UNLOCKING THE EVOLUTIONARY HISTORY OF THE MIGHTY BLUEFIN TUNA USING NOVEL PALEOGENETIC TECHNIQUES AND ANCIENT TUNA REMAINS

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SUMMARY

Using novel molecular techniques, DNA was extracted from Atlantic bluefin tuna vertebrae excavated from late Iron Age and ancient Roman settlements in coastal Iberia (Portugal 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). Comparisons have been made between the amount of DNA contained in each sample (measured via quantitative polymerase chain reactions), their age and the environmental conditions which the bones have been exposed to. A high performance genotyping panel containing SNPs derived from two separate projects funded by the GBYP scientific programme has been designed for the purpose of genotyping all historical samples along with modern samples collected from the same geographic areas. Included in the panel are 76 SNPs with high similarity to a wide variety of genes associated with the musculoskeletal system, development, metabolism, cellular function, osmoregulation and immune response. An additional 20 SNPs that provide significant discrimination between modern populations have been included in the panel.

RÉSUMÉ

En utilisant de nouvelles techniques moléculaires, de l'ADN a été extrait de vertèbres de thons rouges de l'Atlantique provenant de fouilles d'anciennes colonies romaines et de la fin de l'âge de fer sur les côtes de la péninsule ibérique (Portugal et Espagne, 4e-2e siècle avant JC; n=23) et à Constantinople (période byzantine) (4e-15e siècle après JC ;n=6), ainsi que de vertèbres des archives de Massimo Sella conservées à l'Université de Bologne (mers Ionienne, Tyrrhénienne et adriatique, début du 20e siècle; n=150). Des comparaisons ont été réalisées entre la quantité d'ADN contenu dans chaque échantillon (mesuré au moyen des réactions en chaîne de la polymérase quantitative), leur âge et les conditions environnementales auxquelles les os ont été exposés. Un panel de génotypage très performant contenant des SNP issu de deux projets financés par le programme scientifique ICCAT-GBYP a été conçu aux fins du génotypage de tous les échantillons historiques ainsi que d'échantillons modernes prélevés dans les mêmes zones géographiques. On a inclus dans le panel 76 SNP présentant une grande similitude en ce qui concerne la large variété de gênes associés au système squeletto-musculaire, au développement, au métabolisme, à la fonction cellulaire, à l'osmorégulation et à la réponse immunitaire. On a également ajouté dans le panel 20 autres SNP qui créent une discrimination importante entre les populations modernes.

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RESUMEN

Utilizando técnicas moleculares nuevas, se extrajo el ADN de vértebras de atún rojo del Atlántico rescatado de antiguos asentamientos romanos y de la edad de hierro tardía en las costas de la península ibérica (Portugal y España, IV-II a.c.; n=23) y de Constantinopla de la era bizantina (IV-XV d.c.; n= 6), así como de vértebras del archivo Massimo Sella conservado en la Universidad de Bolonia (mares Jónico, Tirreno y Adriático, principios del siglo XX, n=150). Se ha comparado la cantidad de ADN de cada muestra (medida mediante reacciones en cadena de la polimerasa cuantitativas), su edad y las condiciones medioambientales a las que han estado expuestos los huesos. Se ha diseñado un panel de genotipificación de alto rendimiento que contiene los SNP derivados de dos proyectos separados financiados por el programa científico del GBYP, con el fin de genotipificar todas las muestras históricas junto con muestras modernas recogidas en las mismas zonas geográficas. En el panel se incluyeron 76 SNP muy similares a una amplia variedad de genes asociados con el sistema musculo esquelético, el desarrollo, el metabolismo, la función celular, la osmorregulación y la respuesta inmune. También se incluyeron en el panel 20 SNP adicionales que proporcionan una discriminación importante entre poblaciones modernas.

KEYWORDS

Bluefin tuna, DNA, Genetics, Evolution, Coastal fisheries, Bones, Mediterranean Sea, Population dynamics, Population structure, Long-term changes

1. Introduction

Mediterranean civilizations and the Atlantic bluefin tuna (BFT, *Thunnus thynnus*) have shared a dramatic history spanning millennia. Today, traditional fishing techniques are fading into the past and industrial fisheries command the seas. 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 Atlantic BFT, which pre-dates detailed record keeping, this is impossible. However, analysis of ancient genetic material can help to reveal elements of the past necessary for establishing something of a pseudo-baseline (Nielsen *et al.* 1999). Ancient DNA research has accomplished much in the past two decades, particularly for anthropological studies. Much of the research has focused on our own history, such as exploration into our ancient relationship with Neanderthals 40,000 years ago (Briggs et al. 2009), identification of the bacterial culprit behind the Black Death which ravaged Europe's population 660 years ago (Bos *et al.* 2011), and analysis of the dietary habits of Native Americans 2000 years ago (Poinar *et al.* 1998). Anthropological studies aside, dozens of studies have focused on animals as well, such as extinct moas (Cooper et al. 2001), dodos (Shapiro *et al.* 2002) and New Zealand's giant eagle (Bunce *et al.* 2005). Fisheries' scientists have caught the aDNA bug too, recognizing that through the genetic analysis of historical samples (scales, otoliths, vertebrae) geneticists are capable of elucidating the impact of humankind on fish species since the beginning of commercial-scale exploitation. Microsatellite analysis has been used to measure the impacts of restocking on salmon populations in Denmark (Nielsen *et al.* 1997), show genetic loss in New Zealand Snapper (*Pagrus auratus*) after decades of heavy exploitation (Hauser *et al*. 2002), measure genetic integrity and estimate effective population sizes of heavily exploited populations of North Sea Cod (*Gadus morhua*) over a period of seven decades (Hutchinson et al. 2003; Poulsen *et al*. 2005), and reveal genetic adaptation to increasing sea temperatures in Atlantic Cod (*Gadus morhua*) (Nielsen *et al.* 2007). Mitochondrial DNA has been used by researchers to assess the temporal stability of the Danish brown trout (*Salmo trutta*) genome dating back to the early 1900s (Hansen et al. 2002) as well as measure genetic change in Atlantic Salmon (*Salmo salar*) over a 41,000 year interval (Conseguera *et al.* 2002). In a review by Knapp and Hofreiter (2010), Next Generation Sequencing 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. By analyzing the DNA contained in the bones of bluefin tuna recovered from archeological excavations throughout Europe, we hope to reconstruct the species' adaptive responses to fisheries pressure and a changing environment.

2. Collection of historical samples

2.1 Massimo Sella Archive

The Massimo Sella archive is a collection of remains (vertebrae, skulls and fins) belonging to some 5000 fish captured in the Mediterranean Sea during the early decades of the 20th century by Italian professor Massimo Sella. Originally housed at the Adriatic Marine Biology Institute in Rovigno, Croatia, the collection was moved to a University of Bologna facility in Fano, Italy during the Second World War. The vast majority of bones in the collection are Atlantic bluefin tuna (BFT) caught in tuna traps in Istria (Croatia), Messina (Italy) and Sliten (Libya). Several years ago geneticists from Italy used bones from this collection to investigate the temporal dynamics of population structuring using a host of microsatellites (Riccioni *et al.* 2010). In that study, 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. 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 too weak to demonstrate these genetic dynamics and, 4) a more robust sampling design using advanced techniques and more effective genetic markers are required to address these questions (Cannas et al. 2012). For these reasons we have continued with this important work and included 150 samples from the Massimo Sella archive from the same locations used by Riccioni et al. (2010). DNA has been extracted from all of these samples and information concerning the capture site and date, weight and length of animal and sex has been recorded for each sample, whenever the data is available.

2.2 Istanbul, Turkey

Archeological excavations conducted between 2004 and 2013 in the Yenikapi neighbourhood of Istanbul have revealed the location of a Byzantine era harbour. 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 and historical accounts of the Ancient Greek and Roman tuna fishery (Oppian 177BCE; Aristotle 350BCE) 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).

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 whereabouts of their prey which migrated into the warming waters of the Black Sea in the spring, returning to the warmer waters of 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 lammellae), 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 embryos and larvae. 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 isolated tribe, if any have survived. Information concerning the distribution of the remaining Black Sea bluefin 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 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 haven'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).

2.3 Iberian Peninsula

A vast collection of animal bones collected from archeological sites around the world are archived at the Faculty of Archeozoology at the Autonomous University of Madrid. 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.) 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.

3. Challenges of ancient DNA analysis

DNA begins to 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 (35-40C) and humidity also facilitate autolysis. 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 is spared some of the rapid and extensive degradation of soft tissues (Wandeler *et al*. 2007). Genetic molecules contained in older tissues are also subject to increased depurination which is a chemical process by which purines are liberated from the DNA molecule, allowing for hydrolysis of the sugar-phosphate groups, resulting in further fragmentation of DNA adjacent to purine residues (Overballe-Petersen *et al*. 2012).

4. DNA extractions

Sample 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.

4.1 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 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) 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.

4.2 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 the collaboration with the Department of Cultural heritage at the University of Bologna, which hosts specialists in the extraction of DNA from ancient humans, we have developed new protocols that yield higher concentrations of DNA from archeological remains than previously used methods (Riccioni *et al*. 2010). 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.

In order to verify the species of each individual, all samples have had mini-barcodes of the following genes amplified via polymerase chain reaction: cytochrome oxidase subunit 1 (CO1), mitochondrial control region (mtCR) and internal transcribed spacer (ITS1).

4.3 Quantification of historical DNA

All DNA has undergone rigorous quality and quantity analysis using multiple platforms (NanoDrop, Agilent BioAnalyzer, QuBit, qPCR). Most vertebrae from the Massimo Sella archive contained sufficient DNA for SNP genotyping purposes, as determined by the QuBit spectrophotometer (**Table 1**). However, samples older than 500 years possess concentrations of DNA lower than the threshold that can be quantified in this manner. 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 (**Table 2**). 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.

4.4 Quantitative Polymerase Chain Reaction

At the University of Gent (Belgium) a subset of all samples have been analyzed using quantitative polymerase chain reactions (qPCR). Several species-specific primer pairs were designed and used to execute all qPCRs in order to quantify the concentration of mitochondrial and nuclear DNA successfully extracted from each sample. Reactions were carried out using two mitochondrial genes (Control Region [TTCR1] and Cytochrome Oxidase subunit 1 [TTCOX1]) and one nuclear gene (Internal Transcribed Spacer Region 1 [TTITS1]). Comparisons have been made between the amount of DNA contained in each sample and their age and the environmental conditions which the bones had been exposed to. Both nuclear and mitochondrial gene markers performed consistently for all reactions. Primer pairs used to amplify short fragments of CO1 and ITS1 performed better than the CR primers. 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 (**Figure 1**).

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. 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.

5. SNP genotyping panel for 480 samples

A genotyping panel has been developed which contains 96 SNPs that are indicative of genetic population structuring in modern populations as well as SNPs associated with genes that may hold selective or evolutionary significance. Within the ICCAT/GBYP Phase 4 contract for the *Data Recovery* programme, it plainly 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 319 modern individuals and 178 historical individuals, all of which will be genotyped and compared to ensure comprehensive comparisons across all time periods and sampling locations (**Table 4**). 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 GBYP Phase 4, and 2) Transcriptomic SNP development by the BGSA during Phase 2.

5.1 Genotyping-by-Sequencing SNP discovery and comparison with annotated genes of other fish species.

As mentioned above, the 96 SNPs that were discovered and validated in Phase 4 of the BGSA Programme have been analyzed for their potential to be used for the purposes of detecting spatio-temporal shifts in genetic structuring of BFT populations as well as detect adaptation over time to environmental and fishery pressures. All SNP flanking sequences were compared with previously published sequences of annotated genes from other teleost species using the BLAST algorithm (Altschul *et al*. 1990) provided by the National Center for Biotechnology Information. A total of 35 SNP flanking sequences aligned with >100bp of previously annotated genes with an identity rate of >75%. The vast majority of these alignments occurred with well studied teleosts such as: *Dicentrarchus labrax, Takifugu rubripes, Poecilia formosa, Larimichthys crocea, Stegastes partitus, Oreochromis niloticus, Oryzias latipes, Maylandia zebra*, and *Haplochromis burtoni*. The genes with which these SNPs are likely to be associated are listed in **Table 5**.

Several of the SNPs that demonstrate higher capacity for population discrimination have been selected for additional investigation regarding the nature of their impact on gene expression. The flanking region sequences of these SNPs of additional interest have been aligned with several sequences from other teleost species and translated into the expected expression of amino acids (i.e. building blocks of proteins) in an effort to determine whether the different alleles of SNP modify the structure of the protein. For example, one candidate aligned with the *Myosin VIIB (myo7b)* gene which 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 testis. During the development of the 384 SNP panel for Phase 4 BGSA, when SNPs were being selected due to extreme differences in pooled allele frequency differences across populations, this particular SNP was a strong candidate. Alignments of the flanking regions and SNPs of the BFT genotyped in GBYP Phase 4 revealed a non-synonymous base substitution that resulted in a modification of the amino acid sequence. 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). This kind of modification in the genetic code can have a profound influence on the phenotypes of individuals and can be indicative of evolutionary adaptation.

5.2 Transcriptome SNP discovery and comparison with the annotated genome of Atlantic cod

All 384 SNPs discovered by the BGSA Genetic Consortium during Phase 2 were aligned with the publicly available annotated genome of the Atlantic cod, *Gadus morhua*. A total of 41 SNPs of the total number of SNPs derived from the analyzed transcriptome aligned with a high degree of similarity to annotated genes of cod (**Table 6**). These have all been added to the genotyping panel.

To complete the 96 SNP panel, an additional 20 SNPs with high sampling location discrimination power (data derived from GBYP Phase 4 - Validation of 96 SNP panel by BGSA Consortium) were included.

The final results from this study will be submitted to the secretariat on January 31, 2016.

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Table 1. 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
Sliten 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

Table 2. 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

Table 3. Number of samples analyzed with qPCR from each of ten different excavation sites and archives.

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 4. 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 (Massimo Sella Archive)	1755 (Cadiz, Spain)	4^{th}-15^{th} century AD (Istanbul, Turkey)	1^{st} century AD (Cadiz, Spain)	2^{nd} century BC (Tavira, Portugal)	4^{th}-2^{nd} century BC (Portugal + Spain)	**TOTAL**
Adriatic Sea	**50** (2011)	**48** (Istrian traps)						**98**
Tyrrhenian Sea	**50** (2012)	**48** (Traps of Pizzo and Messina-Cialona)						**98**
South of Sicily	**48** (2012)	**48** (Trap of Sliten)						**96**
Gibraltar	**42** (2011)		**2**		**10**	**10**	**5**	**69**
Levantine Sea	**29** (2011)	**2** (Istanbul)		**5**				**36**
Balearic Sea	**50** (2011)							**50**
Bay of Biscay	**50** (2011)							**50**
Total	**319**	**146**	**2**	**5**	**10**	**10**	**5**	**497**

Table 5. Possible genes associated with GBS derived SNPs.

Transmembrane protein 132 (TMEM132)
Leucine-rich repeats and immunoglobulin-like domains 1 (lrig1)
Pre-B-cell leukemia transcription factor-interacting protein 1
rho guanine nucleotide exchange factor 16
Unconventional myosin-VIIa
Zinc finger and BTB domain-containing protein 12
Regulator of G-protein signaling 20
Kappa-type opioid receptor
C4b-binding protein alpha chain
Protein disulfide-isomerase TMX3
Myosin heavy chain 1
Anaphase promoting complex subunit 2 (anapc2)
Bridging integrator 1 (bin1b)
Copper transporting ATPase 2 (ATP7B)
Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase
Excitatory amino acid transporter 1
Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (gbf1)
GTP-binding protein Di-Ras1
HoxBb gene cluster
Immunoglobulin superfamily member 21
Kisspeptin 2 (Kiss2)
Laminin, alpha 5 (lama5)
S-adenosylmethionine mitochondrial carrier protein
Leucine-rich repeats and immunoglobulin
Lysyl oxidase homolog 1
Metabotropic glutamate receptor 2
Mothers against decapentaplegic homolog 4
Myc box-dependent-interacting protein 1
Myosin VIIB (myo7b)
Protein FAM73B
Protein kinase C delta
Retinol dehydrogenase 8
Sodium/myo-inositol cotransporter
Solute carrier family 5 (sodium/myo-inositol cotransporter), member 3 (slc5a3)
T-box brain protein 1 (TBR1)
Dermatan-sulfate epimerase-like protein
Anti-dorsalizing morphogenic protein

Table 6. Genes from the annotated cod genome with flanking region sequences highly similar to transcriptome-derived SNPs from GBYP Phase 2.

Asparaginyl-tRNA synthetase
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c3 (subunit 9) genome duplicate b
ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit
Betaine-homocysteine methyltransferase
Calponin 1, basic, smooth muscle, b
Collagen, type VI, alpha 1
Complement factor D (adipsin)
COP9 constitutive photomorphogenic homolog subunit 5
Elongation factor 1 alpha
Eukaryotic translation elongation factor 1 alpha 1a
Eukaryotic translation elongation factor 2a, tandem duplicate 2
Eukaryotic translation initiation factor 3, subunit G
Fructose-1,6-bisphosphatase 1b
Fructose-1,6-bisphosphatase 2
Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit
Integral membrane protein 2Bb
Myomesin 1
Myosin, heavy chain 13, skeletal muscle
Myosin, light chain 12, genome duplicate 1
Myosin, light chain 9b, regulatory
Myozenin 1
Myozenin 1b
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9
Phosphoglucomutase 1
Ribosomal protein L18a, L27, L8, L28, L3, L31, L7, L9, S14, S18, S2, S23, SA
Thioredoxin interacting protein a
Ubiquinol-cytochrome c reductase hinge protein
Vacuolar protein sorting 28
zgc:92035

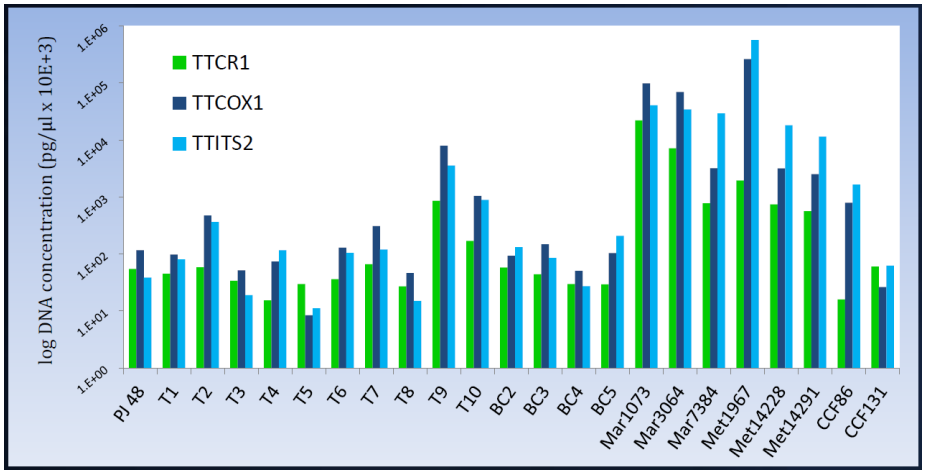

Figure 1. qPCR results for 23 samples of ancient origin using three different molecular markers (CO1, mtCR and ITS1). Samples are arranged chronologically. Name codes for sampling locations are featured in Table 3.