DATA RECOVERY PLAN (GBYP PROJECT 04/2015)
OF THE ATLANTIC-WIDE RESEARCH PROGRAMME ON BLUEFIN TUNA
(ICCAT GBYP – Phase 5)

"HISTORICAL GENETIC SAMPLES COLLECTED IN OLD TIMES IN THE EASTERN MEDITERRANEAN SEA, IN THE MARMARA SEA OR IN THE BLACK SEA, INCLUDING THE GENETIC ANALYSES OF THESE SAMPLES."

FINAL REPORT

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SUMMARY

During ICCAT GBYP Phase 4 and 5 Data Recovery, novel molecular techniques were developed, and DNA has been extracted from Atlantic bluefin tuna (BFT) vertebrae excavated from late Iron Age and ancient Roman settlements in coastal Iberia (Portugal and Spain, 4th-2nd century BC; n=65) and Byzantine-era Istanbul (4th-15th century AD; n=60), as well as vertebrae from the Massimo Sella archive located at the University of Bologna (Ionian, Tyrrhenian and Adriatic Seas, early 20th century; n=145). 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. Comparisons have been made between the amount of BFT DNA contained in each sample (measured via quantitative polymerase chain reactions), their age and environmental conditions. A high performance genotyping panel containing SNPs derived from two separate projects funded by the ICCAT GBYP scientific programme has been designed for the purpose of genotyping historical and modern samples collected from the same geographic areas. SNPs selected for the panel provide significant discrimination between modern populations and/or align with a variety of genes associated with the musculoskeletal system, development, metabolism, cellular function, osmoregulation and immune response. Most historical samples were successfully genotyped; however, the samples from Roman-era Iberia performed poorly. The results revealed a degree of differentiation between modern and historical samples as well as an overall and significant divergence of modern samples from the Western Atlantic and samples from the Eastern Atlantic and Mediterranean. Within the Mediterranean, some pairwise comparisons involving samples from the Adriatic and Levantine Seas were significant. Several loci, distinguished by high F_{ST} values when comparing modern and historical samples, are described in detail.

Sampling

Istanbul, Turkey

Archaeological excavations conducted between 2004 and 2013 in the Yenikapi neighbourhood of Istanbul revealed the location of a Byzantine era harbour. Built by Emperor Theodosius in the 4th century CE, it was one of the largest and most important ports in the Roman Empire for nearly eight centuries. 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*. All vertebrae are very well preserved and suitable for both morphological and molecular investigations. Butchery marks 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 mostly traded abroad. The rapid inundation of the harbour with fine silts has preserved all vertebrae in excellent condition. Growth rings can be easily counted and growth rates estimated with ease. To date, this magnificent collection of BFT bones at the University of Istanbul hasn't been utilized for any research purposes. The historical context of this collection and its scientific significance is discussed in further detail by Puncher *et al*. (2014).

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; Di Natale, 2015).

It has been suggested that a group of ancient BFT, which was distributed between the Black Sea and the eastern Mediterranean Sea (in both the northern Aegean Sea and the Levantine Sea), may have been spawning in isolation. The migratory movements of this "clan" was likely determined by the whereabouts of their prey which migrated into the warming waters of the Black Sea in the late spring, returning to the warmer waters of the Mediterranean Sea in autumn. Aristotle recorded their 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). According to these authors, this would place the local group in the Black Sea during the spawning season, far from all other spawning groups. More recent knowledge of the anticipated spawning season in the eastern Mediterranean, along with detailed information from the trap fishing

operations in the Marmara Sea, suggest that the interpretation of the bluefin tuna movements was at least partly misinterpreted and that spawning was possibly taking place in the Levantine Sea even in these ancient times (Di Natale, 2015). Regardless of the location of their spawning area, both ancient and modern works suggest that at least a part of this group remained in the eastern Mediterranean region throughout entire year, which distinguishes them from the migratory population that spends the majority of the year feeding in the Atlantic Ocean.

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 had 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 100 m (MacKenzie and Mariani, 2012). Moreover, additional adaptations would have been required of the sperm, unfertilized eggs and developing embryo and larvae. If this was the case, the genetic programming for these adaptations could be recorded in the bones uncovered at Yenikapi and might still be traced to the remaining descendants of this potentially isolated group, if any have survived. Information concerning the distribution of the remaining Black Sea BFT can provide information about population structuring, migratory behaviour, population ecology, genetic diversity and adaptive potential.

Bone powder was collected from a total of 65 samples archived at the University of Istanbul. High quantities of DNA have been extracted from all samples, while additional powder has been archived at the University of Bologna at -80C for future analysis if required. An official sub-contract was successfully established between the University of Istanbul and the University of Bologna in order to allow access to these samples.

Iberian Peninsula

The Faculty of Archaeozoology at the Autonomous University of Madrid has provided 20 genetic samples from vertebrae of medium sized adults (150-200 kg) captured by colonists of the Late Iron Age and Republican Rome (IV century BCE – I century CE) 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.

After learning of our research activities through the SCRS, Prof. Dario Bernal-Casasola of the Department of History, Geography and Philosophy at the University of Cádiz invited Dr. Gregory Puncher to assist with an archaeological excavation at Baelo Claudia during July 2015 (**Figure 1**). At this time much information was gathered concerning the ancient methods used for the preparation of salted tuna and fish sauces or *garum*. An additional 44 samples were collected from various areas and strata within the excavation area.

Figure 1: Excavation area at Baelo Claudia and location of ancient salting and fermentation vats.

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 the Italian professor Massimo Sella. Originally housed at the Istituto Italo-Germanico di Biologia Marina/Deutsch-Italienisches Institut für Meersbiologie, Rovigno, Italy (now Center for Marine Research of the Ruđer Bošković Institute, 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) from both markets and fisheries in Istria (Croatia, Adriatic Sea), Messina, Pizzo and Gaeta (Italy, Tyrrhenian Sea) and Zliten (Libya, Central Mediterranean) and likely caught by tuna traps in the area; however, a few vertebrae specimens from Bosporus Strait and non-Mediterranean areas are also included.

SPECIES IDENTIFICATION

All samples were identified as bluefin tuna, based on morphological features (size of vertebrae or span of caudal fin) or DNA mini-barcodes. The protocols used for DNA barcoding and quantification of hDNA by way of quantitative PCRs are described in Puncher *et al*. (2015).

Figure 2: Two caudal fins from which DNA samples were taken, shown *in situ* at Baelo Claudia.

SNP GENOTYPING

A 96 SNP genotyping panel was developed in order to analyze changes in allele frequencies of polymorphic loci that have been used by other ICCAT GBYP research efforts to investigate population structuring in modern populations as well as SNPs associated with genes that may hold selective or evolutionary significance. The SNPs contained in the panel were developed using 2 separate approaches: 1) Genotyping-by-Sequencing SNP development by the Biological and Genetic Sampling and Analysis Consortium (BGSA) during ICCAT GBYP Phase 2 and 4) Transcriptomic SNP development by the BGSA Consortium during Phase 2. Twenty SNPs were selected for their capacity to discriminate among modern populations. The remaining 76 SNPs were selected due to the high similarity between the sequences contained in their flanking regions and genes associated with adaptive traits: musculoskeletal system, development, metabolism, cellular function, osmoregulation and immune response.

The ICCAT GBYP Phase 5 contract states that “The genetic data of these historical samples

must be fully comparable with the existing genetic data of contemporary BFT populations already included in the ICCAT GBYP database and obtained with the programs GBYP - Phase 3 and Phase 4, for maximizing data exploitability and cost reduction." As such, high quality DNA has been extracted from 291 modern individuals and 270 historical/ancient individuals, all of which were genotyped and compared to ensure balanced comparisons across all time periods and sampling locations (**Appendix 1**). Coding conventions used in previous ICCAT GBYP projects were maintained (**Table 1**).

Two negative controls accompanied each set of samples during DNA extractions and genotyping. While genotyping, two "BLANK" wells containing purified water, instead of DNA, were used for each 96-well plate. Three previously genotyped modern individuals were re-genotyped to ensure consistency of results (CMAS11J01, CMAS11J02, CMAS11J03). Similarly, DNA was extracted twice from four historical individuals and fully genotyped (HIST4AD01, HIST4AD03, HIST4AD05, HIST4AD07, HCM192641, HTAV2BC05, HTAV2BC09, HTYR191143, HTYR191105 [x3], HTYR1911-39 [x3]). Genotypes among the modern replicates were identical, while the similarity between historical replicates was acceptable at 97.8 ± 3.6%. Several historical samples needed to be genotyped twice in order to provide sufficient data for analysis (HBCLIA05, HBCLIA06, HIST4AD57, HTAV2BC04, HTAV2BC10, HTYR191131, HTYR191135, HTYR191145).

Table 1: Codes used for samples used throughout the report.

Sampling Location	Historical or Modern	Area code	Age class	Year	Sample code
Central Mediterranean - Adriatic Sea	Modern	CMAS	Juvenile	2011	CMAS11J
Central Mediterranean - Southern Sicily	Modern	CMSI	YOY	2012	CMSI120
East Atlantic - Bay of Biscay	Modern	EABB	Juvenile	2011	EABB11J
East Atlantic - Strait of Gibraltar	Modern	EAGI	Large Adults	2011	EAGI11L
East Mediterranean - Levantine Sea	Modern	EMLS	YOY	2011	EMLS110
West Atlantic - Gulf of Mexico/Cape Hatteras	Modern	WAGM	Larvae/YOY	2008-09	WAGM080-09V
West Mediterranean - Balearic Islands	Modern	WMBA	YOY	2011	WMBA110
West Mediterranean - Tyrrheniann Sea	Modern	WMTY	YOY	2012	WMTY120
Central Mediterranean - Istria, Croatia	Historical	HADR	Adult	1927	HADR1927
East Atlantic - Baelo Claudia, Spain	Historical	HBC	Adult	II BCE	HBC2BC
East Atlantic - Baelo Claudia, Spain	Historical	HBC	Adult	V CE	HBC5AD
East Atlantic - Baelo Claudia, Spain	Historical	HBC	Adult	IV-II BCE	HBCLIA
Central Mediterranean - Zliten, Libya	Historical	HCM	Adult	1926	HCM1926
East Mediterranean - Istanbul, Turkey	Historical	HIST	Adult	1911	HIST1911
East Mediterranean - Istanbul, Turkey	Historical	HIST	Adult	IV-XV CE	HIST4AD
East Atlantic - Cadiz, Spain	Historical	HPJ	Adult	IV-II BCE	HPJLIA
East Atlantic - Tavira, Portugal	Historical	HTAV	Adult	II BCE	HTAV2BC
West Mediterranean - Tyrrhenian Sea (Pizzo, Messina, Gaeta)	Historical	HTYR	Adult	1911	HTYR1911

Most modern (289/291) and a majority of the historical (159/270) samples were successfully genotyped with a great deal of success (**Appendix 2**). A total of 16 individuals were removed from the following samples in the dataset, due to a failure to produce genotypes for any loci: HIST4AD (n=1), HBC2BC (n=11), HTYR1911 (n=1), HPJLIA (n=1), HADR1927 (n=2). Unfortunately, 28/48 historical individual samples from the Adriatic Sea and all results from the

Late Iron Age and Early Roman Republic (from Baelo Claudia) had to be removed from population structuring analyses because of low genotyping success (>30% of all SNPs not genotyped). The overall low genotyping success rate from samples collected from Baleo Claudia may be the results of low concentrations of DNA contained in those samples could be an indication of the presence of some inhibiting agents contained in the environment there. Three SNPs failed to be genotyped among the historical samples, despite successful characterization among all modern samples (**Table 2**). This could be an indication of variable sites contained in the flanking regions of these locations within the genome of historical samples that have since been eliminated.

Table 2: Genotyping success rates of three SNPs that failed to be genotyped among many (SNP_46), if not all historical samples, in addition to the modern sample collected in the Tyrrhenian Sea in 2012 (WMTY120; SNP_45 and SNP_76).

	SNP 45	**SNP 46**	**SNP 79**
CMAS11J	38/40	40/40	40/40
CMSI120	22/38	37/38	25/38
EABB11J	33/40	40/40	40/40
EAGI11L	38/40	40/40	40/40
EMLS110	29/29	29/29	29/29
WAGM080-09V	24/24	22/24	24/24
WMBA110	39/40	39/40	39/40
WMTY120	0/40	40/40	0/40
HADR1927	0/47	8/47	0/47
HBC2BC	0/33	0/33	0/33
HBC5AD	0/4	0/4	0/4
HBCLIA	0/3	0/3	0/3
HCM1926	0/46	9/46	0/46
HIST1911	0/2	0/2	0/2
HIST4AD	0/59	3/59	0/59
HPJLIA	0/3	0/3	0/3
HTAV2BC	0/10	0/10	0/10
HTYR1911	0/47	9/47	0/47
Overall (%)	**60.2**	**43.9**	**57.8**

ANALYSIS OF ALLELE FREQUENCY

HWE analysis was conducted for each locus and population using Arlequin v. 3.5.2.2 (Excoffier and Lischer, 2010). No loci were outside of the HWE; however, the following loci were monomorphic among one or more sets of samples: SNP_59 (EAGI11bL, EMLS110, WMBA110, HADR1927, HCM1926, HTYR1911), SNP_18 (HADR1927, HIST4AD), SNP75 (EMLS110, HADR1927), SNP_81 (EAGI11bL).

An analysis of the frequency of heterozygotes at 92 loci (SNP_44 also removed from population structure analysis because of low genotyping success among HIST4AD individuals) among 441 individuals from 12 populations was performed using a Hardy Weinberg test for He deficiency in Genepop version 4.2 (Raymond and Rousset, 1995; Rousset, 2008). Only 4 historical datasets (HIST4AD, HTY1911, HADR1927, HCM1926) with adequate sample sizes were considered for HWE calculations. Historical samples demonstrated significantly depressed heterozygosity at two loci (SNP_10 and SNP_76; **Table 3**).

Table 3: F_{IS} and p-value estimates for heterozygote deficiency at two SNP loci genotyped among 12 modern and historical sample sets.

	SNP_10		**SNP_76**	
Samples	**P-val**	**F_{IS}**	**P-val**	**F_{IS}**
CMAS11J40	0.1759 ± 0.0033	0.2008	0.9860 ±0.0008	-0.2881
CMSI12042	0.2855 ±0.0039	0.1542	0.5854 ±0.0033	0.0263
EABB11J40	0.0924 ±0.0027	0.2576	0.0480 ±0.0018	0.3158
EAGI11bL40	0.4717 ±0.0040	0.0591	0.0007 ±0.0002	0.6824
EMLS11029	0.5406 ±0.0042	0.0508	0.4753 ±0.0038	0.0808
WAGM09V08	0.0865 ±0.0017	0.3913	0.6291 ±0.0027	0.0143
WMBA11050	0.4878 ±0.0046	0.0591	0.2772 ±0.0042	0.1494
WMTY12b090	0.7135 ±0.0041	-0.0380	0.6579 ±0.0040	-0.0151
HADR192749	0.0211 ±0.0010	0.5238	0.4077 ±0.0023	0.2169
HCM192650	0.0053 ±0.0004	0.4260	0.0022 ±0.0003	0.5506
HIST4AD67	0.0022 ±0.0004	0.4623	0.0016 ±0.0002	0.5835
HTYR191151	0.0892 ±0.0027	0.2755	0.0061 ±0.0005	0.4880

A similar analysis using a dataset containing all historical and modern samples, pooled into two separate groups, revealed three loci (SNP_12, SNP_18_SNP41) with much lower heterozygosity than expected for the historical pool (**Table 4**).

Table 4: Allele frequencies and heterozygosity (expected and observed) for three SNP loci from modern and historical samples (number of individuals in parentheses).

	SNP_12		**SNP_18**		**SNP_41**	
	Modern (n=289)	**Historical (n=141)**	**Modern (n=284)**	**Historical (n=122)**	**Modern (n=287)**	**Historical (n=152)**
f reference allele	0.5640	0.7057	0.1585	0.0205	0.6132	0.2763
f alternative allele	0.4360	0.2943	0.8415	0.9795	0.3868	0.7237
Expected heterozygosity	0.4918	0.4154	0.2667	0.0401	0.4744	0.3999
Observed heterozygosity	0.4913	0.3050	0.2465	0.0246	0.4042	0.4079

Using a separate software (Lositan; Antao et *al*., 2008) that detects outlier loci based on both F_{ST} values and heterozygosity differences, various combinations of sample sets were probed for SNPs with exceptional patterns of allele frequency (**Table 5**). Two of the aforementioned loci (SNP_18, SNP_41) were highlighted multiple times during this process.

Table 5: Lositan analysis of outlier loci using various datasets.

Comparison	**F_{ST}**	**Outliers**
448 individuals, 93 SNPs, all samples considered separate	F_{ST} = 0.108	1, 5, 11, 17, 21, 26, 32, 34, 35, 36, 37, 41, 52, 59, 63, 66, 68, 72, 80, 81, 84, 96
441 individuals, 93 SNPs, datasets with large sample sets only, all samples considered separate	F_{ST} = 0.014	18, 25, 41, 43, 53, 54
441 individuals, 93 SNPs, large samples only, modern and historical samples grouped separately	F_{ST} = 0.004	18, 41

Due to these apparent differences between modern and historical samples, the nature of these polymorphisms and their potential as evidence of selective adaptation was studied in further detail. A nucleotide BLAST was performed for each of the three loci and their flanking regions, using the entire NCBI database, optimizing for somewhat similar sequences (**Table 6**). SNP flanking sequencing were then aligned with similar published sequences contained in the online database and translated into and amino acids. Changes in nucleotides that result in the expression of different amino acids are considered non-synonymous base substitutions (NSBS), which can potentially result in changes to protein conformation and function. Allele and genotype frequencies were plotted in bar graphs to illustrate temporal shifts at each locus (**Figures 3 - 5**). Only one locus (SNP_41) demonstrates characteristics of an NSBS (**Figure 6**).

Table 6: Details of outlier SNPs that aligned with known annotated genes contained in the NCBI

database.

	Non-synonymous base substitution?	Probable associated gene	Species sharing similar sequences in NCBI
SNP_12	N/A	N/A	N/A
SNP_18	No	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit (*HADHB*)	*Salmo salar*, *Epinephelus bruneus*, *Ictalurus punctatus*, *Danio rerio*, *Larimichthys crocea*
SNP_41	**Yes** Shift from histidine (positively charged side chain) to glutamine (polar uncharged side chain)over time.	Synemin, intermediate filament protein (*synm*)	*Larimichthys crocea* *Stegastes partitus* *Notothenia coriiceps* *Haplochromis burtoni* *Pundamilia nyererei* *Oreochromis niloticus* *Neolamprologus brichardi*

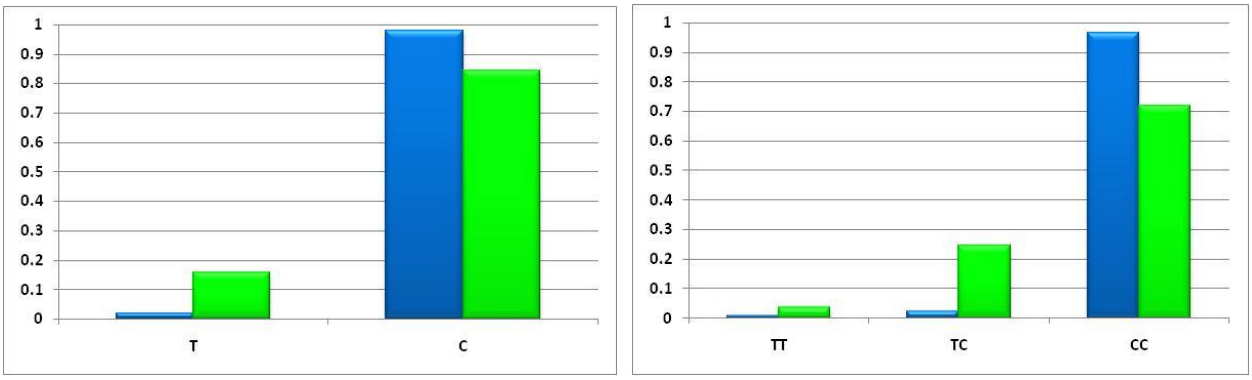

Figure 3: Allele frequency changes at the trifunctional protein subunit beta gene (SNP_18), an essential protein for the metabolism of long chain fatty acids. Blue and green bars represent historical and modern samples, respectively.

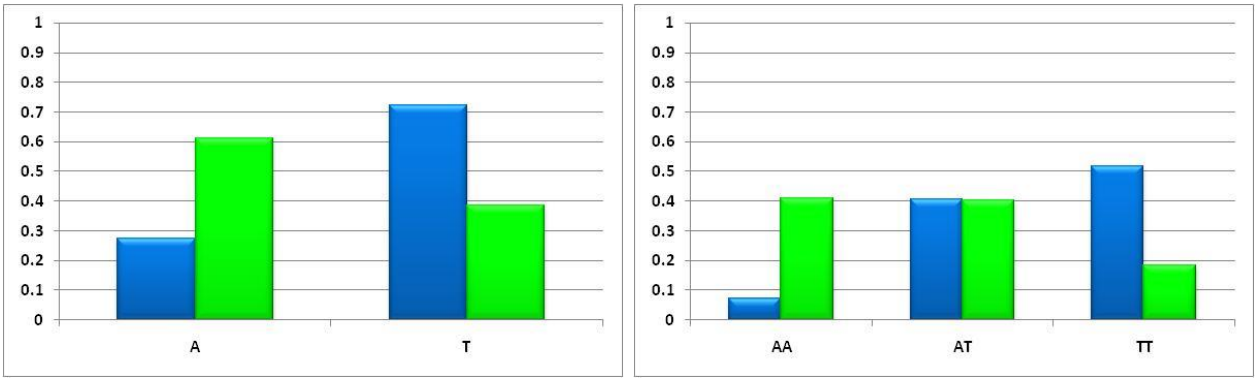

Figure 4: Changes in allele frequencies within the gene sequence coding for synemin (SNP_41), a cytoskeletal protein that provides structural integrity in musculature and resistance to mechanical stress. Blue and green bars represent historical and modern samples, respectively.

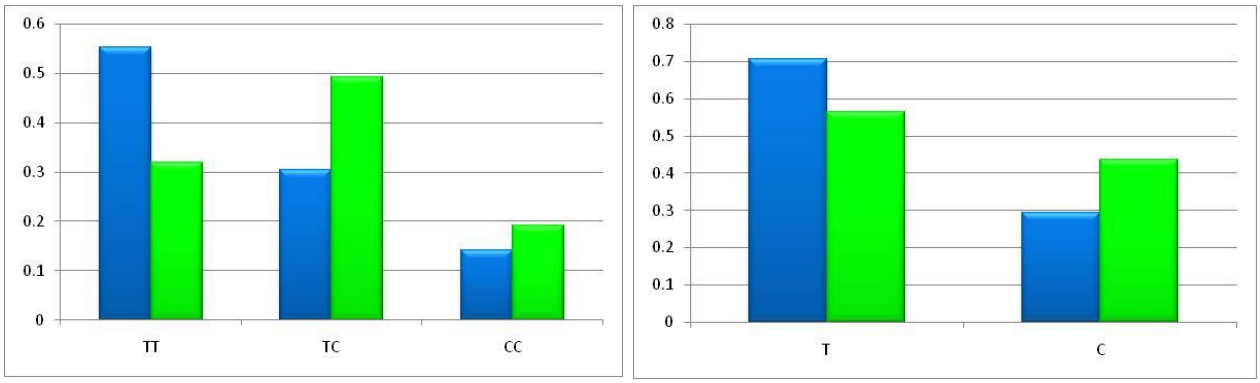

Figure 5: Changes in allele frequencies at SNP_12. Blue and green bars represent historical and modern samples, respectively.

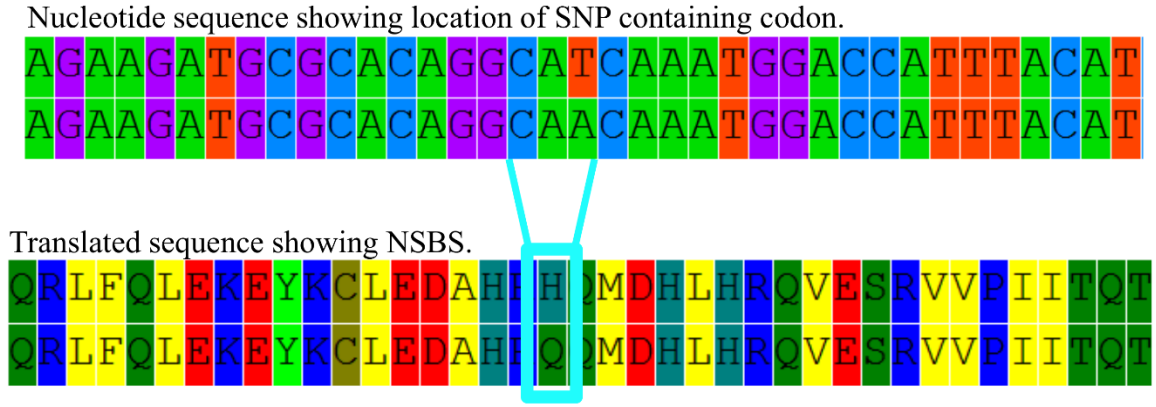

Figure 6: Alignments of synemin gene nucleotide sequence containing SNP and the translated polypeptide molecules containing different amino acids.

POPULATION STRUCTURING

The low number of individual samples acquired from the West Atlantic (n=24) presented a challenge for statistical interpretation, as small sample sizes decrease the power of analyses and consequently reduce the ability to detect significant population structure. Since the larvae and young-of-the-year collected from the Gulf of Mexico and Cape Hatteras were demonstrated to be genetically similar in previous analyses by the Biological and Genetic Sampling and Analysis Consortium, these samples were pooled into a single group for population structure analyses dependent on balanced samples sizes. However, caution should be used when interpreting these results and future ICCAT GBYP research efforts to compare samples from the eastern and western Atlantic should prioritize the collection of additional samples from the West.

The genetic differentiation of various groups of samples was estimated in Arlequin v.3.5.2.2, using several analyses of molecular variance (AMOVA) in order to examine multiple levels of variance in the dataset. Three geographical structuring scenarios were explored by assigning samples to various *a priori* arbitrary groups (**Table 7**). STRUCTURE analysis revealed several individuals that failed to cluster with any group of samples (data not shown), and as such the following 7 individuals were removed from AMOVA analyses: EAG11bL03, EAG11bL06, EAG11bL18, EAG11bL24, WMTY12b052, WMTY12b057, WMTY12b066. Due to low genotyping coverage at several loci (SNP_17, 18, 39, 64, 73, 77, 89) among certain samples, seven loci were not included in the AMOVA population distance computations.

Significant differences between historical and modern samples were detected (F_{ST} = 0.002, p-value = 0.049 ± 0.006), although the F_{ST} values differentiating the two is rather weak. A scenario of three temporally persistent populations in the Mediterranean (east, central and west) was not statistically supported at all (p-value = 0.843 ± 0.010). A final scenario in which modern samples from the Gulf of Mexico, Adriatic and Levantine Seas are differentiated from all other samples, separately, is supported by the data (F_{ST} = 0.015, p-value = 0.007 ± 0.002).

Table 7: Results from various AMOVAs using 85 SNP loci and three different population structuring scenarios.

AMOVA Groupings	% of variation	Fixation indices	p-value
AMOVA 1 – Historical vs. modern			
Among groups	0.23	FCT = 0.002	0.049 ± 0.006
Among samples within groups	0.49	FSC = 0.005	0.000 ± 0.000
Among individuals within samples	3.0	FIS = 0.030	0.004 ± 0.002
Within individuals	96.27	FIT = 0.037	0.002 ± 0.001
AMOVA 2 – West (WMBA110, WMTY12b0) vs. Central (CMAS11J, CMSI120, HADR1927, HCM1926) vs. East (EMLS110, HIST4AD, HTYR1911) Mediterranean			
Among groups	0.00	FCT = -0.002	0.843 ± 0.010
Among samples within groups	0.86	FSC = 0.009	0.000 ± 0.000
Among individuals within samples	2.89	FIS = 0.029	0.009 ± 0.003
Within individuals	96.41	FIT = 0.036	0.001 ± 0.001
AMOVA 3 – WAGM08-9V vs. CMAS11J vs. EMLS110 vs. all other samples (WMBA110, WMTY12b0, CMSI120, HADR1927, HCM1926, HIST4AD, HTYR1911)			
Among groups	1.49	FCT = 0.015	0.007 ± 0.002
Among samples within groups	0.24	FSC = 0.002	0.002 ± 0.001
Among individuals within samples	2.93	FIS = 0.030	0.003 ± 0.002
Within individuals	95.34	FIT = 0.047	0.000 ± 0.000

In order to investigate this structuring further, pairwise comparisons between all samples were executed using FSTAT v.2.9.4 (Goudet, 1995), using a dataset containing 92 SNPs as well as the 31 SNP sub-panel composed of loci that were found to be capable of distinguishing between modern populations of BFT (Phase 4, Biological and Genetic Sampling and Analysis). The resulting 92 SNP-based F_{ST} matrix (**Table 8**) shows significant differences between samples from the Western Atlantic (WAGM080-9V) and most other samples, except for the modern samples from Gibraltar (EAGI11L) and the historical samples from the Adriatic Sea (HADR1927) and Istanbul (HIST4AD). Interestingly, the modern sample from the Adriatic Sea shows the very same pattern of differentiation and similarity to the same three samples. Additional significant differences can be found between the historical samples from the Tyrrhenian (HTYR1911) and samples from the Balearic Islands (WMBA110) as well as the modern Tyrrhenian (WMTY120) and Levantine (EMLS110) Sea samples. The 31 SNP panel provided far fewer pairwise differences. Using this smaller panel, the samples from the Western Atlantic were determined to be significantly different from all other samples, except the historical sample from Istanbul

(HIST4AD). The modern samples from the Levantine Sea and Balearic Sea were also found to be significantly different (**Table 9**).

Principal Coordinate Analysis (PCoA), a multivariate technique, was then employed in order to highlight and plot the major patterns within the dataset. Using a covariance – standardized option and a distance matrix based on genetic distance in GenAlEx 6.5 (Orloci, 1978; Peakall and Smouse, 2006, 2012), it was estimated that the first two axes that define these patterns account for 59% of variation within the dataset. When plotted, the four large historical samples cluster separately from the modern samples, while the modern samples from the Western Atlantic (WAGM080-9V) are clearly differentiated from all others (**Figure 7**). Moreover, the historical sample from Istanbul (HIST4AD) also appears to be quite distinct from the other samples, while the modern Levantine Sea sample (EMLS110) is situated between a main cluster of modern Mediterranean samples and the distal modern Adriatic Sea sample (CMAS11J).

Similar results were provided by discriminate analysis of principal components (DAPC) performed in R using the Adegenet package (Jombart, 2008; Jombart and Ahmed, 2011). Again, the historical samples clustered together while the samples from the Western Atlantic and the Adriatic Sea diverged slightly from the main cluster containing all other samples (**Figure 8**).

Table 8: Pair-wise F_{ST} results using modern and historical Atlantic bluefin tuna samples (n=441) using 92 SNPs. Above the diagonal, statistical significance of results are indicated as significant (*) or non-significant (NS) after correction for multiple testing (adjusted nominal level of 0.05), while F_{ST} values are shown below the diagonal. Sample codes and details can be found in Table 1. Pairwise tests of differentiation were calculated using FSAT v.2.9.3.2 (Goudet, 1995).

	WAGM080-9V	EAGI11L	EABB11J	WMBA110	HTYR1911	WMTY120	HADR1927	CMAS11J	CMSI120	HCM1926	HIST4AD	EMLS110
WAGM080-9V		NS	*	*	*	*	NS	*	*	*	NS	*
EAGI11L	0.0281		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
EABB11J	0.0171	0.0015		NS	NS	NS	NS	*	NS	NS	NS	NS
WMBA110	0.0201	0.0025	0.0014		*	NS	NS	*	NS	NS	NS	NS
HTYR1911	0.0349	0.0132	0.0055	0.012		NS	NS	*	NS	NS	NS	NS
WMTY120	0.0305	0.007	0.0065	0.0062	0.0017		NS	*	NS	NS	NS	*
HADR1927	0.0341	0.0082	0.0044	0.0051	0.0024	0.0037		NS	NS	NS	NS	NS
CMAS11J	0.0383	0.019	0.0148	0.0113	0.0202	0.0182	0.0183		*	*	NS	*
CMSI120	0.0286	0.0062	0.005	0.0062	0.0046	0.002	0.0049	0.0168		NS	NS	NS
HCM1926	0.0305	0.0054	0.0051	0.0045	0.0001	0.0033	-0.0015	0.018	0.0004		NS	NS
HIST4AD	0.0265	0.0143	0.0063	0.0068	0.0028	0.0026	0.0034	0.0204	0.0043	0.002		NS
EMLS110	0.0325	0.0081	0.0031	0.0066	0.0132	0.015	0.0133	0.0142	0.0063	0.0114	0.0128	

Table 9: Pair-wise F_{ST} results using modern and historical Atlantic bluefin tuna samples (n=441) using 31 SNPs that have shown potential for discrimination of modern populations. Above the diagonal, statistical significance of results are indicated as significant (*) or non-significant (NS) after correction for multiple testing (adjusted nominal level of 0.05), while F_{ST} values are shown below the diagonal. Sample codes and details can be found in Table 1. Pairwise tests of differentiation were calculated using FSAT v.2.9.3.2 (Goudet, 1995).

	WAGM080-9V	EAGI11L	EABB11J	WMBA110	HTYR1911	WMTY120	HADR1927	CMAS11J	CMSI120	HCM1926	HIST4AD	EMLS110
WAGM080-9V		*	*	*	*	*	*	*	*	*	NS	*
EAGI11L	0.0361		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
EABB11J	0.0318	-0.0054		NS	NS	NS	NS	NS	NS	NS	NS	NS
WMBA110	0.03	-0.0055	-0.0015		NS	NS	NS	NS	NS	NS	NS	*
HTYR1911	0.0405	0.0036	-0.005	0.0054		NS	NS	NS	NS	NS	NS	NS
WMTY120	0.0303	-0.0061	-0.0004	-0.0022	0.01		NS	NS	NS	NS	NS	NS
HADR1927	0.0502	0.0031	0.0022	0.0025	0.0093	0.0069		NS	NS	NS	NS	NS
CMAS11J	0.0374	-0.0025	0.0019	0.0007	0.0055	0.0021	0.0101		NS	NS	NS	NS
CMSI120	0.0329	-0.0072	-0.0022	0.0006	0.0064	-0.0024	0.0098	-0.0029		NS	NS	NS
HCM1926	0.0367	-0.0019	-0.0023	-0.0062	0.0006	0.0067	0.0011	0.0042	0.0014		NS	NS
HIST4AD	0.0403	0.0032	-0.0015	-0.0005	0.0073	0.0055	0.0082	0.0038	0.002	-0.0012		NS
EMLS110	0.0664	0.011	0.0069	0.0163	0.0075	0.0163	0.0202	0.007	0.0104	0.0184	0.0146	

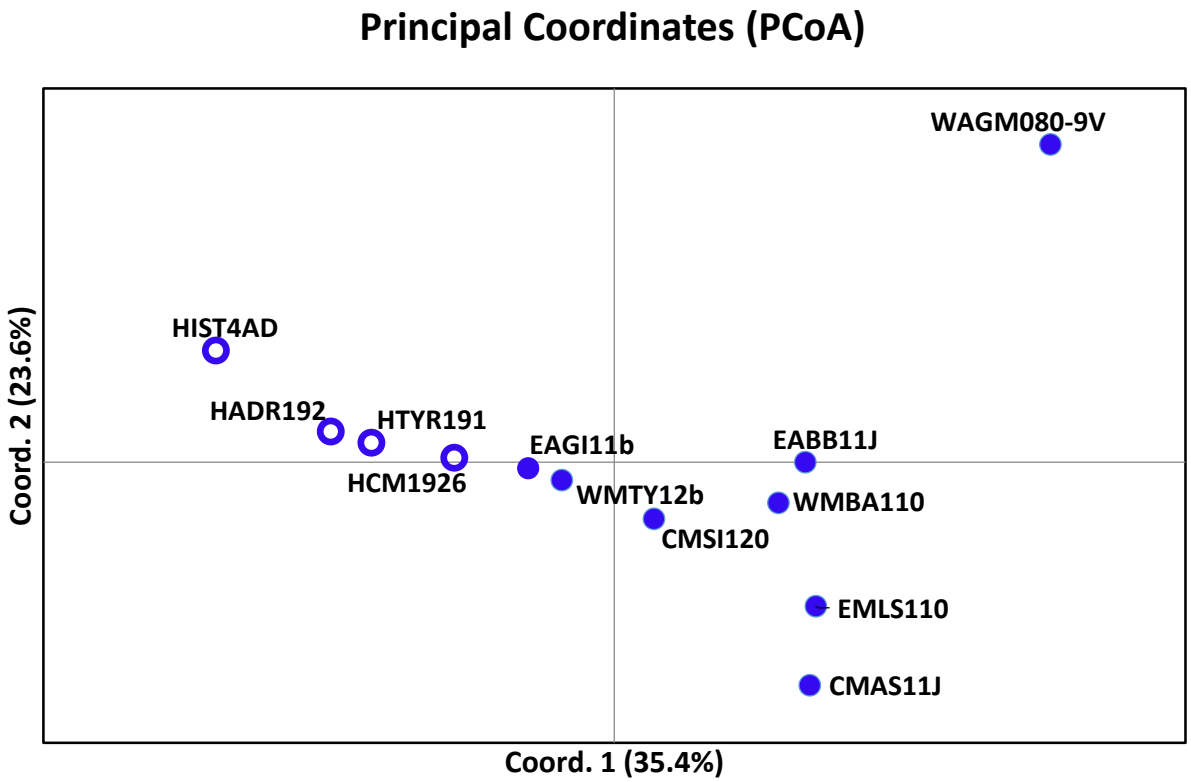

Figure 7: Principal coordinates analysis (PCoA) showing clustering of historical samples and isolation of samples from the Gulf of Mexico and the Levantine and Adriatic Seas. Sample codes and details can be found in Table 1.

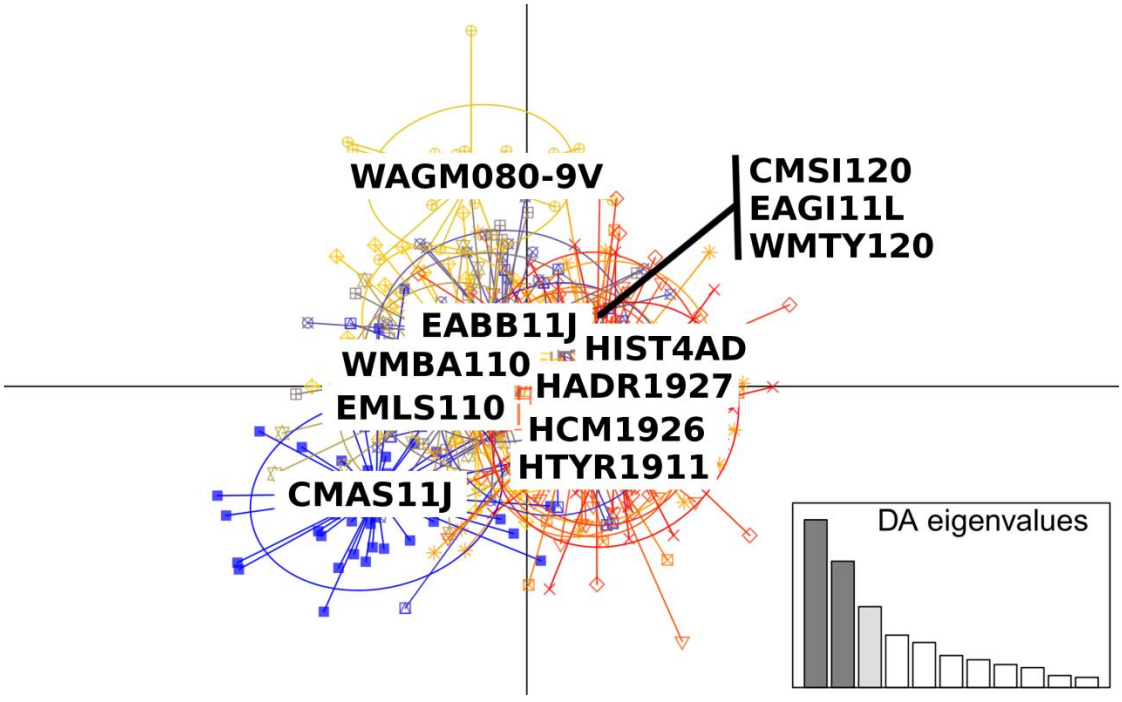

Figure 8: Discriminant analysis showing clustering of historical samples and isolation of samples from the Gulf of Mexico and the Levantine and Adriatic Seas. Sample codes and details can be found in Table 1.

DISCUSSION

Overall, the project aims have been achieved, with a very high success rate of genotyping among modern samples and an impressive number of ancient samples effectively genotyped. The failure of several loci to be genotyped by historical samples may be related to other changes in the genome that are not associated with the SNP loci themselves but with the sequence regions that flank the polymorphic sites. Additional sequencing of these regions may be warranted, as they seem to be diagnostic of both historical samples as well as individuals from the Tyrrhenian Sea.

When comparing modern and historical samples several loci showed significant differences in allele frequency between groups of samples. So far locus **SNP_41** has been identified as a non-synonymous base substitution (modification in amino acid sequence). Alignments with annotated genes have revealed that this polymorphic locus is located within the synemin, or desmuslin, gene sequence. Our results suggest that over the past 100 years a significant shift from histidine (amino acid), which has a positively charged side chain, to glutamine with a polar uncharged side chain has occurred. This change may affect the function of the target protein, which is an intermediate filament and cytoskeletal protein that confers resistance to mechanical stress and important structural support in muscles.

Additional analysis of the SNP associated with the HADHB gene may be warranted due to its potential as an adaptive trait. The HADHB gene codes for an enzyme complex, the mitochondrial trifunctional protein, which is composed of a total of 8 subunits. The enzymes are essential for the metabolism of long chain fatty acids and subsequent production of energy from said reaction. Fatty acids are stored in the adipose tissues of animals and are a major source of energy for the heart and muscles as well as the liver during periods of fasting. The interaction of estrogen receptors and the trifunctional protein is suspected of playing an important role in estrogen-mediated lipid metabolism (Zhou *et al*., 2012). A changing prey assemblage within the Mediterranean Sea, due to overfishing of some species and climate change, has fundamentally changed the marine community in the last century (Bănaru *et al.* 2013; Colloca *et al.* 2013; Tsikliras et al. 2013; Van Beveren *et al.* 2014; Corrales *et al.* 2015; Marbà *et al.* 2015). Such modifications to BFT trophic uptake could be capable of bringing about adaptive change.

Further effort will be dedicated to the exhaustive characterization of all SNP loci, with the collaboration of the BioComputing Group from the University of Bologna, which has outstanding expertise in bioinformatics with a special focus on protein modelling and prediction. Improved annotation is already ongoing for all 92 loci and all results will be explored to identify and assess the structural and functional changes associated to the different genotypes observed in modern and historical samples.

Comparisons between modern and historical samples revealed genetic divergence of the two groups over time. The 4^{th}-15^{th} century Istanbul sample is the most distal of the historical samples and is substantially different from the modern Levantine Sea sample, although the pairwise comparison between the two samples is not significant. As such, these results seem not to support the hypothesis that the BFT currently frequenting the Levantine Sea are direct descendants of an ancient Black Sea tribe.

Genetic differences were also found among modern samples collected in the Mediterranean Sea. The results using the complete set of 92 loci provide evidence of 5%-significant divergence of two modern samples (Adriatic Sea and Levantine Sea) from the main cluster of samples containing all other modern and historical samples, even if these significant pairwise Fst values (Adriatic Sea: 0.011-0.020; Levantine Sea: 0.014-0.015) are overlapping the range of Fst values observed in the comparisons among the other Mediterranean samples without any statistical significance (0.000-0.020). This pattern with patched and sporadic significant differences does not solidly support the existence of temporally persistent subpopulations within the Mediterranean.

Given the limited temporal window between modern and historical sampling events, factors other than passive genetic drift may be responsible for the observed level of genetic differentiation. A recent study using 8 microsatellite loci, for the analysis of population structuring in the same region, provided evidence that BFT within the Mediterranean are a meta-population with complex sub-population dynamics subject to partial reproductive isolation (Riccioni *et al.* 2010, 2013). Similar to our results, that study uncovered differentiation of samples collected in the Adriatic Sea from other Mediterranean sampling locations (Algeria, Liguria, Cyprus, Tyrrhenian, Alboran, Sardinia; Riccioni *et al.*, 2013). The authors also uncovered evidence of genetic structuring along two environment gradients associated with temperature (west-to-east) and salinity (north-to-south). Their results suggest that preference for some spawning habitat conditions may be contributing to genetic structuring in the Mediterranean Sea. Our results show additional structuring between samples collected in the Adriatic and Levantine Seas, providing further evidence of differentiation along a longitudinal gradient.

Long-term fluctuations in BFT catches occurred in the Mediterranean across the last centuries (Ravier and Fromentin 2001) and even if further and deeper analyses are needed to disentangle reliable patterns (Di Natale *et al.*, 2016), it appears that long-term BFT fluctuations are linked with long-term trends in temperature (Ravier and Fromentin 2004). Overall temperatures as well as a pattern of longitudinal temperature gradients within the Mediterranean have been increasing in magnitude at a rapid pace over the past century (Mariotti and Dell'Aquilla 2012). These changes are likely the result of the combined influence of the Atlantic Multidecadal Oscillation (AMO) index and global climate change (Mariotti and Dell'Aquilla 2012). Climate change has also caused a progressive salinisation of

intermediate and deep waters and increased stratification of the water column (Calvo *et al.* 2011). Overall, the impacts of climate change appear to be having a much greater impact in the eastern part of the Mediterranean than in the western part (Figure 9; Coll et al. 2012). Combined with changing oceanographic conditions, heterogeneous changes in prey availability, might also have led to changes in BFT migratory routes, feeding grounds and spawning locations (Fromentin 2009).

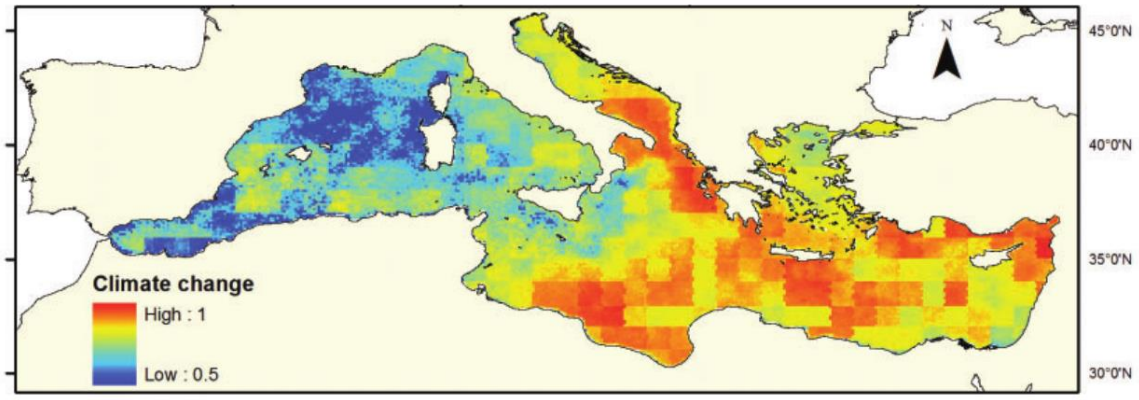

Figure 9: Map detailing the potential impacts of climate change (sea surface temperature anomalies, UV increase and ocean acidification) within the Mediterranean Sea (from Coll et al. 2012).

The observed increase in genetic heterogeneity of BFT within the Mediterranean may indicate the presence of additional groups that were not represented among the samples contained in the Massimo Sella archive. Similarly, the proportional representation of each of these groups may have changed over time. The historical samples could be dominated by one particularly abundant group that migrated between North Atlantic feeding grounds and the Mediterranean (Group 1 in Fromentin 2009). This group appears to have experienced a dramatic reduction in size during the 1950-1960s, due to environmental and fisheries factors in the North Sea and North West Atlantic (Ravier and Fromentin 2004; Fromentin 2009). Following this reduction, a more heterogeneous structure may have surfaced, in which the demographic contribution of different genetic groups is more evident.

As proposed by Ravier and Fromentin (2004), the reproductive strategy of BFT in the past may have been a mix of opportunistic and natal homing, wherein individuals were capable of spawning in multiple locations throughout their range, even while migrating from feeding areas in the Atlantic Ocean. This explains the presence of fertile adults in the Atlantic during the spawning season, outside of the most widely recognized and studied spawning areas (Lutcavage et al. 1999). This proclivity towards errant release of gametes would have encouraged a great deal of mixing among metapopulations within the two large spawning areas. As environmental conditions and prey biomass continues to change, the geographic range of suitable spawning habitats may change over time.

The lack of genetic differentiation among analyzed samples may be a result of the age class of the subjects studied. Juvenile and adult individuals are capable of moving between sampling locations and therefore cannot be assumed to have an origin in the location from which they were collected. This applies to several of the modern samples analyzed (EAGI11L, EABB11J, CMAS11J) as well as the large vertebrae collected from tuna traps situated along migratory routes. Additional larvae and young-of-the-year samples, particularly from the West Atlantic, are necessary for further analysis.

The DNA extracted from the historical samples has shown a great deal of promise and should continue to be studied. Ample powder was collected from each bone for several more extractions. Bones, extracted DNA and bone powder remain archived at the University of Bologna in ideal conditions for long term storage.

COMMUNICATION OF RESULTS

Specific aspects of the above described research (sampling, aDNA extractions, barcode identification of ancient remains) have been presented as oral presentations and posters at several international conferences in Italy, Spain, Canada, Portugal and USA. At each of these events ICCAT was thoroughly acknowledged for the funds provided for the research.

Presentations

Puncher, G.N., Cariani, A., Cilli, E., Massari, F., Martelli, P.L., Morales, A., Onar, V., Toker, N.Y., Moens, T., Arrizabalaga, H., Tinti, F. (2015) Unlocking the evolutionary history of the mighty bluefin tuna, *Thunnus thynnus*, using novel paleogenetic techniques and ancient tuna remains (Oral presentation). **Climate Impacts on Oceanic Top Predators Symposium in San Sebastian, Spain 14-18 September, 2015**.

Puncher, G.N., Massari, F., Cariani, A., Cilli, E., Leone, A., De Fanti, S., Martelli, P.L., Morales, A., Onar, V., Toker, N.Y., Moens, T., Arrizabalaga, H., Tinti, F. (2015) Unlocking the evolutionary history of the mighty bluefin tuna, *Thunnus thynnus*, using novel paleogenetic techniques and ancient tuna remains (Oral presentation). **VI congress of the Italian Society for Evolutionary Biology in Bologna, Italy August 31 - September 3, 2015**

Puncher, G.N., Cariani, A., Cilli, E., Massari, F., Morales, A., Onar, V., Toker, N.Y., Moens, T., Tinti F. (2015) Species identification of ancient tuna remains using a novel archaeogenetic protocol and barcoding techniques (Oral presentation). **6th International Barcode of Life Conference in Guelph, Ontario, Canada 18-21 August, 2015**.

Posters

Puncher, G.N., Cariani, A., Cilli, E., Massari, F., Martelli, P.L., Morales, A., Onar, V., Toker, N.Y., Bernal-Casasola, D., Moens, T., Tinti, F. (2016) Unlocking the evolutionary history of the mighty bluefin tuna using novel paleogenetic techniques and ancient tuna remains (Poster). **Bluefin futures symposium, Monterey, California, USA January 18-20, 2016.**

Puncher, G.N., Morales, A., Onar, V., Massari, F., Cariani, A., Cilli, E., Arrizabalaga, H., Tinti, F. (2014) Unlocking the evolutionary history of the mighty bluefin tuna using novel paleogenetic techniques and ancient tuna remains (Poster). **Mares Conference Marine Ecosystems Health and Conservation, Olhao, Portugal November 17-21, 2014.**

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

Geographic region	**Modern samples**	**Historical Samples**				
		1911-1926 (Massimo Sella Archive)	IV-XV CE (Istanbul)	IV BCE – V CE (Baelo Claudia)	II BCE (Tavira)	**TOTAL**
Adriatic Sea	**40** (2011) CMAS11J	**49** (Istria) HADR1927				**89**
Tyrrhenian Sea	**40** (2012) WMTY120	**48** (Messina/Pizzo/Gaeta) HTYR1911				**88**
Central Mediterranean Sea	**38** (2012) CMSI120	**46** (Zliten) HCM1926				**84**
Strait of Gibraltar	**40** (2011) EAGI11L			**55** HBC2BC, HBC5AD, HBCLIA	**10** HTAV2BC	**105**
Levantine Sea	**29** (2011) EMLS110	**2** (Istanbul) HIST1911	**60** HIST4AD			**91**
Balearic Sea	**40** (2011) WMBA110					**40**
Bay of Biscay	**40** (2011) EABB11J					**40**
Cape Hatteras and Gulf of Mexico	**24** (2008-09) WAGM080-09V					**24**
Total	**291**	**145**	**60**	**55**	**10**	**561**

Appendix 2: Individuals with <70% of SNPs satisfactorily genotyped and subsequently removed from population structure analyses. Percentage of SNPs that were not genotyped for each individual are provided in parentheses.

Sample	n	Individuals codes
EAGI11bL	1/42 (2.4%)	EAGI11bL04 (31)
WMBA110	1/50 (2.0%)	WMBA11020 (37)
HADR1927	28/48 (58.3%)	HADR192705 (100), HADR192706 (97), HADR192709 (94), HADR192710 (86), HADR192711 (95), HADR192713 (95), HADR192714 (100), HADR192715 (93), HADR192717 (69), HADR192720 (66), HADR192723 (64), HADR192724 (74), HADR192725 (76), HADR192726 (32), HADR192728 (82) , HADR192730 (68), HADR192732 (54), HADR192733 (95), HADR192734 (97), HADR192735 (83), HADR192736 (78), HADR192737 (93), HADR192738 (80), HADR192740 (90), HADR192742 (53), HADR192746 (38), HADR192747 (64), HADR192750 (89)
HBC2BC	45/45 (100.0%)	HBC2BC01 (95), HBC2BC02 (81), HBC2BC03 (81), HBC2BC04 (95), HBC2BC05 (93), HBC2BC06 (88), HBC2BC07 (69), HBC2BC08 (81), HBC2BC09 (80), HBC2BC10 (95), HBC2BC11 (96), HBC2BC12 (98), HBC2BC13 (97), HBC2BC14 (98), HBC2BC16 (86), HBC2BC17 (97), HBC2BC18 (96), HBC2BC19 (98), HBC2BC20 (100), HBC2BC21 (98), HBC2BC22 (94), HBC2BC23 (98), HBC2BC24 (96), HBC2BC25 (97), HBC2BC26 (94), HBC2BC27 (96), HBC2BC28 (98), HBC2BC29 (97), HBC2BC30 (97), HBC2BC31 (93), HBC2BC32 (90), HBC2BC33 (100), HBC2BC34 (93), HBC2BC35 (100), HBC2BC36 (98), HBC2BC37 (98), HBC2BC38 (97), HBC2BC39 (96), HBC2BC40 (96), HBC2BC41 (83), HBC2BC42 (88), HBC2BC43 (96), HBC2BC44 (96), HBC2BC45 (92)
HBC5AD	4/4 (100%)	HBC5AD1 (69), HBC5AD2 (88), HBC5AD3 (37), HBC5AD4 (44)
HBCLIA	2/3 (66.7%)	HBCLIA02 (31), HBCLIA03 (55)
HIST4AD	13/60 (21.7%)	HIST4AD13 (92), HIST4AD15 (73), HIST4AD17 (53), HIST4AD22 (100), HIST4AD23 (51), HIST4AD31 (41), HIST4AD49 (41), HIST4AD51 (83), HIST4AD55 (75), HIST4AD59 (94), HIST4AD61 (62), HIST4AD63 (96), HIST4AD64 (32)
HPJLIA	3/4 (75%)	HPJLIA01 (100), HPJLIA03 (96), HPJLIA04 (53)
HTAV2BC	7/10 (70%)	HTAV2BC01 (91), HTAV2BC02 (76), HTAV2BC03 (91), HTAV2BC04 (47), HTAV2BC06 (81), HTAV2BC07 (88), HTAV2BC08 (96)
HTYR191	9/50 (18%)	HTYR191115 (50), HTYR191120 (44), HTYR191122 (58), HTYR191123 (44), HTYR191125 (83), HTYR191127 (31), HTYR191146 (73), HTYR191148 (37), HTYR191149 (75), HTYR191118 (100)