platform by comparing *de nov*o and reference-based assembly of sequences produced by GBS. They found that errors generated by Illumina HiSeq platforms are typically A-C and G-T conversions, as the fluorophores attached to these bases are excited by the same laser and the emission

spectra may not be perfectly separated (Minoche et al. 2011). Errors in Illumina generated sequences are far more likely to be substitutions (99.5% of errors), rather than insertions and deletions, with the majority of incorrect base calls occurring after guanine and before adenine and thymine (Minoche et al. 2011).

Recent GBS studies have used alternative REs with longer base pair cut sites (*Pst*I, *EcoT22I*) for studies of wild non-model species (Alcaide et al. 2014; Kort et al. 2014; Li et al. 2014). In fact, the majority of GBS studies that have used *Ape*KI as RE have focused on SNP discovery in highly domesticated agricultural crops (Elshire et al. 2011; Morris et al. 2013; Sonah et al. 2013; Reddy et all. 2014). Ironically, the GBS protocol using *Ape*KI was first introduced as a robust and simple NGS approach for high diversity species in a study that focused on highly inbred populations of maize and barley (Elshire et al. 2011). *Ape*KI is simply an inappropriate choice of RE for wild, non-model, genetic diverse species with large effective population sizes.

The data was greatly improved by the development of a genome reference which allowed for the improvement of SNP discovery due to the rough template that it provided for assembly and alignment. The trimming algorithm executed by the GBSX programme (Herten et al. 2015) greatly improved the quality of merged reads and increased the overall length of fragment that could be aligned with the genome reference. Both of these improvements to the protocol overcame the ambiguities and daunting challenges of *de novo* SNP discovery. For considerably lower library construction and sequencing efforts, pooling of genotype data from NGS sequenced individuals can allow for accurate estimations of population allele frequencies with similar accuracy to individual-based analyses (Gautier et al. 2013). Since we chose to pool all of the GBS data, due to the weaknesses described above, individual allele frequencies were lost and the resulting analysis focused on read counts instead. Admittedly, this could have introduced some bias into the SNP discovery process; however, the results seem to speak for themselves. Technical validation of *in silico* discovered SNPs showed a 75% conversion rate to functional SNP arrays.

4.5 Conclusion

This study outlines several shortcomings of the GBS approach when analysing a non-model species with high genetic diversity and effective population sizes. By digesting our DNA with the *Ape*KI restriction enzyme, sequencing with the Illumina platform, and analyzing the resulting data with the

UNEAK pipeline, we have effectively written a protocol for errors to be avoided when using NGS technology. Despite these technical challenges, we managed to salvage the data achieve the intended goal of developing a high throughput genotyping panel. The 96 SNP panel developed in this chapter was used to genotype an additional 556 BFT. The details of that exercise are explained in Chapter 5.

Acknowledgements

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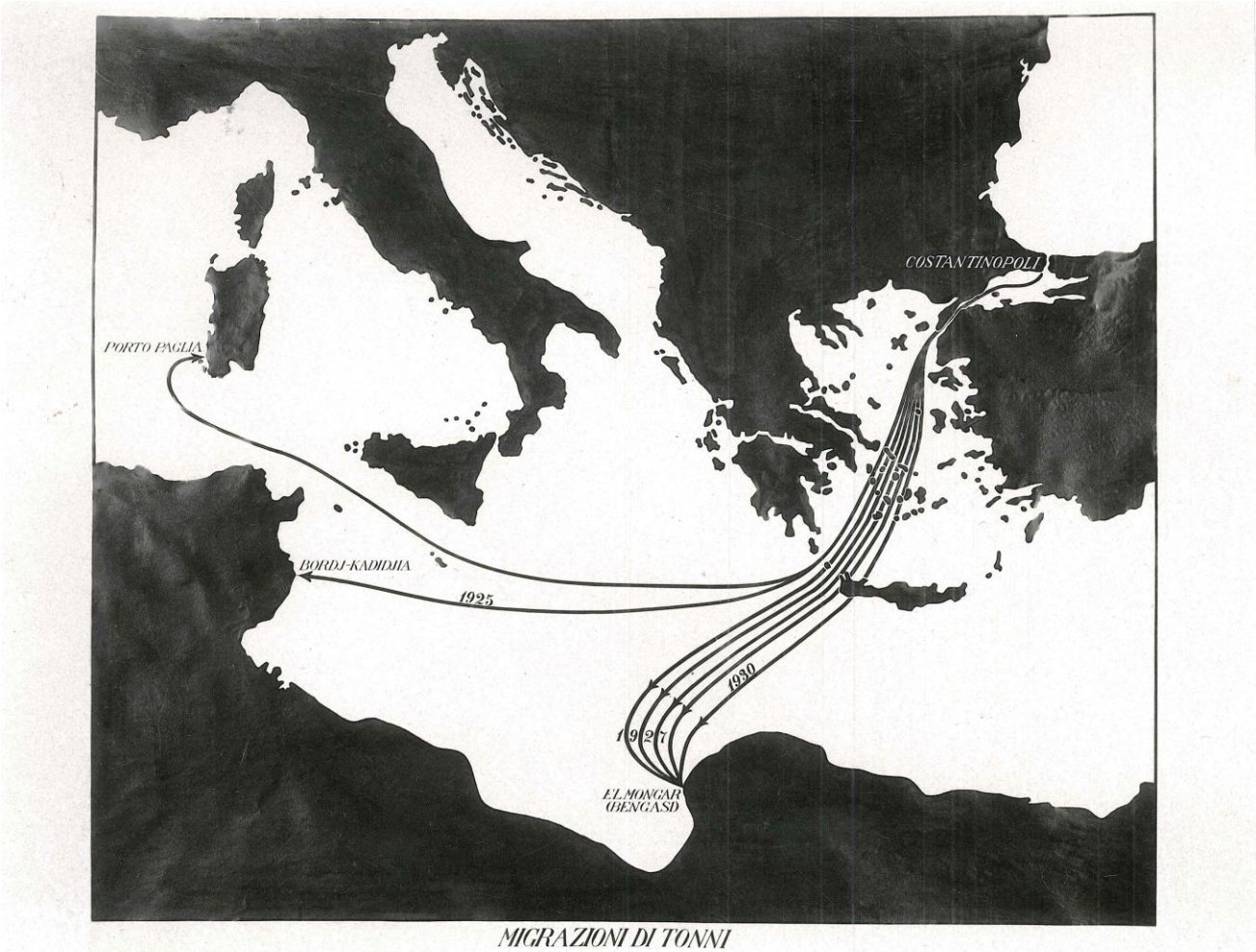
Appendix1: Summary of all BFT genetic population structuring studies to date. Numbers in parentheses reflect the number of samples

Year	Marker	Sample size	Resultp	Reference
1999	mtDNA CR	Mediterranean Sea and western Atlantic Ocean (140)	Allele frequency revealed no differentiation within Mediterranean (p = 0.3721) Allele frequencies revealed differentiation of pooled MED and W. Atlantic (p = 0.0428)	Alvarado-Bremer JR, Naseri I, Ely B (1999) Col Vol Sci Pap ICCAT 49:127–129
2002	mtDNA CR	Western Mediterranean 1990 (31), 1992 (32), 1993 (37), 1998 (38) Atlantic Large Adults 1994 (34) Atlantic Small Adults 1994 (38)	No differentiation	Ely B, Stoner DS, Bremer A.J, Dean JM, Addis P, Cau A and Quattro JM (2002) Mar Biotech 4: 583-588.
2002	*ldhA* nuclear gene	Western Mediterranean 1990 (31), 1992 (32), 1993 (37), 1998 (38) Atlantic Large Adults 1994 (34) Atlantic Small Adults 1994 (38)	Differentiation of MED 1998 and all other strata No other differentiation of other strata combinations	Ely B, Stoner DS, Bremer A.J, Dean JM, Addis P, Cau A and Quattro JM (2002) Mar Biotech 4: 583-588.
2003	37 allozyme loci	NW Atlantic 1996 (39) NE Atlantic 1994 (49) Western Mediterranean (601) Central Mediterranean (133)	No differentiation within MED No differentiation of E Atlantic and MED Significant allele frequency differences for *SOD-1* enzyme between pooled E Atlantic / MED and pooled W estern Atlantic	Pujolar JM, Roldán MI and Pla C (2003) Mar Biol 143: 613-621.
2004	9 microsatellites	Young of the Year only Balearic 1998 (74) Balearic 1999 (60) Tyrrhenian 1998 (28) Tyrrhenian 1999 (33) Tyrrhenian 2002 (63) Ionian 1998 (9) Ionian 1999 (16)	No temporal differentiation within WMED (F_{ST} = −0.0013, p = 0.735) No differentiation between Balearic and Tyrrhenian (F_{CT} = 0.0013, p = 0.196) No temporal differentiation between Baleriac, Tyrrhenian and Ionian (F_{SC} = −0.002, p = 0.883) Differentiation of Balearic, Tyrrhenian and Ionian Sea (F_{CT} = 0.0032, P =0.019) No differentiation of temporally pooled Balearic and Tyrrhenian (F_{ST}=0.0007, p = 0.226) No differentiation of temporally pooled Balearic and Ionian (F_{ST}=0.0046, p = 0.103) Differentiation of temporally pooled Ionian and Tyrrhenian (F_{ST}=0.0087, p = 0.015) Differentiation of temporally pooled Balearic, Ionian and Tyrrhenian (F_{ST}=0.0023, p = 0.038)	Carlsson J, McDowell JR, Diaz-Jaimes P, Carlsson JEL, Boles SB, Gold JR and Graves J E (2004) Mol Ecol 13: 3345-3356.
2004	mtDNA CR (868bp)	Young of the Year only Balearic (24) Ionian (23) Tyrrhenian (22)	Differentiation of Ionian, Balearic and Tyrrhenian (Φ_{ST}= 0.0239, P = 0.0314) No differentiatioan of Balearic and Ionian Seas (Φ_{ST}=0.0085, P=0.25) No differentiation of Balearic and Tyrrhenian Seas (Φ_{ST}= 0.0270, P=0.053) Differentiation of Ionian and Tyrrhenian Seas (Φ_{ST}=0.0366, P = 0.03)	Carlsson J, McDowell JR, Diaz-Jaimes P, Carlsson JEL, Boles SB, Gold JR and Graves J E (2004) Mol Ecol 13: 3345-3356.
2005	mtDNA CR (450bp)	Western Atlantic (50) Eastern Atlantic (24) Mediterranean (Gulf of Mersin, Aegean Sea, Ionian Sea, Libyan coast, Tyrrhenian Sea, Tunisian coast, Ligurian Sea, Gulf of Valencia) (323) Total n=607* *Additional samples from Ely et al. (2002)	No differentiation of Atlantic and MED (Φ_{ST} = 0.002, P = 0.245)	Alvarado Bremer JR, Viñas J, Mejuto J, Ely B and Pla C (2005) Mol Phylo Evol 36: 169-187.
2006	8 microsatellites	Iceland EEZ (800)	Differentiation of pooled early season (1999+2002) and pooled late season ABFT (1999+2002) (F_{CT}=0.00154, P= 0.000) No differentiation between alternative temporal or spatial combinations	Carlsson J, McDowell JR, Carlsson, JEL, Ólafsdóttir D and Graves JE (2006) ICES J Mar Sci 63: 1111e1117

2007	8 microsatellites	Gulf of Mexico Larvae 2003 (40) WMED YOY 1998-2002 (255) EMED YOY 1998-2002 (25)	Global differentiation (F_{ST}= 0.0059, p = 0.0005) Differentiation of WMED and GOM (F_{ST}= 0.0048, p = 0.0260) Differentiation of WMED and EMED (F_{ST} = 0.0067, p = 0.0279) Differentiation of GOM and EMED (F_{ST}= 0.0117, p = 0.0236)	Carlsson J, McDowell JR, Carlsson, J.E and Graves JE (2007) J Heredity 98: 23-28.
2007	mtDNA CR	Gulf of Mexico Larvae 2003 (40) WMED YOY 1998-2002 (255) EMED YOY 1998-2002 (25)	Global differentiation (Φ_{ST}= 0.0129, p = 0.0139) Differentiation of WMED and GOM (Φ_{ST} = 0.0104, p = 0.0359) Differentiation of WMED and EMED (Φ_{ST} = 0.0174, p = 0.0482) No difference of GOM and EMED (Φ_{ST} = 0.0134, p = 0.1105)	Carlsson J, McDowell JR, Carlsson, JE, Graves JE (2007) J Hered 98: 23-28.
2008	mtDNA CR	Gulf of Mexico 1995-2005 (61) Western Mediterranean 1997-2004 (47) Eastern Mediterranean 1997-2003 (62) Range of size classes	Differentiation of GOM and MED (Φ_{ST} = 0.01116, p = 0.03029) No significant differentiation of GOM and WMED (Φ_{ST}= 0.01223, p = 0.06554) No significant differentiation of GOM and EMED (Φ_{ST}= 0.00699, p = 0.12019) No significant differentiation of WMED and EMED (Φ_{ST}= -0.00419, p = 0.6504)	Boustany AM, Reeb CA and Block BA (2008) Mar Biol 156: 13-24.
2010	8 microsatellites	Adriatic 2003-2005 (73) S. Tyrrhenian 2007 (39) Ligurian 1999-2000 (36) SW Sardinia 2005 (29) Algeria 2006 (39) Alboran 2005 (40) Historical Adriatic 1926-1927 (69) Historical Tyrrhenian 1911 (39)	Global F_{ST} contemporary (F_{ST} = 0.014, P < 0.0001) Differentiation of historical samples (F_{ST} = 0.020, P < 0.0001) Differentiation among all pairwise comparisons except contemporary populations of Algeria and S Tyrrhenian, Ligurian and SW Sardinia, and Adriatic and Ligurian.	Riccioni G, Landi M, Ferrara G, Milano I, Cariani A, Zane L, Sella M, Barbujani G and Tinti F (2010) Proc Natl Acad Sci 107: 2102–2107.
2011	7 microsatellites	Turkey 2008 (48) Balearics 2008 (48)	No differentiation of EMED and WMED (F_{ST} = 0.002, P = 0.2)	Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü and Araguas RM (2011) Rev Fish Biol Fish 21: 527-541.
2011	mtDNA CR	Turkey 2008 (48) Balearics 2008 (48) *Augmented by samples from Ely *et al.* (2002) and Bremmer *et al.* (2005) Total MED of known origin = 516 (1990-2010)	No global differentiation (Φ_{ST} = -0.004, P = 0.618) No differentiation of EMED and WMED (n = 426) (Φ_{ST}= 0.002, P = 0.135) No temporal differentiation across all 516 samples (Φ_{ST} = 0.004, P =0.111)	Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü and Araguas RM (2011) Rev Fish Biol Fish 21: 527-541.
2013	17 SNPs	Bay of Biscay (46) Balearic Sea (46) NW Atlantic (Virginia) (15)	Differentiation of NW Atlantic, Bay of Biscay and Mediterranean Sea F_{ST} = 0.029±0.024, P < 0.05 He = 0.272 ± 0.178 F_{IS} = 0.096 ± 0.133 BB–NWA: F_{ST}= 0.120±0.091, P < 0.01 MED–NWA: F_{ST}=0.116±0.078, P < 0.01 BB-MED: F_{ST} = 0.004±0.007, P>0.01	Albaina A, Iriondo M, Velado I, Laconcha U, Zarraonaindia I, Arrizabalaga H, Pardo MA, Lutcavage M, Grant WS and Estonba A (2013) Animal Genetics. doi: 10.1111/age.12051

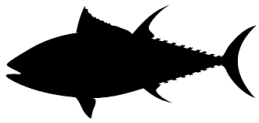


MIGRAZIONI DI TONNI

Chapter 5: Assessment of the genetic population structure of modern bluefin tuna using a genotyping-by-sequencing approach.

When buxom spring's luxuriant airs inspire
The softer wish, and blow the genial fire,
The tunnies, rushing from th'Atlantic Deep,
In Midland seas with us their nuptials keep.
Them first Iberia's hardy sons detain,
Skill'd in the labours of the bloody plain;
Next, near the Rodan's Mouth, the swain that boasts
Maffilia's Pleasures, and Phocean Coasts.
Next Ætna's Isle, and rich Etruria's soil
Dismiss their tillers to the wat'ry toil.
To wider deeps beyond the Tuscan shore
The shoal disperses, and the sport's no more.
Prodigious draughts enrich experienced swains,
When am'rous tunnies lead their vernal trains,
Some likely coast of fit extent they find.
With mossy caves and verdant herbage lin'd ;
Steep be the shore, and gentle be the wind.
A faithful spy some neighboring mount ascends,
And gives the timely signal to his friends.
With watchful look the coming shoal defies,
Recounts their numbers, and remarks their size.
Nets, like a city, to the floods descend,
Their gates, their bulwarks, and their streets extend.
Distinguisht by their families and years
With swift advance the marshall'd troop repairs,
Crowds unsuspicious thro the fatal way,
And loads the closing net with copious prey.

- Oppian's Halieuticks, 2nd century C.E.



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5.1 Introduction

Currently, ICCAT manages the Atlantic bluefin tuna stocks (BFT) as if they were compsed of two populations: one which spawns in the Gulf of Mexico and forages in the North Western Atlantic and a second that spawns in the Mediterranean Sea and forages in the Mediterranean and North Eastern Atlantic (Block et al. 2005). Several tagging and otolith studies have demonstrated that these two populations mix extensively and form large feeding aggregations in the Atlantic Ocean, particularly in the vicinity of the Mid-Atlantic Bight (see Chapter 1). Despite this mixing, spawning seems to occur in isolation and some evidence suggests that the two populations appear to have developed pronounced differences in life history traits and physical characteristics (Waples 1998; Fromentin and Powers 2005; Heinisch et al. 2008). It has been a widely held assumption among ICCAT's Standing Committee on Research and Statistics that BFT from the north eastern Atlantic and Mediterranean Sea mature after approximately 4 years (~110cm/35kg), while those from the north western Atlantic aren't mature until they reach 8 years of age (~200cm/150kg; Fromentin and Powers 2005). However, this assumption is increasingly criticized and recently, at the 2015 ICCAT BFT Data Preparatory Meeting (Madrid, 2-6 March 2015) several committee members expressed doubts about its validity. Regardless of these doubts, the theory of isolated spawning occurring in the Gulf of Mexico and the Mediterranean is well established and it is rarely called into question, if ever. Now, managers are asking for a genetic tool that can be used to identify the origin of individuals in order to detect seafood fraud as well as monitor rates of mixing and recruitment.

Recent evidence from an array of technologies including satellites tags, genetic markers and microchemical analysis of otoliths suggest that the population structure of BFT is even more complex than the current two population management model (reviewed in Chapter 1). The eastern BFT may actually consist of a nomadic population that spawns in the western Mediterranean and forages in the Atlantic Ocean and another population that remains within the Mediterranean, spawning in isolation in the Levantine Sea (Fromentin 2009). Historical accounts of fish migrations in the Mediterranean refer to large herds of BFT in the vicinity of the Black Sea at the end of the summer when modern adult BFT are expected to be feeding outside of the Mediterranean Sea in the Atlantic Ocean (Badham 1854). Moreover, Aristotle wrote about the meat of BFT caught near Constantinople being much more enjoyable in the fall, after the annual plague of parasitic gadflies

(probably copepods) abates (Aristotle 350 BCE). BFT in the western and eastern basins of the Mediterranean Sea also show geographic differences in life history traits. It is well known that BFT spawn in the eastern Mediterranean in late May, in the central Mediterranean in June and as late as July in the western Mediterranean. A geographic gradient of sea surface temperatures is likely responsible for this chronological cascade of spawning, as the Mediterranean warms up earlier in the east than the west (Heinisch et al. 2008). A temporal difference in spawning times would reinforce the separation of populations if BFT are indeed returning to the locations of their natal origin for spawning (Rooker et al. 2008). If BFT in the Mediterranean Sea are indeed two separate populations spawning in isolation, molecular tools should be able to detect genetic differences between the two. These differences may already be reflected in the phenotypes of BFT captured throughout the Mediterranean. A survey of adult fish captured in the western and eastern Mediterranean between May and July of 2003-2005 revealed that approximately 95% of fish caught in the west were large fish (>100kg) while 40% of the eastern BFT could be considered small (<100kg; Heinisch et al. 2008). Differences in physical characteristics may simply reflect the species' phenotypic plasticity or selective habitat use by different demographic groups. Alternatively, they may be the expression of genetic adaptations to localized environmental conditions (temperature, salinity and water circulation; Reeb 2010). Several studies have demonstrated weak signals of populations structuring within the Mediterranean Sea (Carlsson et al. 2004, 2005; Riccioni et al. 2010, 2013; see Chapter 4 for review); however, these studies can be criticized for not having adequate sample sizes and a lack of representation from the eastern Mediterranean (Levantine Sea). Moreover, similar studies using the same techniques and markers have been unable to detect differentiation of populations within the Mediterranean (Boustany et al. 2008; Viñas et al. 2008). As such, the issue clearly remains unresolved.

As described in Chapter 4, a 96 SNP genotyping panel was developed and technically validated. Here we use the same SNP panel for the genotyping of an additional 556 BFT, bringing the total number of genotyped individuals up to 723. This represents the most ambitious number of samples to be analyzed since Pujolar et al. (2003) and Carlsson et al. (2006) who each genotyped 800 and 822 individuals, respectively. This study is also the most geographically comprehensive genetic analysis of BFT, with samples coming from as far away as Libya, Turkey, the Gulf of Mexico and the Gulf of St. Lawrence. Samples were collected over

a six year period with several samples captured at the same geographic location over mutliple years, thereby allowing for an analysis of inter-annual genetic variation. This ambitious project allowed for a rigorous assessment of the genotyping panel and contributed to a more complete understanding of the spatial dynamics of the species. An accurate understanding of the population structure of BFT and the factors that affect their distribution is essential for the development of management plans capable of ensuring the conservation of the species.

5.2 Materials and Methods

5.2.1 Sample collection

A total of 731 Atlantic bluefin tuna (BFT, *Thunnus thynnus*) from different age classes were captured in 14 different gepgraphical locations throughout the species' range (Table 1; Fig. 1, Appendix 1). All larvae were collected using plankton nets and sacrificed via immersion in 96% ethanol, according to standard larval survey practices. Beside morphological identification, all larvae specimens were barcoded as described in Chapter 3 to ensure proper species assignment.

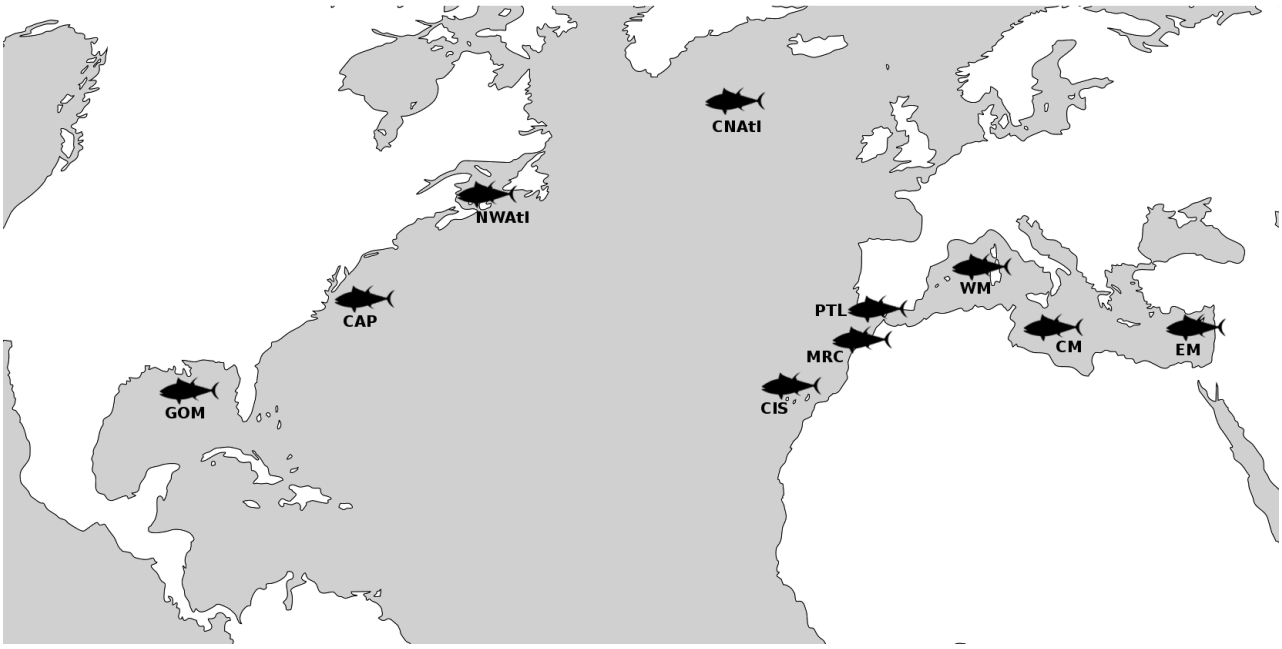

Fig. 1. Sampling locations of Atlantic bluefin tuna (medium and large adults, young-of-the-year, and larvae) genotyped (GOM = Gulf of Mexico; CAP = Cape Hatteras; NWAtl = North West Atlantic; CNAtl = Central North Atlantic; CIS = Canary Islands; PTL = South Portugal; MRC = North West Morocco; WM = Western Mediterranean or Balearic Islands, Tyrrhenian, Ligurian; CM = Central Mediterranean or Sicily, Malta, Gulf of Syrta; EM = Eastern Mediterranean or Levantine Sea). See **Appendix 1** for a detailed map of Mediterranean Sea sampling locations.

All YOY were captured with handlines and trolling lines. Adults were captured by long line and purse seine. Permission to conduct sampling surveys and all legal permits were granted by ICCAT. All research was coordinated through the "Atlantic-wide research programme for bluefin tuna" (GBYP) and ICCAT issued a recommendation (Rec. 11-06) allowing the parties involved in this research to collect and sacrifice young Atlantic BFT for the purposes of genetic research and transport samples from one country to another (Cert. No. ICCAT RMA12-049).

Table 1: Samples genotyped with the selected 96 SNP panel. Integers in parentheses in N. Individuals column represents samples that were eventually removed from the analysis due to low genotyping success (<70%SNPs genotyped). Samples used for SNP development are indicated with an asterisk.

Sampling location	Geographic region	Sample code	Age class	Year	N. individuals
Gulf of Mexico	GOM	WA-GM07-V*	Larvae	2007	2
	GOM	WA-GM08-V*	Larvae	2008	8
	GOM	WA-GM09b-V*	Larvae	2009	10
	GOM	WA-GM09-V*	Larvae	2009	8
	GOM	WA-GM10-V	Larvae	2010	40 (4)
Cape Hatteras	CAP	WA-CH08-0*	Young-of-the-Year	2008	16
Gulf of St. Lawrence	NWAtl	WA-GSL13-L	Large Adult	2013	23 (1)
Central North Atlantic	CNAtl	CNAT12-M	Medium Adult	2012	24
	CNAtl	CNAT12-L	Large Adult	2012	47 (1)
Canary Islands	CIS	EA-CI13-L	Large Adult	2013	24
Morocco	MRC	EA-MO12-L	Large Adult	2012	24
	MRC	EA-MO13-L	Large Adult	2013	19
Portugal	PTL	EA-PO11-L	Large Adult	2011	23
Balearic Sea	BAL	WM-BA11-0*	Young-of-the-Year	2011	24
	BAL	WM-BA12-V	Larvae	2012	40
	BAL	WM-BA13-0	Young-of-the-Year	2013	34
Ligurian Sea	LIG	WM-LI13-0	Young-of-the-Year	2013	33
Tyrrhenian Sea	TYR	WM-TY12-0*	Young-of-the-Year	2012	24
	TYR	WM-TY13-0	Young-of-the-Year	2013	40
Malta	MLT	CM-MA13-0	Young-of-the-Year	2013	40
Strait of Sicily	STS	CM-SI08-V	Larvae	2008	25 (2)
	STS	CM-SI11-0*	Young-of-the-Year	2012	21
	STS	CM-SI12-0*	Young-of-the-Year	2012	27
	STS	CM-SI13-0	Young-of-the-Year	2013	40
Gulf of Syrta	SRT	CM-SY12-M	Medium Adult	2012	24
	SRT	CM-SY12-L	Large Adult	2012	24
Levantine Sea	LEV	EM-LS11-0*	Young-of-the-Year	2011	27
	LEV	EM-LS13-0	Young-of-the-Year	2013	40
	Total				731 (8)

5.2.2 DNA extractions and SNP-typing

Approximately 20mg of muscle tissue/finclip from post-larval BFT and complete or partial (caudal fin or eyeball) larvae were digested overnight in a Proteinase K solution. Genomic DNA was extracted from all individuals and purified using Promega's Wizard®SV96 Genomic DNA Purification kit and vacuum manifold following the manufacturers' protocols. DNA was eluted with 60 μL of distilled water heated to 60°C.

SNP-typing was carried out with the Biomark™ HD platform (Fluidigm) and run with ROX as the passive reference dye and FAM and HEX SNPtype™ Assays for specific allele calling.

Basic quality control analysis was performed on the raw data using summary statistics produced with Arlequin v3.5.2 **(**Excoffier and Lischer 2010). Individuals with low genotyping coverage (<70% SNPs genotyped) and loci with an excess of missing data (>10%) were removed from the analysis. SNPs with minor allele frequencies (MAF) of less than 0.01 among all samples were removed from the dataset.

5.2.3 Genetic diversity and outlier detection

BFT captured in the Gulf of Mexico between 2007 and 2009 were pooled for all calculations, due to the limited number of larvae in each sample. Values of expected and observed heterozygosity were calculated in Genetix v. 4.05 (Belkhir et al. 1996-2004). The inbreeding coefficient (F_{IS}; Weir and Cockerham 1984) was calculated for each sample using Arlequin v3.5. 2. All loci were checked for departure from HWE across the entire dataset and among each sample separately, using Genepop on the Web v.4.2. (Raymond and Rousset 1995; Rousset 2008) computing a locus by locus test for Hardy Weinberg equilibrium, performed with one million Markov chain steps and 100k dememorization steps.

Identification of loci under selection was performed using Arlequin v3.5.2 with the following settings: finite island model, 100 simulated demes per group, 20000 coalescent simulations performed. Outlier loci were again identified using the Beaumont and Nichols (1996) FDIST approach, implemented in Lositan (Antao et al. 2008) with a 0.995 confidence interval. Sequences of all identified outlier loci were submitted to BLAST alignments (Altschul et al. 1990) against all sequences contained in the NCBI database using the blastn algorithm.

After optimization of panel of SNPs to be used for analysis, loci selected for intra-Mediterranean and

trans-Atlantic discrimination of groups (see Chapter 4) were divided into SNP panels containing 43 and 46 loci, respectively.

5.2.3 *Population genetic structure*

The genetic heterogeneity and differentiation of YOY and larvae samples was estimated in Arlequin 3.5.2, using several analyses of molecular variance (AMOVA) in order to examine multiple levels of variance in the dataset. The following population structuring scenarios were explored by assigning samples to various *a priori* arbitrary groups: 1) Western Atlantic (GOM, CAP) vs. Mediterranean, 2) Western Mediterranean (BAL) vs. Central West Mediterranean (TYR, LIG) vs. Central Mediterranean (STS, MLT) vs. Eastern Mediterranean (LEV), 3) Temporal replicated pooled by geographic region (see Table 1), and 4) All samples analyzed separately. Additional analyses were performed using genotypes generated by the 46 and 43 SNP sub-panels for tran-Atlantic and intra-Mediterranean differentiation. All AMOVAs were performed with 1000 permutations and a significance level of 0.05. Pairwise comparisons between samples were executed using FSTAT v.2.9.4 (Goudet 1995), testing for significance with the adjusted nominal level (α=0.05) for multiple comparisons (Bonferroni correction).

5.2.4 *Detection of genetic clusters*

Genetic admixture and population structuring was investigated using STRUCTURE v2.3.4 (Pritchard et al. 2000), a software that employs a Bayesian model-based clustering method. During all STRUCTURE simulations, the LOCPRIOR model was used because of the low F_{ST} values provided by population differentiation analysis. STRUCTURE provides an option to use geographic sampling locations as prior information to assist with the clustering of groups in datasets that have genuine population structure but the signal is too weak for standard structure models to detect (Pritchard et al. 2010). The LOCPRIOR model is suitable when one is assuming that the sampling locations may be informative about ancestry (Hubisz et al. 2009). STRUCTURE analysis was conducted with the following settings: 10k Burn In, 50k Reps, Admixture Model, LOCPRIOR using the geographical regions defined in Table 1 and lambda inferred for each run. A plot of the mean likelihood L(K) and variance per K value from all STRUCTURE analyses was obtained using Structure Harvester (Earl and von Holdt 2012) and used to infer the optimal K value or number of ancestral clusters. Individual clustering was further assessed using the Adegenet package v1.4-2 in R, which

provides a newly dedicated tool Discriminant Analysis of Principal Components (DAPC) that perform Discriminant Analysis (DA, defining groups), based upon a dataset of Principal Components (PC, reducing data to several dimensions) constructed from multi-locus genotypes (Jombart 2008; Jombart and Ahmed 2011).

5.2.5 Population assignment tests

Individuals from two datasets containing 1) young and 2) adult BFT were assigned to the most likely spawning areas of origin using Geneclass2 software (Piry et al. 2004), considering as reference for assignment individuals divided in two broad classes: the Western Atlantic and the Mediterranean. In an effort to avoid upward bias of assignment scores, an additional unbiased method for the calculation of assignment scores was performed (Anderson 2010). A random selection of 50% of young individuals were selected from each sample and pooled by the two geographic regions to serve as assignment references, while the other 50% were used as queries. The Rannala and Mountain (1997) criterion was used along with a threshold of 0.05 and a minimum threshold of 70% for the scores of assignment was implemented throughout all population assignment analyses.

5.3 Results

5.3.1 SNP genotyping

Eight individuals were removed from the dataset due to low genotyping coverage (Table 1). The success rate for genotyping of the remaining 723 individuals across all loci was high (99%). Two loci (SNP_010, SNP_329) with high missing data (>10%) and fourteen SNPs had MAF < 0.01 across all populations were excluded from calculations.

5.3.2 Genetic diversity and outlier detection

Observed and expected heterozygosity ranged from 0.377 to 0.398 (mean = 0.391 ± 0.007; Table 2) and from 0.365 to 0.395 per sample (mean = 0.386 ± 0.010; Table 2), respectively. Seven SNPs significantly deviated from HWE (low He) when analyzed among all samples; however when analyzed separately none of these loci were consistently divergent from HWE among all samples.

Calculations of the inbreeding coefficient (Table 2) showed that the sample from Cape Hatteras (n=16) deviated from HWE due to a deficit of heterozygotes (F_{IS} = 0.092, p-value = 0.042).

Table 2. Summary statistics for 80 SNP loci among eight samples pooled by geographic sampling locations. Number of individuals (n), expected heterozygosity (H_E) and observed heterozygosity (H_O).

Pooled Geographic Samples	n	H_E	H_O	F_{IS}	P (rand F_{IS}>=Obs F_{IS})
Gulf of Mexico	64	0.385	0.359	0.040	0.080
Cape Hatteras	16	0.365	0.340	0.092	0.042
Balearic Sea	98	0.395	0.384	0.025	0.139
Ligurian Sea	33	0.378	0.368	-0.017	0.674
Tyrrhenian Sea	64	0.391	0.379	0.028	0.169
Malta	40	0.393	0.378	0.046	0.110
Sicily	111	0.389	0.381	0.013	0.287
Levantine Sea	67	0.395	0.381	0.033	0.125

Outlier loci analysis revealed that only two loci (SNP161 and SNP385) are candidates for positive selection (F_{ST} = 0.057 and 0.117, respectively; Fig. 2). Western Atlantic samples appear to have a higher proportion of cytosine (0.451-0.467) at SNP_161, when compared to the samples from the Mediterranean (0.176-0.248; Appendix 2). Conversely, at SNP_385 Mediterranean samples have a lower proportion of cytosine (0.406-0.667) than samples from the Mediterranean (0.718-0.95). Neither of these loci aligned with any published sequences from the NCBI database.

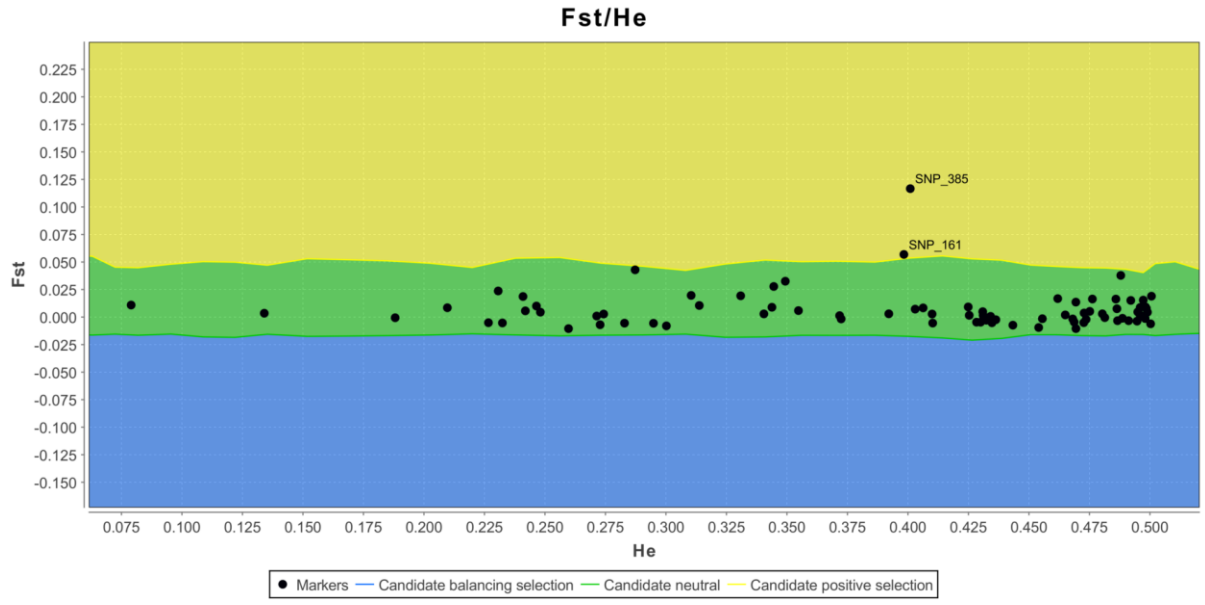

Fig. 2: Comparison of F_{ST} and heterozygosity (H_E) in 80 polymorphic loci from 723 individuals to identify outliers and potential candidates for selection using Lositan. Loci highlighted in yellow are candidates for positive selection, in blue candidates for balancing selection and in green are selectively neutral.

5.3.3 Genetic differentiation

Analysis of the fixation indices of all samples (F_{IS}), pairs of samples (pair-wise F_{ST}) and individuals (F_{IT}) revealed that the majority of variation in the dataset could be attributed to between individual differences (Table 3). Among the different sample groupings analyzed via AMOVA, the only scenario that was provided with significant statistical support was that of a population in the Mediterranean and another in the Gulf of Mexico (Table 3, AMOVA 1; Table 4, AMOVA 5). The 46 SNP sub-panel provided higher F_{ST} values (FST = 0.013, p = 0.004 ± 0.002) than the full 80 SNP panel (FST = 0.008, p = 0.010 ± 0.004) for the pairwise comparison between these two regions.

Table 3. Results from various AMOVAs using 80 SNP loci and three different populations structuring scenarios. For sample codes refer to **Table 1.**

AMOVA Groupings	**% of variation**	**Fixation indices**	**p-value**
AMOVA 1 – All samples, two groups: GOM, CAP vs. all Mediterranean samples			
Among groups	0.75	FCT = 0.008	0.010 ± 0.004
Among samples within groups	0.56	FSC = 0.006	0.000 ± 0.000
Among individuals within samples	2.23	FIS = 0.023	0.018 ± 0.004
Within individuals	96.45	FIT = 0.035	0.004 ± 0.002
AMOVA 2 – All samples, eight pooled groups: WAGM, WACH, BAL, LIG, TYR, STS, MLT, LEV.			
Among groups	-0.08	FCT = -0.001	0.512 ± 0.014
Among samples within groups	0.85	FSC = 0.009	0.000 ± 0.000
Among individuals within samples	2.24	FIS = 0.023	0.019 ± 0.004
Within individuals	96.98	FIT = 0.030	0.001 ± 0.001
AMOVA 3 – Mediterranean samples only, four groups: (BAL) vs. (TYR, LIG) vs. (STS, MLT) vs. (LEV)			
Among groups	-0.28	FCT = -0.002	0.985 ± 0.004
Among samples within groups	0.80	FSC = 0.008	0.000 ± 0.000
Among individuals within samples	1.79	FIS = 0.018	0.065 ± 0.010
Within individuals	97.69	FIT = 0.023	0.020 ± 0.005

Table 4. Results from various AMOVAs using two SNP sub-panels designed to elucidate differentiation of western Atlantic and Mediterranean samples (46 loci) and sub-groups within the Mediterranean (43 loci). For sample codes refer to **Table 1.**

AMOVA Groupings	% of variation	Fixation indices	p-value
AMOVA 5 – 46 SNP WAtl vs. MED sub-panel: GOM, CAP vs. all Mediterranean samples			
Among groups	1.31	FCT = 0.013	0.004 ± 0.002
Among samples within groups	0.58	FSC = 0.006	0.000 ± 0.000
Among individuals within samples	1.99	FIS = 0.020	0.044 ± 0.008
Within individuals	96.11	FIT = 0.039	0.010 ± 0.003
AMOVA 6 – 43 SNP MED sub-populations : BAL vs. LIG vs. TYR vs. MLT vs. STS vs. LEV			
Among groups	-0.31	FCT = -0.003	0.864 ± 0.012
Among samples within groups	0.82	FSC = 0.008	0.000 ± 0.000
Among individuals within samples	1.86	FIS = 0.019	0.079 ± 0.007
Within individuals	97.63	FIT = 0.024	0.029 ± 0.006

Pairwise comparisons of all samples showed differences between geographic areas that were not persistent between years (Appendix 3). For this reason, multi-year sets of data were combined where sampling had taken place in the same areas over multiple years. Young tuna caught during different sampling seasons in the Gulf of Mexico and Cape Hatteras showed very little variation between samples, providing further support to pool temporal samples in this region in order to increase statistical power (F_{ST} = 0.006 – 0.0439; Fig. 3 A,B). After pooling, Western Atlantic samples significantly differed from all other Mediterranean samples (F_{ST} = 0.006 – 0.0278; Fig. 3 C,D). The F_{ST} matrices using data from the 80 SNP panel also show significant differences between samples from the Strait of Sicily and Malta (F_{ST} = 0.0057). F_{ST} matrices plotting the divergence of genotypes from the 46 SNP panel show significant differentiation of the sample from Malta and samples from the Strait of Sicily (F_{ST} = 0.0068), Balearic (F_{ST} = 0.006) and Tyrrhenian Sea (F_{ST} = 0.0077).

5.3.4 Detection of genetic clusters

Results from the Bayesian cluster analysis performed with Structure v.2.3.4 showed a distinct divergence of the Western Atlantic and Mediterranean samples as well as subtle but temporally stable differences among Mediterranean samples (Fig. 4). The results from the DAPC analysis in Adegenet showed a weaker degree of differentiation of Western Atlantic samples from the Mediterranean samples, which are

completely overlapping, suggesting migration of individuals between geographical areas and interbreeding (Fig. 5).

A

	GOM	CAP	BAL	LIG	TYR	MLT	STS	LEV
GOM		NS	*	NS	*	*	*	*
CAP	0.0004		*	NS	NS	*	*	*
BAL	0.0084	0.0149		NS	NS	NS	NS	NS
LIG	0.0052	0.014	0.0001		NS	NS	NS	NS
TYR	0.006	0.0124	0	0.0005		NS	NS	NS
MLT	0.0119	0.0253	0.0038	0.0029	0.0028		*	NS
STS	0.0076	0.0132	0.0005	0	0.0012	0.0057		NS
LEV	0.0087	0.0169	-0.0012	-0.0003	0.0004	0.0033	-0.0002	

B

	GOM	CAP	BAL	LIG	TYR	MLT	STS	LEV
GOM		NS	*	NS	*	*	*	*
CAP	0.0035		*	NS	*	*	*	*
BAL	0.0123	0.0285		NS	NS	*	NS	NS
LIG	0.0071	0.0266	-0.0022		NS	NS	NS	NS
TYR	0.0098	0.0233	-0.0024	0.0008		NS	NS	NS
MLT	0.0239	0.0439	0.006	0.0027	0.0077		*	NS
STS	0.0106	0.0249	-0.0014	-0.0005	-0.0008	0.0068		NS
LEV	0.013	0.0292	-0.0026	-0.0019	-0.0009	0.0048	-0.0013	

C

	WAtl	BAL	LIG	TYR	MLT	STS	LEV
WAtl		*	NS	*	*	*	*
BAL	0.0096		NS	NS	NS	NS	NS
LIG	0.0067	0.0001		NS	NS	NS	NS
TYR	0.0072	-0	0.0005		NS	NS	NS
MLT	0.0147	0.0038	0.0029	0.0028		*	NS
STS	0.0085	0.0005	-0	0.0012	0.0057		NS
LEV	0.0103	-0.0012	-0.0003	0.0004	0.0033	-0.0002	

D

	WAtl	BAL	LIG	TYR	MLT	STS	LEV
WAtl		*	NS	*	*	*	*
BAL	0.0152		NS	NS	NS	NS	NS
LIG	0.0106	-0.0022		NS	NS	NS	NS
TYR	0.0122	-0.0024	0.0008		*	NS	NS
MLT	0.0278	0.006	0.0027	0.0077		*	NS
STS	0.0131	-0.0014	-0.0005	-0.0008	0.0068		NS
LEV	0.0161	-0.0026	-0.0019	-0.0009	0.0048	-0.0013	

Fig. 3: Pair-wise F_{ST} results using young tuna (larvae and young-of-the-year) samples pooled by geographic region. Statistical significance of results are indicated as significant (*) or non-significant (NS) after correction for multiple testing (adjusted nominal level of 0.05) above the diagonal and F_{ST} values are shown below the diagonal. F_{ST} values >0.005 and < 0.01 are highlighted in light blue. F_{ST} values >0.01 are highlighted in dark blue.Matrices summarize results from pairwise comparisons using 80 SNPs (A,C) and 46 SNPs (B,D), as well as pooled (C,D) and unpooled (A,B) western Atlantic samples. For sample codes refer to Table 1.

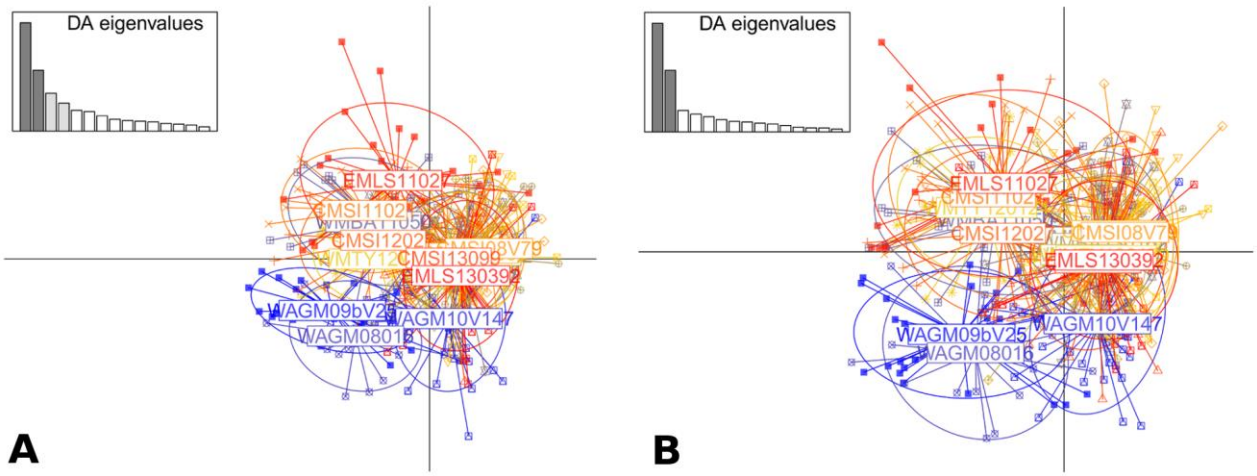

Fig. 5: Discriminant analysis of principal components of 493 young tuna individuals considering 80 SNP (A) and 46 SNP panels (B).

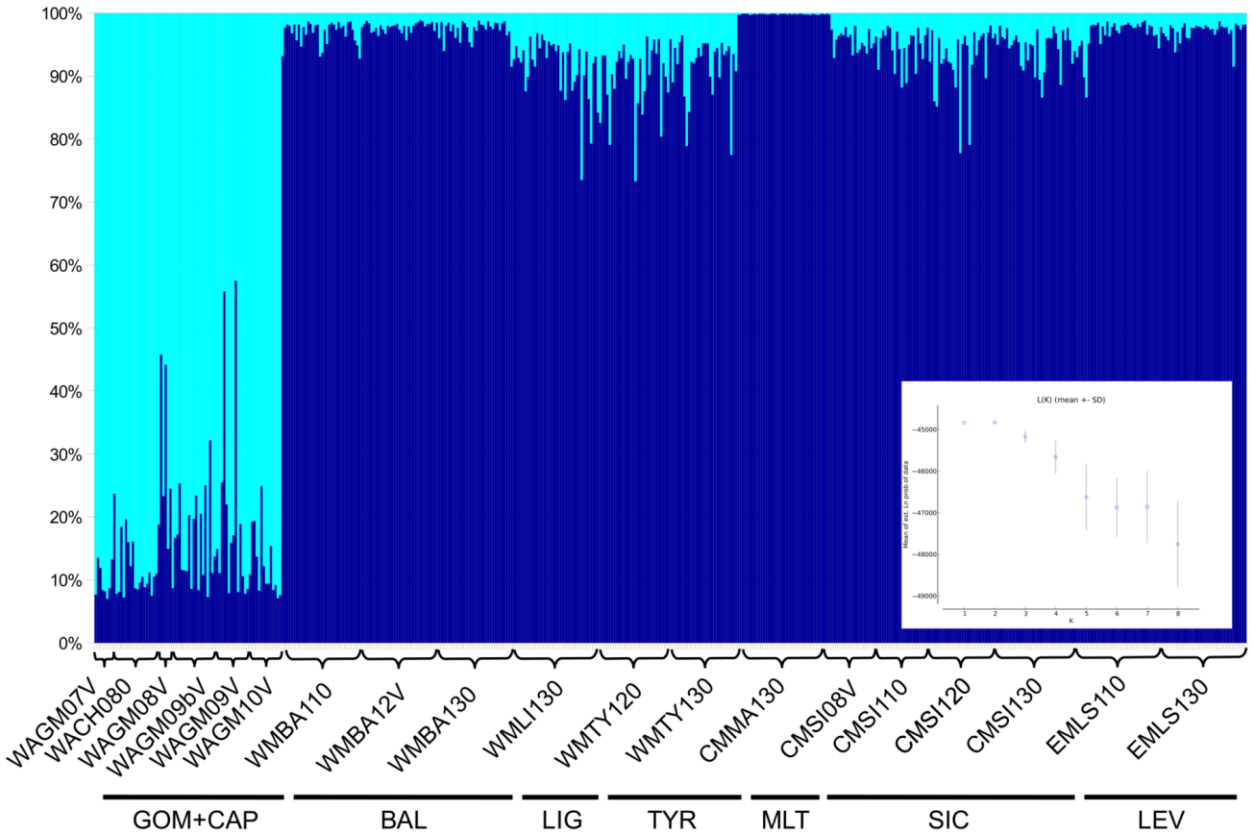

Fig. 4: STRUCTURE plot of 493 young tuna samples genotyped at 80 SNPs. Individual columns represent the proportion of membership from two inferred ancestral clusters. Plot of the mean likelihood L(K) and variance per K value from all STRUCTURE analyses, produced by Structure Harvester is inset.

5.3.5 Assignment tests

A total of 80.3% of young tuna and 79.1% of adults were assigned to one of the two regions of origin, using the reference datasets composed of young tuna genotypes. Results from the direct self assignment of all young tunas to the full reference datasets (Appendix 4) were very similar to the unbiased method for assignment that used 50% of young tuna as references and the remaining 50% as queries (Table 5). The proportion of individuals assigned to the Western Atlantic (16.0%, average score = 87%) and Mediterranean Sea (64.3%, average score = 91.6%) using the unbiased method changed little from the results using the full self assignment datasets (19.1%, average score = 88.4% and 60.2%, average score = 92.2%, respectively). The average scores for assignment of medium and large adult samples to the Western Atlantic and Mediterranean were 87% and 92%, respectively (Table 6). Same assignment tests performed using the 46 SNP sub-panel resulted in an overall decrease in both assigned individuals (n=14) and average assignment

score (~1%) and an increase of unassigned individuals. In total, the proportion of adults assigned to the Western Atlantic and Mediterranean was 10% and 67.4%, respectively. When the proportion of individuals from each sample assigned to the two spawning areas are visualized onto maps (Fig. 6; Fig. 7), rates of mixing throughout the range become more obvious.

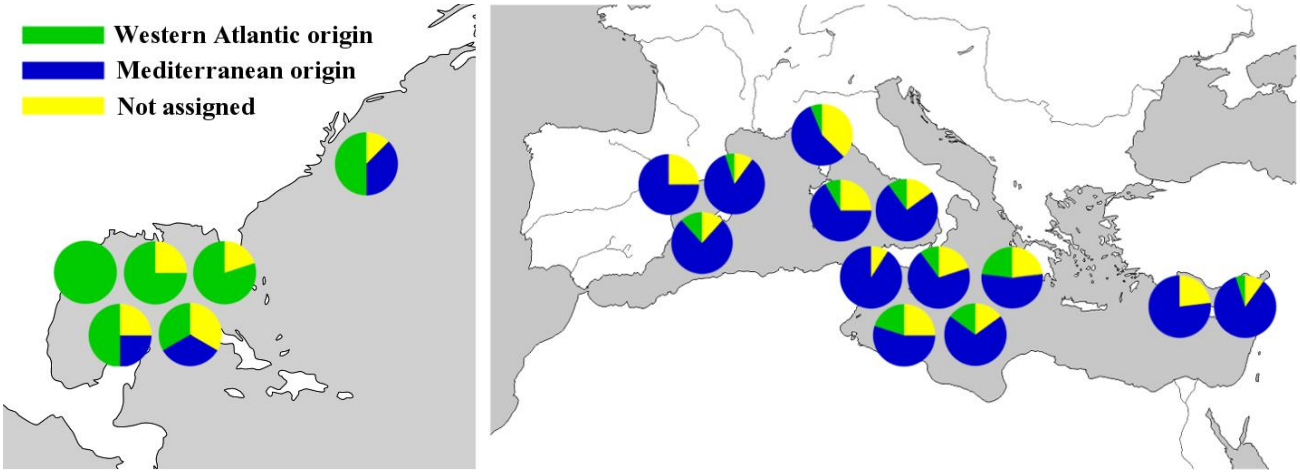

Fig. 6: Proportional assignment of 50% of randomly selected larvae and young-of-the-year samples (n=244) to Western Atlantic and Mediterranean spawning areas of origin as defined by the genotypes of the remaining 50% of individuals in each sample, calculated using GeneClass2. Individuals with an assignment score <70% were considered NOT ASSIGNED.

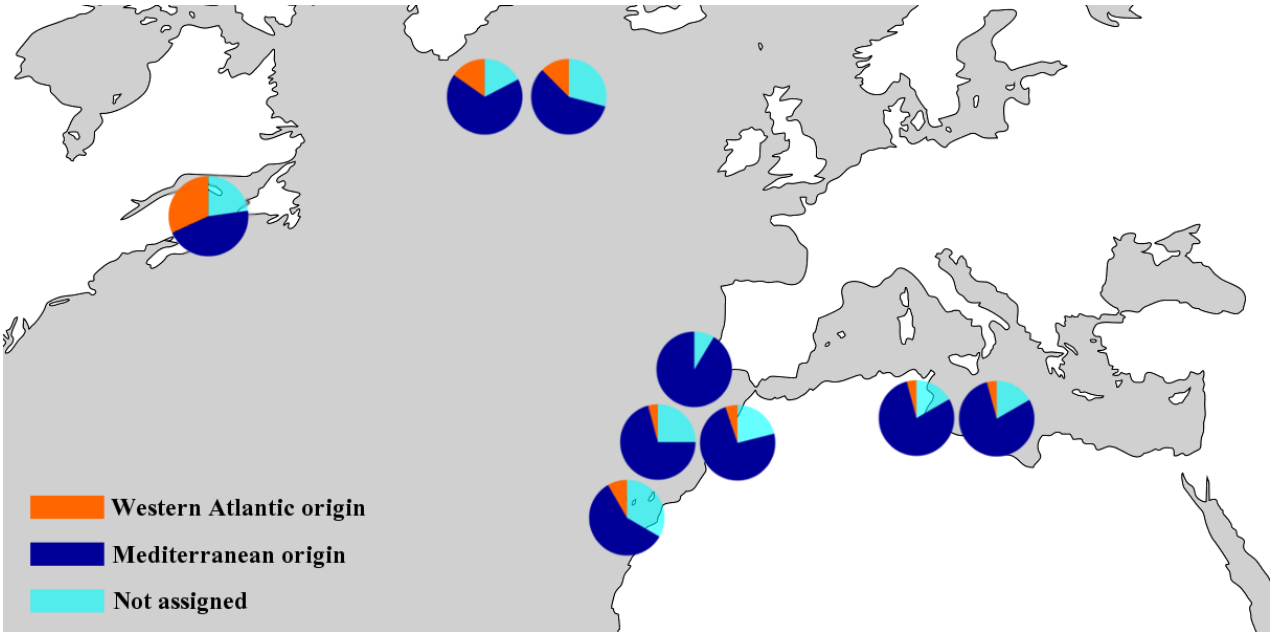

Fig. 7: Proportional assignment of adult samples (n=230) to Western Atlantic and Mediterranean spawning areas of origin, calculated using GeneClass2.

Table 5. Proportional assignment of 50% of randomly selected larvae and young-of-the-year samples (n=247) to Western Atlantic and Mediterranean spawning areas of origin as defined by the genotypes of the remaining 50% of individuals in each sample. Individuals with an assignment score <70% were considered NOT ASSIGNED.

SAMPLE	N	Proportion Assigned to Western Atlantic (%)	Avg. Score (%)	Proportion Assigned to Mediterranean (%)	Avg. Score (%)	NOT ASSIGNED	Avg. total assignment score (%)
WAGM07V	1	100.0	73.4	0.0	0.0	0	73.4
WAGM08V	4	75.0	95.7	0.0	0.0	1	85.0
WAGM09bV	5	80.0	83.9	0.0	0.0	1	79.1
WAGM09V	4	50.0	84.0	25.0	87.4	1	78.4
WAGM10V	18	33.3	93.8	33.3	86.0	6	79.3
WACH080	8	50.0	94.8	50.0	91.9	1	88.6
WMBA110	12	0.0	0.0	75.0	91.6	3	84.8
WMBA12V	20	5.0	78.6	85.0	90.3	2	87.1
WMBA130	17	11.8	85.9	76.5	90.2	2	85.5
WMLI130	16	6.0	77.1	56.3	94.8	6	81.3
WMTY120	12	8.0	73.7	66.7	92.3	3	82.9
WMTY130	20	10.0	92.6	75.0	93.8	3	88.5
CMMA130	20	20.0	84.0	55.0	90.7	5	80.9
CMSI08V	11	0.0	0.0	90.9	89.8	1	86.9
CMSI110	10	10.0	78.6	70.0	92.7	2	84.9
CMSI120	13	23.0	89.5	53.8	96.3	3	87.0
CMSI130	20	15.0	76.9	70.0	90.9	3	84.1
EMLS110	13	0.0	0.0	77.0	97.1	3	88.4
EMLS130	20	5.0	87.0	85.0	88.9	2	85.6
Total	244	16.0	87.0	64.3	91.6	48	84.6

Table 6. Proportional assignment of adult samples (n=230) to Western Atlantic and Mediterranean spawning areas of origin. Individuals with an assignment score <70% were considered NOT ASSIGNED.

SAMPLE	n	% assigned to Western Atlantic	Avg. score %	% assigned to Mediterranean	Avg. score %	Not assigned	Avg. total assignment score
WAGSL13L	22	31.8	77.2	45.4	91.14	5	79.0
CACA12L	46	15.2	89.0	67.4	90.3	8	84.3
CACA12M	24	12.5	74.1	58.3	92.3	7	80.8
EACI13L	24	8.3	86.8	58.3	93.3	8	81.7
EAMO12L	24	4.2	80.4	70.8	93.7	6	84.6
EAMO13L	19	5.3	93.0	73.7	91.3	4	85.3
EAPO11L	23	0.0	0.0	91.3	88.1	2	85.7
CMSY12L	24	4.2	81.1	79.2	92.4	4	86.5
CMSY12M	24	4.2	81.5	79.2	91.4	4	86.1
Total	230	10.0	82.4	69.1	91.3	48	83.8

5.4 Discussion

Population structuring analyses using young tuna samples (larvae and YOY) showed consistent differentiation between Western Atlantic and Mediterranean Sea samples, supporting the generally accepted hypothesis of maintained natal homing or spawning group fidelity, and the current paradigm of the management policy. The F_{ST} values presented here (F_{ST} = 0.008 – 0.013) that define the separation of these two large areas are similar to the values calculated by previous genetic studies using mtDNA (Φ_{ST} = 0.011 – 0.013; Boustany et al. 2008, Carlsson et al. 2007, respectively) and microsatellites (F_{ST} = 0.0059; Carlsson et al. 2007). Young tuna caught during different sampling seasons in the Gulf of Mexico and Cape Hatteras showed very little variation between samples, suggesting that these locations are connected as spawning and nursery areas. A higher proportion of individuals assigned to eastern origin in the Cape Hatteras samples suggests that there may be trans-Atlantic migration of young fish. These results may not have been detected by tagging studies in the past due to the simple difficulty of attaching tags to smaller fish.

Results from the Bayesian clustering and pairwise differentiation analyses suggest that there are subtle differences among spawning areas within the Mediterranean. The only significant F_{ST} value generated by pairwise comparisons among pooled samples is between Malta and several other groups from the western and central Mediterranean (Strait of Sicily, Tyrrhenian and Balearic Sea). Since the sample from Malta consists of a single season, additional sampling is required to determine if this signal is temporally stable. Statistically significant F_{ST} values detected among single sampling seasons and not others, suggest that some structuring is not persistent over time and that differences exist among cohorts. This raises doubts about previous studies that have used samples from one year only and should act as a testament for the need for multi-year sampling.

Considering the highly migratory nature of the species, the weak signal of differentiation (low yet statistically significant F_{ST} values) between populations is not surprising. Only a handful of individuals migrating between spawning areas each generation can eliminate most genetic evidence of stock structuring (Waples 1998). Although this number of migrants is insignificant for stock management purposes, it is significant for the delineation of stocks, and can thus interfere with the optimization of management policies based on genetic information. The F_{ST} values generated here are still relatively low when compared with other

taxa; however, these values are consistent with what is to be expected from a highly migratory marine species with a larval phase and recently generated populations (Alvarado Bremer et al. 2005). Ward et al. (1994) analyzed population differentiation values for 49 species of freshwater fish, 57 species of marine fish and seven anadromous species and determined that lowest levels of differentiation occurred among marine species. In this regard, the results concerning differentiation within the Mediterranean Sea should not be discarded simply because they lack statistical significance.

The SNP panel described herein has provided novel insights into the population structuring and spatial dynamics of Atlantic Bluefin Tuna. The high proportion of individuals assigned with high assignment scores to probable spawning areas of origin serves as a validation of our methods. The high proportion of "unassigned" and "cross-assigned" (fish identified as Gulf of Mexico origin found in the Mediterranean Sea and vice versa) is likely a result of the low number of individuals used in the reference pool and this should be overcome with addition of samples. This could also be attributed to the unexplored possibilities of genetic flow and unidentified alternative spawning areas in the Atlantic, however, deeper assessment of the statistical power of the developed set of genetic markers has to be properly assessed with simulation studies and comparison between different methods.

Results showing a high proportion of individuals in the west assigned to eastern origin are similar to the results of Rooker et al. (2008) who assigned BFT to origin using otolith micro-constituents. They determined that between 35 – 57.4 % of 154 mature BFT captured in the mid-Atlantic Bight were of eastern origin. Of the 132 adults they captured in the Mediterranean and analyzed, only ~4% were found to be of Western origin (Appendix 5). In Rooker et al. (2014), they too determined that a high fraction of YOY in the eastern Mediterranean (Cyprus) were of western origin using carbon and oxygen isotopes ($\delta^{13}C$ or $\delta^{18}O$).

5.5 Conclusion

Our SNP panel has provided novel insights into the population structuring and spatial dynamics of Atlantic Bluefin Tuna. The proportion of individuals assigned with high assignment scores to probable spawning areas of origin was high, serving as an indication of the high performance of the selected SNPs. In order to improve the analysis of assignment to Western Atlantic and Mediterranean spawning areas, additional

young tuna samples are required from the Gulf of Mexico and adult samples are required from the eastern Mediterranean. With further development and increased sampling, our capacity to trace individuals back to a Western or Eastern origin is expected to improve. The results from the analysis of young tunas provide further evidence of persistent population structuring across broad geographic areas, they can be used to estimate mixing rates and, with further statistical development, may lead to traceability tools. Efforts should be made in the future to combine analysis using this SNP panel with other techniques being used to investigate population structuring of BFT. Using the same individuals for multiple analyses (i.e. microchemical analysis of otoliths, different genetic approaches and otolith shape) would allow for reconciliation of inference between the different approaches.

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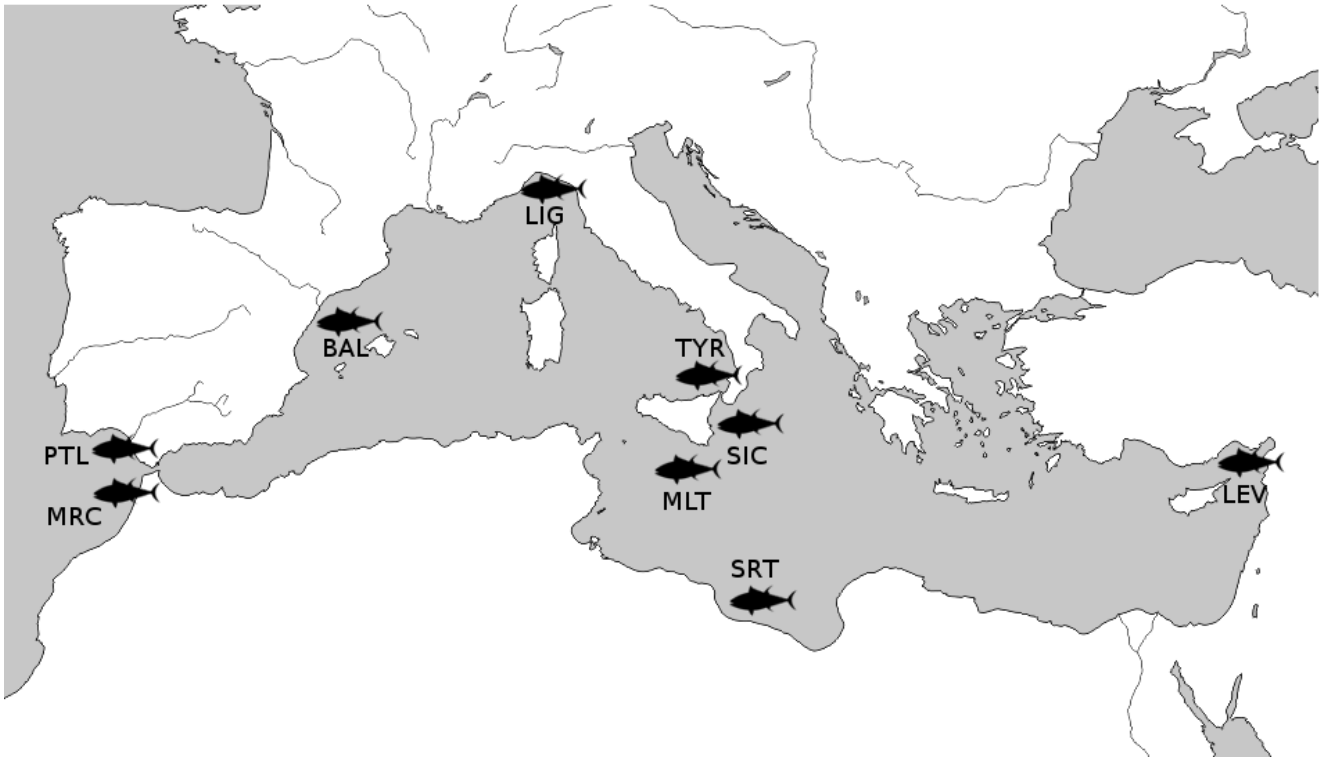
Appendices

Appendix 1. Higher resolution details of geographic locations of individuals captured within the Mediterranean Sea genotyped using a 96 SNP panel from Figure 2. (PTL = South Portugal; MRC = North West Morocco; BAL = Balearic Islands; LIG = Ligurian Sea; TYR = Tyrrhenian Sea; SIC = South Sicily; MLT = Malta; SRT = Gulf of Syrta; LEV = Levantine Sea).



Appendix 2. Outlier loci allele frequencies for pooled young tuna samples.

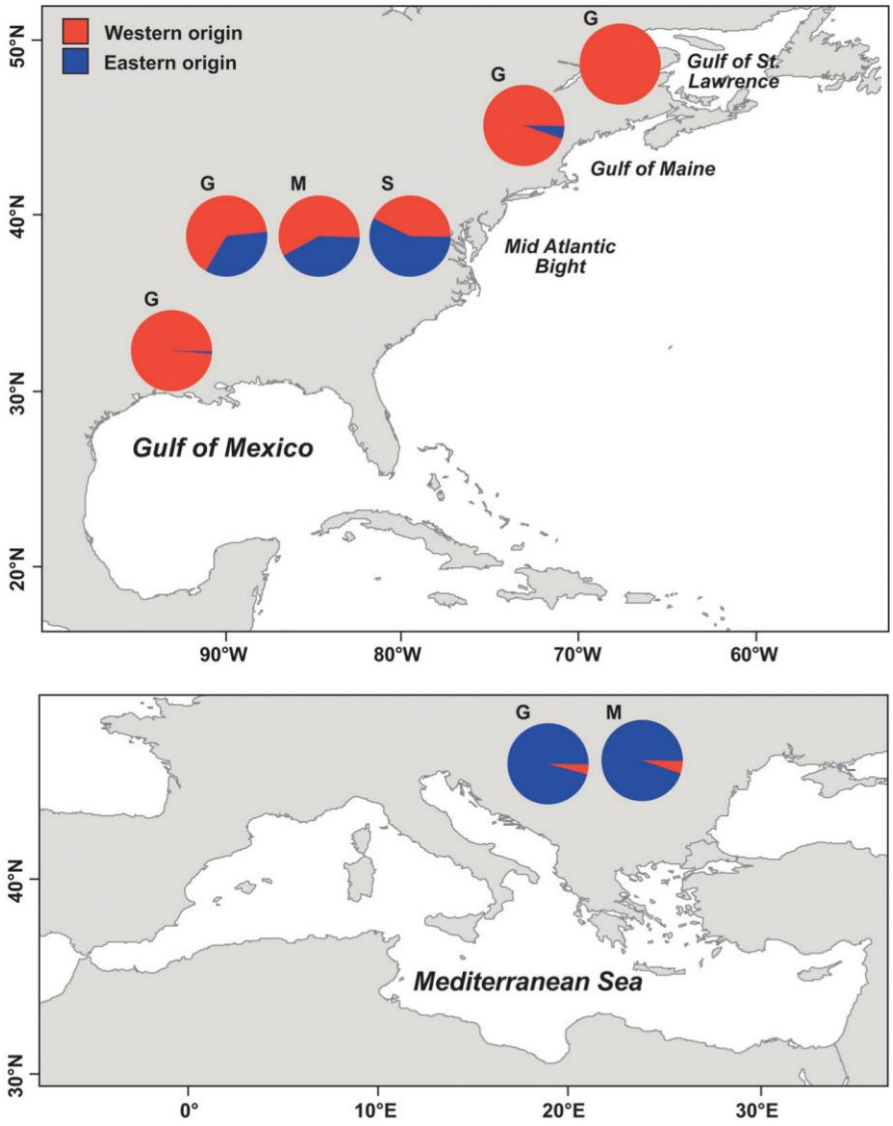
	SNP_161		**SNP_385**	
	Cytosine	Thymine	Cytosine	Thymine
Gulf of Mexico	0.451	0.549	0.667	0.333
Cape Hatteras	0.467	0.533	0.406	0.594
Balearic Sea	0.224	0.776	0.786	0.214
Ligurian Sea	0.234	0.766	0.864	0.136
Tyrrhenian Sea	0.198	0.802	0.722	0.278
Malta	0.176	0.824	0.95	0.05
Sicily	0.211	0.789	0.718	0.282
Levantine Sea	0.205	0.795	0.724	0.276

Appendix 3. Pair-wise F_{ST} results using young tuna (larvae and young-of-the-year) samples. For sample codes refer to **Table 1.** Statistical significance of pairwise comparisions are shown above the diagonal (NS = not significant, * = significant) and F_{ST} values are shown below. Statistically significant values have been corrected for multiple testing (adjusted nominal level of 0.05). F_{ST} values >0.005 and < 0.01 are highlighted in light blue. F_{ST} values >0.01 are highlighted in dark blue.

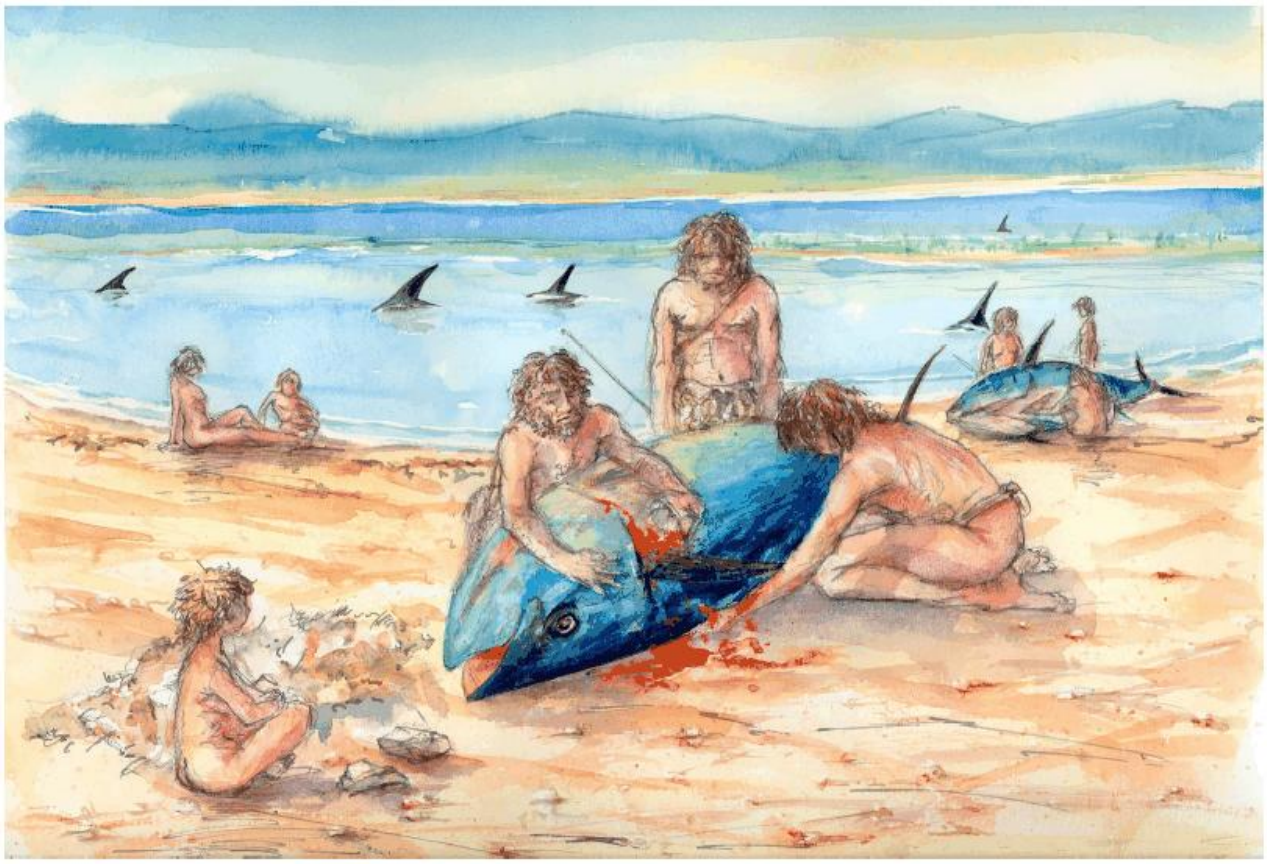
	WA-GM07-09-V	WA-GM10-V	WA-CH08-0	WM-BA11-0	WM-BA12-V	WM-BA13-0	WM-LI13-0	WM-TY12-0	WM-TY13-0	CM-MA13-0	CM-SI08-V	CM-SI11-0	CM-SI12-0	CM-SI13-0	EM-LS11-0	EM-LS13-0
WA-GM07-09-V		*	NS	*	*	*	NS	NS	*	*	*	NS	NS	*	*	*
WA-GM10-V	0.0099		NS	NS	NS	NS	NS	*	NS	NS	NS	*	NS	NS	*	NS
WA-CH08-0	-0.0028	0.0074		NS	NS	*	NS	NS	NS	*	NS	*	NS	NS	*	NS
WM-BA11-0	0.0151	0.0164	0.0134		*	NS	NS	NS	NS	*	*	NS	NS	NS	NS	NS
WM-BA12-V	0.0215	0.0041	0.015	0.0086		NS	NS	*	NS	NS	NS	*	NS	NS	NS	NS
WM-BA13-0	0.0228	0.0013	0.0203	0.0091	-0.0012		NS	*	NS	NS	NS	*	NS	NS	NS	NS
WM-LI13-0	0.0147	0.0028	0.014	0.0071	-0.0006	0.0005		NS	NS	NS	NS	NS	NS	NS	NS	NS
WM-TY12-0	0.0128	0.0188	0.0198	0.015	0.0111	0.0148	0.0158		*	NS	*	*	NS	*	*	NS
WM-TY13-0	0.0199	0.0024	0.0142	0.0074	-0.0025	-0.001	-0.0023	0.0162		NS	NS	*	NS	NS	NS	NS
CM-MA13-0	0.0242	0.0067	0.0253	0.0117	0.0037	0.0023	0.0029	0.0174	0		NS	*	*	NS	*	NS
CM-SI08-V	0.027	0.0058	0.0265	0.0178	-0.0007	-0.0017	0.0019	0.0197	-0.0014	0.0037		NS	NS	NS	NS	NS
CM-SI11-0	0.0194	0.0265	0.0234	0.0073	0.0172	0.0191	0.0096	0.0158	0.0122	0.0169	0.0171		NS	NS	NS	*
CM-SI12-0	0.0093	0.0113	0.0072	0.0067	0.0056	0.0049	0.0077	0.0094	0.0084	0.0131	0.011	0.0092		NS	NS	NS
CM-SI13-0	0.0159	0.0007	0.0114	0.0046	-0.0039	-0.0018	-0.0036	0.0203	-0.0046	0.0023	0.0008	0.0096	0.0044		NS	NS
EM-LS11-0	0.0261	0.0266	0.0311	0.0074	0.0089	0.0108	0.0081	0.0208	0.0124	0.0168	0.017	0.0096	0.0082	0.0089		*
EM-LS13-0	0.0161	0.0006	0.0144	0.0113	-0.002	-0.0025	0.0012	0.0107	-0.001	0.0007	-0.0009	0.0147	0.0078	-0.0017	0.0168	

Appendix 4. Proportional self-assignment of larvae and young-of-the-year samples (n=493) to Western Atlantic and Mediterranean spawning areas of origin. Individuals with an assignment score <70% were considered NOT ASSIGNED.

Samples	n	Proportion Assigned to Western Atlantic (%)	Avg. score%	Proportion Assigned to Mediterranean (%)	Avg. Score %	NOT ASSIGNED	Avg. total assignment score (%)
WAGM07V	2	100	94.0	0.0	0.0	0	94.0
WAGM08V	8	87.5	96.0	0.0	0.0	1	92.0
WAGM09b	10	100	86.7	0.0	0.0	0	86.7
WAGM09V	8	100	94.3	0.0	0.0	0	94.3
WAGM10V	36	52.8	90.7	11.1	96.3	13	80.1
WACH080	16	87.5	93.7	0.0	0.0	2	89.2
WMBA110	24	16.7	82.8	66.7	92.9	4	86.1
WMBA12V	40	7.5	80.0	77.5	92.9	5	86.7
WMBA130	34	5.9	80.0	76.4	92.2	6	86.1
WMLI130	33	3.0	91.4	63.6	91.0	11	81.6
WMTY120	24	12.5	81.7	54.2	91.9	8	79.7
WMTY130	40	7.5	77.4	72.5	92.7	8	84.7
CMMA130	40	7.5	85.7	72.5	92.7	8	86.1
CMSI08V	23	0.0	0.0	78.3	93.2	5	86.3
CMSI110	21	4.8	70.9	76.2	91.8	4	85.3
CMSI120	27	18.5	86.2	55.6	91.6	7	83.0
CMSI130	40	10.0	79.3	72.5	90.7	7	84.2
EMLS110	27	3.7	72.4	85.2	94.6	3	90.3
EMLS130	40	10.0	82.8	67.5	90.2	9	82.5
Total	493	19.1	88.4	60.2	92.2	102	85.0



Appendix 5. Estimates of natal origin for school (S), medium (M), and giant (G) category Atlantic bluefin tuna from spawning areas (Mediterranean Sea, Gulf of Mexico) and foraging areas (Gulf of St. Lawrence, Gulf of Maine, Mid-Atlantic Bight). Size classes were approximated based on weight or age (actual or derived from length): giant (> 140 kg, > age 10), medium (60-140 kg, age 5-9) and school (< 60 kg, < age 5) category bluefin tuna. Percent contribution of "western population" and standard deviation (SD) around estimated proportion per region and size category: Gulf of Mexico (giant: 99.3% [SD 1.7%], N = 42), Mediterranean Sea (giant: 4.2% [SD 3.1%], N = 94; medium: 4.2% [SD 4.4%], N = 38), Gulf of St. Lawrence (giant: 100% [SD 0.0%], N = 38), Gulf of Maine (giant: 94.8% [SD 5.3%], N = 72), Mid Atlantic Bight (giant: 64.9% [SD 21.9%], N = 12; medium: 55.7% [SD 10.4%], N = 56; school: 42.6% [7.2%], N = 86). From Rooker et al. (2008).



Neanderthals with bluefin tuna **by Jose Luis Cort**

Chapter 6 – Palaeogenetics of bluefin tuna

Strait of Gibraltar (28,000 BCE) – A lone brown figure stands in bright sunlight surveying a churning blue sea, silhouetted against the black iris of Gorham's cave carved into the eroding cliffs of Gibraltar. Refelexively, the figure swats at a fly annoying her forehead, careful not to smudge the paint decorating her brow. She peers into the darkness of the cave, trying to make out the forms of others sleeping at the back of the cave. It was the time of the tuna and the packs of massive black and white sea wolves that chase the big fish out of the sea. In the late afternoon the family will wake and leave the safety of the cave for the daily walk along the coastline, hoping to come across a big bluefin stranded on the beach. They used to enjoy eating fish in the sun but now they hide themselves in the cave, dragging what they can scavenge inside. Many things had changed since the arrival of the hairless and quick-tempered strangers. Despondently, and in an effort to distract herself from the hunger gnawing at her insides the lone figure carves a cross into the soft limestone wall. Waiting for nightfall.

Publication note:

Aspects of the work detailed in Chapters 6 and 7 have been published in the following ICCAT documents:

Puncher, G.N., Onar, V., Toker, N.Y., Tinti, F. (2014) A multitude of Byzantine era bluefin tuna and swordfish bones uncovered in Istanbul, Turkey. ICCAT SCRS/2014/167.

Puncher, G.N., Cariani, A., Cilli E., Morales, A., Onar, V., Toker N.Y., Massari, F., Moens T., Martelli, P.L., Tinti F. (2015) Unlocking the evolutionary history of the mighty bluefin tuna using novel paleogenetic techniques and ancient tuna remains. ICCAT SCRS/2015/049.

The entire contents of Chapters 6 and 7 are currently being drafted for publication in a peer-reviewed journal with the following details:

Title Rediscovering our relationship with the sea: Unlocking the evolutionary history of the mighty bluefin tuna using novel paleogenetic techniques and ancient tuna remains.

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6.1 Introduction

6.1.1 A historical context

The history of the bluefin tuna and humankind stretches as far back as the Neanderthal (Morales-Muñiz and Roselló-Izquierdo 2008). In Gorham's Cave at Gibraltar, bluefin tuna remains have been found associated with a Neanderthal dwelling (Brown et al. 2011). Prehistoric peoples immortalized their reverie of the mighty bluefin in cave paintings on the Egadi Island of Levanzo some four thousand years ago. During the 5th century BCE, two trading super powers in the Mediterranean, the Carthaginians and Phoenicians stamped their coins with images of bluefin tuna (as the Croatians continue to do today). Aristotle, Aeschylus, Pliny the Elder, Strabo the Greek, Plutarch and Oppiano all wrote about the bluefin tuna in poetry, legends and humanity's earliest natural history texts. The ancient Greeks enjoyed eating thick slices of grilled tuna, they consumed it dried, pickled and slated (Levine 2006). During the Iron Age, the Phoenicians managed to enjoy bluefin tuna wherever they settled (Morales-Muñiz and Roselló-Izquierdo 2008). The best tuna was refuted to come from the entrance of the Hellespont, in the waters surrounding the ancient cities of Troy and Byzantium, or modern day Çanakkale and Istanbul (Athenaeus, 3rd century B.C.E.).

The ancient bluefin tuna fishery was a successful commercial enterprise with rich merchants organizing the hunt and employing the lower class during migratory periods when the bluefins came close to shore for brief periods of time. The relics of this booming industry litter the coastlines of the Mediterranean, particularly around the Strait of Gibraltar where the remains of fishing factories and scores of amphorae, which were used by Iron Age peoples to transport fish products, can be found (summary in Morales-Muñiz and Roselló-Izquierdo 2008). Certainly, the bluefin tuna fishery was unique in many regards at the time. Capturing such large animals and only during a short time period in the spring would have required a complex social organization, requiring groups of people to coordinate for a single time every year. Indeed, the necessity or desire for this sort of seasonal social organization for mutual benefit could have represented a major milestone in civilizing people of the area. As explained by Morales-Muñiz and Roselló-Izquierdo (2008), most fishing operations during the Late Iron Age were small scale and concentrated around estuaries where individuals or small family units could take advantage of river currents and narrow water ways. Some evidence suggests that the Phoenicians facilitated this shift from small subsistence littoral fisheries to large-

scale commercial fisheries with an organized work force and factories to process the catch. From the second century B.C.E. to the first century C.E. the bluefin tuna fishery thrived in places like Baelo Claudia and Cerro del Mar.

Bluefin tuna once again achieved widespread notoriety among gastronomers during the early Roman Republic who's passion for the fermented fish sauce (*garum*) bordered on fanaticism. Many parallels can be drawn between the acclaim of the fish sauces of ancient Romans and the current popularity of *toro* sushi, which has reached global levels of celebrity and prestige. Both have created thriving corporations of fishermen, merchants and middle men, while the commodity itself is reserved for the wealthy alone, due to prohibitive costs. As such, imitations abound, only adding to the original's prestige (Badham 1854). The most highly valued sauces came from Carthage and Spain where coastal fisheries targeted migratory schools of tuna that passed close to the shore. One highly esteemed sauce was made from the decomposed blood and entrails of bluefin tuna. Another class of fish sauce took the form of a liqueur that was enjoyed by the rich and distributed among wealthy friends as a gift. This was illustrated by 1st century poet Martial, “Of scomber's precious blood I send a *garum*'s bottle to my friend; costly and thick, the last that dript, from bleeding gills and entrails ript.” In the latter half of the first century, something changed in the kitchens of the Roman Empire and demand for the big tuna waned until mackerel became the dominant catch in the third century C.E. The preference for smaller fish continued to gain favour into the third century, at which time sardines and anchovies became favoured for the manufacture of fish sauces (Morales-Muñiz and Roselló-Izquierdo 2008). It remains unclear whether these changes in trade were a result of market demand or shifting fish community assemblages due to “fishing down of the food web”.

At the other end of the Mediterranean Sea, built at the nexus of the east and west, Constantinople thrived as a commercial center during the 4th century C.E. and quickly became one of the largest cities in the world. Due to its rapid commercial growth, Emperor Theodosius I commissioned the development of the Harbour of Eleutherius or Theodosius Harbour between 379-395 C.E. (Muller-Wiener 1998). It was to become one of the largest and busiest ports in Byzantium, if not the world and it had no rival for nearly a millennium. Through this port flowed the commerce of an empire: grains from Egypt, spices and precious stones from India, silk from China, metals from Europe and slaves from throughout the territories of the

expanding empire. Between the 5th and 6th century C.E., the city's population grew from 300,000 to some half a million inhabitants. Providing this number of people with food was certainly a challenge for the rulers of the time. Certainly, the bounty of the sea provided a necessary source of protein for the Byzantines. To the west lies the Sea of Marmara, the Dardanelles and the Mediterranean Sea and to the east, the turbulent waters of the Bosporus Strait and the Black Sea. These waters teemed with fish and porpoises feeding on the riches provided by the collision of the two water bodies. Each year massive herds of bluefin tuna passed by the city of Troy, through Hellespont (now Dardanelles) and into the Bosporus Strait on their annual migration to and from the Black Sea and the fishery thrived. Fourth century B.C.E. food fan, Archistratus had the following to say about the bluefn tuna caught near Byzantium: "Let it come to you from lovely (*erateinou*) Byzantium if you desire the best, yet you will get what is good even if it is caught somewhere near this place here. But it is poorer (*kheiron*) the farther you go from the Hellespont (near Byzantium), and if you journey over the glorious courses of the briny Aegean main, it is no longer the same, but utterly belies my earlier praise." The tuna traps that harnessed the annual bounty of migrating tuna were eventually appropriated by the state and leased to fishermen, providing substantial revenue for both Constantinople and Rome. This practice was such a success that the regulations spread throughout the rest of the empire. By the 9th century CE, a new law was required to limit the propagation of tuna traps along the coast and better organize the fishery. Byzantine Emperor Leo VI introduced legislation dictating the spacing between traps, the development of factories for processing of fish and storage of equipment between seasons and property rights over tuna traps and their location in the Bosporus (Bekker-Nielsen and Bernal Casasola 2007; Marzano 2013). This was to become the first law to infringe on the public domain, as defined by Justinian Law, and grant private ownership to stretches of coastline and sea floor.

After the Roman period and into the Middle Ages, coastal communities appear to have abandoned hunting in the open seas for large pelagic tunas. The centuries following the collapse of the Western Roman Empire were punctuated with invasions, plagues and a constant reshuffling of the centres of power and economy. During this turbulent time tuna merchants lost wealthy customers, factories fell into disrepair and new borders were drawn across trade routes. Coastal communities returned to unspecialized inshore fishing, using the same techniques used by their ancestors centuries earlier. During the 11th century, the tuna traps in

Sicily were so lucrative that the fishermen in Trapani were granted a dispensation, from the bishop, allowing them to work on Sundays during the trapping season. Moreover, they were threatened with excommunication and eternity in hell if they failed to deliver 10% of their profits to the taxman (Maggio 2001). Royalty, Italian bankers and the church were all very much invested in the operations of tuna traps during the 15th -18th centuries. During the 16th century, images portraying the design and function of beach seines and set tuna traps started to appear (Fig. 1; Di Natale 2002). At this time set traps had become common throughout Sicily, Sardinia and Napoli in Italy and at Cadíz and Barbate in Spain. Some historians postulate that the use and design of set traps had been communicated to Europeans by Arabs as many of the names used to identify trap structures and the roles of the various participants in the slaughter are Arabic in origin (Maggio 2001).

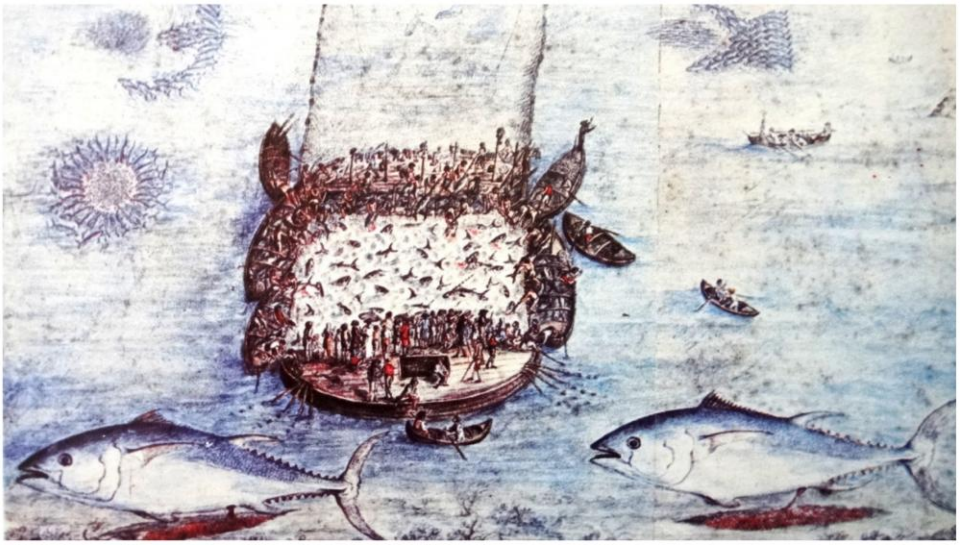

Figure 1: One of the first illustrations of a typical "mattanza" or tuna slaughter at Trapani in Sicily painted by an anonymous artist in the 17th century.

During the 18th century, we have an example of one of the greatest accounts of fishery record keeping in the form of a letter written by a monk, addressed to a Duke. In 1757, Martín Sarmiento sent a communication to the Duke of Medina Sidonia, in a desperate attempt to explain why the catches of bluefin tuna had been in a steady state of decline for several decades (López González and Ruiz Acevedo 2012). According to his records, the Duke's operations at Conil and Zahara (north and south of Barbate) had averaged a little over 6,000 BFTs per year during the previous five seasons (1752-1756). In contrast, over 100,000 BFT had been captured in the same locations eleven of the thirteen years between 1558 and 1570. The monk Sarmiento suggested that the decline was likely due to overfishing but may also be related to

changes in the climate (López González and Ruiz Acevedo 2012). A comparison of this dataset with long term climate conditions suggested that the reduction in catches was at least partly influenced by lower temperatures during the so-called "Little Ice Age" during 1640 and 1715 (Ganzedo et al. 2009). Moreover, metanalysis of historical records detailing the number of bluefin caught in coastal traps in the Mediterranean has shown short term (20 and 100 years) fluctuations in abundance (Ravier and Fromentin 2001). War and civil unrest resulting from the bubonic plague, typhus, smallpox, and other maladies ravaging the Iberian Peninsula during the 17th century would also have had a hug impact on the performance of fishery. Its estimated that during this particularly troubling period in Spain's history, 1.25 million of the 8.5 million citizens fell victim to the various illnesses (Payne 1973). Regardless of the cause of the decline, catches have never recovered to the same levels as the 16th century, despite the steady increase in temperatures. Ganzedo et al. (2009) suggested that the continuous levels of intense exploitation since the Industrial Revolution have never allowed the stock to recover. If this hypothesis holds any truth, then the current baseline and estimates of potential productivity are vastly underestimated.

Today, traditional fishing techniques are fading into the past and industrial fisheries threaten the survival of the world's largest tuna species. Effective fishery management benefits from the existence of baseline information detailing population size and demographics before the onset of exploitation. Unfortunately, due to the long history of human exploitation of BFT, which pre-dates detailed record keeping, this is impossible. Analysis of DNA extracted from historical samples recovered from archives, museums and archaeological excavations can help to reveal elements of the past necessary for establishing something of a pseudo-baseline (Nielsen et al. 1999).

6.1.2 Analysis of ancient DNA

The first museum specimen to be genetically analyzed was the quagga (*Equus quagga*), a zebra-like species that was declared extinct in 1883 (Higuchi et al. 1984). For that study, DNA was extracted from salt preserved dried muscle (proteinase k and detergent digestion, phenol extraction and ethanol precipitation), DNA fragments were cloned (most of which were <500bp) and a 229 bp fragment of mitochondrial DNA was sequenced. When compared with sequences from modern horses, the number of base pair substitutions suggested that the quagga and horse shared an ancestor 3-4 mya, confirming the results of fossil record

estimates. Of course, Higuchi et al. (1984) found that the DNA preserved in the quagga's tissues was in short supply. Aside from the novelty of the methods used, this study did little to expand our knowledge of the species; however, its impacts on biological sciences have been considerable. Following this study, many studies were quickly published, making use of the well preserved and relatively contaminant free tissues of museum specimens (Pääbo 1985, 1989; Hofreiter et al. 2001). Much of the research to date has focused on terrestrial taxa because of the availability of samples and a long held belief that DNA is better preserved in dry environments. Within 15 years, many extinct taxa were quickly analyzed with the new techniques: marsupial wolf, sabre-toothed cat, moa, mammoth, cave bear, blue antelope, giant ground sloth, aurochs, mastodon, New Zealand coot, South Island piopio, Steller's sea cow, Neanderthal, Shasta ground sloth and the pig-footed bandicoot (Hofreiter et al. 2001). The stated motivation behind many of these studies was the resolution of phylogenetic relationships. The media exposure, scientific capital, public curiousity and interest of funding organizations were also valuable acquisitions. As techniques developed, older specimens were analyzed, particularly those originating from the permafrost. Specimens from these locations have yielded DNA of highest molecular mass, due to the cold preservation of tissues and reduced microbial degradation. Among the species studied from these areas are brown bears from 14,000-42,000 years before present (yBP; Leonard et al. 2000), cave bears from 26,500-49,000 yBP (Hofreiter et al. 2002), Adélie penguins 6,000 yBP (Lambert et al. 2002; Ritchie et al. 2004), woolly mammoths from 28,000-43,000 yBP (Krause et al. 2006; Poinar et al. 2006; Römpler et al. 2006) and an early Pleistocene horse from 700,000 yBP (Orlando et al. 2013).

Historical DNA studies have also given us a glance into our own past. Studies focused on the analysis of the DNA of ancient modern man and Neanderthals didn't appear until after the methods had been developed and optimized by zooarchaeologists. This delay in the development of anthropocentric ancient DNA (aDNA) studies is likely due to a hesitation on the part of museum curators to sacrifice relatively rare ancient human specimens and in part to the esteem in which we hold the remains of our ancestors. Among the many studies that have been published following the first genetic analysis of Neanderthal remains (Krings et al. 1997) are explorations into our ancient relationships with neanderthals, the introgression of neanderthal genetic material into our own genome and the evolution and migration of modern humans out of Africa

(Briggs et al. 2009; Stewart and Stringer 2012; Callaway 2014; Sankararaman et al. 2014). Additional publications of note that demonstrate the increasing potential of aDNA research are an analysis of the pre-Columbian dietary habits of Native Americans 2000 yBP (Poinar et al. 1998; Luciani et al. 2006) and characterization of the genome of the bacterial culprit behind the Black Death which ravaged Europe's population 660 years ago (Bos et al. 2011).

In general, little use has been made of museum specimens in aDNA studies of fish. Many historical collections of fishes have used formalin as a preservative, which thoroughly degrades DNA (Miething et al. 2006; Zimmermann et al., 2008). Although some researchers have managed to make use of formalin preserved tissues (Wirgin et al. 1997; Klanten et al. 2003; Chakraborty et al. 2006) most researchers prefer to use dried hard tissues like scales, otoliths, spines and vertebrae. The type of tissue, time required for full desiccation of said tissue and the conditions in which it is stored, all have a significant influence on the quality, quantity and potential use of DNA extracted from historical specimens (Burrell et al. 2014). The oldest DNA to be extracted from fish remains has come from bones excavated from temperate archaeological sites, with the exception of one case in which DNA was extracted from 1,500 year old (y.o.) catfish pectoral spines from the Levantine Sea coast of Turkey (Arndt et al. 2003). From the temperate sites, aDNA has been successfully extracted from 9,000 y.o. salmonid vertebrae from Washington state, USA (Butler and Bowers 1998), 3, 250-41,000 y.o. Salmonid vertebrae from northern Spain (Consuegra et al. 2002), 500,000 y.o. whitefish vertebrae from Poland (Brzuzan et al. 2004), 2,000-7,000 y.o. salmonid vertebrae from the Pacific coast of Canada (Yang et al. 2004; Moss et al. 2014), and 100-500 y.o. Atlantic cod vertebrae excavated from historical fishing sites in Iceland (Ólafsdóttir et al. 2013). Other non-vertebrae extractions that have yielded amplifiable DNA have come from 1,000 y.o. jaw bones (*os cleithrum*) of common bream from northern Poland (Ciesielski et al. 2002), and various salmonid bones from 1,100-1,500 yBP excavated from sites along the Fraser River in British Columbia, Canada (Speller at al. 2005). All of these studies have taken advantage of the unique properties of bone, which is composed of collagen and hydroxyapatite. DNA binds to and is stabilized by hydroxyapatite and the degree to which DNA is preserved in bone is a function of the crystallinity of this mineral (Götherström et al. 2002). Collagen is associated with higher concentrations of DNA in ancient bones and, when present with hydroxyapatite, appears capable of preserving DNA for

extremely long periods of time (Götherström et al. 2002).

In addition to bones, many aDNA studies of fishes have used scales (Nielsen et al. 1997; Hansen et al. 2002, 2010; Hauser et al. 2002; Coughlan et al. 2006; Iwamoto et al. 2012) and otoliths (Hutchinson et al. 2003; Poulsen et al. 2005; Nielsen et al. 2007; Cuveliers et al. 2011; Poulsen et al. 2011; Smith et al. 2011; Johsnton et al. 2013; Therkildsen et al. 2013) archived by fisheries scientists in the past (1900s - 1950s). Due to the relative youth of fisheries science, these specimens are rarely more than a hundred years old. Previous to the present work, only one study has made use of historical BFT DNA (Riccioni et al. 2010). In that study, researchers extracted DNA from 219 vertebrae from the Massimo Sella Archive at the University of Bologna which is described at length in Section 2.1.7.1. A description of the analysis and conclusions from that study are provided in Chapter 7.

6.1.3 Properties of ancient DNA

Although DNA is constantly undergoing degradation and repair in metabolically active cells, it begins to rapidly degrade shortly after death (Bär et al. 1988). The decay of an animal begins with autolysis and putrefaction, followed by aerobic and bacterial decomposition. High temperatures (35-40°C) and humidity also facilitate autolysis (Bär et al. 1988). Two groups of enzymes released during autolysis facilitate DNA fragmentation: 1) endonucleases, which shear DNA into shorter fragments, and 2) exonucleases which remove nucleotides one at a time from fragment ends. DNA originating in hard tissues are spared some of the rapid and extensive degradation of soft tissues (Wandeler et al. 2007). Exposure of the remains to the environment and oxidative stressors can result in a loss of nucleotides and fragmentation of DNA. After the days immediately following putrefaction of tissues and degradation by microorganisms, DNA can become stabilized in hard tissue, after which two chemical processes have the most significant impact on the integrity of the molecule. Deamination and depurination are the leading causes for misinterpretation of aDNA sequences (Lindahl 1993; Pääbo et al. 2004). Depurination is a chemical process by which purines are liberated from the double helix, due to hydrolysis of the unstable DNA glycosyl bond, resulting in fragmentation of DNA adjacent to purine residues (Fig. 2; Overballe-Petersen et al. 2012). This is particularly common in historical DNA which often has a higher representation of A and G adjacent to the 5' ends of recovered DNA fragments (Sawyer et al. 2012). This

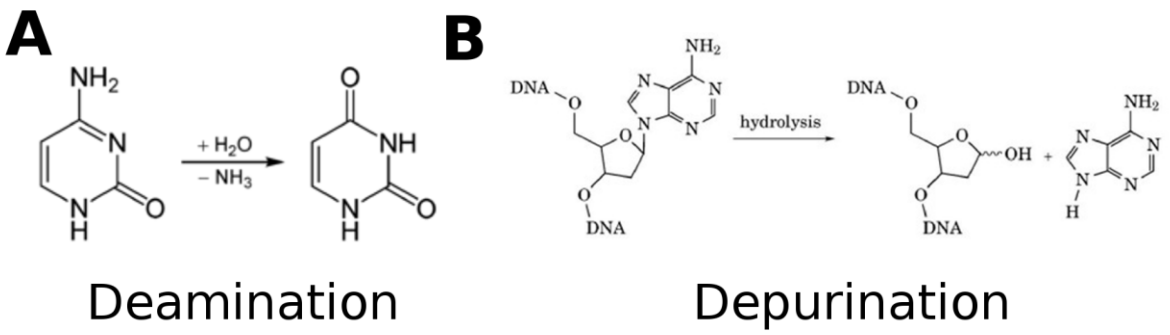

Fig. 2: Two chemical processes that influence the quality of DNA extracted from historical specimens.

type of DNA fragmentation occurs in the decades following mortality and does not appear to continue at a constant rate among samples that have been preserved under similar conditions (Sawyer et al. 2012). In other words, thousand year-old samples can contain longer fragments of DNA than twenty year-old samples, given adequate storage conditions. Conversely, pyrimidines (cytosine and thymine) are liberated from the DNA double helix at 5% of the rate of purines (Lindahl 1993). Among purines, this appears to occur slightly more rapidly with guanine than adenine residues among samples younger than 100 years and for samples 500-2000 y.o. the two purines occur at approximately the same frequency (Sawyer et al. 2012).

In addition to depurination, DNA can also degrade through a process called deamination, which is a hydrolytic reaction that converts cytosine into uracil (Fig. 2). At 37°C and pH=7.4, the half life of individual cytosine residues is 200 years in single-stranded DNA and 30,000 years in double stranded DNA, due to the added protection afforded by the double helix (Federico et al. 1990). Deamination is most commonly detected at the 5' and 3' tails of DNA fragments, suggesting that exposed portions of DNA are more susceptible due to exposure to water molecules which catalyze the loss of amine residues. Since uracil is nearly chemically synonymous with thymine (uracil lacks the 5' methyl group), this can result in erroneous complimentary strand assembly during PCR amplification.

Degradation of long strands of DNA into short fragments is another one of the major characteristics of ancient DNA that determine the way in which it is analyzed. All chemical processes described above contribute to the highly fragmentation of ancient DNA (Hofreiter et al. 2001). Surprisingly, time isn't a consistent or significant factor in the determination of fragment size of historical DNA (Sawyer et al. 2012). For example, 20,000 y.o. mammoth hairs and 6,000 y.o. penguin remains recovered from the permafrost have

been measured at 164 bp and 352 bp, respectively (Miller et al. 2008; Ritchie et al. 2004) which cannot be analyzed with SNP genotyping arrays which require at least 120bp fragment length. DNA of this quality can, however, be analyzed with NGS platforms without requiring the use of restriction enzymes, which can do more damage than good (Debruyne et al. 2008). Fragments of this length can be analyzed with the PacBio platform which provides high quality data and confident genome assemblies due to the length of fragments that can be sequenced. Regardless of the challenges associated with these two examples of DNA, researchers have managed to construct genomes for both species (Poinar et al. 2006; Green et al. 2010).

DNA is typically of lower concentration in historical specimens due to leaching processes (if specimen is buried in sediments) and the chemical process described above. Aside from some examples of aDNA studies that have managed to extract high quantities of DNA from well-preserved samples, most efforts to extract DNA from ancient tissues yield approximately one nanogram of DNA per milligram of tissue (Millar et al. 2008). The amount of DNA that can be extracted from historical specimens determines how they can be analyzed (NGS, Sanger sequencing, SNP-typing) and with which platform, if they are to be analyzed using NGS technology or SNP-typing. For example, only a few copies of template sequences in DNA extractions of low concentration are required for Sanger sequencing of PCR amplified products. Illumina paired-end sequencing requires a minimum of 2-5µg of double stranded DNA with fragment lengths of at least 500bp suspended in a buffer solution with DNA concentrations greater than 20ng/µL. Shotgun sequencing with the Roche 454 sequencing platform requires at last 1.3µg of template DNA (70-500bp) for library construction, after which >500ng of double stranded DNA in TE buffer at a concentration greater than 50 ng/uL is required. Therefore, careful consideration of the expected quality and quantity of DNA from samples is required before proceeding with analysis. Moreover, each of these methods have vastly different costs associated with analysis, which is an important factor in project planning.

Ancient specimens recovered from archeological digs have literally been saturated in sources of exogenous DNA (microbes, scavengers, detritivores) for centuries or millennia. In a study analyzing the remains of neanderthals (44,450 ± 550 years old) DNA extractions were found to be composed of 95-99% non-primate DNA (Green et al. 2010). Specimens can also be contaminated by the archeologists handling them. Samples being processed by aDNA researchers can also become contaminated with the DNA of

laboratory staff or other modern DNA being analyzed in the same building via equipment, reagents or aerosols in the air. Willerslev and Cooper (2005) warned that microscopic aerosol droplets released from PCR tubes can contain over a 250,000 copies of template DNA fragments per microlitre.

In 2000, Cooper and Poinar published what they surely hoped would become the ten commandments for ancient DNA research in an article entitled "Ancient DNA: do it right or not at all" (**Box 1**). In that publication they appeal to journal editors, reviewers, granting agencies, and researchers to promote and follow their strict guidelines for paleogenetics in order to maintain credibility as a recognized area of evolutionary research. Surely they had a persuasive argument after it had been revealed that several studies had based their results on exogenous DNA or contaminants (Pääbo et al. 2004; Willerslev and Cooper 2005).

Adherence to all ten criteria is both time consuming and costly and were developed with anthropological studies in mind, in so far as laboratory technicians can be a source of contaminating DNA. Not mentioned in this list of precautions are the sample surface decontamination measures that are standard for any paleogenetic work. Among the methods used to clean surfaces of samples and avoid contamination of inner tissues are treatment with bleach and UV light. Ethanol is used by some but only has the capacity to disinfect and sterilize surfaces and falls short of destroying DNA. Also important is decontamination of all reagents, tubes, vials and pipette tips to be used in the processing of samples. Autoclaving of these items can only fragment DNA, making it indistinguishable from ancient DNA. Bleach is effective in this regard; however, it also causes depurination of exogenous DNA which also makes it appear like historical DNA aged over time (García-Garcerà et al. 2011).

Box 1: Ten requirements for ancient DNA studies as proposed by Cooper and Poinar (2000).

1) Ancient DNA extractions should take place in an isolated laboratory reserved for this purpose.
2) Use negative controls during PCR amplifications. Avoid postive controls as they can be a source of contamination.
3) Report any unexpected results and justify any deviations from the norm (phylogenetic, amplicon size).
4) Results should be reproducible.
5) Results from sequencing of PCR products should be verified by cloning experiments used to determine the relative proportions of exogenous and endogenous sequences, damaged DNA and numts.
6) Extractions and sequencing should occur in at least two different laboratories, especially for anthropological studies.
7) Information concerning the quality and quantity of extracted DNA should be published alongside results.
8) Attempts should be made to estimate the initial number of extracted DNA templates (i.e. quantitative PCRs).
9) Associated faunal remains can be used as negative controls.
10) Multiple primer pairs should be used to verify sequences and account for numts.
*This last point was originally featured as part of #5 but stands separate here because the topics are so unrelated.

6.1.4 Mediterranean collections of historical BFT remains

6.1.4.1 Massimo Sella Archive

The Massimo Sella archive is a collection of remains (vertebrae, skulls and fins) belonging to some 5000 fish (*Thunnus thynnus*, *T. alalunga*, *Euthynnus alletteratus*, *Sarda sarda*, *Xiphias gladius*) captured in the Mediterranean tuna traps during the early decades of the 20th century and collected by Italian professor Massimo Sella. Originally housed at the Istituto Italo-Germanico di Biologia Marina/Deutsch-Italienisches Institut für Meersbiologie, Rovigno, Italy (now Institute Center for Marine Research, Rovinj, Croatia), the collection was moved to a University of Bologna facility in Fano, Italy after the Second World War. The vast majority of bones in the collection are Mediterranean bluefin tuna (BFT) caught in tuna traps in Istria (Croatia), Messina (Italy) and Zliten (Libya); however, a few vertebrae specimens from Bosporus Strait and non-Mediterranean areas are also included.

6.1.4.2 Istanbul, Turkey

Archeological excavations conducted between 2004 and 2013 in the Yenikapi neighbourhood of Istanbul have revealed the location of a Byzantine era harbour (Fig. 4). Built by Emperor Theodosius in the 4th century C.E., it was one of the largest and most important ports in the Roman Empire for nearly eight centuries. The city itself thrived until the 13th century, after which point it weakened under the strain of the crusades and costly conflicts with the Venetians. Then in the 15th century C.E. the city was taken by the Ottoman Empire. At the same time, the Bayrampasa River, the Lykos slowly filled in the harbour with sediments and waste from the city until it became useless for the large commercial fleet in the late 12th century C.E. (Kadir Eriş et al. 2009). Princess Anna Comnena (1083-1153), a Byzantine historian, wrote "Time in its irresistible and ceaseless flow carries along on its flood all created things and drowns them in the depths of obscurity". One must wonder whether her words were inspired by the Lykos River and the slow burial of a once bustling and magnificent harbour. The remains of the time were buried in the mud, preserved and forgotten until excavations began in 2004.

Fig. 4: Marmaray project archeological excavations at Yenikapi (Buket et al. 2009).

Many animal remains have been uncovered, including 150 vertebrae of Atlantic bluefin tuna, *Thunnus thynnus*, as well as a multitude of swordfish rostra, *Xiphias gladius*. The rapid inundation of the harbour with fine silts has preserved all vertebrae in excellent condition (Fig. 5). Butchery marks and historical accounts of the Ancient Greek and Roman tuna fishery (Oppian 177 B.C.E.; Aristotle 350 B.C.E.) in the region suggest that the vertebrae belonged to tunas caught in the immediate vicinity (Marmara or Black Sea). After the meat was removed from the bone, it was likely sold fresh to a local buyer or preserved in salt or processed into *garum* (fermented fish sauce) and traded abroad. Since a viable population of BFT has been absent from the Black Sea since the mid 1980s (Karakulak and Oray 2009), these vertebrae are of particular interest for the scientific community. The cause of their disappearance from the Black Sea remains unknown; however, hypotheses abound, including eutrophication, overfishing, noise pollution, lack of prey and changing environmental conditions (Papaconstantinou and Farrugio 2000; Daskalov 2002; Sara et al. 2007; MacKenzie and Mariani 2012).

To date, this magnificent collection of BFT bones at the University of Istanbul hasn't been used for any research purposes. Due to restrictions imposed by the Ministry of Culture in Turkey, all archeological

specimens used for scientific purposes must be subjects of an official research agreement between Istanbul University, ICCAT, University of Istanbul and the Turkish Ministry of Culture and as such, only six samples were made available at the time of sampling. Since that time a new contract, paired with sufficient funding, has been signed with ICCAT and additional sampling of the collection will commence in the summer of 2015.

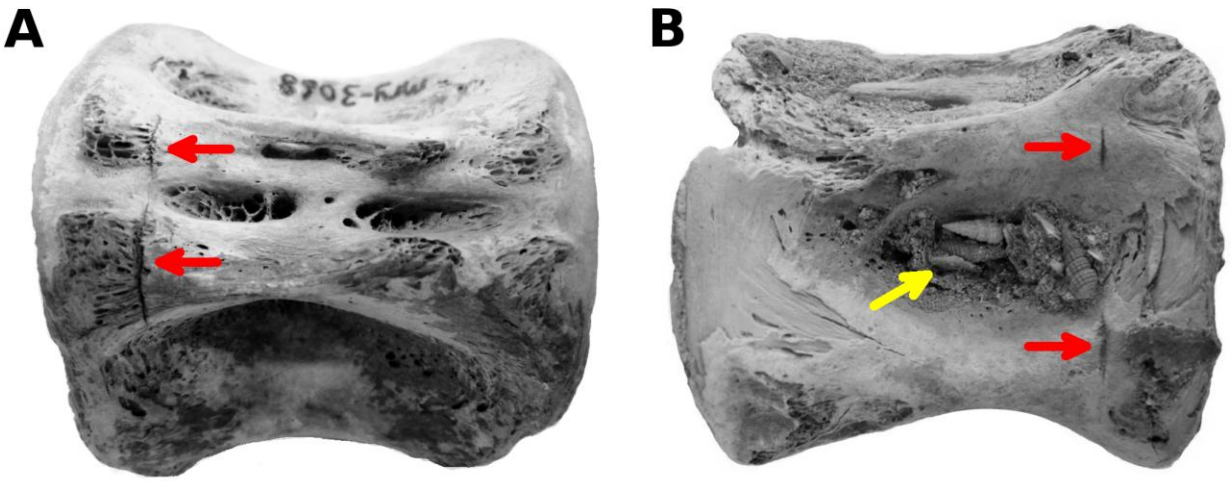

Fig. 5: *Thunnus thynnus* vertebrae recovered from excavations at Yenikapi, Istanbul, Turkey. Butchery marks (red arrows) and gastropod shells (yellow arrow) are indicated.

6.1.4.3 Iberian Peninsula

In the archives of the Faculty of Archeozoology at the Autonomous University of Madrid, Professor Arturo Morales watches over a vast collection of animal bones collected from archaeological sites around the world (Fig. 6). From this collection, we were able to acquire 30 DNA genetic samples from "giant" tuna (~400-500kg) vertebrae captured during the 1985 *almadraba* (annual tuna slaughter) at Barbate (Spain, n=10), vertebrae from medium sized adults (150-200kg) captured by Late Iron Age and republican roman colonists (2^{nd}-1^{st} century B.C.E.) in Tavira (Portugal, n=10) and Baelo Claudia (Spain, n=10). The samples collected from Tavira had been used by home builders as building materials and were incorporated into the walls of the buildings within the small Iberian roman colony. This was done in an effort to provide an organic matrix that strengthens instead of weakens architectural structures. Conversely, the bones from Baelo Claudia seemed to have been buried in a refuse pile or midden.

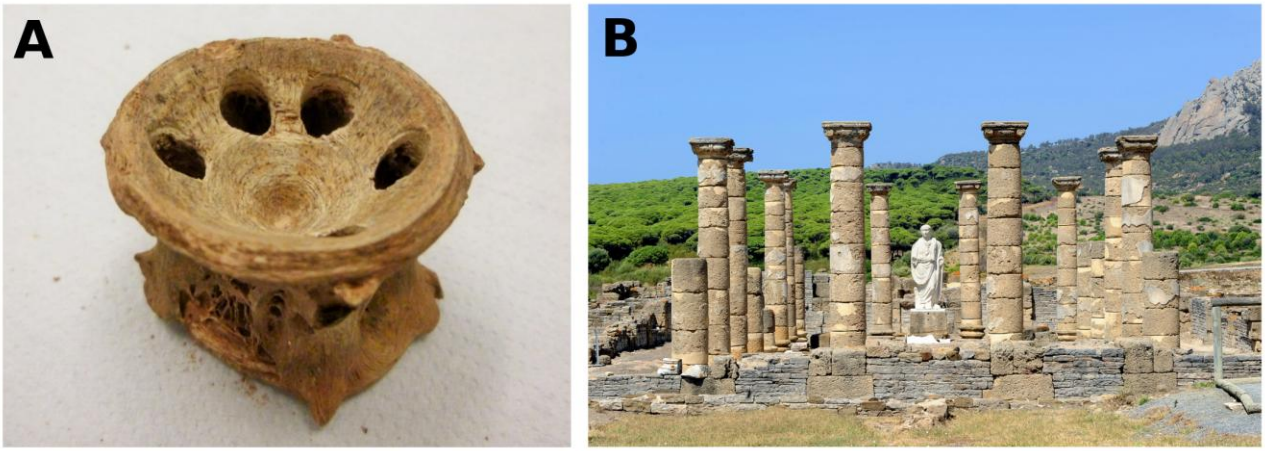

Figure 6: A) BFT vertebrae from late iron age settlement at Castro Marim with drill holes after powder removal. **B)** Archeological site of ancient Roman fishing town of Baelo Claudia.

6.1.5 Study Aim

DNA was extracted from a total of 186 specimens taken from the collections described above, using a newly developed aDNA extraction protocol. Performance of this protocol was optimized through a series of extractions and evaluations of DNA quality and quantity using various platforms (Gel electrophoresis, NanoDrop, QuBit). A proportion of ancient and modern DNA contained in each sample was also estimated using a combination of electrophoresis and flow cytometry (Agilent BioAnalyzer). In an effort to prioritize future efforts, the concentration of endogenous DNA from a subset of samples from each sampling location was estimated using quantitative PCR techniques. Comparisons have been made between the amount of BFT DNA contained in each sample and their age and the environmental conditions which the bones have been exposed to. The species identity of all samples was verified using newly developed mini-barcodes of the following genes: mitochondrial cytochrome oxidase subunit 1 (CO1), mitochondrial control region (CR) and nuclear internal transcribed spacer (ITS1).

6.2 Materials and Methods

6.2.1 Sample collection

Access was granted to three historical collections archived at the Autonomous University of Madrid (Spain), University of Bologna (Italy) and Istanbul University (Turkey). From these collections were gathered bluefin tuna vertebrae excavated from late iron age and ancient roman settlements in coastal Iberia (Portugal

and Spain, 4th-2nd century BC; n=28) and Byzantine-era Constantinople (4th-15th century AD; n=6), as well as vertebrae from the Massimo Sella archive located at the University of Bologna (Ionian, Tyrrhenian, Adriatic Seas, and Levantine Seas early 20th century; n=152). The approximate geographic origin for each group of historical samples is shown in Figure 7 and the number of samples from each location and time period is summarized in Table 1.

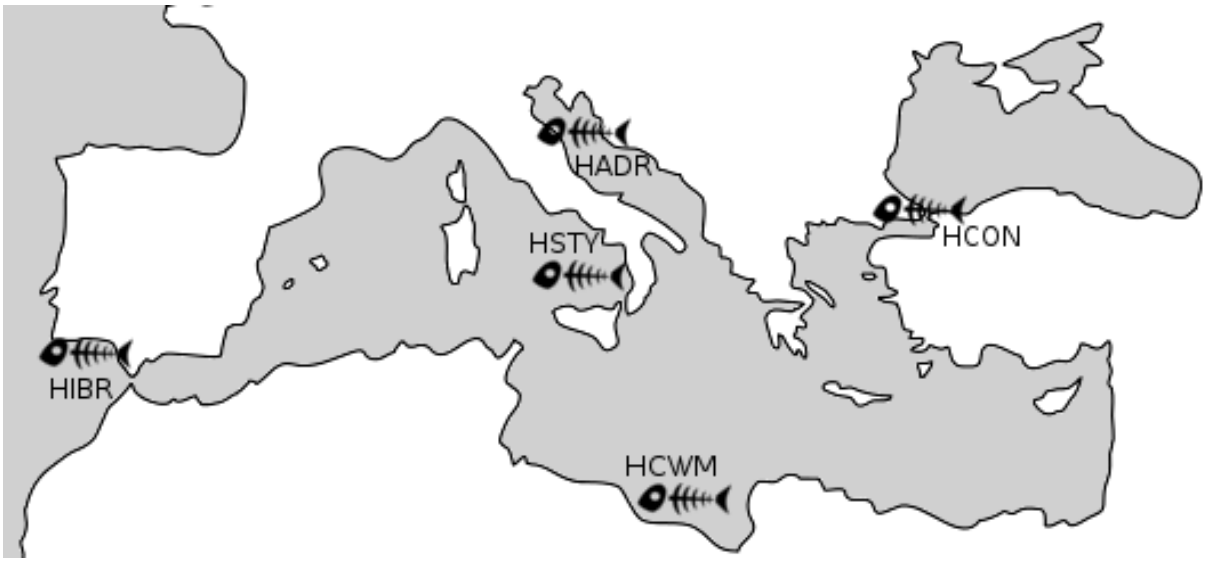

Figure 7: Locations in the Mediterranean Sea, Atlantic Ocean and Sea of Marmara from which historical bluefin tuna vertebrae were located: HIBR = Iberia (Castro Marim, Baelo Claudia, Cadíz in Spain and Tavira in Portugal), HSTY = Tyrrhenian Sea, HADR = Adriatic Sea, HCWM = Central Western Mediterranean (Zliten in Libya), HCON = Constantinople/Istanbul.

Table 1: Geographic and temporal origin and quantity of historical samples from various locations in the Mediterranean Sea, Atlantic Ocean and Black Sea.

Geographic region	1911-1926	1755	4th - 15th century	1st century	4th - 2nd century BCE	Total
Adriatic Sea	**50** (Istria)					**50**
Tyrrhenian Sea	**50** (Pizzo & Messina)					**50**
Ionian Sea	**50** (Zliten)					**50**
Gibraltar and Portugal		**2** (La Chanca de Conil)		**10** (Cádiz)	**16** (Baelo Claudia, Castro Marim, Tavira)	**28**
Levantine Sea	**2** (Istanbul)		**6** (Istanbul)			**8**
Total	**152**	**2**	**6**	**10**	**16**	**186**

6.2.2 DNA extractions

*S*ample preparation (bone surface decontamination, drilling and powder collection) was conducted at the location of each collection, using a portable sampling set and UV lamp. Enzymatic and chemical extraction of DNA from all samples occurred in Ravenna (Italy) at the Ancient DNA Laboratories at the University of Bologna's Environmental Science campus and Department of Cultural Heritage.

6.2.2.1 Sample preparation

All bones were sprayed with 1.5 - 2% sodium hypochlorite (diluted commercial bleach) and left to soak for up to ten minutes, following published protocols for sterilization of bone surfaces (Kemp and Smith 2005). Some of the very old samples were much more porous and absorbent and were therefore exposed to less bleach for a shorter duration of time. Following this treatment, bones were rinsed with distilled water, wiped clean with paper towel, rinsed with sterilized water and air-dried. Once dry, a thin surface layer was removed using sand paper and the surface was once again rinsed with bleach and distilled water. All bones were left to dry for a minimum of 15 minutes under UV light (254nm wave length; Fig. 8) and holes were drilled into each bone to simultaneously gain access to the internal matrix and produce bone powder. For bones that were too small for drilling, surfaces were diligently cleaned and a hand saw was used to bisect the bone, at which time the internal matrix of the bone was scraped free and pulverized.

Fig. 8: A cleaned vertebra prepared for drilling and bone powder pulverized with a mortar and pestle.

6.2.2.2 Chemical/Enzymatic DNA extraction

Various protocols for the chemical extraction of DNA from bones were developed and compared in order to maximize DNA yields. Through collaboration with Dr. Elisabetta Cilli at the Department of Cultural heritage at the University of Bologna, improved protocols were developed. Based on a previously published protocol by Dabney et al. (2013), the new protocol uses the physicochemical properties of silica filter columns and guanidine to isolate DNA from digested bone lysates. All extractions were performed in a laboratory dedicated to historical DNA analysis in which PCR products are forbidden and protective clothing is worn by all technicians (Fig. 9). All work surfaces were decontaminated with ethanol and bleach after the processing of each specimen. All tools and consumables were sterilized with UV light (254nm) before every extraction, with the exception of Qiagen's MiniEluteTM Spin Column (guaranteed sterile by the manufacturer). A blank control (i.e. microtube containing UV sterilized Milli-Q water) accompanied all extractions throughout processing to detect cross sample contamination if it were taking place.

Approximately 100 mg of bone powder from each sample was split into two equal amounts and placed into separate microtubes. After adding 432 µL of EDTA (0.5M, pH 8.0) to each microtube, the samples were incubated for 16-24h, in a shaker at 37°C. After 70 µl of Proteinase K was added to each microtube, the samples were incubated for an additional 16-24h. The following binding buffer was prepared directly before each extraction exercise: guanidine thiocyanate (5M), Tween 20 (0.05%), isopropyl alcohol (40% vol/vol), sodium acetate (90mM, pH 5.2) and distilled water. Samples were centrifuged for 3 minutes at 10,000 rpm and the supernatant was transferred to new microtubes. Then 1004 µL of binding buffer was added to each microtube and vortexed. Equal aliquots of solution from each sample duplicate pair were transferred to single sample spin columns and centrifuged for 4 min at 14,000 rpm. This was repeated until all samples had passed through the columns and supernatant discarded. 750 µL of PE Buffer from the Qiagen MiniElute kit was added to each spin column, centrifuged for 2 min at 6,000 rpm, supernatant discarded and repeated once. The column was then dry spun for 1 min at 14,000 rpm in order to eliminate any trace ethanol. A maximum of 60 µL of pre-heated (60-70°C) distilled water was then added to each column, incubated at room temperature for 10 minutes, centrifuged for 1 minute at 8,000 rpm and again for 2 minutes at 10,000 rpm.

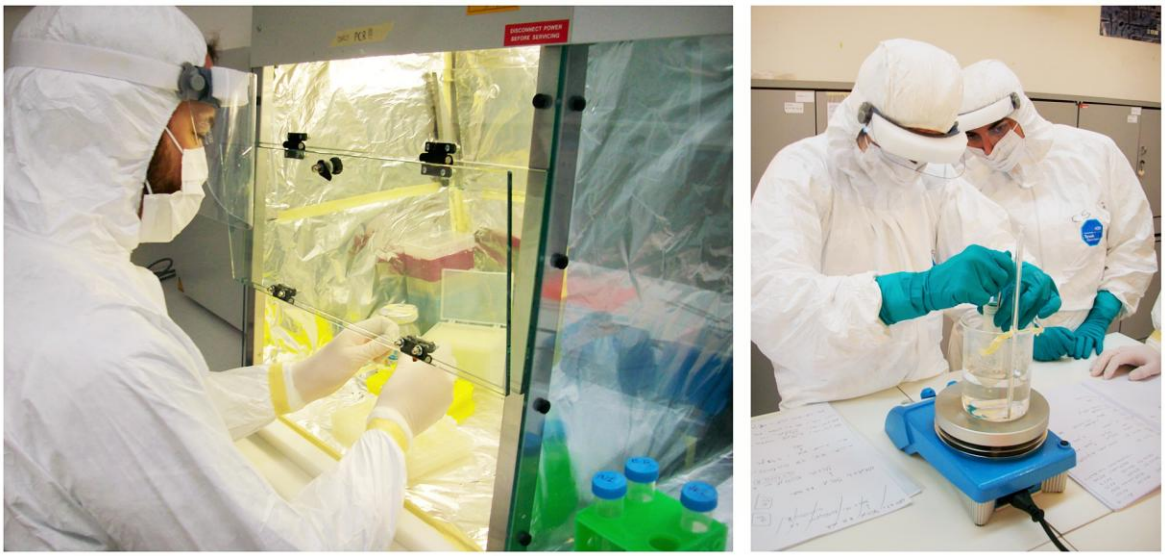

Fig. 9: Researchers wearing protective gear during DNA extractions of historical tuna remains.

6.2.3 Quantification of historical DNA

The concentration of total DNA contained in each extraction was measured using a Qubit® dsDNA HS (High Sensitivity) Assay Kit. Four samples were randomly selected and analyzed with the Agilent BioAnalyzer High Sensitivity DNA kit. Finally, a subset of randomly selected samples (Table 2) were analyzed via several quantitative PCR (qPCR) using a Roche Light Cycler® 480 at the Department of Marine Biology at Gent University and an Applied Biosystems® 7500 Real Time PCR system at the Department of Cultural Heritage at the University of Bologna. Three species specific primer pairs (Table 3) were developed to amplify 81-120bp fragments of DNA from the cytochrome oxidase subunit 1 (*CO1*), mitochondrial control region (CR) and internal transcribed spacer (*ITS1*) using Primer3 (Koressaar and Remm 2007; Untergrasser et al. 2012). The BioLine SensiMix™ SYBR® No-ROX Kit was used according to the manufacturer's instructions, aside from a reduced reaction volume of 20µl to conserve reagents. The qPCR conditions were as follows: An initial incubation at 95°C for 10min, then 40 cycles of denaturation at 95°C for 15sec, annealing at 60°C for 15sec and extension at 75°C for 15sec, followed by melting curve ramping at 2.2°C/s to a final temperature of 97°C.

Table 2: Samples from ten different excavation sites and archives analyzed with qPCR.

Samples	Location	Time period
5	Ionian Sea, Italy	1925-1926
5	Tyrrhenian Sea, Italy	1911
2	La Chanca de Conil de la Frontera, Spain (CCF)	1755
2	Marmaray, Yenikapi, Istanbul, Turkey (Mar)	300-1400 AD
3	Metro station, Yenikapi, Istanbul, Turkey (Met)	300-1400 AD
10	Baelo Claudia, Tarifa, Spain (BC)	100-200 BC
10	Roman Balsa, Tavira, Portugal (T)	200 BC
2	Palacio de Justicia, Cadìz, Spain (PJ)	300-100 BC
1	Castro Marim, Portugal	200 BC
2	Castro Marim, Portugal	400-300 BC

Table 3: Species specific primers used for qPCR and mini-barcoding of historical samples.

Primer pair name	Genome	Sequence	T_M	Product size (bp)
TTCOX1	Mitochondrial	Forward: 5'- CCCACGAATGAACAACATGA -3' Reverse: 5'- CTCCAGCCTCAACTCCTGAA -3'	60.37 60.52	83
TTCR1	Mitochondrial	Forward: 5'- AAATCGTCTAAGCCATACCAAGT -3' Reverse: 5'- TGGACTGGATGGTAGGCTCT -3'	58.31 59.68	81
TTITS2	Nuclear	Forward: 5'- GGGGGTTCAATGTCTCC -3' Reverse: 5'- TTTACACCGCACAGAGGTTG -3'	55.93 59.76	120

6.2.4 Species verification

In order to verify that all samples to be analyzed in additional downstream applications (**Chapter 7**) once belonged to living BFTs, sequencing of mini-barcodes was carried out. Each sample was PCR amplified (conditions described above in 6.2.3) using the aforementioned newly developed mini-barcode primers. Sequencing of amplicons was out-sourced to Macrogen Europe and all alignments were conducted at UNIBO. Due to the short length of sequences analyzed, unambiguous alignments with standard reference sequences was impossible. As such, samples were identified based on characteristic attributes (methods described in Chapter 3) shown in Box 2.

6.3 Results

Most vertebrae from the Massimo Sella archive contained sufficient DNA for SNP genotyping purposes, as determined by the QuBit spectrophotometer (Table 4). However, samples older than 500 years possess concentrations of DNA lower than the threshold that can be quantified in this manner.

Box 2: Locations of primer annealing sites and characteristic attributes for three gene sequences used to identify historical fish remains. Forward primers are highlighted in green text, reverse primers are highlighted in red text and characteristic attributes are highlighted in grey. Interliner text and dashes indicate amplified sequence.

Mitochondrial gene: *Cytochrome oxidase subunit 1* primers (83bp)

GCATT**CCCACGAATGAACAACATGA**GCTTCTGACTCCTTCCCCCTTCTTTCC
TTCTGCTCCTAGCTTC**TTC**
TTCOX1F>----------

AGGAGTTGAGGCTGGAGCCGGAACCGGTTGAACAGTCTACCCTCCCCTTG
CCGGCAACCTAGCCCACG
----------<**TTCOX1R**

Mitochondrial gene: *Control region* primers (81bp)

AATTCAGGCGATTAAACGAGATTTAAGACCTAACATAAATCT**AAATCGTCTA**
AGCCATACCAAGTCTCCT
TTCR1F>----------

CATCTCTGACATCTCGTAAACTTAAGCGCAGTA**AGAGCCTACCATCCAGTC**
CATTTCTTAATGCATACGGT
----------<**TTCR1R**

Table 4: Concentration of DNA extracted from samples from the Massimo Sella archive collected between 1911 -1926. DNA has been extracted from 50 vertebrae from each location.

	Average [DNA] (ng/µl)	Maximum [DNA] value	Minimum [DNA] value
Zliten trap samples	5.2 ± 3.24	15.7	0.37
Istrian trap samples	1.0 ± 1.61	10.3	0.05
Messina trap samples	5.4 ± 5.78	20.8	0.42

Spectrophotometry platforms (i.e. QuBit, NanoDrop) measure a sample's total DNA concentration, not the separate proportions of DNA belonging to the specimen (endogenous) and that which can be attributed to sources of contamination (exogenous). Preliminary analysis of historical DNA extractions, using the far more expensive Agilent BioAnalyzer platform, revealed that a large proportion of the total DNA in a handful of extractions consisted of long chains of nucleic acids (Fig. 10, Table 5). Since DNA is expected to fragment and degrade with time (exposure to catalytic enzymes, chemical decomposition and digestion by fungi and bacteria), longer strands of DNA are likely from exogenous sources.

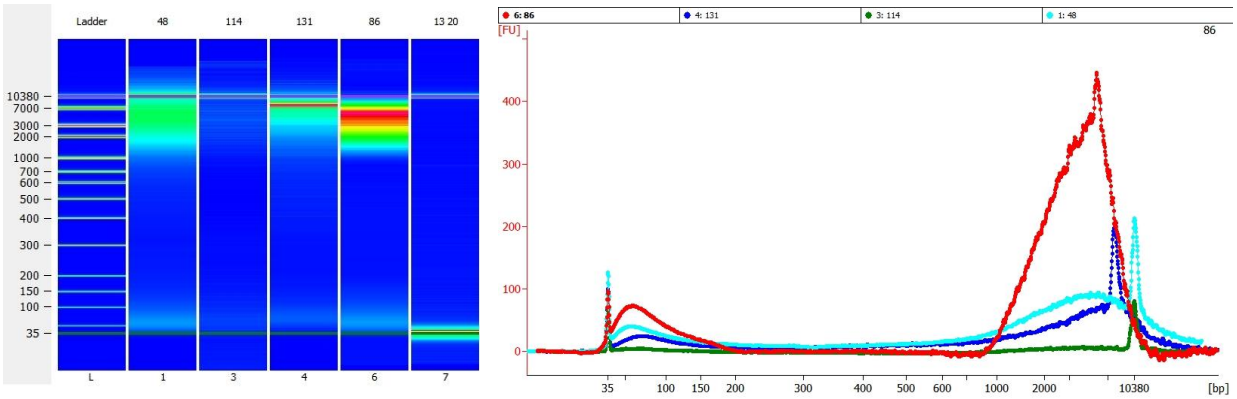

Fig. 10: Results from the analysis of four ancient samples from Spain (2 samples from La Chanca de Conil de la Frontera, 1755 and two samples from Palacio de Justicia, Cadìz, 300-100 BCE) using the Agilent BioAnalyzer. The picture on the left shows the length of DNA fragments on the y-axis with concentrations of DNA in columns, expressed as a gradient of colours. The picture on the right shows a peak of short fragment DNA (historical DNA; 40-300bp) and much larger peaks of long fragments of DNA (modern exogenous DNA; 400-10000bp).

Table 5: Proportion of total DNA belonging to historical sources as measured by the Agilent BioAnalyzer in four historical samples (CCF=La Chanca de Conil de la Frontera, 1755; PJ= Palacio de Justicia, Cadìz, 300-100 BCE).

Sample	Time period	% of total DNA	[aDNA] (pg/µl)	Average size (bp)
PJ48	300-100BC	39%	517.87	103
PJ114	300-100BC	48%	119.42	74
CCF86	1755	26%	7338.65	89
CCF131	1755	29%	1706.42	124

6.3.1 Quantification of endogenous DNA using qPCR

All three nuclear and mitochondrial gene markers performed consistently for all reactions. TTCOX1 and TTCR1 primer pairs performed identically during the amplification of target fragments, as shown by the overlapping standard curves in Fig. 11a. The melting curve of the nuclear marker fragments was more scattered than the mitochondrial marker curves (Fig. 11b,c). Amplification of mitochondrial fragments occurred sooner and more completely among all samples (Fig. 11d,e,f). Overall, both mitochondrial primer pairs appeared to be slightly more efficient than the TTITS2 primers probably because of the longer targetted amplicon length and inherently lower concentration of nucler genome DNA.

Bones from the twentieth century collection yielded such high concentrations of DNA (mean yield = 1.8ng/µL) that they were excluded from further comparisons among the more ancient samples. Samples from

Istanbul contained the highest concentration of DNA, while samples from Tavira yielded the lowest amount of DNA among the bones of ancient origin (Fig. 12).

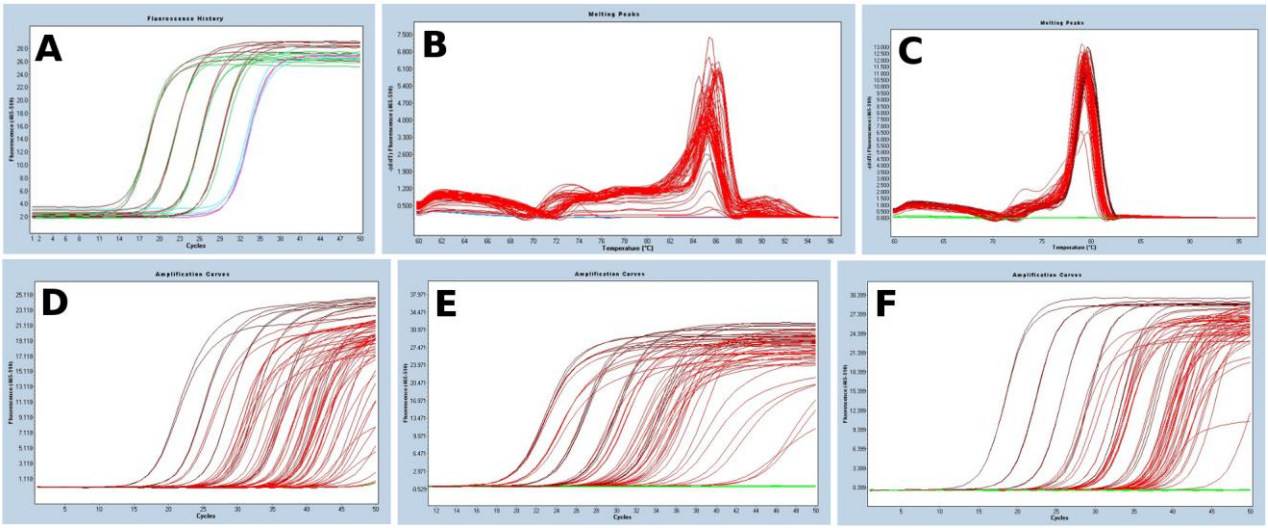

Fig. 11: Overlapping amplification curves of TTCOX1 (purple and pink) and TTCR1 (green and blue) primer pair standards (**A**). Melting curves of TTITS2 (B) and TTCOX1 (C) primer pair amplicons. Note: TTITS2 amplicons have a more scattered distribution around a central peak. Amplification curves of 30 (x2 replicates) historical samples (light red) with 5 (x2 replicates) serial dilution standards (dark red) using TTITS2 (**D**), TTCOX1 (**E**) and TTCR1 (1) primer pairs. Note: the range in which the majority of amplifications take place for TTITS2 is between 25-45 cycles, TTCOX1 between 26-35 cycles and TTCR1 between 30-40 cycles.

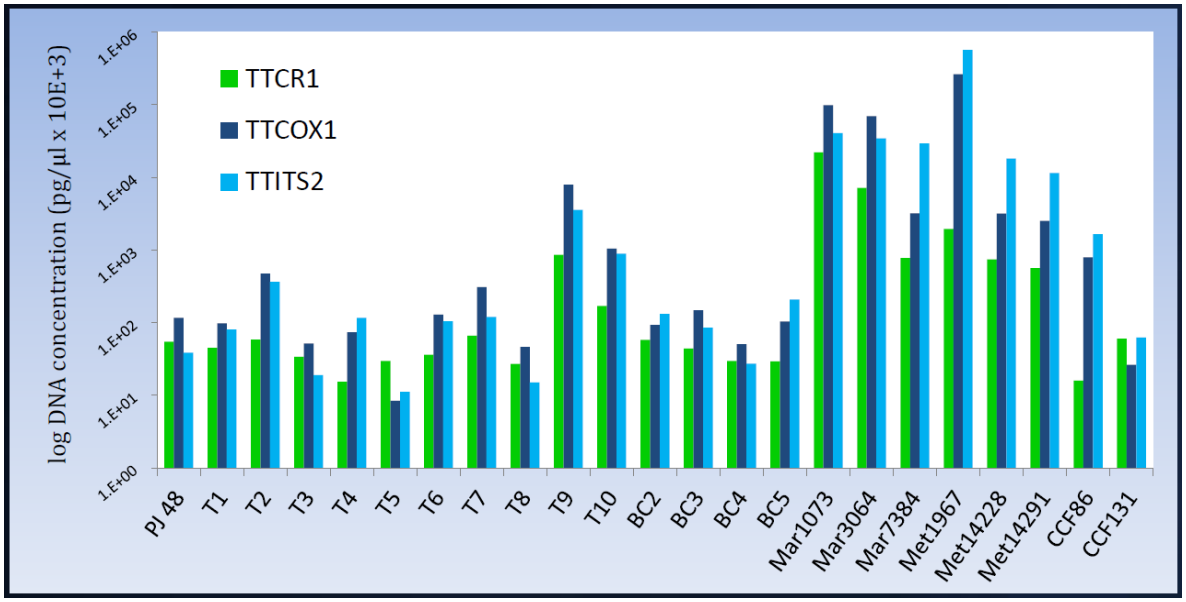

Fig. 12: qPCR results for 23 samples of ancient origin using three different molecular markers (CO1, mtCR and ITS1). Samples are arranged chronologically. Letters in sample names refer to geographic origin: Palacio de Justicia, Cadìz, Spain (PJ), Roman Balsa, Tavira, Portugal (T), Baelo Claudia, Tarifa, Spain (BC), Marmaray, Yenikapi, Istanbul, Turkey (Mar), Metro station, Yenikapi, Istanbul, Turkey (Met), La Chanca de Conil de la Frontera, Spain (CCF).

6.4 Discussion

6.4.1 qPCR quantification of ancient DNA

The qPCR results were consistent across samples (location and age), thereby validating the approach for relative comparisons of DNA yields between sample types. The approach might benefit from an adjustment to the way in which the standards were constructed. Campbell and Narum (2009) created their standards by amplifying target DNA fragments and inserting these into vectors using restriction enzymes. The molecular mass of each plasmid construct was calculated and 1:10 dilutions were prepared. Dilutions were then left to evaporate overnight and rehydrated with PCR mix the following day in an effort to reduce the impact of imprecise pipetting.

Focusing strictly on the Iberian samples, extractions with the highest concentration of DNA are perhaps the oldest amongst the collection from Castro Marim. Sample ROM542 is estimated to date back to the early roman republic (3rd-2nd century BC) and samples IA504 and IA535 were butchered by Iron Age fishermen. Samples with the lowest quantity of DNA are the vertebrae from the Roman settlement of Balsa in the Algarve, close to Tavira. Dried tuna vertebrae were incorporated into the building materials of the ancient Romans in the Algarve, presumably to add structural integrity as a strong organic brick of sorts. The low concentration of DNA in these bones may be a result of the way in which the bones were treated (boiling and drying in the hot summer sun) before being cemented into walls and floors. Alternatively, once inside of the walls they would have been exposed to extreme temperature variations between very high temperatures during the summer as the clay walls heated up during the day and cooled down at night, all of which is damaging for DNA. The age of bones clearly appears to have an influence on DNA yield; however, the DNA extracted from ancient sources using our new technique is of sufficient quality and quantity to achieve our next challenge: high throughput SNP genotyping. Various studies have shown that the degradation of DNA is not only a function of a specimen's age but is also significantly dependent on the environment conditions (temperature, humidity, pH, microorganisms) to which it has been subjected since the death of the animal (Miller et al. 2008; Zimmermann et al. 2008; Sawyer et al. 2012). This certainly seems to be the case with the samples analyzed here.

A similar comparative study focused on the preservation of DNA in human teeth (approximately 3000 years old) recovered from various archeological sites that differed in three factors: exposure to microorganims, pH and temperature (Burger et al. 1999). They found that remains extracted from a rather damp cave (Lichtenstein Cave, Germany) still contained high concentrations of quality DNA, likely due to the low temperatures (8°C) within the cave. In comparison, similar samples recovered from an archaeological site exposed to very low amounts of precipitation and higher temperatures (19-35°C) contained much less DNA (Shimal, United Arab Emirates).

The samples from Istanbul contained a relatively high DNA content. It was assumed that these samples would contain a reduced concentration of DNA due to exposure to coastal biogeochemical conditions for hundreds of years. However, the bones were buried in sediments that were likely alkaline due to the adjacent body of seawater (pH=8.1) and high carbonate content of the sediments owing to the myriad of shells found in the same layer of sediments. These conditions are likely responsible for the preservation of the 38 wooden ships found alongside the tuna bones.

6.4.2 Species identification

Only 40 of the 150 samples from the Massimo Sella archive were identified to as *T. thynnus*. An additional 60 samples were identified as *Thunnus* spp. and the remaining 50 samples could not be identified due to failures to amplify sequences or sub-standard sequencing results. However, since a majority of samples were identified as BFT or at least to genus level, were captured in the same area and at the same time, and had the same morphological characteristics, it is assumed that all are *T. thynnus*. Samples from the Istrian trap had the highest failure rates, which did not come as a surprise because also have very low concentrations of DNA. The colour and lack of dirt and dried tissue on the surface of these samples suggest that they have been treated in some way which might have also decreased the concentration of DNA in each sample.

6.5 Conclusion

The techniques developed during this study have potential for future use in both ancient and modern (traceability of cooked or canned food products) forensic investigations. Used for archaeological purposes, our methods can help to identfy damaged and incomplete animal remains and provide information about historical human diets, trade, species distribution and biodiversity.

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Chapter 7
Genotyping of ancient DNA

Vext with the puny foe the thunnies leap,
Flounce on the stream, and toss the mantling deep,
Ride o'er the foaming seas, with torture rave,
Bound into air, and dash the smoking wave.
Oft with imprudent haste they fly the main
And seek in death a kind release from pain,
Vault on some ship, or to the shore repair,
And gasp away their hated lives in air.
- John Jones, trans. Opp.(from Prose Halieutics)

Publication note:

As mentioned in Chapter 6, the contents of this chapter are being drafted for publication in a peer-reviewed journal with the following details:

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7.1 Introduction

7.1.1 Basics of Fishery Induced Evolution

Prior to having the genetic evidence of fisheries-induced evolution (FIE), fisheries scientists had proof that artificial selection by humans was capable of causing longlasting changes to species (Kuparinen and Merilä 2007; Allendorf et al. 2008; Allendorf and Hard 2009). Artificial selection of fishes occurs when fisheries harvest a specific fraction of a population based upon particular traits such as size, age, or sex. This selection can occur as a result of fishing gear (mesh size, gear type), areas and habitats targeted and timing of harvest. Changes exhibited by populations subject to fisheries induced evolution (FIE) are maturation at younger ages, reduced size at maturity, decreased egg volume and larvael size at hatching, increased/decreased fecundity, reduced annual growth rate and loss of genetic diversity (Jørgensen et al. 2007; Heino and Dieckman 2009; Audzijonyte et al. 2012). An extreme example of rapid FIE documented in a wild population was a 10 cm reduction in the age-specific maturation length for North Atlantic cod in only 7 years (Olsen et al. 2004). A review focused on fishery induced evolution by Jørgensen et al. (2007), suggested a possible range of 15-33% change in annual growth and a 21-22% loss of genetic diversity for some commercially exploited species within 13-125 years. They said: “The question is not whether such evolution will occur, but how fast fishing practices bring about evolutionary changes and what the consequences will be.” Examples of commercial species with traits indicative of FIE are Atlantic cod, many salmonids, whitefish, plaice, sole, herring, orange roughy and snapper (Jørgensen et al. (2007).

Targeting of large BFT in the Mediterranean Sea may already have caused profound changes to the demographics, life history strategies and genetics of the species. The fleet, which is dominated by purse seiners, targets the first large schools that enter into the Mediterranean when the season begins in the spring. Analysis of the size distribution of migrating BFT entering the Mediterranean has revealed that large fish are the first to enter, followed by smaller adults and juveniles (Badham 1854). A comparative morphometric study of modern BFT and vertebrae excavated from ancient Roman settlements in Iberia has revealed a 30% reduction in growth rates (Morales and Roselló 2007). More recently, fisheries pressure has been linked to demographic changes in the north eastern Atlantic BFT population(s), such as decreased age at maturity, reduced proportion of older stock and a reduced number of repeat spawners (Mackenzie et al. 2009). The

probability that the genetics of BFT has been altered in response to fisheries pressure is high; however, evidence of these changes has yet to surface.

Modern evolutionary changes in commerical species in response to fisheries pressure have the potential to cause negative irreversible impacts on stock biomass, demography and economic yield. Beyond the single species perspective, FIE can also impact trophic dynamics, competitive interactions, and community composition among other ecological relationships (Jørgensen et al. 2007). Demographic changes can increase instability of yields, leading to reduced catches or overestimations of stock size and increased quotas. These changes can reduce a species' resilience and confuse stock assessments. Evolution appears to have a real-time economic impact and as a result, evolutionary impact assessments have been proposed as a means by which changes in the utility of a stock, resulting from FIE, can be evaluated (Jørgensen et al. 2007).

Ancient DNA research has accomplished much in the past three decades with the number of publications increasing exponentially covering subjects linked with archaeology, anthropology, evolutionary biology, conservation, and climate research (Hagelberg et al. 2015). Fisheries' researchers have caught the aDNA bug as well, recognizing that through the genetic analysis of historical samples (scales, otoliths, and vertebrae) we're now capable of elucidating the impact of humankind on fish species, since the beginning of commercial-scale exploitation. Comparisons between genetic information collected from existing populations and historical samples from museums and archives has led to new reveations concerning the loss of genetic variation and the distribution of species and populations over time (Roy et al.1994; Taylor et al.1994; Vallianatos et al. 2002; Burrell et al. 2015).

7.1.2 Use of historical samples following the PCR Revolution

The first study to provide genetic infromation derived from museum samples was that of Higuchi et al. (1984) who directly sequenced a 229 bp fragment of cloned mitchondrial DNA. This was followed soon after by a study that cloned a short fragment of DNA from an Egytpian mummy (Pääbo 1985). When a protocol for the polymerase chain reaction (PCR) was published in the late 1980s it revolutionized biology and opened up a universe of opportunities for the study of historical samples (Saiki et al. 1985; Mullis and Faloona 1987). In short, PCRs make use of the enzyme DNA polymerase to amplify millions of copies of a chosen template of DNA through multiple temperature cycles (denaturation of DNA at 94-96°C, annealing of

primers to template DNA at 3-5°C lower than the melting temperature of the primers and elongatin or assembly of a complementary strand of DNA using deoxynucleotide triphosphate building blocks). This simple recipe, that earned Kary Banks Mullis the Nobel Prize, allows for the replication of small amounts of DNA by several magnitudes of order. Unfortunately, the use of PCRs in aDNA studies isn't a simple and straightforward solution for low concentrations of template DNA. Shortly after death, crosslinks begin to form within and between DNA molecules through condensation reactions that bind the sugar and primary amino-groups in nucleic acids (Pääbo et al. 2004). These crosslinks, called Maillard products, inhibit PCR amplification of DNA, even when present in abundance (Willerslev and Cooper 2005). Maillard products can be elminated if DNA extractions are treated with N-phenacylthiazolium bromide (Poinar et al. 1998). PCRs also introduced the potential for amplification of contaminants; a problem made clear when authors started releasing studies containing breakthroughs in the analysis of million-year-old plant materials, amber preserved insects and dinosaur bones, all of which have been discredited since (reviewed in Willerslev and Cooper 2005). Despite these early errors, palaeogenetics has become a respected and widely used method for investigating processes of evolution and changes in population genetics.

7.1.3 Mitochondrial DNA as a tool in paleogenetics

The earliest investigations into FIE using genetic tools focused on mitochondrial DNA (mtDNA), because of the relative ease with which genotypes can be obtained due to the abundance of copies per cell, reduced size and haploidy of the mitchondrial genome (Millar et al. 2008). The purpose of the majority of studies that have focused on sequencing of historical mtDNA has been for the identification of damaged ancient remains. While this research hasn't provided a great deal of information regarding evolution of species, they have served to clarify the distribution of historical populations, human patterns of resource use and ancient trade routes. For example, short fragments (119-250 bp) of mitochondrial DNA have been used to ascertain the distribution of several salmon species in the North East Pacific from 1100-9000 yBP (Yang et al. 2004; Butler and Bowers 1998; Speller at al. 2005; Moss et al. 2014). In central Poland, short fragments of 102bp were used to identify 500,000 year old whitefish bones (Brzuzan et al. 2004) and in the north of Poland 1,000 year old sea bream bones were identified using DNA fragments of 172 bp (Ciesielski et al. 2002). In the Levantine Sea, sequencing of DNA extracted from ancient catfish pectoral spines (1,500 yBP) confirmed

commercial trade of the species between Egypt and ancient Anatolia (modern day Turkey; Arndt et al. 2003). Short sequences of mitochdrial DNA have also been used to estimate temporal changes in the genetic diversity and populations sizes of Atlantic cod in Iceland (Ólafsdóttir et al. 2009), salmon in northern Spain (Consuegra et al. 2002), and brown trout in Denmark (Hansen et al. 2002).

7.1.4 Microsatellites

Hypervariable microsatellites, or short tandem repeats, in the nuclear genome have been used in several ichthyological studies exploring temporal changes in population structuring and effective population sizes using collections of historical and modern specimens. Microsatellites have been used to measure the impacts of restocking on salmon populations in Denmark (Nielsen et al. 1997), show genetic loss in New Zealand snapper after decades of heavy exploitation (Hauser et al. 2002), reveal genetic adaptation of Atlantic cod to increasing sea surface temperatures (Nielsen et al. 2007), and uncover evidence of extirpated hiistorical populations of salmon in the Columbia River basin (Iwamoto et al. 2012). They have provided evidence of depressed genetic integrity and decreases in effective population sizes of heavily exploited populations of North Sea cod, brown trout and sole during the past century (Hansen et al. 2002; Hutchinson et al. 2003; Poulsen et al. 2005; Cuveliers et al. 2011). Microsatellites have also been used to demonstrate that hybridization of wild trout with aquaculture farm escapees results in depressed fitness and reduced genetic diversity in wild populations (Coughlan et al. 2006; Hansen et al. 2010).

Two aDNA studies have been published that have utilized the the Massimo Sella vertebrae: Landi et al. (2007) and Riccioni et al. (2010). Both studies used a limited number of microsatellite loci and were able to elucidate a weak population structure. According to both studies the genetic diversity of the species remains virtually unchanged from the past 90 years, despite a reduction of biomass of >70% and a 46% contraction in their geographic range (Worm and Tittensor 2011). Riccioni et al. (2010) focused on 8 microsatellite loci which revealed no significant differences in allelic richness and gene diversity across contemporary and historical samples. They found weak signatures of population differentiation among modern ($Fst = 0.014$, $P < 0.0001$) and two historical samples ($FST = 0.020$, $P < 0.0001$); although there remained no significant evidence of genetic structuring when Bayesian tools were employed. From the limited number of sites analyzed, they described a heterozygote deficit that could indicate either a Wahlund effect or a pronounced

variation in reproductive success. Taking these results into consideration, researchers were left with the following conclusions: 1) Data derived from the historical Libyan samples suggest that spatiotemporal shifts in BFT population structure and dynamics have occurred in the Mediterranean, 2) there is a weak signal of genetic structuring within the Mediterranean Sea, 3) the genetic markers used to date are likely to weak to demonstrate these genetic dynamics and, 4) a more robust sampling design using advanced techniques and higher performing genetic markers are required to address these questions (Cannas et al. 2012).

Admittedly, mtDNA and microsatellite studies have made a great deal of valuable insights in the field of evolutionary genetics in the past two decades; however they both suffer from several limitations. Inferences made from mtDNA are limited because the target genetic material is inherited from a single parent while microsatellite loci suffer from null alleles and a variable mutation rate that make inference difficult (Wandeler et al. 2007). Both are susceptible to homoplasy which poses severe limitations. Conversely, Single Nucleotide Polymorphisms (SNPs) are abundant throughout the genome and their evolution can be described by simple mutation models (Morin et al. 2004).

7.1.5 Next Generation Sequencing

In a review by Knapp and Hofreiter (2010), Next Generation Sequencing (NGS) was credited with revolutionizing all fields of genetics and no other field has profited from its advent more than ancient DNA research, a field which they describe as now being at the centre of evolutionary biology. Since its inception the technology has developed at an alarmingly rapid pace and its sequencing capabilities are beyond what could have been imagined just years ago. That being said, NGS protocols cannot be applied directly to aDNA studies, due to the inherently low concentration of template DNA in historical samples. Most NGS protocols require multiple filtration steps during library preparation which greatly reduces DNA yields by as much as 99% (Maricic and Paabo 2009). As such, specialized protocols have been developed for aDNA research using NGS techniques. Shotgun sequencing executed with NGS platforms can be credited with many of the advances in palaeogenomics, as it has been used for the reconstruction of the genomes of many extinct and non-model species. The higher number of copies and short length of the mitchondrial genome increases the likelihood of completing the entire sequence using high throughput platforms; however, the number of genes contained in the genome is limited and thus offer limited scope for evolutionary inference.

Shotgun sequencing exectured on NGS platforms begins with random shearing of extracted DNA into short fragments which are then sequenced. The resulting sequences are then joined together by aligning them *in silico* where their sequences overlap, leading to the assembly of longer fragments in a bridge-like fashion. Thresholds ensure that alignments are legitimate by requiring a minimum number of reads per base. This method requires high quality, relatively pure and abundant endogenous DNA. Shearing already fragmented aDNA results in a surplus of short fragments which can be highly similar with many other short fragments from different regions of the genome (repeats). Incorrectly aligning these short repeat fragments results in a confused and erroneous genome full of artificial sequences (artefacts or paralogs). All the same, Knapp and Hofreiter (2010) counted 16 ancient DNA studies that used next generation shotgun sequencing partly or exclusively for sequence data collection between 2006 and 2010 for various taxa. Researchers can overcome low template concentrations via PCR target enrichment; however, this results in a reduced spectrum of analysis. Pre-amplification of target sequences in aDNA studies can also be problematic if low amounts of exogenous or damaged DNA are amplified in the first cycles of PCR. A single erroneous sequence can be amplified millions of times, becoming indistinguishable from the endogenous target DNA. Hybridization capture approaches (like primer extension capture) can overcome some of the difficulties associated with PCRs and focus more on the enrichment of endogenous DNA (Noonan et al. 2006; Briggs et al. 2009). In this approach, target DNA hybridizes with specialized probes that are either immobilized on microarrays or capture beads. Combinations of these techniques have led to the sequencing of complete mitochondrial genomes from the following extinct species: New Zealand moa (Cooper et al. 2001), mammoth (Krause et al. 2006), Neanderthal (Green et al. 2008), cave bear (Krause et al. 2008), woolly rhinoceros (Willerslev et al. 2009), auroch (Edwards et al. 2010), and Tasmanian tiger (Miller et al. 2008).

7.1.6 SNP genotyping

The process by which SNPs are discovered, validated and incorporated into genotyping arrays has been described in chapters 4 and 5. Due to the nature of SNP arrays and microsatellite sequences, poor quality samples are genotyped with a higher success rate using the SNP array approach. A minimum of 1000 starting copies of template DNA have been advised for successful genotyping of microsatellites while as few as 50 copies are required for genotyping of SNPs (Campbell and Narum 2009). A 2012 study attempted to genotype

60 herring bones recovered from various archaeological sites in British Columbia, Canada (100-3,800 yBP) using mitochondrial DNA, microsatellites and SNPs (Speller et al. 2012). The authors found that the mtDNA marker had limited power for population discrimination and microsatellites provided low quality data because of allele drop-out and stuttering. The SNP assay, however, provided low error rates and high discriminatory power, thereby motivating the authors to conclude that SNPs were the most effective approach for the analysis of genetic population structuring in aDNA studies. In addition to higher versatility with DNA of varying quality and quantity, the potential of SNP genoytping platforms for high throughout processing is impressive. In 2011, Smith et al. (2011) conservatively estimated that a single researcher was, at that time, capable of generating up to 72,960 genotypes in a single day (760 samples genotyped with 96 SNP loci).

SNP genotyping panels have been used in aDNA studies to ascertain temporal changes in the population structuring of Atlantic cod (Poulsen et al. 2011) and herring (Speller et al. 2012). Researchers have also used SNPs to determine how populations of Atlantic cod (Poulsen et al. 2011; Therkildsen et al. 2013), Atlantic herring (Limborg et al. 2012) and Pacific chum salmon (Seeb et al. 2011) have been influenced by temporal changes in environmental conditions.

7.1.7 Study aim

A SNP panel is developed for the genotyping of a collection of modern (n=269) and historical (n=186) BFT samples (described in Chapter 6) in order to ascertain whether the population structure and spatial dynamics of the species in the Mediterrranean Sea has changed over time. If significant differences in geographic and/or temporal allele frequencies are revealed, then they will be compared with fishery time series data and environmental parameters in order to determine whether selective responses to a changing environment and industrial fisheries have occurred. Due to the absence of an extant population of BFT in the Black Sea region, the historical samples from the Black Sea region are of particular interest in this study. There is a strong possibility that the BFT of the Black Sea were spawning in isolation, since their migratory movements were likely determined by the distribution of their prey which migrated into the warming waters of the Black Sea in the spring, returned to the Mediterranean Sea in autumn. Aristotle recorded this migratory behaviour in 350 BCE and his observations have been verified by 20th century marine scientists (Akyuz and Artuz 1957; Mather et al. 1995, Karakulak and Oray 2009). This would place the local population in the Black

Sea during the spawning season, far from all other spawning groups. If spawning was taking place in the Black Sea, then adaptations to the local hydrographic conditions would have been required. During the spawning season, salinity, density and temperature are lower in the Black Sea than all other BFT spawning areas (Mackenzie and Mariani 2012). Unless physical adaptations provided increased egg buoyancy (increased size, decreased dry weight and thinner chorions with fewer lamellae), the eggs of BFT would quickly sink into the Black Sea's hypoxic waters below 100m (Mackenzie and Mariani 2012). Additional adaptations would have been required of the sperm, unfertilized eggs and developing embryo and larvae. A genetic record of these adaptations could be recovered from the Byzantine-era bones uncovered in Istanbul and might still be traced to the remaining descendants of this isolated tribe, if any have survived. Information concerning the distribution of the remaining Black Sea BFT can provide information about population structuring, migratory behaviour, genetic diversity and adaptive potential. By comparing the genetic code of modern and ancient BFT we can shed light on the evolution of the species genome in response to nearly two millennia of fisheries pressure, a changing climate and pollution of the sea.

The SNPs selected for the genotyping panel were developed using 1) a Targeted Gene Approach, 2) Genotyping-by-Sequencing SNP discovery, and 3) Transcriptome SNP discovery. The SNPs that were discovered using the GBS approach (Chapter 4) will provide information concerning the temporal continuity of genetic population structuring, while SNPs derived from the targeted gene approach and transcriptomics are associated with genes that may hold selective or evolutionary significance.

7.2 Materials and Methods

7.2.1 Targeted Gene Approach

An array of primers were designed to amplify regions of DNA that are likely to be associated with genes subject to selective pressures, such as those that influence metabolism, growth and immune response (myosin, heat shock protein, interleukin factor, hemoglobin, methylmercury binding protein). Several genes already had primer sequences published for *T. thynnus* or sister taxa, while others had to be designed using previously published sequences of Atlantic or Pacific BFT and Primer 3 v. 0.4.0 software (Koressaar and Remm 2007; Untergrasser et al. 2012; Appendix 1). Gradient PCRs were conducted for all primer pairs, in order to determine the optimal annealing temperature for each. PCR reactions were performed in 25 µL

volume consisting of 1x PCR Buffer, 1.0 µM of each primer, 160 µg/mL of BSA, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$, 2.5 U/mL of Invitrogen Taq polymerase and ~100 ng of template DNA. Gradient PCR conditions consisted of 94°C for 3 min, 35 cycles of 30 sec at 94°C, 30 sec at 12 different temperatures (full range of 10°C with a median temperature 5°C below the lowest primer's melting temperature), and 30 sec at 72°C, with a final extension at 72°C for 3 min. Once PCR conditions were optimized for a handful of primer pairs, DNA fragments were amplified using a group of 77 modern samples from 5 different locations in the species' geographic distribution (Table 1). The PCR products of the three most successful gene amplifications (heat shock protein 70, interleukin factor region 1 and 2) have been sequenced, aligned and analyzed for SNP loci using Mega v. 6 (Tamura et al. 2013).

Table 1: Samples used in the search for SNPs in a targeted gene amplification approach.

Geographic Origin	Age Class	Year of Capture	No. of Samples
Strait of Sicily	Young-of-the-Year	2012	14
Balearic Sea	Young-of-the-Year	2011	10
Tyrrhenian Sea	Young-of-the-Year	2012	10
Levantine Sea	Young-of-the-Year	2011	2
Levantine Sea	Medium Adult	2011	10
Levantine Sea	Large Adult	2011	16
Gulf of Mexico	Larvae	2007	2
Gulf of Mexico	Young-of-the-Year	2008	13
			Total: 77

7.2.2 Genotyping-by-Sequencing SNP loci

The process by which SNPs were discovered using Genotyping-by-Sequencing techniques is the topic of **Chapter 4**. The performance criteria by which each of these SNPs were evaluated and subsequently used for the selection of loci for the current temporal investigation are explained in Chapter 5.

7.2.3 *Transcriptome derived SNPs*

In 2012, researchers at the GenMAP laboratory at the University of Bologna with funding through ICCAT GBYP Phase 2 used combined approach of transcriptome sequencing and shallow genome sequencing to discover SNPs within the BFT genome (Pintus 2013). In that study, the reseachers used GS FLEX Titanium sequencing technology to obtain >1 million cDNA sequences from ten BFT from different

geographic locations. Concurrently, the Illumina HiSeq2000 was used to generate 833 million reads from four BFT which were used to construct a partial reference genome consisting of 500,000 contigs via *de novo* assembly. The cDNA reads were then mapped to the reference and the best 4000 contigs were selected for *in silico* SNP discovery, based on number of reads (minimum 10) and sequence length (>200bp). From these 384 candidate SNPs were selected based on strict quality criteria. Following validation of the selected SNPs, 919 individuals from 23 populations throughout the species' range were genotyped with all 384 SNPs using Illumina Golden Gate assays. Some 287 SNPs and 848 individuals proved to be satisfactory for use in genetic structuring analysis. Various statistical analyses revealed extremely low and non-significant levels of genetic differentiation among bluefin tuna population samples.

7.2.4 Comparison of GBS and EST SNPs with published annotated genes

All 220 validated SNPs decribed in Chapter 4 along with the 384 transcriptome-derived SNPs described above were submitted to BLAST alignments (Altschul et al. 1990) with several selected reference sequence libraries. Sequences contained in these libraries were publicly available annotated gene sequences for Atlantic cod (*Gadus morhua*; Star et al. 2011), sea bass (*Dicentrarchus labrax*; Tine et al. 2014), BFT (Chini et al. 2008; Gardner et al. 2012), and an umbrella set of sequences containing data from all other teleosts. Query matches with referencee sequences were only recognized if alignment coverage was > 80% and identity scores >80%. Several SNPs highly discriminatory SNPs were selected for additional investigation regarding the nature of their impact on gene expression. The flanking region sequences of these loci were aligned with sequences from other teleost species and translated into the corresponding amino acid sequences in an effort to determine whether base substitutions resulted in changes to the amino acid sequence.

7.2.5 Sample collection

All 186 historical samples from which DNA was extracted (described in Chapter 6) will be genotyped using the 96 SNP panel described here and compared with 269 modern samples collected from the same geographic locations or adjacent areas (Appendix 1, Fig. 1). Two additional groups of samples have been added to the analysis in order to complete geographic coverage (Balearic Sea) and include at least one group from outside of the Mediterranean Sea (Bay of Biscay). DNA extractions have been outsourced to the

University of the Basque Country (UPV/EHU) where they will undego DNA extraction in plate format with Macherey-Nagel Nucleospin kits, quantification (spectrophotometry) and normalization.

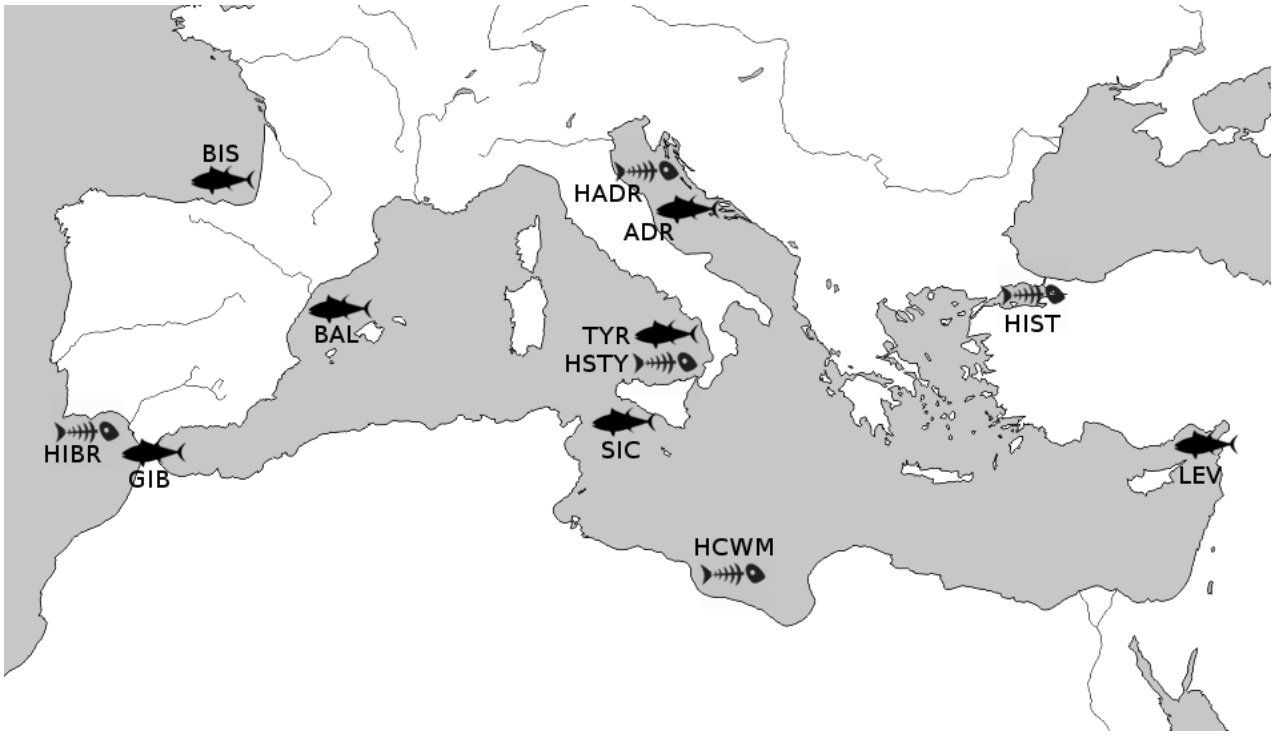

Fig 1: Geographic origins of modern and historical samples to be genotyped using a 96 SNP panel. Historical samples: HIBR = Iberia (Castro Marim, Baelo Claudia, Tavira), HSTY = Tyrrhenian Sea, HADR = Adriatic Sea, HCWM = Central Western Mediterranean (Zliten, Libya)), HCON = Constantinople. Modern samples: BIS = Bay of Biscay, GIB = Strait of Gibraltar, BAL = Balearic Sea, TYR = Tyrrhenian Sea, SIC = Strait of Sicily, ADR = Adriatic Sea, LEV = Levantine Sea.

7.3 Results

7.3.1 Targeted Gene Approach for SNP selection

Only half of the eighteen primer pairs provided successful PCR amplifications. Details concerning the primers that provided satisfactory amplicons are featured in Table 2. Primer pairs that consistently produced multiple bands and smears were TTHSP27, TTMER1, TTMER2, TTMER3, TTMER4, TTIL1, TTTNF1, TTTNF3, TTMYO. Additional amplifications and sequencing of three gene loci (TTIL2, TTIL3 and TTHSP70; example of gels in Fig. 2) were conducted in order to provide an adequate number of sequences for comparative alignments. A total of 66, 42, and 28 individuals were successfully sequenced with TTHSP70 (501bp) TTIL2 (622bp), and TTIL3 (594bp) primer pairs. Alignments of TTHSP70 and TTIL2 sequences revealed too many variable sites for suitable construction of SNP probes (minimum of 60bp on either side of SNP). The TTIL3 alignment was less variable and provided no evidence of geographic differences.

Table 2: Primers developed to amplify targeted genes associated with selective traits.

Primer Pair Name	Optimized Annealing Temp.	Comments
TTNKATP	64.0	Single band at high temp. Two bands at low temp.
TTHSP70	63.7	Clear consistent amplification.
TTTNF2	63.8	Clear consistent amplification.
TTTNF4	65.0	Three bands at low temp. Persistent primer dimers.
TTTNF5	56.0	Clear consistent amplification.
TTHEM	59.1	Multipe bands. Requires additional optimization.
TTMER5	58.2	Multiple bands. Requires additional optimization.
TTIL2	59.0	Clear consistent amplification.
TTIL3	57.3	Clear consistent amplification.

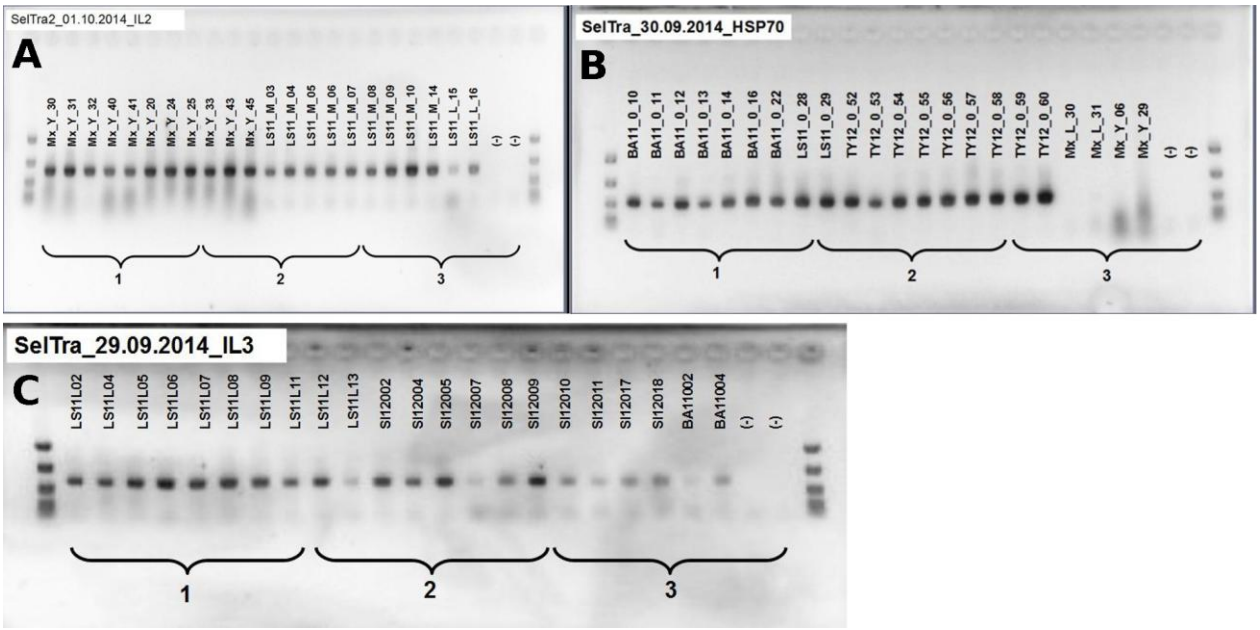

Fig. 2: Examples of electrophoresis gels showing successful amplification of targeted genes from ABFT samples collected throughout the species range. Images **A** and **C** are are gels containing stained amplicons of interleukin factor region 1 and 2, and image B is a gel of heat shock protein 70 amplicons. Unlabelled columns are molecular weight ladders and each gel contains two columns with negative controls (-).

7.3.2 Candidate SNP loci alignments with published annotated genes

Of the 604 candidate loci queried, 10 matched with published BFT sequences (Appendix 2), 51 with seabass sequences (Appendix 3), 12 with Atlantic cod annotated genes (Appendix 4) and 30 with annotated gene sequences of other teleosts (Appendix 5). The vast majority of "other teleost" matches correspnded with

well studied species like *Takifugu rubripes, Poecilia formosa, Larimichthys crocea, Stegastes partitus, Oreochromis niloticus, Oryzias latipes, Maylandia zebra*, and *Haplochromis burtoni*. Figure 3 is a Venny diagram illustrating the distribution of all candidate loci – reference sequence matches. All 68 SNPs that were matched with annotated genes were added to 28 highly discriminatory SNPs to complete the 96 SNP panel.

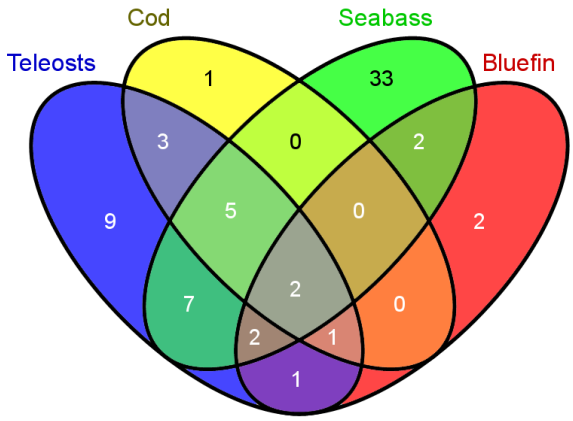

Fig. 3: Venny diagram (Oliveros 2007) of the distribution of 68 published sequences from various teleost species that aligned with EST and GBS derived SNP containing oligonucleotides.

During the development of the GBS-derived 384 SNP panel (**Chapter 4**), SNPs were selected based on extreme differences in pooled allele frequency differences across populations. One particular locus (SNP_150) was a strong candidate (Table 3) for the SNP panel and upon closer analysis during the work detailed in this chapter, was shown to align with the myosin VIIB gene. The expressed product of this gene is an actin-based motor molecule with ATPase activity that plays an important role in intracellular movements. In humans it is expressed in the eye and inner ear, the olfactory epithelium, brain, choroid plexus, intestine, liver, kidney, adrenal gland, and testes (Chen et al. 2001). While guanine was the dominant nucleotide expressed in the Levantine Sea samples (60%), adenine was by far the dominant allele for samples captured in the Strait of Sicily and the Tyrrhenian Sea (92% and 93%, respectively). After the validation of a high performance 96 SNP panel, SNP_150 continued to show significant differences in allele frequency across sampling sites (Table 4). Nucleotide and amino acid sequences of the myosin VIIB gene from various other teleost species were aligned with the flanking sequence of SNP_150. Substitution of the reference allele (A) with the alternative allele (G) results in a non-synonymous base substitution.

Table 3: Allele frequency of SNP associated with the Myosin VIIB gene sequence (SNP_150) from 555 samples pooled by geographic area from GBS SNP discovery by BGSA Genetics Consortium during GBYP Phase 4.

Pop	SNP read count	Allele A frequency
Western Atlantic	205	0.77
Balearic Islands	816	0.55
Ligurian Sea	39	1.00
Tyrrhenian Sea	268	0.93
Strait of Sicily	188	0.92
Levantine Sea	498	0.40

Table 4: Allele frequency of SNPs associated with the Myosin VIIB gene from 167 samples pooled by sampling location from GBS SNP discovery by the BGSA Genetics Consortium during GBYP Phase 4 - 96 SNP panel validation.

Population	Year		A	G
Cape Hatteras	2008	Young-of-the-Year	0.588	0.412
Gulf of Mexico	2008	Larvae	0.688	0.312
Gulf of Mexico	2009	Larvae	0.500	0.500
Strait of Sicily	2011	Young-of-the-Year	0.857	0.143
Strait of Sicily	2012	Young-of-the-Year	0.542	0.458
Levantine Sea	2011	Young-of-the-Year	0.654	0.346
Balearic Islands	2011	Young-of-the-Year	0.604	0.396

7.4 Discussion

7.4.1 Technical considerations

It's difficult to anticipate the outcome of the genotyping of efforts currently underway (July 2015) using the selected SNP panel described in this chapter. However, since all SNPs have been validated and used to genotype modern tuna samples in the past two years as part of other projects, it can be expected that the success rate will be high. As described in Chapter 6, the historical samples to be genotyped are of varying concentration and quality. Undoubtedly these factors will play a role in the number of samples that are successfully genotyped. According to Sawyer et al. (2012), it should be expected that thymine residues will have replaced cytosine at the 5' end of some of the historical DNA fragments. However, since all SNPs are located 60 bp from fragments ends, according to the requirements of the Fluidigm genoytping platform, this should not cause errors with SNP genotyping but may present a problem with the binding of damaged

fragments to probes. As such, we may expect to see reduced efficiency in older samples, not as a consequence of reduced DNA concentration, but rather reduced binding efficienty due to errors in the 5' end fragments. This may prove to be a significant problem for specimens that have been exposed to heat or cleaning agents (Sawyer et al. 2012), as may be the case with the samples from the Adriatic Sea.

7.4.2 Alternative options for investigations into temporal shifts in allele frequencies

Briggs et al. (2009) developed a technique called Primer extension capture (PEC) which they have used to capture targeted fragments of DNA in contaminated historical samples. Basically, a species specific 5' biotinylated oligonucleotide primer is allowed to bind to the target fragment during a single *Taq* DNA polymerase extensions. Excess primers are removed from the mix using spin column purification. The target fragment and primer duplex are then allowed to bind to magnetic beads which are then washed of all other elements within the solution (other DNA and reagents). Finally the target DNA is eluted from the beads and the result is pure target DNA which can be used for a number of downstream applications (amplification and sequencing, 454 emulsion sequencing etc.). This protocol was used to isolate neanderthal DNA from 38,000-70,000 year old remains.

7.4.3 Potential applications

If the historical samples from the Black Sea prove to be genetically unique from the other historical and modern samples, then further investigation is certainly warranted. The BFT bones excavated from the Yenikapi site may eventually reveal the nature of local adaptations to conditions in the Black Sea suggested by Mackenzie and Mariani (2012). These revelations will be required for any efforts to restock the Black Sea with spawning BFT, if isolated spawning was indeed taking place there in the past. Attempts can be made to screen individuals from the modern stock for these traits or genetically modify BFT at the various culturing facilities in Japan, Spain, Italy and Israel (de la Gandara et al. 2010).

If the residential group of BFT in the eastern Mediterranean proves to be descended from a unique Black Sea population, then this should be reflected in future conservation efforts. Results of this nature would justify additional genetic sampling, a more comprehensive tagging campaign in the Sea of Marmara and Aegean Sea and modelling experiments to determine the impacts of different management initiatives.

7.4.4 Future Aims

Manufacture of the final genotyping SNP panel will be completed by Fluidigm in May 2015 and all samples are expected to be genotyped shortly thereafter. We expect to have the final deliverable for this project completed by the end of June 2015. Beyond the preliminary results expected from this short contract, it will take some time to fully exploit the potential of all of the expected results from this ambitious project.

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Appendices

Appendix 1: Samples to be genotyped using a high performance 96 SNP panel containing loci associated with population structuring and selective traits.

Geographic region	Modern samples	1911-1926	1755	4th-15th century	1st century	4th-2nd century BCE	Total
Adriatic Sea	**40** (2011)	**50** (Istria, Croatia)					**90**
Tyrrhenian Sea	**40** (2011)	**50** (Pizzo and Messina, Italy)					**90**
Strait of Sicily / Ionian Sea	**40** (2012)	**50** (Zliten, Libya)					**90**
Gibraltar / Portugal	**40** (2011)		**2** (Cádiz, Spain)		**10** (Cádiz, Spain)	**16** (Baelo Claudia, Castrom Marim, Tavira)	**68**
Levantine Sea	**29** (2011)	**2** (Istanbul, Turkey)		**6** (Istanbul, Turkey)			**37**
Balearic Sea	**40** (2011)						**40**
Total	**269**	**152**	**2**	**6**	**10**	**16**	**455**

Appendix 1: Primers used to amplify portions of targeted genes associated with selective traits. Asterisk denotes new primers that were designed using previously publshed sequences and Primer 3 software (Koressaar and Remm 2007; Untergrasser et al. 2012).

Name	Gene	Sequence	Species	Reference
TTNKATP	Na+/K+ ATP-ase	F: 5'- GAGCAYTTCATCCACATCCATCAC -3' R: 5'- ATCTGGTTGTCRAACCAC -3'	*Thunnus orientalis*	Mladineo and Block (2009)
TTMYO	Myoglobin	F: 5'- AATCAGACGGGATATATTAC -3' R: 5'- TTTTAAAGCAACAGAGAG -3'	*Thunnus thynnus*	Marcinek et al. (2001)
TTHSP27	Heat shock protein 27	F: 5'- ACCACGCCACTTTTGTTTTC -3' R: 5'- CAGCAG CCATGACAGAGAGA-3'	*Thunnus orientalis*	*Ojima and Oohara (2008)
TTHSP70	Heat shock protein 70	F: 5'- CGACCAGGGCAACAGGAC -3' R: 5'- GGTCAGGATGGACACGTCGAAGG -3'	*Thunnus orientalis*	Mladineo and Block (2009)
TTTNF1	Tumour necrosis factor alpha, Section 1	F: 5'- CGACTGGAGCACGAGGACACTGA -3' R: 5'- CAACAAGGAGAGCAGTAGCAGCCG -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTTNF2	Tumour necrosis factor alpha, Section 2	F: 5'- GCTGGAGTGGAGAGTTGAT -3' R: 5'- GCTGTCAACGATACGCTACGTAACG -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTTNF3	Tumour necrosis factor alpha, Section 3	F: 5'- GAGAGAAGTATCACCACAGAGCG -3' R: 5'- TGGCTGTAGACGAAGTAGAGGC -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTTNF4	Tumour necrosis factor alpha, Section 4	F: 5'- TCTTGGTGCCGTGTTTCAG -3' R: 5'- CTTCGTATCCTCTCAATTAGTATCACAGC -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTTNF5	Tumour necrosis factor alpha, Section 5	F: 5'- CCAGGCRGCCATCCATTTAGAAG -3' R: 5'- TTTCCCGCTCCCTGCTCGTCG -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTHEM	Beta chain hemoglobin	F: 5'- TCATTGAGGAGACAAACCACA -3' R: 5'- TCAAGAGCCATCATGGTTGA -3'	*Thunnus thynnus*	*Yokoyama et al. (2004)
TTMER1	Methylmercury binding protein, Section 1	F: 5'- ACGGATCGTGAAAACCAGTC -3' R: 5'- GCTTTGTGTC CAGCATCTCA-3'	*Thunnus thynnus*	*GenBank: DM189309.1
TTMER2	Methylmercury binding protein, Section 2	F: 5'- GGTCTGCTGGGTTTACTGGA -3' R: 5'- TCAAGATCGTCCACTT GCTG -3'	*Thunnus thynnus*	*GenBank: DM189309.1
TTMER3	Methylmercury binding protein, Section 3	F: 5'- CCAGGCAGAGGAAGACAAAG -3' R: 5'- ACTCACCATTCTCCGTCTGG -3'	*Thunnus thynnus*	*GenBank: DM189309.1
TTMER4	Methylmercury binding protein, Section 4	F: 5'- GCTCTTGTTTCCCAGCTGAC -3' R: 5'- GCAAGCTTCCTGTCAACCTC-3'	*Thunnus thynnus*	*GenBank: DM189309.1
TTMER5	Methylmercury binding protein, Section 5	F: 5'- TGACGATGCTGTCAGAGGAC -3' R: 5'- GATCACGGCTCTTTGCTCTC -3'	*Thunnus thynnus*	*GenBank: DM189309.1
TTIL1	Interleukin 1 beta, Section 1	F: 5'- GCTGGGATAACCAACCAAAC -3' R: 5'- GTCCAGCAAGATGTTGAGCA -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTIL2	Interleukin 1 beta, Section 2	F: 5'- GCACCGAGTTCAGAGATGAAA -3' R: 5'- ATTAAGAGTTTTACCTCCAGATGC -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)
TTIL3	Interleukin 1 beta, Section 3	F: 5'- TCTATCGCATGCACCACATT -3' R: 5'- CACACTCACACAGCGTAAGGA -3'	*Thunnus thynnus*	*Lepen Pleic et al. (2014)

Appendix 2: 10 SNPs that aligned with published bluefin tuna sequences (Chini et al. 2008).

SNP	Reference code	Protein description	%ident	%cover	E-value
SNP_023_EST	EG630677	Asparaginyl-tr synthetase	95.28	100	3e-42
SNP_147_EST	EL610929	Elongation factor 1 alpha	95	99.17	6e-47
SNP_027_EST	EL611421	Eukaryotic translation initiation factor subunit g	99.12	93.39	1e-57
SNP_144_EST	EG999382	Gamma-aminobutyric acid receptor-associated 1	91.87	100	3e-33
SNP_059_EST	EC919262	High-mobility group box 1	100	93.39	2e-59
SNP_220_EST	EC919270	Integral membrane protein 2b	100	100	3e-64
SNP_305_EST	EC917824	Myosin regulatory light chain smooth muscle isoform	95.93	100	1e-50
SNP_163_EST	EH000371	Platelet-derived growth factor receptor-like	93.97	94.21	1e-38
SNP_086_EST	EC092247	TGF beta-inducible nuclear protein 1	99.17	100	6e-50
SNP_125_EST	EC918814	Thioredoxin interacting protein	100	100	3e-64

Appendix 3: 51 SNPs that aligned with *Dicentrarchus labrax* annotated genes. Four genes aligned with more than one SNP-containing oligonucleotide.

SNP	Reference code	Protein description	%ident	%cover	E-value
SNP_165_EST	LG12:9448943-9449064	Alpha cardiac-like isoform 1	93.44	100	2e-41
SNP_199_EST	LG17:12724116-12724212	Alpha cardiac-like isoform 1	94.85	80.17	6e-36
SNP_223_EST	LG10:1491505-1491623	Amylo-6-4-alpha-glucanotransferase isoform 1	84.03	98.35	3e-16
SNP_316_EST	LG20:4801689-4801801	Betaine-homocysteine s-methyltransferase 1-like	89.38	93.39	8e-32
SNP_309_EST	UN:33920969-33921082	Calumenin isoform x2	85.09	94.21	2e-17
SNP_210_EST	LG17:8208563-8208666	Cation transport regulator-like protein 1	95.19	85.95	4e-40
SNP_381_EST	LG2:5701470-5701587	Cofilin-2	95.76	97.52	2e-48
SNP_026_EST	UN:47791637-47791753	Collagen alpha-1 chain	87.18	96.69	5e-24
SNP_356_EST	LG12:21422927-21423050	Complement factor d-like	89.52	100	8e-32
SNP_334_EST	LG7:27011773-27011876	Cyclic amp-dependent transcription factor atf-4-like	93.33	86.78	1e-33
SNP_147_EST	LG9:21157592-21157711	Elongation factor 1-alpha	97.5	99.17	2e-54
SNP_283_EST	LG10:10016955-10017062	Elongation factor 2	93.52	89.26	9e-38
SNP_027_EST	LG8:11023666-11023786	Eukaryotic translation initiation factor 3 subunit g	93.39	100	2e-44
SNP_018_EST	LG20:18424838-18424949	Fructose-bisphosphatase isozyme 2-like	89.29	92.56	3e-28
SNP_185_GBS	LG6:16013746-16014145	Gamma-tubulin complex component 6	90	100	1e-132
SNP_226_EST	LG8:21804879-21804986	Heat shock protein 25 variant 1	90.74	89.26	1e-30
SNP_059_EST	LG14:17296613-17296730	High mobility group-t	92.37	97.52	6e-39
SNP_111_EST	LG1A:12106316-12106433	Homeobox protein tgif2	83.33	99.17	1e-12
SNP_014_EST	LG6:5722288-5722410	Lactate dehydrogenase-a	92.68	100	4e-40
SNP_052_EST	LG1B:11884835-11884939	Lim domain-binding protein 3-like isoform 2	89.52	86.78	2e-26
SNP_109_EST	LG10:16715626-16715744	M-striated muscle-like isoform 1	91.6	98.35	4e-37
SNP_319_EST	LG13:16731815-16731935	Myeloid leukemia factor 1-like	84.3	100	7e-17
SNP_087_EST	LG22-25:12829639-12829736	Myosin light polypeptide 6	90.82	80.99	3e-31
SNP_305_EST	LG1A:12105077-12105192	Myosin regulatory light chain smooth muscle isoform	93.97	95.87	1e-43
SNP_280_EST	LG1B:12032167-12032287	Myozenin 1	96.69	100	1e-52
SNP_300_EST	LG1B:12030692-12030790	Myozenin 1	91.92	81.82	5e-30
SNP_195_EST	LG4:6467652-6467749	Phosphoglucomutase 1	95.92	80.99	6e-39
SNP_230_EST	LG4:6461135-6461249	Phosphoglucomutase 1	86.09	94.21	4e-18
SNP_307_EST	LG4:6464452-6464568	Phosphoglucomutase 1	90.6	96.69	1e-33
SNP_081_EST	LG13:15806738-15806834	Phosphorylase kinase gamma subunit 1	87.63	80.17	3e-19
SNP_251_EST	LG13:15808866-15808980	Phosphorylase kinase gamma subunit 1	87.83	95.04	5e-27
SNP_163_EST	LG2:14697136-14697256	Platelet-derived growth factor receptor-like	91.74	100	6e-39
SNP_321_EST	LG2:14696671-14696785	Platelet-derived growth factor receptor-like	84.35	95.04	1e-15
SNP_259_EST	LG7:10231075-10231184	Polycystin-1 isoform x2	81.51	98.35	6e-11

SNP_084_EST	LG8:13049346-13049453	Polymerase i and transcript release factor-like	84.26	89.26	7e-14
SNP_329_EST	LG16:4979463-4979579	Proline-rich nuclear receptor coactivator 2	88.24	98.35	8e-26
SNP_263_GBS	LG24:2370971-2371370	Protein FAM171b-like	90	100	1e-132
SNP_263_EST	LG11:15886279-15886382	Protein kinase c inhibitor aswz variant 5	88.46	85.95	5e-24
SNP_297_EST	LG11:22112661-22112778	Reticulon-4-like isoform 1	94.12	98.35	6e-42
SNP_327_EST	UN:30827233-30827353	Ribosomal protein s14	98.35	100	2e-57
SNP_228_EST	LG1A:15592644-15592747	RUVb-like 1-like	85.58	85.95	3e-16
SNP_021_EST	LG17:12874139-12874248	Ryanodine receptor 3-like	86.73	93.39	2e-20
SNP_345_EST	LG14:23358400-23358517	Sarcoglycan delta	90.83	97.52	8e-29
SNP_070_EST	LG14:26383715-26383831	Smoothelin-like protein 2-like	89.74	96.69	3e-31
SNP_143_EST	LG6:9865641-9865761	Synemin isoform x1	85.95	100	1e-21
SNP_292_EST	LG18-21:6432507-6432628	T-complex protein 1 subunit epsilon	91.8	100	4e-37
SNP_125_EST	LG9:3159022-3159137	Thioredoxin-interacting protein	89.66	95.87	1e-30
SNP_253_EST	LG17:12286073-12286189	Trifunctional enzyme subunit mitochondrial	92.31	96.69	6e-39
SNP_119_EST	LG9:13694258-13694378	Vacuolar protein sorting-associated protein 28 homolog	91.74	100	1e-39
SNP_160_EST	LG18-21:11024585-11024705	Very-long-chain -3-hydroxyacyl	90.08	97.52	2e-29
SNP_031_EST	LG15:12489701-12489817	Xin actin-binding repeat-containing protein 2-like	88.03	96.69	2e-26

Appendix 4: 12 SNPs that aligned with *Gadus morhua* genes.

SNP	**Reference code**	**Protein description**	**%ident**	**%cover**	**E-Value**
SNP_023_EST	ENSGMOP00000013477	Asparaginyl-tRNA synthetase	90	99.17	9e-16
SNP_100_EST	ENSGMOP00000016108	Calponin 1, basic, smooth muscle	91.67	89.26	3e-13
SNP_026_EST	ENSGMOP00000004553	Collagen, type VI, alpha	82.5	99.17	2e-15
SNP_356_EST	ENSGMOP00000005890	Complement factor D (adipsin)	82.5	96.69	3e-15
SNP_191_EST	ENSGMOP00000018264	COP9 constitutive photomorphogenic homolog subunit 5	100	84.3	2e-13
SNP_147_EST	ENSGMOP00000012846	Elongation factor 1 alpha	90	99.17	7e-16
SNP_283_EST	ENSGMOP00000012542	Eukaryotic translation elongation factor2	82.5	99.17	5e-14
SNP_027_EST	ENSGMOP00000003671	Eukaryotic translation initiation factor 3, subunit G	100	99.17	8e-17
SNP_018_EST	ENSGMOP00000015427	Fructose-1,6-bisphosphatase 2	89.19	91.74	3e-15
SNP_253_EST	ENSGMOP00000019175	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	95	99.17	2e-18
SNP_365_EST	ENSGMOP00000015062	Keratin 23 (histone deacetylase inducible	94.59	91.74	1e-13
SNP_276_GBS	ENSGMOP00000011833	Solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	87.22	99.75	1e-56

Appendix 5: 30 SNPs that aligned with teleost genes.

SNP	Reference code	Protein Description	%ident	%cover	E-value
SNP_023_EST	B5X340_SALSA	Asparaginyl-tRNA synthetase, cytoplasmic	92.5	99.17	5e-15
SNP_183_EST	V9HXV7_SPAAU	Betaine homocysteine methyltransferase isoform 3 OS	97.14	86.78	3e-12
SNP_316_EST	V9HZ12_SPAAU	Betaine homocysteine methyltransferase isoform 4	92.31	96.69	5e-16
SNP_100_EST	M4A2Z2_XIPMA	Calponin	88.89	89.26	4e-12
SNP_011_EST	B3IYI3_SOLSE	Calsequestrin	88.57	86.78	2e-11
SNP_210_EST	C1BJW9_OSMMO	Cation transport regulator-like protein 1	84.62	96.69	8e-13
SNP_026_EST	W5UTL9_ICTPU	Collagen alpha-1(VI) chain	85	99.17	2e-14
SNP_191_EST	C1BF76_ONCMY	COP9 signalosome complex subunit 5	100	84.3	5e-12
SNP_147_EST	A0A087XDA8_POEFO	Elongation factor 1-alpha	100	99.17	1e-16
SNP_283_EST	Q7ZVM3_DANRE	Eukaryotic translation elongation factor 2, like	95	99.17	1e-15
SNP_027_EST	G3PKM0_GASAC	Eukaryotic translation initiation factor 3 subunit G	100	99.17	2e-15
SNP_018_EST	B5DGL9_SALSA	Fructose-1,6-bisphosphatase	89.19	91.74	1e-13
SNP_263_EST	E3TGF9_ICTPU	Histidine triad nucleotide-binding protein	82.86	86.78	2e-09
SNP_253_EST	G1FKK0_EPIBR	Hydroxyacyl-Coenzyme A dehydrogenase	97.5	99.17	2e-17
SNP_220_EST	C3KJN1_ANOFI	Integral membrane protein 2B	95	99.17	1e-16
SNP_356_EST	Q2Z1R4_ORYLA	Kallikrein like protein	85	96.69	4e-15
SNP_109_EST	W5UM88_ICTPU	M-protein, striated muscle	82.05	96.69	2e-11
SNP_150_EST	B9V3X3_EPICO	Muscle-specific beta 1 integrin binding protein 2	85.71	86.78	2e-11
SNP_010_EST	E1CPX3_THUMA	Myoglobin	100	81.82	3e-11
SNP_123_EST	E6ZJ46_DICLA	Myomesin-1	82.5	99.17	1e-11
SNP_185_EST	E6ZJ46_DICLA	Myomesin-1	91.18	84.3	9e-12
SNP_091_EST	Q98TQ4_9TELE	Myosin heavy chain	87.18	96.69	6e-13
SNP_305_EST	B5DGS9_SALSA	Myosin regulatory light chain 2, smooth muscle isoform	95	99.17	1e-14
SNP_280_EST	E3TCH3_ICTFU	Myozenin-2	82.5	99.17	5e-12
SNP_307_EST	S4S3W7_SPAAU	Phosphoglucomutase 1	97.5	99.17	1e-17
SNP_379_EST	W0M2F7_SINCH	SET and MYND domain containing protein 1a	85	99.17	4e-15
SNP_125_EST	I7HH00_OPLFA	Thioredoxin-interacting protein	97.5	99.17	5e-16
SNP_155_EST	A0JMJ0_DANRE	Ttnl protein	83.33	89.26	4e-09
SNP_365_EST	C0LMQ3_SPAAU	Type I keratin-like protein (Fragment)	97.3	91.74	8e-13
SNP_119_EST	C1BLI6_OSMMO	Vacuolar protein sorting-associated protein 28 homolog	94.29	86.78	3e-11

Tuna Fishing by Salvador Dalí

Chapter 8: Summary

The loud Americans bubbled with enthusiasm, jostled each other about the boat and snapped selfies of pouting lips and flexing muscles while the stoic German tourists sat rigid and uncomfortable, surveying the sea around Favignana. Salvatore stood at the helm like an ancient statue carved from stone. He'd been making this trip out to the tuna traps every spring morning since he was a young fourteen year old. As they cleared the cliff wall marking the entrance to the harbour, rays of golden sunshine warmed his leathery skin and a cool morning breeze played with his tangled mop of silver hair. Twenty years ago, he and his friends would have been hard at work at this hour, singing songs of the hunt and heaving at the heavy nets that descended into the deep. Giant tunas, each the size of his turquoise Fiat Cinque Cento, would be desperately dashing about the enclosing network of ropes and nets, splashing the fishermen with vengeful blades of water. They would fill their boats with thrashing tuna and bathe in their blood. Times were different now and Salvatore was exhausted. As they neared the artificial nets that were forever closed and permanently fixed in a place where the hunt had never taken place, Salvatore looked overboard into the black eye of a large bluefin rolling into the wave of his wake before disappearing into the dark blue waters below. The boat's occupants hadn't noticed the mighty fish, they were all too busy taking pictures and searching for birds in the sky. They always were. Only he was haunted by the ghosts of the past.

Despite the great amount of economic and political resources investmented in BFT fisheries, there is still much to be understood regarding their biology, reproductive behaviour and population structuring. Uncertainties concerning these key elements facilitated one of the most notorious stock depletions in the late 20th century and can be found at all levels of fisheries research, beginning with taxonomic errors that have led to the misidentification of larvae. Clearly, this issue must be resolved before conservation and management efforts can be optimized. Recent advances in genetics has greatly decreased the cost and improved the capabilities of molecular tools to identify unknown specimens. As such, both morphology-based taxonomists and conservation geneticists would benefit from closer collaboration. Efforts to resolve the genetic population structure of the species have been met with both success and increased uncertainty. BFT are a particularly difficult species to study in terms of population genetics, given that they are extremely fecund, long-lived, highly migratory, and have overlapping cohorts, high genetic diversity and effective population sizes.

The goal of this PhD project was to provide soultions to some of these issues and has managed to do so. Although not every task accomplished during the research period was included in the original study plan, the entirety of the research forms a cohesive and comprehensive work. Each chapter of this dissertation builds upon the lessons of the previous chapter, following a logical path while answering questions of increasing complexity. This work begins with a task as fundamental as the proper identification of BFT larvae and concludes with the design of a tool for the analysis of evolutionary change. Topics such as fisheries, commerce, spatial dynamics, life history strategies and adaptation are given a modern and historical context. This dissertation also provides the most comprehensive BFT population structure analysis to date, in geographic, temporal and genomic scope. An analysis of the composition of feeding aggregations using genetic characteristics has also been provided for the first time. The milestones reached during the study period are detailed in Table 1, which highlights both the conclusions and actions taken to resolve each challenge.

Single nucleotide polymorphisms (SNPs) have been demonstrating a great deal of promise for fisheries science in recent years. Since other molecular markers (mtDNA control region and microsatellites) have provided mixed results concerning the population structure of BFT, it was expected that the increased resolution of SNPs and genome coverage provided by the Genotyping-by-Sequencing technique could

provide some clarity to the issue. As such, a high throughout SNP genotyping panel has been developed and validated. Overall FST values are comparable with those provided through the analysis of other genetic markers. Substantial genetic differences between cohorts were revealed, thereby highlighting the need for temporal replicates in genetic structuring investigations.

Table 1: Milestones reached during the PhD study period.

CONCLUSIONS	ACTIONS TAKEN
Scombrid larval taxonomy is plagued with confusion and requires modernization.	A review of long trusted taxonomic keys has revealed sources of errors.
Taxonomic confusion has resulted in the misidentification of Atlantic bluefin tuna larvae in the Mediterranean.	A molecular identification key based on characteristic attributes was developed for the identification of scombrids in the Mediterranean.
The population structure of Atlantic bluefin tuna remains uncertain.	A high throughput SNP panel for population assignment was developed.
Combined with an inappropriate restriction enzyme, the Genotyping-by-Sequencing pipeline performed poorly when analyzing BFT samples.	A new work pipeline based on allele read frequencies was developed.
Temporal variation in SNP allele frequencies within sampling locations was detected.	Multiple years of samples were analyzed in an effort to eliminate inter-annual variation.
Young Atlantic bluefin tuna from the Gulf of Mexico and the Mediterranean can be distinguished from one another.	
Composition analysis of adult feeding aggregations shows annual variation in recruitment from the two spawning areas.	
Ancient bones retrieved from archaeological excavations contain DNA in low concentrations.	A new protocol for the extraction of DNA from ancient samples was developed.
DNA extracted from 2000 year-old bones can be analyzed using advanced genetic techniques and could provide a genetic baseline from thepre-industrialized era.	A genotyping panel containing SNPs from two separate projects (transcriptomics and genotyping-by-sequencing) was designed for evolutionary analysis and population structuring studies.

The data provided by the techniques used for SNP discovery were unsatisfactory at first, until a solution was developed, using pooled allele read counts. The SNP panel should continue to be used for the

genotyping of samples for several years in order to accurately characterize the inter-annual variation and migration rates between areas. The newly developed SNP discovery work pipeline was dependent on the genome reference developed during this period. Efforts to construct and annotate a reference genome for BFT should continue, as it will provide a road map for all future Next Generation Sequencing studies. The approach developed herein can be applied to similar studies focusing on other tuna species.

The changing composition of adult feeding aggregations detected during this research should be taken into consideration for stock assessment purposes and for the allocation of quotas. A ton of BFT captured in Portugal is quite different from one caught in Iceland. Unfortunately, the existing ICCAT management plan doesn't recognize that BFT cross the 45th meridian and as such a large proportion of BFT hailing from the Gulf of Mexico are harvested in European waters. Moreover, the biomass of BFT caught on the east coast of North America can no longer be considered indicative of the health of the western population, since many of these fish are migrants from the Mediterranean. The results from this thesis suggest that the composition of these feeding aggregations should be assessed using genetic tools, in order to establish a more accurate estimate of the recruitment and mortality rates of both populations.

Historical collections of bones preserved in favourable conditions have allowed for an expansion of temporal genetic analyses. A new protocol for ancient DNA extractions has been described as well as an in depth characterization of extracted DNA from several collections. A new panel of SNPs, taking elements from two SNP-discovery approaches has been developed for analysis of these samples has been designed and the results of this analysis should be available shortly. Unfortunately, because the research conducted within the PhD study period requires a great deal of time, the results from this last analysis won't be available for publication for several months.

Currently, two articles have been published from this work and two more are currently in preparation. Due to the continuous nature of the research projects discussed herein, numerous additional manuscripts are likely to be written in the coming year before all results are properly communicated to the public.

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