ALMA MATER STUDIORUM

UNIVERSITÀ DI BOLOGNA

SCUOLA DI SCIENZE

Corso di laurea magistrale in BIOLOGIA MARINA

First assessment on genetic structure and phylogeography of Mediterranean blue shark (*Prionace glauca*, L. 1758) population using mitochondrial gene variation: a comparison with the Atlantic.

Tesi di laurea in Struttura e Connettività delle Popolazioni Marine

Relatrice

Dott.ssa Alessia Cariani

Presentata da

Ilenia Urso

Correlatori

Agostino Leone

Fausto Tinti

III sessione

Anno Accademico 2014/2015

Table of contents

1. Introduction

1.1 Morphology, ecology and biology of the blue shark *Prionace glauca*

The blue shark (*Prionace glauca*, Linnaeus 1758) (**Fig.1**) is one of the most abundant carcharhinid shark and it is the only one species of *Prionace* genus. According to the "FAO species catalogue – Vol.4, Sharks of the world" (Compagno, 1984), the blue shark can be classified as follows:

- Phylum: Chordata
- Subphylum: Vertebrata
- Superclass: Gnathostomata
- Class: Chondrichtyes
- Subclass: Elasmobranchii
- Superorder: Galeomorphi
- Order: Carcharhiniformes
- Family: Carcharhinidae
- Genus: Prionace
- Species: *P.glauca*

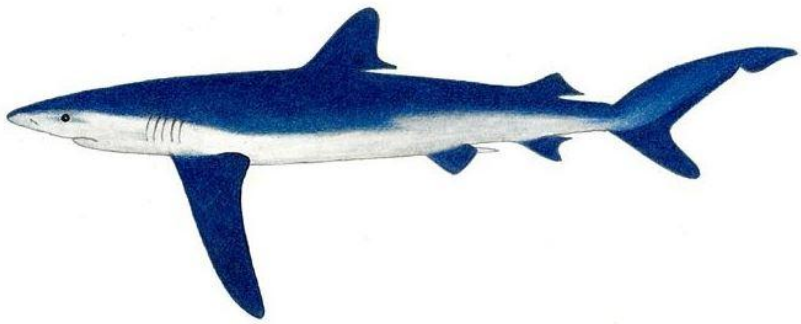

Fig.1 Blue shark *Prionace glauca* [Illustration by Ann Hecht ©]

The blue shark is widely distributed in the world's oceans; it is an oceanic and epipelagic shark and one of the few known shark species to be common in all tropical, subtropical and temperate seas

and both in deep and coastal waters. Blue sharks prefer water temperature between 7 and 16 °C, even if they can also tolerate warmer temperatures, up to 21 °C (Compagno, 1984). It has a unique morphology, which allows us distinguish blue sharks from any other shark: it has a long and slim body, with long pectoral fins and first dorsal fin set backward compared to the pectoral ones, second dorsal less than a third the size of the first dorsal. Blue sharks show pectoral fins long, narrow and tapered, caudal fin non-lunate with the upper lobe longer than the inferior one; this shark has also large eyes without posterior notches, short labial furrows, and weak keel on caudal peduncle. Finally, it is characterized by absence of inter-dorsal ridge, extended and sharp snout longer than the width of the mouth, well differentiated teeth in upper and lower jaws, upper teeth with broad, triangular, curved cusps - between straight and oblique – but not sharp, or small cusps except in very young specimens and each tooth is usually replaced every 8 to 15 days (Compagno, 1984; Nakano & Seki, 2003; Valeiras & Abad, 2009). All these unique characteristics make the blue shark body morphology highly-hydrodynamic and it provides them the possibility for fast and strong swimming; this body shape enables this species to make long migrations, fast hard turns during predation and it also permits them to get away from other fast swimming fish (Nakano & Seki, 2003; Sampaio da Costa, 2013).

Its common name, "blue shark", derives from the peculiar body coloration, which also represents a specific adaptation of these sharks to the open ocean habitat. In fact, the body shows a deep and brilliant blue color on the dorsal side, while the flanks appears of a grey-blue and the ventral side becomes brightly white; because of this adaptation, if seen from above blue sharks exhibit a dark back which matches the dark surrounding water, while, if seen from below, the white belly matches the bright lightened surface (Karleskint *et al.*, 2009).

1.2 Reproductive Cycle

Blue sharks can reach a maximum size of 400 cm, but the common length is around 334 cm. Age and growth of blue shark was analyzed from embryos to adults using length frequency data and vertebra samples in Nakano (1994). It is a placental viviparous species, so embryos develop inside

the female's uterus with a gestation period between 9 and 12 months, size at birth is about 36 cm body length, referring to pre-caudal length, and it is believed that mating and fertilization occur in early summer (Pratt, 1979; Castro & Mejuto, 1995; Nakano, 1994). Blue shark is moderately productive characterized by a rapid growth, growing up to 30 cm annually until maturity, being this the reason why it can be considered the species with the fastest growth rate of all sharks. Male specimens reach sexual maturity around a 183 body size and an average age of 6 years. It has been seen that males smaller than 125 cm were immature presenting non-calcified claspers that did not reach the posterior end of the pelvic fins (Pratt, 1979; Nakano & Stevens, 2008; Megalofonou *et al.*, 2009). Females are ready for mating at 2 years old and it can be considered a sub-adult life period during which they are still developing sexual organs needed for gestation; in fact, observations on the reproductive organs in relation to body length showed that females smaller than 120 cm have immature ovaries with no maturing oocytes, while mature ovaries with visible yolky oocytes have been seen in specimens larger than 203 cm (Megalofonou *et al.*, 2009). Full female sexual maturity comes at a 185 cm body length and a average age of 4-5 years. Once pregnant, females migrate north to birthing and pupping grounds in the sub-arctic boundary, where juveniles usually stay until they reach maturity.

Approximately, 30% shark species show an oviparous reproduction (which means that females spawn in the outer space), 50% of sharks have a a-placental viviparous (which means that the development and spawning of the embryos occurs inside the maternal uterus and pups come out fully formed) while the remaining 20% exhibit a placental viviparous reproduction. Blue shark belongs to this latter category; in this kind of reproduction pups are in contact with mothers through a kind of primitive placenta and, after development has been completed, they come out in the outer space. Blue sharks have a gestation period of 9 to 12 months (Pratt, 1979; Castro & Mejuto, 1995) and, at the moment of parturition, the number of pups consists of 4 to 135, with an average number of 50-60 pups with a 35-50 cm length. Male specimens will reach maturity at 4 to 6 years, with a 250 cm body length, while female ones at 5 to 7 years. The average age for this species seems to be

about 20 years, while the generation time is 8.2 and 9.8 for South African and North Atlantic stocks respectively, which means that it takes around 8.2/9.8 years between two consecutive generations in the lineages of its population (Cortès *et al.*, 2015). In some areas, blue shark reproduction seems to be seasonal, occurring during spring and summer in the Atlantic Ocean, immediately followed by mating (Pratt, 1979).

In the Mediterranean Sea, according to what has been speculated in the first study documenting biological information of this species within this geographical area (Megalofonou *et al.*, 2009) the sex ratio seems to be constantly in favor of males and it is similar to that reported in the eastern North Atlantic Ocean and the Strait of Gibraltar. Nevertheless, sex ratio seems to be inverse to that reported in the western North Atlantic (Pratt, 1979) and eastern North Atlantic in British and Irish waters (Buencuerpo *et al.*, 1998; Megalofonou *et al.*, 2009; Stevens, 1976; Henderson *et al.*, 2001), where it was strongly in favor of females, according also to the idea that male blue shark move inshore only upon attaining sexual maturity (Whelan, 1991; Pratt, 1979). The Mediterranean Sea can be considered a reproduction area for blue sharks, as supported by the results of the observations on the catch composition, according to which there's a substantial presence of mature male and females (Megalofonou *et al.*, 2009). In addition to these observations, previous studies have shown that the Adriatic Sea could be a nursery area and gravid females have been observed both in the Adriatic and in the Ionian Sea (Bianchi *et al.*, 1997; Pomi, 1997).

1.3 Geographical distribution

Blue sharks distribution is circumglobal, both in temperate and tropical waters, and it is reasonably due to their excellent capability to make transoceanic movements. The blue shark geographic distribution (**Fig.2**) shows that these elasmobranchs can be found in Indian, Pacific and Atlantic ocean at a latitude between 60°N and 50°S (Steven *et al.*, 2010); in Western Atlantic blue sharks can be found from Newfoundland, Canada to Argentina, while in the Eastern part they live from

Norway to South Africa, including Mediterranean Sea (Kohler *et al.*, 2002; Clarke *et al.*, 2006; Megalofonou *et al.*, 2009).

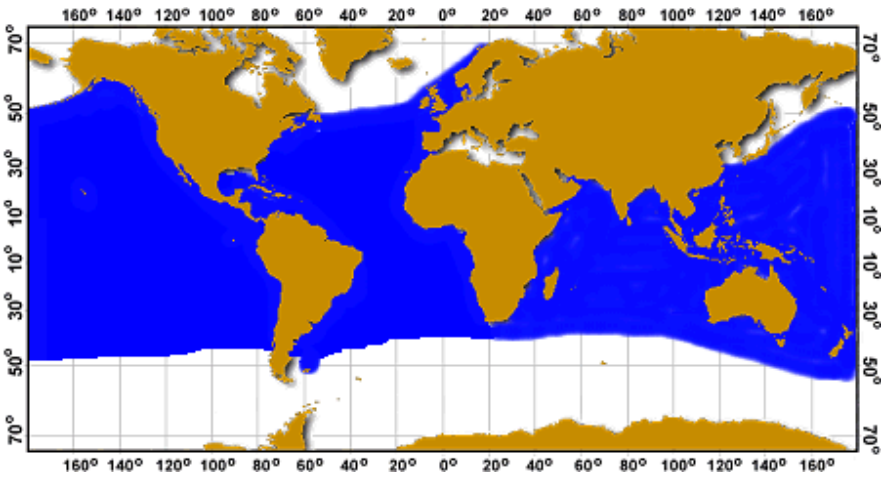

Fig.2 World geographical distribution for the blue shark *Prionace glauca* (downloaded from www.fao.org)

We know that, as other elasmobranch species, blue sharks are characterized by spatial segregation due to body size, sex and reproductive stage. In case of segregation by reproductive stage, male and female sharks live in different areas until they reach sexual maturity and mating can occur (Simpfendorfer *et al.*, 2002; Nakano & Seki, 2003; Robbins, 2007). Moreover, it seems that body length increases moving from temperate and sub-arctic to tropical latitudes, but few information are available about the complex population structure of this species.

As already said, blue shark is a pelagic species, but it can be occasionally found close to the continental shelf. These elasmobranchs seem to prefer swimming in surface, but their morphology and biological characteristic allow them to reach deeper waters up to 190 m., and some evidence show how these sharks are able to dive up to 350 m., searching for preys.

According to what reported by the recreational fishery data, this species was once abundant, while in the last 30 years a 75% decline in its abundance has been registered, even though this evidence seems to be more related to the Ionian Sea rather than the Adriatic Sea (Ferretti *et al.*, 2008). Considering specifically the Mediterranean Sea, *P. glauca* is the most common shark species observed in both Thyrrenian and Ionian sides of Strait of Sicily (Sperone *et al.*, 2012) and this data

is consistent with other studies referred to the Mediterranean area (De Metrio *et al.*, 1984; Megalofonou *et al.*, 2005; Psomadakis *et al.*, 2009). However, it seems that Mediterranean blue sharks are generally declining in abundance, probably more than elsewhere in the world (Cavanagh & Gibson, 2007), and this event can be attributed to different factors, such as the life history characteristics of the species in combination with the intense fishing activity throughout these waters (Megalofonou *et al.*, 2009). In fact, blues sharks represent a major by-catch of long line fisheries targeting swordfish or tuna (Megalofonou *et al.*, 2005a, 2005b), every year approximately 20 millions of these specimens are caught and these datas give us an idea of how this species is highly exposed to the commercial pressure. Comparing historical data from Italian swordfish fisheries in the Gulf of Taranto with recent data we can see how the catch rates in the Mediterranean Sea over the last 20 years have decreased by an average of 38,5% (De Metrio *et al.*, 1984; Filanti et *al.*, 1986; Cavanagh & Gibson, 2007).

Prionace glauca, as top predator, represents a key component of the marine ecosystem, regulating the community structure. The loss of apex predators, due to the loss of entire functional groups of the marine ecosystem, might lead to enormous consequences for the ecosystem structure with a cascaded downward effect (Myers *et al.* 2007). One of the major damage is observed in the modification of population dynamics and population size of many species and this evidence suggests how the loss of top-predators can have dramatic consequences. For all these reasons blue shark is listed among the “protected fauna species” of the Bern convention (Appendix III - Protected fauna species) and it is also considered as "Near Threatened" on the IUCN red list assessment for Mediterranean chondrichthyans (Stevens J., 2009). Even if Blue Shark *P. glauca* (BS) has been categorized as "Near Threatened" in the IUCN (Stevens 2009; IUCN 2014) global assessment, the further assessments may require a revision because of the tremendous fishing effort to which the species is globally subjected, approximately 20 million specimens per year. In contrast, the Mediterranean BS population has been categorized as "Vulnerable" A2b category because a combination of biological and ecological characteristics of the species in a potentially insulator

basin. The UNEP RAC/SPA Action Plan for the Conservation of Cartilaginous Fishes in the Mediterranean Sea (2003) aims at promoting the general conservation of the chondrichthyan populations of the Mediterranean and the protection of selected chondrichthyan species, whose populations are considered endangered. This document lists *Prionace glauca* among the main species for which it is recommended the development of national and regional programs for sustainable fisheries either as they are target and accessory species (Cavanagh & Gibson, 2007).

1.4 Blue shark population and stock structure

1.4.1 Tagging data and baselines

Thanks to their peculiar and highly hydrodynamic body shape, blue sharks are able to perform great movements, travelling for about 3000 km and making often transoceanic movements, moving between northern and southern hemispheres and overcoming 6000 km distances, with the maximum recorded distance travelled by a blue shark specimen of 7176 km (Stevens, 1990; Kohler *et al.*, 2002; Quieroz *et al.*, 2010; Costa *et al.*, 2012). A representation of trans-regional movements and distances travelled for blue sharks according to the cooperative shark tagging program can be found in **Fig.3** (Kohler *et al.*, 2002).

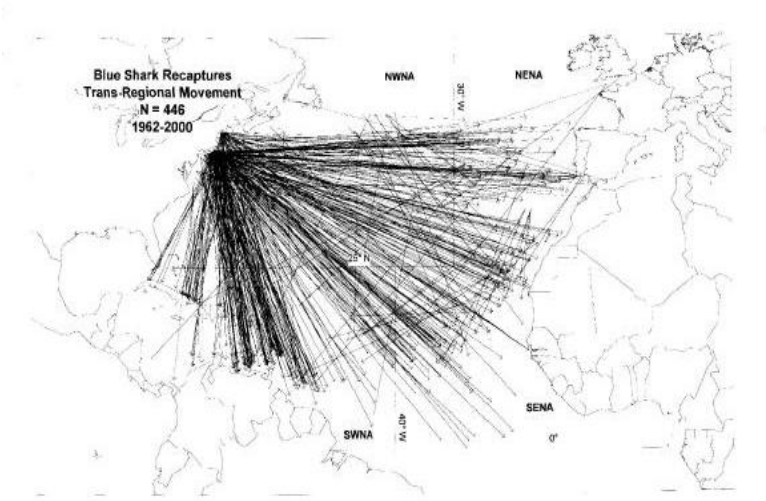

Fig.3: Recapture distribution for trans-regional movements of the blue-shark, *Prionace glauca*, from the cooperative shark tagging program. 1962-2000. Area definitions can be found in the text. From Kohler *et al.*, 2002.

Nonetheless, there is evidence of a site fidelity to coastal or pelagic oceanic locations, such as seamounts, oceanographic fronts, continental shelves. This behaviour can be related to the presence, in these regions, of an high nutrient concentrations supplied by runoff and thermal front boundaries which provide high primary productivity (Bigelow *et al*., 1999; Litvinov *et al*., 2006; Quieroz *et al*., 2012).

Considering a review of tagging data from different tag-and-release programs carried out by different ICCAT members (Green *et al*., ICCAT, 2009; SCRS/2008/130), data regarding Irish recreational fisheries for blue sharks show how only 789 individuals of the 16804 sharks tagged in the period of time between 1970 and 2006 have been recaptured, resulting in a recapture rate of 4.7%. Results from this studies report that no recapture occurred in the South Atlantic, while only one Mediterranean blue shark specimen has been recaptured and this data supports the idea of a separation between the Atlantic blue shark stock and the Mediterranean one (Kohler *et al*., 2002).

Although several studies have been developed in order to define the patterns of the movements and migrations of this species and, contextually, to understand the connectivity between different areas, the complex structure of blue shark populations, as showed by different tag-recapture studies (Kohler *et al*., 1998; Fitzmaurice *et al*., 2005; Mejuto *et al*., 2005; Green *et al*., 2009) is still uncertain and, specifically for the Mediterranean Sea, it is still unknown.

Several works focused on this aspect have been carried out for the Atlantic and especially North-Atlantic blue shark populations; according to the results of these studies, we know that the locations having high incidence of juvenile blue sharks (those with a<150 cm body length) are located off mainland Portugal, off the Azores and off western South Africa (Kohler et al., 2002; Silva et al., 2010; Quieroz et al., 2012). Data showed that both female and male small juvenile blue sharks tend to remain for extended period of time in a general area delimited by the Azores, the Atlantis – Great Meteor seamount complex and the Mid-Atlantic Ridge (Vandeperre *et al*., 2014). There are also different and contrasting opinion about the spatio-temporal segregation preference: the movement

patterns found by Vandeperre et al. (2014) didn't show any indications of segregations between the two sexes at that spatial scale and found that segregation does not occur before the second year of life (Vandeperre *et al.*, 2014). This result was in agreement with data from conventional tagging programs, which suggested that juveniles of 100-130 cm do not perform extensive latitudinal migrations. On the contrary, Quieroz et al. (2012) found that juvenile blue sharks made more extensive movements closer to continental shelves and others state that males and females segregate during the first year (Litvinov, 2006). Moreover, according to Vandeperre et al. (2014) probably there's no connectivity between the central North-Atlantic and other juvenile grounds like the continental shelves of the Iberian Peninsula and Northern Africa, in agreement with the hypothesis that parturition may take place in this area of the North-Atlantic. The collected data provide also the evidence of the existence of a nursery area for blue sharks in the central North-Atlantic.

We have information about the tendency of North-Atlantic large juvenile female to dominate summer catches off south-west England (Simpfendorfer *et al.*, 2002; Pratt, 1979; Campana *et al.*, 2006), while in winter the major presence of female specimens seems to be recorded off Portugal (Quieroz *et al.*, 2005) and around the Azores. Female also exhibit a preference to surface water, with a spatio-temporal segregation between 15°C and 16°C throughout the year (Vandeperre *et al.*, 2014). In regard of adult female blue sharks, results from tagging studies in North Atlantic waters showed that these specimens display direct movements to tropical waters, some of them carrying embryos. This data reasonably indicates a relation between these movements and the reproductive cycle, supporting the fact that specimens of different ages and reproductive stage move in different directions and occupy separate areas (Vandeperre *et al.*, 2014).

As declared by the DELASS project (Development of Elasmobranch Assessment – Heessen, 2003), which aims at improving the scientific basis for the management of fisheries affecting elasmobranch species, and confirmed by the ICCAT Shark Assessment Working Group (ICCAT, 2009), there's one stock of blue shark *Prionace glauca* in the North Atlantic Ocean (Fitzmaurice *et al.*, 2005) and the most probable division between North and South Atlantic stocks of blue shark

would be located near the 5°N parallel (ICCAT, 2009). All the information available regarding the blue shark stock structure are mainly based on data and results from tag-recapture studies, assuming that there are, at least, three stocks in the Atlantic Ocean, consisting of one Mediterranean blue shark stock, another one in the North Atlantic and a third stock in the South Atlantic (ICCAT, 2009). Previous studies have shown how the North Atlantic stock individuals would probably be characterized by a cyclical migration in a clockwise direction between 30-50°N (Kohler *et al.*, 2002; Skomal & Natanson, 2002; Fitzmaurice *et al.*, 2005), while results from tag-recapture investigations have reported a limited movement of blue sharks between the Atlantic and the Mediterranean Sea. On the basis of these few information, a single Mediterranean stock is reported, separated from the two stocks of the Atlantic Ocean (Kohler & Turner, 2008; ICCAT, 2009).

1.4.2 Genetic structure and diversity

Genetic studies have been carried out on the Atlantic and Pacific populations using microsatellite nuclear markers. Fitzpatrick et al. (2010) analyzed approximately 1,000 individuals collected worldwide at 16 microsatellite loci. This study revealed an inter-oceanic genetic structuring with gene-flow generally restricted within ocean. A population genetic analysis carried out on Brazilian BS populations with the same biparental markers indicated a moderate population structure among samples of Rio Grande do Norte, São Paulo and Rio Grande do Sul (Ussami et al. 2011). These results may suggest the existence of two South American local populations even if to assess population boundaries further and deeper analyses in terms of sampling design and type of markers to be used are required. The analysis of juvenile specimens (<2 yr) from Atlantic Ocean nurseries (Western Iberia, Azores and South Africa) using both mitochondrial and microsatellite markers showed a significant heterogeneity among nursery areas, and a temporal structuring within as well as between nurseries, suggesting the existence of different reproductive units in time and space within Atlantic Ocean (Verissimo *et al.*, 2013). The genetic structure and demographic history of Pacific BS populations was inferred using mitochondrial DNA. A variation analysis of Cytochrome b sequence in 404 specimens from 10 different locations of Indo-Pacific indicated weak or no

genetic differentiation across the Indo-Pacific, and phylogenetic analysis has shown a historical demographic expansion, suggesting that this population has not suffered past climatic fluctuations (Taguchi et al. 2014).

Most of the works concerning the population and stock structure of this species are mainly based on aspects including geographic range, age and growth, movements and migrations, while the data about the blue shark *Prionace glauca* genetic population structure in still poor. A recent study has tried to understand and describe the genetic stock structure of blue sharks within the Atlantic ocean, considering three known Atlantic nurseries and using mitochondrial and nuclear markers in order to estimate the genetic diversity of the individuals and targeting the most resident component of blue shark populations (Sampaio da Costa, 2013). As a result of the analysis carried out in this study, similar levels of genetic diversity among blue shark nurseries in the Atlantic were found by the two marker types considered, nuclear microsatellites and mitochondrial Control Region. However, the two markers detected genetic heterogeneity among juveniles sampled in distinct nurseries (Sampaio da Costa, 2013). In agreement with previous studies Sampaio da Costa (2013) suggested the presence of a North Atlantic group of blue sharks and a Southern one; however, on the basis of results from FCA (Factorial Correspondence Analysis) among nurseries, two genetic stocks seem to be present within the North Atlantic Ocean. This data seems in contrast with the absence of genetic differences between the North Atlantic nurseries observed with Fst values. The two markers gave also different results to the null hypothesis of Atlantic panmixia in *Prionace glauca* proposed by Sampaio da Costa (2013). As previously suggested, this contrast between the absence of a genetic structure according to bi-parentally inherited nuclear markers and the existence of a genetic structure according to the maternally inherited mtDNA could be related to the existence of a female philopatry to nurseries in presence of male mediated gene flow, as suggested in other shark species (Pardini *et al.*, 2001; Portnoy *et al.*, 2010; Tillett *et al.*, 2012).

Another study focused on the analysis of four shark species co-distributed in the Indo-Pacific, including blue shark *Prionace glauca*, was carried out using the variation of the mtDNA sequence

control region (Ovenden *et al.*, 2009). Here, they found no evidence of genetic structuring in the Indo-Pacific blue shark populations (Ovenden *et al.*, 2009).

Summarizing, results from previous works in Pacific and Atlantic Ocean highlighted a poor genetic structure even between very far blue shark populations (Ovenden *et al.*, 2009; Sampaio da Costa, 2013).

No genetic studies were carried out on Mediterranean blue shark population and, at this moment, it is extremely important to assess them in order to preserve their status from the increasing threat of by-catch fishing. Unfortunately, at the moment, we still don't know *P.glauca* real population size, geographic distribution within the Mediterranean basin and its possible connections with the Atlantic population.

The Mediterranean Sea represents the largest semi-closed European Sea, and this particular morphology is one of the most influential and important characteristics to be considered talking about the general abundance and the status of many marine populations. The Strait of Sicily (400 km) connects the western Mediterranean Sea, including the Alborán Sea, the Balearic or Iberian Sea, the channel of Sardinia, the Sardinian Sea and the Corsica Sea, to the eastern Mediterranean Sea, consisting of the Adriatic Sea, the Ionian Sea, the Aegean Sea, the Libyan Sea and the Levantine Sea, while there's only a restricted water exchange with the Atlantic Ocean through the Strait of Gibraltar. On the basis of fisheries management purposes the Mediterranean blue shark population has been considered as independent from the North Atlantic one, nevertheless it is still unknown if there is any exchange between the two populations (Megalofonou *et al.*, 2009). Kohler et al. (2002) suggested that probably the Mediterranean blue sharks are local residents rather than occasional visitors, but today there's a lack of knowledge about the real genetic structure and phylogeographic characteristics of these individuals.

In this context the European funded project "MedBlueSGen" aims to create the largest datasets of biological samples of the species and to analyze the single nucleotide polymorphisms (SNPs) throughout the blue shark genome using the NGS technology, RAD sequencing.

The project involves different national and international academic institutions (Alma Mater Studiorum – University of Bologna; UNIPD – University of Padova; UNICAL – University of Calabria; NKUA – National and Kapodistrian University of Athens, GR; IEO – Instituto Español de Oceanografia, ESP; Queen's University of Belfast, UK) and the European Joint Research Centre (JRC, EU). The research project of this thesis is included in this European plan for the identification the genetic structure of Mediterranean BS population.

2. AIM

The fundamental goals of this work consist in providing new molecular data about the Mediterranean blue shark populations. Moreover, the genetic characterization of the Mediterranean blue shark population has been implemented with a comparison with the North-Eastern Atlantic population, using a dataset of samples coming from this geographical area. This approach derives from the necessity to estimate the extent of connectivity among these populations as they appear to be subjected to ever increasing intense fishing effort and different environmental conditions.

Four specific objectives have been fixed:

1) To create a new and unique dataset of Mediterranean blue shark mtDNA sequences available for further investigations.
2) To assess the genetic diversity and phylogeographic structure of the Mediterranean blue shark, testing the hypothesis of genetic structure within the basin.
3) To test the presence of an Atlantic-Mediterranean connection.
4) To provide informations about the historical demographic trend of the blue shark populations defined by this study.

3. Materials and methods

3.1 Blue shark tissue samples from different Mediterranean areas

The map visualization of samples collected for MedBlueSGen project can be visualized in **Fig.4**. In this context, with the final goal to cover as much as possible the entire extent of the Mediterranean Sea and a relevant part of the North-Eastern Atlantic Ocean, we have collected and analysed samples from multiple locations both in the Eastern Mediterranean Sea, such as Central Adriatic, Ionian and Aegean Sea, in the Western Mediterranean Sea, consisting of Thyrrenian and Balearic Sea, and in the North-Eastern Atlantic Ocean. The sampling dataset includes both female (F) and male (M) specimens such as large (L) and juvenile (J) sharks. The dataset consists of 195 blue shark samples (**Tab.1**).

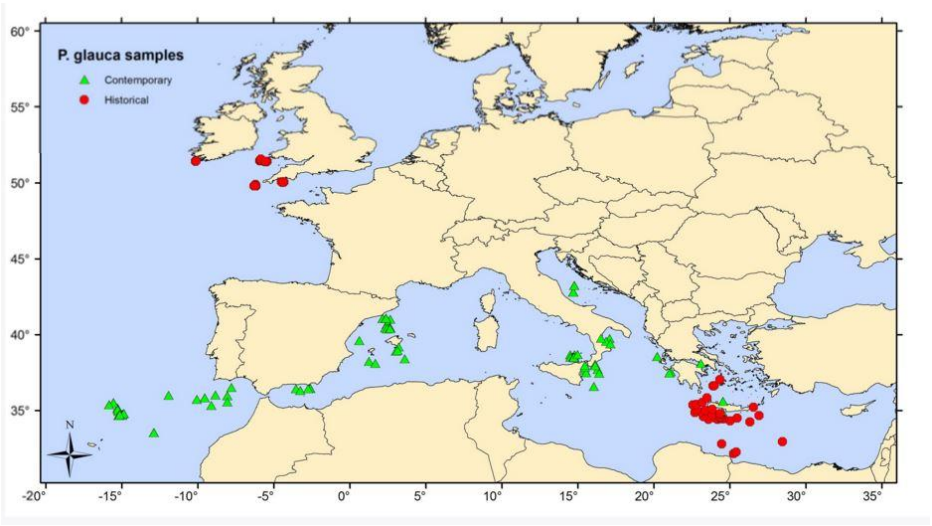

Fig.4 Capture location of historical and contemporary samples (2003-2015), downloaded from https://fishreg.jrc.ec.europa.eu/web/medbluesgen/index.html

Tab.1 Dataset of samples available separated into six geographical locations. For each area, information about number of samples available, successfully extracted mtDNA, successfully processed and sequenced at each selected locus are reported, with the addition of biological characteristics (reproductive stage) and year.

						Stage			CR		Cyt-b	
Sample area	Sample code	Available samples	Partner	Year	Tissue type	J	L	Successfully extracted mtDNA	Successfully amplified at CR locus	Successfully sequenced at CR locus	Successfully amplified at Cyt-b locus	Successfully sequenced at Cyt-b locus
North-Eastern Atlantic Ocean	**EATL**	34	IEO	2014-2015	muscle/fin	28	6	34	34	33	34	32
Western Mediterranean Sea/Balearic	**WMED**	42	IEO	2014	muscle/fin	22	20	42	42	39	42	39
Western Mediterranean Sea/Thyrrenian	**THYR**	10	UNICAL	2015	muscle/fin	10	n.a.	10	10	8	10	10
Eastern Mediterranean Sea/Central Adriatic	**CADR**	21	UNIBO	2015	muscle/fin	21	n.a.	21	21	21	21	21
Eastern Mediterranean Sea/Ionian	**IONI**	15	UNICAL	2012-2015	muscle/fin	13	2	15	15	14	15	15
Eastern Mediterranean Sea/Aegean	**AEGE**	56	NKUA	2003-2005	muscle/fin	13	43	6	6	5	6	5
		14		2015	muscle/fin	6	8	14	14	12	14	14
	TOT	192				113	79	142	142	132	142	136

3.2 DNA extraction

DNA extraction was carried out using two different extraction and purification kit, following the manufacturers' protocols:

- Invisorb® Spin Tissue Kit, Invitek (© STRATEC Molecular)
- Wizard® Genomic DNA Purification Kit, Promega

Comparing the two procedures used to extract DNA, we have to consider some important differences and highlight their advantages and disadvantages; as I've already explained, the use of any DNA extraction kit depends on various factors, including the aim of the investigation we're going to carry out and, therefore, the analysis we want to develop using the DNA solution obtained; each DNA extraction procedure will give us DNA solutions differing each other in terms of nucleic acid quantity and quality. In this case, we can say that Wizard® Genomic DNA Purification Kit (Promega) definitely allow the DNA extraction from much more samples in less time than Invisorb® Spin Tissue Kit (Invitek), nevertheless it would probably have a major risk of contamination among samples. However, the extracted DNAs have been later analyzed using microdrop-MultiskanGO (© Thermo Scientific Fisher Corporation) by UNIPD partners (University of Padova) in order to carry out a DNA quality and quantity check; results from microdrop analyses show a majority of DNA samples exhibiting =/> 1.2 ratio, a part of samples with 1.0<ratio<1.2 while a part of samples, corresponding to those collected from Aegean Sea between 2003-2005, shows </=1.0 ratio; for this reason, we decided to exclude them from successive analyses.

3.3 Design of species-specific primers for mitochondrial markers "control region" and "cyt-b"

The "control region" marker is a non-coding mtDNA region, extremely variable, associated with the initiation of DNA replication, and it has already been used for *P. glauca* in previous work together with three to five microsatellite loci (Ovenden *et al.*, 2009). Ovenden et al. (2009) amplified and sequenced the 5' end of mtDNA control region using the forward primer ProL2 and the reverse primer PheCacaH2 designed by Pardini et al. (2001). Approximately 1145 bp (base

pairs) were amplified and 400 bp of the control region were sequenced in one direction using the forward primer. However, I decided to design new species-specific primers for the amplification of mtDNA "control region" and to test both the previous and the new primers to understand which pair would work best.

No primer specific for the blue shark were available in literature for the mtDNA cytochrome b region, so I designed them *ex-novo*. The mtDNA is easily to isolate and it is characterized by a high number of copies, a long sequence and a turnover of highly preserved and variable regions. It also shows more frequent evolutionary variations rather than the nuclear DNA and they are independent from it, so it appears much more appropriate to use it in order to analyse phylogenetic variations both within the same population and between different populations. We decided to analyze control region and cyt-b markers because of their variability: blue sharks are characterized by great vagility, so it would not be advantageous, in order to find a genetic diversity and a real genetic structure and phylogeography, to consider and analyze mtDNA regions highly preserved, such as CO1 region.

To design primers, I initially used the complete database of sequences of *P. glauca* available in GenBank, NCBI®. I downloaded all the available sequences of control region and cyt-b in FASTA format. Once obtained control region and cyt-b sequences, they have been aligned using ClusterW algorithm implemented in MEGA ver.6.0 (Tamura *et al.*, 2013). Then primer pair for control region and cyt-b were designed using the online software "PRIMER3input (ver.0.4.0)" (Rozen *et al.*, 2000). Primer pairs for the two selected markers, each one with its characteristics, are in Table 2.

Libraries and functions in "PRIMER3input" calculate oligonucleotide melting temperature (Tm), the propensity of oligos to form hairpins or dimers or to hybridize or prime from unintended sites in the genome, performing an exhaustive search for the best primer pairs given the specified template sequence (Untergasser *et al.*, 2012).

The designed primers were tested using the simulation "AmplifX" software version 1.44 (Nicolas Jullien [http://ifrjr.nord.univ-mrs.fr/AmplifX-Home-page]) generating the hypothetical fragments

that will be amplified on the hypothetical annealing positions of the gene and the hypothetical primer annealing temperatures.

Also the available primer pair for "control region" "ProL-2 – PheCacaH2" (Pardini *et al.*, 2001) was tested in order to compare it with the new primers designed and choose the best one.

The primer pairs were first tested in order to identify optimal amplification conditions. Consequently, a first test was carried out by setting a 10°C temperature gradient to identify the most suitable melting temperatures (Tm from 50°C to 60°C) using the same PCR cycling conditions used by Ovenden et al. (2009) for CR primer pair "ProL2-PheCacaH2".

For this assessment a DNA from only one blue shark sample, casually chosen, was tested for the two primer pairs "CR-Blues-F – CR-Blues-R" and "ProL2 – PheCacaH2" for control region marker. Once identified the optimal melting temperature of primer pairs, the PCR thermal profile was adjusted as described in Fig.5 and the PCR reaction is composed by:

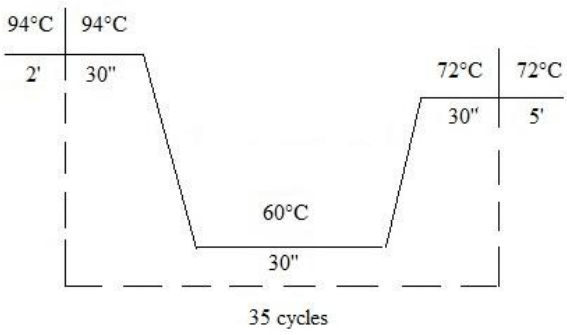

Fig.5 PCR definitive thermal profile and conditions.

PCR reaction final volume of 50 µL containing 31.75 µL of H2O, 8 µL of Buffer 10x (Tris-HCl), 3 µL of $MgCl_2$, 2 µL of dNTPs, 2.5 µL of each primer, 0.25 µL of Taq polymerase and 2 µL of template DNA. The temperature profile included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30s, elongation at 72°C for 30 s and a final elongation step at 72°C for 5 min.

The same evaluation was carried out for the amplification of cyt-b marker.

Primer pairs for the two selected markers, each one with its characteristics, are in **Tab.2**.

Tab.2 Control region and Cytochrome b primer pairs with associated characteristics of primer sequence, GC% (guanine and cytosine content) and melting temperature (Tm).

Marker	Primer	Primer Sequence	GC%	Tm(C°)
Control region (CR)	**CR-Bues-F**	5'-AAA CAC ATC AGG GGA AGG AG-3'	50	58.4
Control region (CR)	**CR-Blues-R**	5'-CAT CTT AGC ATC TTC AGT GCC-3'	47.62	59.4
Cytochrome b (cytb)	**cyt-b-Blues-F**	5'-TCC TCA CAG GAC TCT TCC TAG C-3'	54.55	64
Cytochrome b (cytb)	**cyt-b-Blues-R**	5'-GTC GAA AGA TGG TGC TTC GT-3'	50	58.4

Comparing the gradient test results for the amplification of control region marker, I decided to use "CR-Blues-F – CR-Blues-R" primer pair for further amplifications. Both "control region" and "cyt-b" primer pairs gave positive results in PCR amplifications and amplicons have been sequenced using the sequencing service "EZ-seq V2.0" provided by MACROGEN® Europe.

4. Data analysis

Once obtained CR and Cyt-b sequences, different analyses were carried out in order to identify and describe genetic diversity and the extent of differentiation among and within Mediterranean and NE Atlantic populations. In addition to these analyses, the demographic trends of the populations and the phylogeographic patterns were investigated.

First, all sequences obtained were validated with the homologous gene sequences deposited in the GenBank using the BLAST algorithm implemented in MEGA 6.0 (Tamura *et al.*, 2013).

The sequence datasets have been analyzed with DNAsp software (Librado & Rozes, 2009), in order to assess the genetic diversity in terms of the number of haplotype, the haplotype diversity (Hd), and the nucleotide diversity (Pi) with their associated standard deviation (sd) and the number of polymorphic sites. The genetic diversity analyses have been carried out considering each sequence dataset divided into six populations (East-Atlantic, EATL; West-Mediterranean/Balearic, WMED; Tyrrhenian Sea, THYR; Calabria Ionian Sea, IONI; Central Adriatic Sea, CADR; Aegean Sea, AEGE). In order to investigate at a more specific level the "juvenile" (J) and "large" (L) members of each population were also considered separately. In addition, the Aegean Sea sample (AEGE) dataset was splitted separating individuals sampled in different period of time (2002 and

2005/2015), in order to evaluate possible differences in terms of genetic diversity between these temporal groups. Finally, each value has been calculated for the entire Mediterranean (MED) to directly compare it with Eastern Atlantic (EATL) values.

To investigate haplotype relationships, the parsimony network was created using HAPLOVIEWER (http://www.cibiv.at/~greg/haploviewer) and the *dnapars* program of the PHYLIP package version 3.6 (Felsenstein, 2005).

The software Arlequin ver 3.5.2.2 (Excoffier & Lischer, 2010) has been used to calculate the haplotype frequencies and pairwise Φst matrix with the relative p-values after 10000 permutations, setting up a 0,05 significance level; however, the significance was later adjusted using various methodology of correction for multiple testing implemented in SGoF+ (Carvajal-Rodriguez & de Uña-Alvarez,. 2011). The best-fit model of nucleotide substitution, generated in MEGA6, resulted Tamura-Nei (1993) distance model (TN93).

To perform the Principal Component Analysis (PCoA), pairwise Φ_{ST} distances were transformed into Euclidean matrices through the addition of a smallest positive constant (Cailliez, 1983) and were used to construct scatter plots using the ade4 (Dray & Dufour, 2007) and ape (Paradis, *et al.* 2004) packages in R 3.0.2 (R Development Core Team. 2013).

AMOVA analysis (Analysis of Molecular Variance), implemented in Arlequin software, has been used in order to estimate the extent of variance occurring among and within imposed groups. On the basis of the results of PCoA analysis and pairwise Φst matrix, we tested different combination of groupings from our sample dataset, described as follows:

- AMOVA1 - All Samples, NO groups;

- AMOVA2 - All Samples, three groups: EATL vs WMED (Balearic, Thyrrenian Sea) vs EMED (Central Adriatic, Ionian, Aegean Sea). These groupings were tested considering the separation into the three major geographical areas, as seen for other similar species;

- AMOVA3 - All Samples, four groups: EATL vs WMED (Balearic, Thyrrenian Sea) vs CADR vs IONI+AEGE. This additional combination was tested on the basis of results observed in pairwise Φst matrix.

The AMOVA analysis defines the significance of the variance within and among imposed groups that can be used to detect if the structures considered are significant or not, observing which combination best represents the general distribution pattern of our samples.

Successively, the historical demographic trend of the Mediterranean (MED) samples - obtained considering its different sub-geographical areas together (Balearic Sea, WMED; Thyrrenian Sea, THYR; Ionian Sea, IONI; Central Adriatic Sea, CADR; Aegen Sea, AEGE) and the North-Eastern Atlantic one (EATL) have been investigated using Bayesian Skyline Plot implemented in the software BEAST v.1.8.2 (Drummond & Rambaut, 2007; Drummond *et al.*, 2012), using the best evolutionary model calculated with MEGA6. At the same time, we decided to carry out an historical demographic analysis using also the "Mismatch distribution" analysis implemented in the DNAsp software (Librado & Rozas, 2009), with the aim to assemble the results obtained by the two software. Together with mismatch distribution analysis, Tajima's *D* and Fu's *Fs* neutrality tests (Holsinger, 2013) were carried out in order to assess if any pattern generated could be addressed to a random evolution trend or one under a non-random process, such as demographic expansion or contraction.

Finally, in order to define the phylogeny of the Mediterranean and Eastern Atlantic blue shark populations, we used the software BEAST v.1.8.2 to construct a plausible phylogenetic tree of the species using the evolutionary rate estimated for both markers by Martin et al. (1992). Associated software TreeAnnotator was used to identify a single tree that best represents the distribution, and FigTree software was used as a graphical viewer of the phylogenetic tree produced.

5. Results

Mitochondrial DNA amplifications and consecutive sequencing carried out using our new primer pairs appeared successful in terms of sequence quality and length, and it allows us to affirm that the primer pairs "CR - Blues" and "cytb - Blues" designed *ex novo* are highly performing.

The number of sequences available for all planned data analysis resulted lower than the effective number of amplified samples sequenced (**Tab. 1)**; this difference was due to the fact that some sequences, both for CR and Cyt-b mitochondrial markers, produced suboptimal results in terms of information quality, so we decided to exclude them from the two sequence datasets in order to avoid the risk of adding artefacts as inappropriate polymorphic sites.

For the Cyt-b and CR markers were created respectively a 762 bp alignment of 136 sequences with 15 polymorphic sites (2%), and a 857 bp alignment of 132 sequences with 51 polymorphic sites (6%).

5.1 Genetic diversity

Considering Cyt-b data, the highest haplotype diversity was shown by Ionian Sea - IONI - samples (0.886) - **Tab.3**. Generally, no considerable differences in haplotype and nucleotide diversity were reported within Mediterranean locations (WMED; THYR; CADR; IONI; AEGE), but they differ from the North-Eastern Atlantic group, which show the lowest haplotype diversity (0.613). No relevant differences were detected between juvenile and large individuals within its geographical sample, except for North-Eastern Atlantic and Ionian Sea haplotype diversity values, but these differences between the two size classes have to be interpreted in the light of a disproportion in the number of available sequences for each size class (**Tab. 3)**. Haplotype diversity analysis carried out for CR dataset showed higher values compared with those obtained for the Cyt-b marker, with AEGE group exhibiting the lowest, but anyway high, values. Higher number of haplotypes was obtained at the CR locus, a data presumably coherent to the fact that it is a highly variable region of mitochondrial DNA. Substantially, results obtained from this analysis suggested, especially for CR,

the presence of a low diversification between the population considered, both within Mediterranean groups and among Mediterranean and Eastern Atlantic specimens.

Tab.3 Haplotype (Hd) and nucleotide diversity (Pi) with standard deviation (sd) and number of polymprphic sites (Pol.Sites) of the Mediterranean and North-Eastern Atlantic individuals at the two mtDNA loci (CR and CYT-B). Individuals were subdivided in six geographical locations and each value was calculated also for the entire MED area, for each size class within the locations and the two temporal classes within the AEGE area. Values corresponding to each total geographical location were grey highlighted, while values calculated considering the entire dataset of samples were red highlighted.

		CR							CYT-B						
Sample	Year	n	n° haplotypes	Hd	sd	Pi	sd	Pol. sites	n	n° haplotypes	Hd	sd	Pi	sd	Pol. sites
EATL - TOT		33	23	0.966	0.018	0.00527	0.00048	23	32	5	0.613	0.070	0.00114	0.00021	5
EATL - J		27	20	0.969	0.021	0.00541	0.00058	22	26	4	0.563	0.084	0.00104	0.00024	4
EATL - L		6	6	1.000	0.096	0.00477	0.00089	9	6	4	0.867	0.129	0.00157	0.00036	3
WMED - TOT		39	25	0.966	0.015	0.00495	0.00046	26	39	10	0.812	0.042	0.00202	0.00026	9
WMED - J		22	15	0.952	0.029	0.00451	0.00049	17	20	9	0.847	0.061	0.00218	0.00034	8
WMED - L		17	15	0.985	0.025	0.00540	0.00082	19	19	7	0.772	0.075	0.00184	0.00037	7
THYR - TOT		8	7	0.964	0.077	0.00729	0.00096	15	10	5	0.800	0.100	0.00140	0.00030	4
THYR - J		8	7	0.964	0.077	0.00729	0.00096	15	10	5	0.801	0.101	0.00141	0.00031	4
THYR - L		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
CADR - TOT		21	14	0.962	0.024	0.00454	0.00043	14	21	8	0.833	0.055	0.00196	0.00029	7
CADR - J		21	14	0.962	0.024	0.00454	0.00043	14	21	8	0.834	0.056	0.00197	0.00030	7
CADR - L		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a	n.a	n.a	n.a	n.a	n.a	n.a
IONI - TOT		14	13	0.989	0.031	0.00706	0.00108	19	15	9	0.886	0.069	0.00185	0.00032	8
IONI - J		13	12	0.987	0.035	0.00613	0.00085	17	13	9	0.910	0.068	0.00195	0.00034	8
IONI - L		1	-	-	-	-	-	-	2	2	1.000	0.500	0.00131	0.00066	1
AEGE - TOT		17	9	0.860	0.068	0.00380	0.00078	13	19	9	0.778	0.096	0.00216	0.00042	7
AEGE - TOT	2003-2005	5	5	1.000	0.126	0.00586	0.00183	12	5	4	0.900	0.161	0.00262	0.00054	4
	2015	12	6	0.818	0.096	0.00311	0.00048	6	14	6	0.747	0.111	0.00172	0.00040	5
AEGE - J - TOT		6	5	0.933	0.122	0.00336	0.00063	6	7	6	0.952	0.096	0.00312	0.00064	6
AEGE - J	2003-2005	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	2015	5	4	0.900	0.161	0.00375	0.00073	6	6	5	0.933	0.122	0.00254	0.00063	5
Aegean Sea - L - TOT		35	23	0.963	0.017	0.00524	0.00063	29	12	6	0.682	0.148	0.00167	0.00049	6
Aegean Sea - L	2003-2005	4	4	1.000	0.177	0.00723	0.00207	12	4	3	0.833	0.222	0.00219	0.00058	3
	2015	7	4	0.810	0.130	0.00301	0.00088	6	8	4	0.643	0.184	0.00122	0.00044	3
MED - TOT		99	55	0.963	0.009	0.00556	0.00036	46	104	20	0.818	0.027	0.00197	0.00016	14
TOTAL		132	70	0.968	0.007	0.00564	0.00029	51	136	21	0.787	0.025	0.00181	0.00014	15

5.2 Haplotype Network and Haplotype frequencies in populations

The CR (**Fig.6**) and Cyt-b (**Fig.7**) haplotype networks didn't show any structuring between geographical locations analysed. The reconstruction based on Cyt-b data better show the presence of two major haplogroups more structured than the other, which present a simple star-like pattern. The network based on the CR haplotypes shows the presence of a major number of haplotypes, most of them made up by only one specimen, with less structuring than Cyt-b and a pattern more coherent with results from Φst pairwise analysis as most of IONI samples tend to diverge from other locations.

The relative amounts of each haplotype in the different geographical samples are listed in **Tab.4** (in Appendix). As shown by the two networks, haplotype inference also highlighted the presence for for the Cyt-b marker of two haplogroups shared by the majority of specimens from each location

considered (Hap2, Hap3). Observing CR occurrence of haplotype in each location, three are the most numerous haplotypes, although they are not shared by individuals from all geographical area.

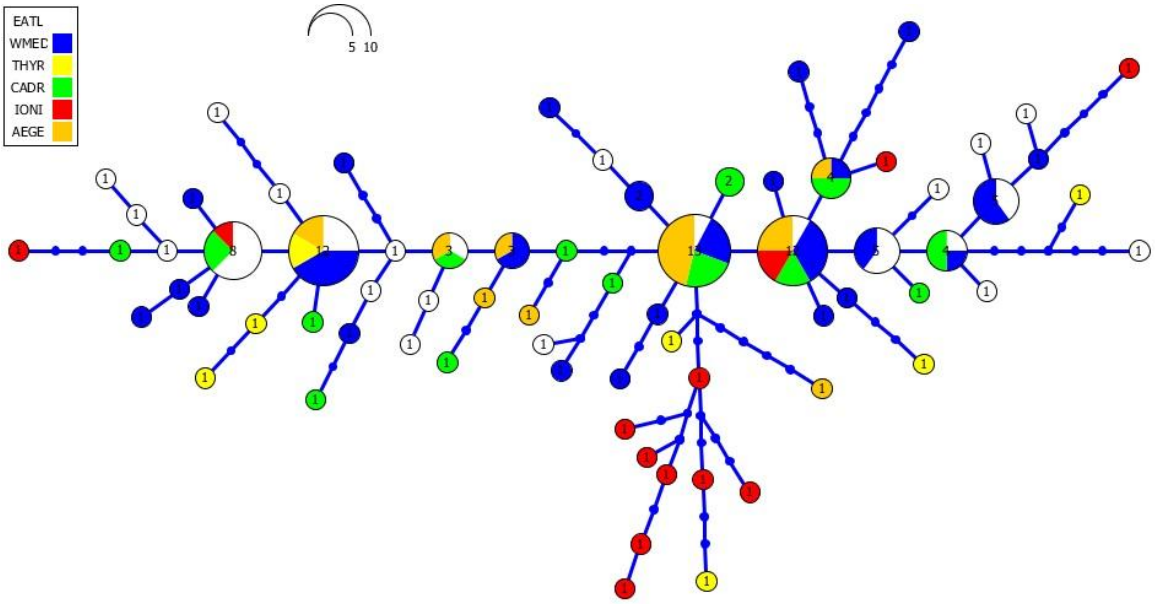

Fig.6 Control region (CR) Haplotype Network form Mediterranean and Eastern Atlantic blue shark populations. Each node represents a haplotype, with its frequency related to the node diameter. Numbers indicate the amount of specimens sharing the haplotype.

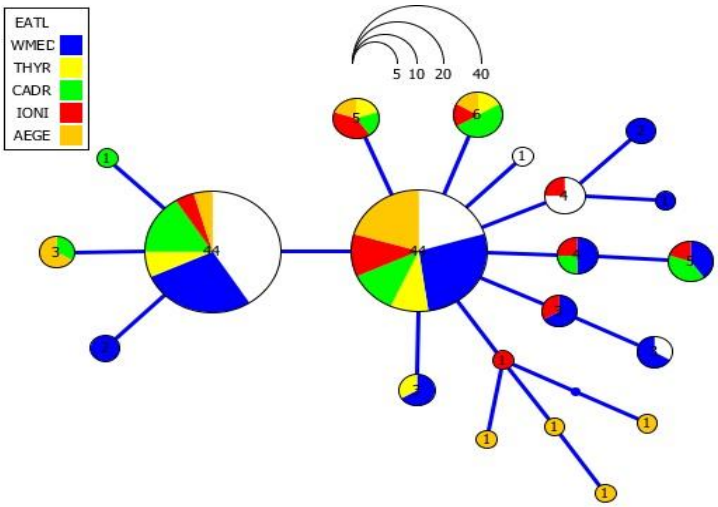

Fig.7 Cytochrome b (cyt-b) Haplotype Network form Mediterranean and Eastern Atlantic blue shark populations. Each node represents a haplotype, with its frequency related to the node diameter. Numbers indicate the amount of specimens sharing the haplotype.

5.3 Population pairwise ΦSTs

The Cyt-b Φst pairwise matrix (**Tab. 5)** showed significant comparisons only between comparisons between Norht-Eastern Atlantic ad Ionian and North-Eastern Atlantic and Aegean samples also after Benjamini-Hockberg (B-H) correction, so between locations more geographically distant. In CR Φst pairwise matrix more significant results were detected, with differentiation values between 4% and 25%, due to higher level of variability between locations (**Tab.5**).

Same analysis was carried out considering exclusively juvenile specimens from each location for both CR and Cyt-b marker, in order to verify possible different patterns in terms of differentiation ascribable to young individuals and so depending to the reproductive stage (**Tab.6**). This additional analysis was performed considering previous evidences reported by Quieroz et al. (2012) and Vandeperre et al. (2014), suggesting that a different reproductive stage shows different migration behaviour and tend to occupy different area.

Tab.5 Cyt-b and CR Φst pairwise matrix. Values in bold were significant only before BH correction (P value<0,05), while underlined values remained significant also after the correction.

Cyt - b						
	EATL	**WMED**	**THYR**	**CADR**	**IONI**	**AEGE**
EATL	*	0.06950+-0.0022	0.10702+-0.0032	0.09148+-0.0028	0.00386+-0.0006	0.00406+-0.0007
WMED	0.02910	*	0.61331+-0.0049	0.28255+-0.0045	0.20018+-0.0038	0.03732+-0.0018
THYR	0.05243	-0.01719	*	0.64261+-0.0046	0.68290+-0.0044	0.59331+-0.0053
CADR	0.03658	0.00656	-0.02672	*	0.20028+-0.0040	0.22087+-0.0042
IONI	**0.15216**	0.01603	-0.02459	0.02388	*	0.66696+-0.0044
AEGE	**0.11449**	**0.04038**	-0.01740	0.01695	-0.01688	*
CR						
	EATL	**WMED**	**THYR**	**CADR**	**IONI**	**AEGE**
EATL	*	0.02693+-0.0015	0.00297+-0.0006	0.09920+-0.0029	0.00000+-0.0000	0.00139+-0.0004
WMED	**0.04114**	*	0.00426+-0.0006	0.68261+-0.0047	0.00000+-0.0000	0.07742+-0.0023
THYR	**0.16552**	**0.15020**	*	0.01228+-0.0012	0.03465+-0.0018	0.01208+-0.0010
CADR	0.02873	-0.01213	**0.14885**	*	0.00030+-0.0002	0.24809+-0.0038
IONI	**0.25534**	**0.18621**	**0.09376**	**0.18630**	*	0.00010+-0.0001
AEGE	**0.14161**	0.03566	**0.17815**	0.01257	**0.18102**	*

Tab.6 Cyt-b and CR Φst pairwise matrix calculated only for juvenile specimens from each location. Values in bold were significant only before B-H correction (P value<0,05), while underlined values remained significant also after the correction.

Cyt - b - Juvenile specimens						
	EATL	**WMED**	**THYR**	**CADR**	**IONI**	**AEGE**
EATL	*	0.16078+-0.0037	0.09316+-0.0028	0.06970+-0.0024	0.00050+-0.0002	0.03534+-0.0018
WMED	0.02119	*	0.52411+-0.0047	0.37214+-0.0047	0.04079+-0.0019	0.13316+-0.0031
THYR	0.08303	-0.01218	*	0.64627+-0.0041	0.46124+-0.0051	0.48470+-0.0052
CADR	0.04563	0.00145	-0.02671	*	0.10256+-0.0028	0.34323+-0.0050
IONI	**0.22714**	**0.06663**	-0.00384	0.04431	*	0.24087+-0.0041
AEGE	**0.13260**	0.04904	-0.00511	0.00622	0.02905	*
CR - Juvenile specimens						
	EATL	**WMED**	**THYR**	**CADR**	**IONI**	**AEGE**
EATL	*	0.05475+-0.0023	0.00505+-0.0007	0.07673+-0.0025	0.00000+-0.0000	0.09039+-0.0029
WMED	0.04427	*	0.01614+-0.0013	0.31957+-0.0042	0.00000+-0.0000	0.46025+-0.0047
THYR	**0.15772**	**0.12795**	*	0.01238+-0.0010	0.01445+-0.0012	0.10831+-0.0027
CADR	0.03644	0.00377	**0.14885**	*	0.00000+-0.0000	0.71716+-0.0047
IONI	**0.31182**	**0.24014**	**0.13123**	**0.23938**	*	0.00851+-0.0010
AEGE	0.07622	-0.01037	0.10265	-0.04682	**0.19317**	*

Observing the two tables, it is clear how the pattern related to Φst pairwise matrix calculated for the entire dataset of samples and the one related only to juvenile specimens are largely overlapped, so we can suggest that in this analysis no considerable differences in the extent of structuring related to the reproductive stage were reported.

5.4 Principal Coordinates Analysis (PCoA)

Both CR (**Fig. 8**) and Cyt-b (**Fig.9**) PCoA, according to the results of the previous analysis, confirmed the apparent absence of a structuring between blue sharks populations, particularly between the two major groups on which we decided to focus attention (Mediterranean Sea and North-Eastern Atlantic). Each PCoA was performed illustrating the resulting plot both in individual genetic distances (plot 1.) and haplotype distances (plot 2.).

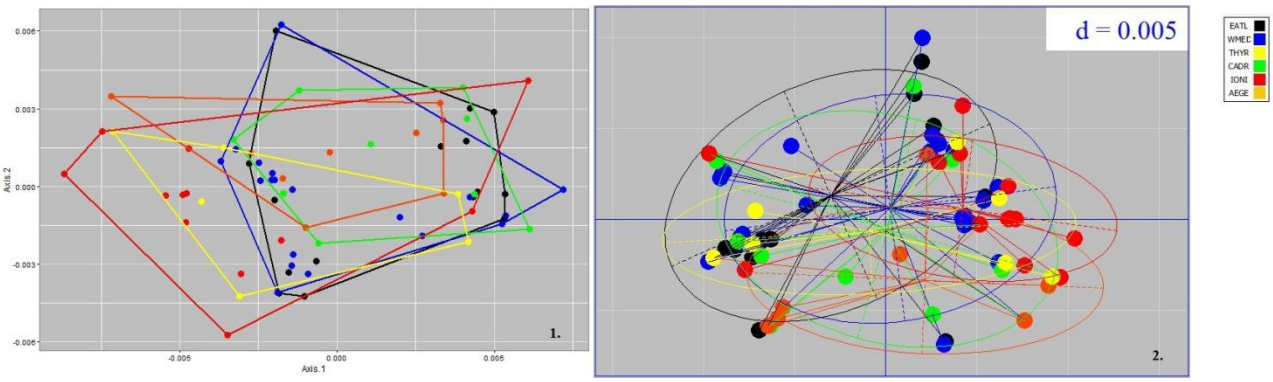

Fig.8 Principal Coordinates plots for CR marker. Plot 1. describes individual genetic distances, while plot 2. describes haplotype distances. Polygons and ellipses represent the 95% confidence interval of samples (points) distribution.

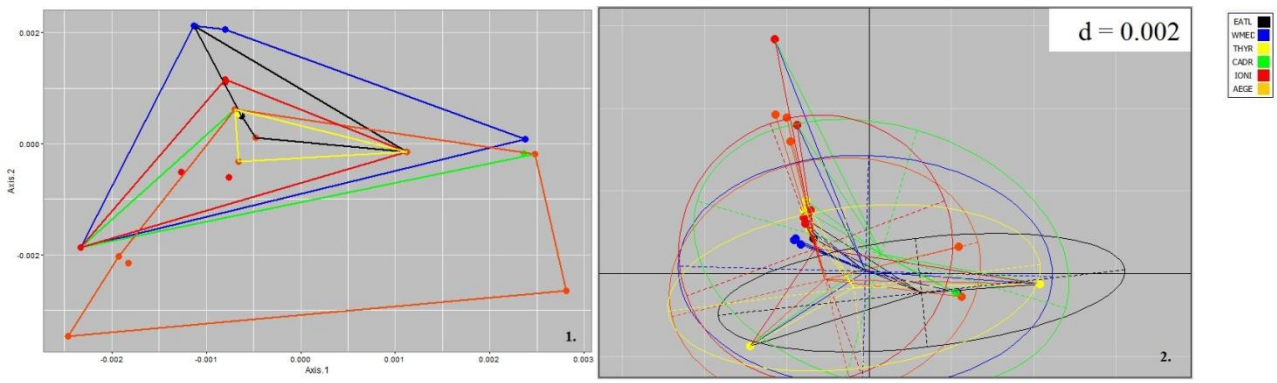

Fig.9 Principal Coordinates plots for Cyt-b marker. Plot 1. describes individual genetic distances, while plot 2. describes haplotype distances. Polygons and ellipses represent the 95% confidence interval of samples (points) distribution.

5.5 AMOVA analysis

AMOVA results are shown in **Tab.7**. We tested different combination of groups with the aim of identifying the one that best summarize and represents the variance among groups imposed on the basis of our assumptions. The first assumption ("NO groups") was tested on the basis of the results obtained from the other analysis, suggesting a lack of structuring between samples, while the second assumption (EATL vs WMED vs EMED) was made considering the entire dataset divided into the three major geographical area, making the hypothesis that the Strait of Gibraltar and Strait of Sicily could represent geographical boundaries as for other similar species. The third assumption was made to test the results of Φst pairwise difference analysis, which showed a more significant differentiation between samples geographically distant, such as EATL and IONI and AEGE.

Tab.7 AMOVA analysis for three different combination samples groups. Results in terms of total amount of variation (%) for each source of variance, F statistics and associated P-values were reported for both CR and Cyt-b marker.

AMOVA–Groupings	CYT-B				AMOVA–Groupings	CR			
AMOVA1-All samples, NO groups					AMOVA1-All samples, NO groups				
		Total variation (%)	*F* statistics	P			Total variation (%)	*F* statistics	P
Among populations		3,23	FST 0,03226	0.01663+-0.00124	Among populations		10,37	FST 0,10375	0.00000+-0.00000
Within populations		96,77			Within populations		89,63		
AMOVA–Groupings	CYT-B				AMOVA–Groupings	CR			
AMOVA2- All samples, 3 groups: EATL vs WMED vs EMED		(North-Eastern Atlantic) vs (Balearic, Thyrrenian Sea) vs (Central Adriatic, Ionian, Aegean Sea)			AMOVA2- All samples, 3 groups: EATL vs WMED vs EMED		(North-Eastern Atlantic) vs (Balearic, Thyrrenian Sea) vs (Central Adriatic, Ionian, Aegean Sea)		
		Total variation (%)	*F* statistics	P			Total variation (%)	*F* statistics	P
Among groups		2,9	FCT 0,029	0.15010+-0.00353	Among groups		-4,27	FCT -0,04268	0.69584+-0.00439
Among population within groups		0,85	FSC 0,00877	0.40911+-0.00530	Among population within groups		13,98	FSC 0,13406	0.00010+-0.00010
Within population		96,25	FST 0,03752	0.01594+-0.00128	Within population		90,29	FST 0,0971	0.00000+-0.00000
AMOVA–Groupings	CYT-B				AMOVA–Groupings	CR			
AMOVA3-All samples, 4 groups: EATL vs WMED vs CADR vs IONI+AEGE		(North-Eastern Atlantic) vs (Balearic, Thyrrenian Sea) vs (Central Adriatic) vs (Ionian, Aegean Sea)			AMOVA3-All samples, 4 groups: EATL vs WMED vs CADR vs IONI+AEGE		(North-Eastern Atlantic) vs (Balearic, Thyrrenian Sea) vs (Central Adriatic) vs (Ionian, Aegean Sea)		
		Total variation (%)	*F* statistics	P			Total variation (%)	*F* statistics	P
Among groups		4,77	FCT 0,04765	0.04535+-0.00195	Among groups		-8,93	FCT -0,08925	0.77386+-0.00443
Among population within groups		-1,12	FSC -0,01176	0.74337+-0-00438	Among population within groups		18,66	FSC 0,17134	0.00010+-0.00010
Within population		96,35	FST 0,03646	0.01574+-0.00137	Within population		90,26	FST 0,09738	0.00000+-0.00000

As we can see in **Tab.7**, the combination which show significant values both for CR and Cyt-b markers is the one in which samples have not been divided into pre-established groups, a result that confirm the evidence already shown by previous analysis of a lack of structuring among the geographical locations considered.

5.6 Demographic Analysis

The Bayesian Skyline Reconstruction was performed for both EATL and MED samples separately, although all previous analysis showed that there's no structuring between them. We initially tried to construct the bayesian plot considering EATL and MED samples together, but the software produced non optimal results in terms of ESS values (Effective Sample Size) which couldn't produce a reliable plot. For this reason we considered the two locations separately, and the Bayesian Skyline plot obtained corresponded to a general stable demographic pattern in Cyt-b (**Fig.10**). Nevertheless, the control region showed a slight demographic increase dated between 250 and 150 Kya, during Pleistocene epoch (**Fig.11**).

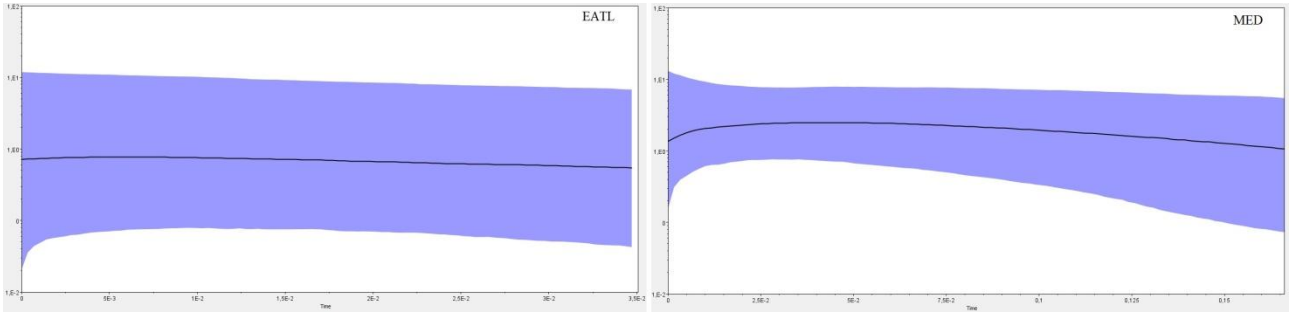

Fig.10 Bayesian Skyline Plot of Cyt-b marker from North-Eastern Atlantic (EATL) and Mediterranean (MED) area. Blue areas represents the 95% confidence intervals.

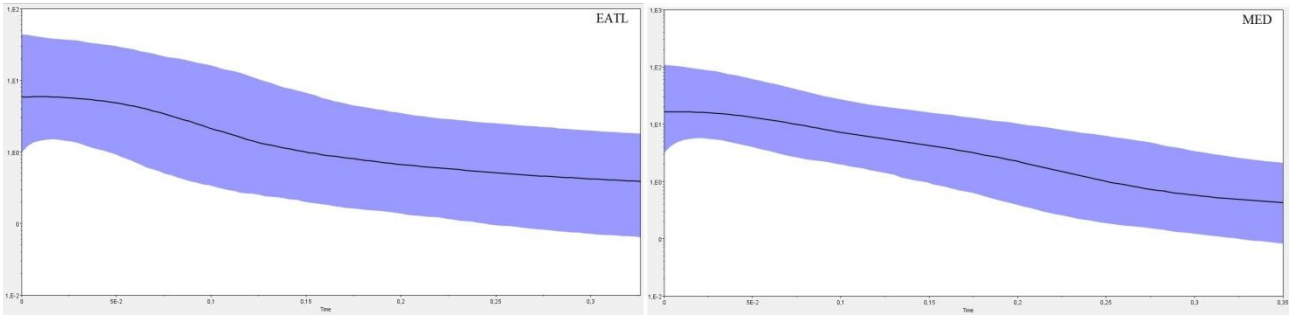

Fig.11 Bayesian Skyline Plot of CR marker from North-Eastern Atlantic (EATL) and Mediterranean (MED) area. Blue areas represents the 95% confidence intervals.

The mismatch distribution trends of the Cyt-b dataset showed a ragged distribution associated with constant population size (**Fig.12**). In contrast, mismatch distribution trends of the control region dataset show a skewed unimodal distribution related to recent bottleneck or sudden population

expansion (**Fig.13).** However, in all cases the values of mismatch distribution and the neutrality tests (Fu's, Tajima) gave no significant results.

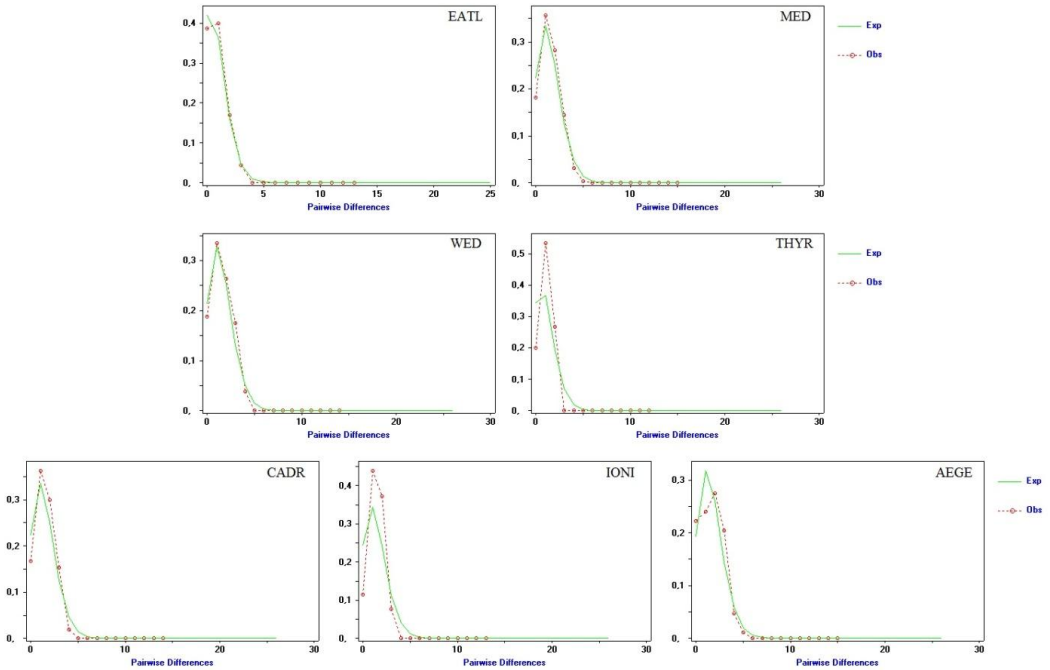

Fig.12 Cyt-b Mismatch distribution constructed for each geographical location, with expected (green) and observed (red) values of distribution.

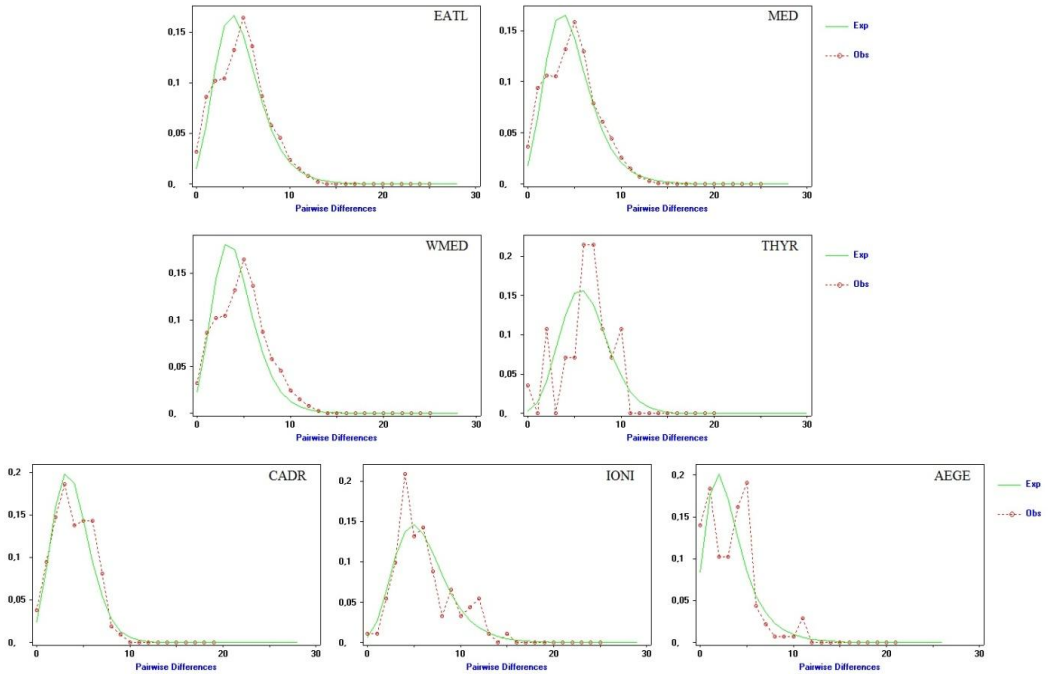

Fig.13 CR Mismatch distribution constructed for each geographical location, with expected (green) and observed (red) values of distribution.

The Bayesian tree showed as expected a topology not defined by populations, with clusters shared by all populations of the study area, stressing the lack of genetic structure among populations.

The divergence time analysis revealed an highly significant separation between the two major clades for both markers, dated at 1,2/1,3 Mya, during Pleistocene epoch **(Fig.14**; **Fig.15** - In Appendix**)**.

6. Discussions and Conclusions

The research project developed in this thesis concerned with the identification of the genetic structure characteristics of blue shark, *Prionace glauca*, population within the Mediterranean Sea, a geographical area still unexplored for this species. Therefore, the principal goal was to fill a gap in the available knowledge relative to this particular aspect.

The evaluation of blue shark biological productivity reported by ICCAT showed the highest values of susceptibility to capture and mortality in longline pelagic fisheries for blue shark Atlantic stocks (ICCAT, 2009). We know that the blue shark is rarely included in target commercial species, and it represents a major bycatch of longline and driftnet fisheries. The majority of this bycatch is often unrecorded, causing problems in the assessment of the effective stock size and its real availability. In this perspective the results of the analyses developed in this work can be useful especially because of the fact that the lack of data regarding the status of the Mediterranean population could probably expose this species to the risk of an underestimated overexploitation.

The lack of previous information about Mediterranean blue sharks, specifically in terms of genetic diversity and population structure, limits the possibility of having an extensive dataset to analyse and compare with new results, but on the other hand gave us the opportunity to create a new and unique data resource (mtDNA control region and cytochrome-b sequences) available for future investigations.

Generally, observing the results obtained in this work we can immediately identify a lack of structuring and differentiation between samples analysed, as found in previous works for other blue shark populations.

Concerning the genetic diversity, results obtained from the analysis of haplotype and nucleotide diversity parameters seems to be in agreement with evidences from studies carried out investigating the genetic composition of blue sharks in other geographical areas, such as Pacific, Indian and Atlantic Ocean (Ovenden *et al.*, 2009; Raquel & Sampaio, 2013), highlighting the absence of a considerable genetic differentiation also within the Mediterranean Sea. In addition to this, comparison between haplotype and nucleotide diversity values obtained for North-Eastern Atlantic group and the whole Mediterranean location gave the same result, leading to the hypothesis that presumably these blue shark specimens could have similar fishing pressure **(Tab.3)**. Furthermore, the high values of genetic diversity observed suggest, in contrast with previous modeling based on fishing data (Ferretti, *et al.*, 2008), that the populations has not yet reached levels of exploitation that could erode its genetic diversity. Probably, the conservation of the genetic diversity can also be due to the short generation time, compared with that of other shark species, and the high productivity of the species in terms of pups (Pratt, 1979; Nakano & Stevens, 2008).

The majority of significant pairwise differentiations between samples were observed in the CR marker, rather than the Cyt-b, due to a higher value of variability on a non-coding genetic marker.

The Φst values of differentiation are consistent with the geographical distances between locations, however the Ionian Sea population, IONI, is the more differentiated from the others, with a maximum divergence of 25% from the Atlantic population and a minimum divergence from the population of the South Tyrrhenian (9%). The low values of Φst among populations of EATL and WMED, and between IONI and THYR, indicate that the maternal gene exchanges between these two geographical groups, through the Strait of Gibraltar and the Strait of Messina respectively, is greater than exchanges between the others areas. The additional analysis of Φst values of

differentiation performed only on juvenile specimens from each geographical area, carried out in order to verify a possible different pattern related to the reproductive stage, gave similar results to those obtained for the entire dataset of samples. This result is not coherent with what has been found earlier (Quieroz *et al.*, 2012; Vandeperre *et al.*, 2014), regarding the tendency of juvenile individuals to move in different directions compared with large specimens. However, this result has to be observed in the light of a considerable difference in the number of juvenile and large blue shark samples available in this work.

In addition to this, it is clear from the analysis of the network that, although there is a minimum genetic differentiation between populations, no structure between MED and EATL areas, and within the MED, are observed, enabling us to formulate the total lack of phylogeographic barriers for this extremely vagile species, as already observed in previous researches (Ovenden *et al.*, 2009; Raquel & Sampaio, 2013). It is possible to hypothesize that the Central Atlantic nursery area highlighted by Sampaio da Costa, (2013) is a structuring part of the reproductive cycle of Mediterranean specimens, with a maternal gene flow between these two areas.

It is clear from the network analyses that one of the two largest clusters, in both markers, is more differentiated, suggesting it as the ancestral cluster where all the geographical groups and the sub-areas sampled in the present study share haplotypes (**Fig.6**; **Fig.7**). The same pattern was observed in the phylogenetic tree. The major haplotype is shared by all samples from different and distant geographical areas and particularly between those from North-Eastern Atlantic and Mediterranean Sea highlighting a lack of structuring among them, as observed also in the PCoA in both markers, with a total overlap of the locations considered **(Fig.8**; **Fig.9)**. This overlap of information between EATL and MED populations corroborates that the Gibraltar doesn't represent a phylogeographic barrier.

Results from these preliminary analyses immediately highlight an inconsistency between the genetic approach and data from previous tag-and-recapture works (Green *et al.*, 2009). For

example, data from the Irish recreational fishery, consisting of fish tagged and/or recaptured from 1970 to 2006 reported only one recapture from the Mediterranean Sea, suggesting that there's a separation between the Mediterranean and the Atlantic stocks, specifically the Northern one, but evidence obtained from the genetic and phylogeographic approach developed in this work suggests that they could probably be considered as a unique population. However, all results reported in this work have to be interpreted in the light of our dataset nature, which doesn't cover the whole Atlantic Ocean, so it would be appropriate to include, in further investigations, also specimens from other Atlantic areas, especially from the Northern ones (e.g. UK, Ireland, ecc.) and from Western Atlantic.

The analysis of the historical demographic trend, enhanced by the results of the different approaches we decided to take such as a Bayesian Skyline Reconstruction and a Mismatch Distribution Analysis, together with the construction of a phylogenetic tree for both CR and cyt-b sequence datasets, gave us important and unexpected information. The images of the demographic trend from mismatch distributions (**Fig.12**; **Fig.13**) suggest a constant population size and a recent bottleneck or sudden population expansion for control region and Cyt-b respectively; however, these analyses and the neutrality test are not significant, invalidating these assumptions. The Bayesian skyline reconstruction seemed to be coherent to a general stable demographic pattern for the Cyt-b marker (**Fig.10**), and show a slightly population expansion for the control region, both in MED and EATL (**Fig.11).** Both these possible expansions are dated between 250 and 150 Kya, during Pleistocene epoch.

Due to the fact that we observe two major haplogroups, and taking into account these demographic analyses, it is plausible that the populations of blue shark have gone through a bottleneck process with a subsequent expansion that generated the second haplogroup observed in the network (**Fig.7**). It is plausible that during an epoch rich of climatic shifts, alteration of food availability and extreme weather conditions, as the Pleistocene, an event of bottleneck can affected the blue shark populations of the EATL/MED areas.

The divergence time analysis succeeds to date this separation event between haplogroup using an evolutionary rate estimated as 0.62%/site/My and 0.32%/site/My for control region and Cyt-b respectively. The analysis dated an highly significant and supported separation between the two major clades for both markers, dated at 1,2/1,3 Mya, during Pleistocene epoch, supporting the hypothesis of a past event that formed the two haplogroups. The fact that both markers associated with different evolutionary rates gave the same significant dating at 1,2/1,3 Mya, is a sign of a good calibration of the molecular clock of the species for these two markers.

Further investigations are needed in order to better understand these predator populations.

Although still a major bycatch of longline fishery instead of a commercial target and the extraordinarily high number of specimens globally registered, due also to the reproductive cycle and general biologic characteristics, blue shark *P. glauca*, as other elasmobranch, is characterized by a high vulnerability. Therefore, t is easy to understand the importance of increasing the source of information related to its population extent, genetic pattern and migratory possibilities throughout different areas in order to plan specific conservation actions and facilitate its stock management. In this perspective this thesis, especially because of the gap in knowledges regarding Mediterranean blue shark populations, can be considered a starting crucial point for planning further investigations, combining these results with the development of genomic resources for the blue sharks, providing genetic markers for this species by identifying genome-wide informative Single Nucleotide Polymorphism (SNP) markers.

References

Aires-da-Silva, A. M. & Gallucci, V. F. (2007). Demographic and risk analyses applied to management and conservation of the blue shark (*Prionace glauca*) in the North Atlantic Ocean. *Marine and Freshwater Research*, *58*(6), 570–580.

Bianchi I., Clò S. and Costantini M. (1997). Med-tag and baby shark: two projects for tagging sharks. First results, considering and prospects. Quaderni della Civica Stazione Idrobiologica di Milano. 22, 137–146.

Bigelow, K. a., Boggs, C. H., & He, X. (1999). Environmental effects on swordfish and blue shark catch rates in the US North Pacific longline fishery. *Fisheries Oceanography*.

Buencuerpo, V., Ríos, S., & Morón, J. (1998). Pelagic sharks associated with the swordfish, *Xiphias gladius*, fishery in the eastern North Atlantic Ocean and the strait of Gibraltar. *Fishery Bulletin*.

Cailliez F. 1983 The analytical solution of the additive constant problem. *Psychometrika* **48**, 305-308.

Campana, S. E., Dorey, A., Fowler, M., Joyce, W., Wang, Z., Wright, D., & Yashayaev, I. (2011). Migration Pathways, Behavioural Thermoregulation and Overwintering Grounds of Blue Sharks in the Northwest Atlantic. *PLoS ONE*, *6*(2), e16854.

Campana, S. E., Marks, L., Joyce, W., & Kohler, N. E. (2006). Effects of recreational and commercial fishing on blue sharks (*Prionace glauca*) in Atlantic Canada, with inferences on the North Atlantic population. *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(3), 670–682.

Carvajal-Rodriguez, A.,de Uña-Alvarez, J. (2011). Assessing Significance in High-Throughput Experiments by Sequential Goodness of Fit and q-Value Estimation. PLoS ONE 6(9): e24700.

Carvalho, F. C. (2011). Spatial predictions of blue shark catch rate and catch probability of juveniles in the Southwest Atlantic. *ICES Journal of Marine Science: Journal du Conseil* 68.5 (2011): 890-900.

Castro J., Mejuto J. (1995) Reproductive parameters of blue shark, *Prionace glauca*, and other sharks in the Gulf of Guinea. *Marine and Freshwater Research* 46(6) 967 - 973.

Cavanagh, R. D., & Gibson, C. (2007). *Overview of the Conservation Status of Cartilaginous Fishes (Chondrichthyans) in the Mediterranean Sea*. *Specialist*. No. 3. IUCN, 2007.

Chiabrera, A., Bianco, B., Bruna, S., & Giordano, S. (1998). Bioelectromagnetics: The state of the science. *RivBiolBiol Forum*, *91*(2), 233–245.

Clarke S.C., McAllister M.K., Milner-Gulland E.J., Kirkwood G.P., Michielsens C.G.J., Agnew D.J., Pikitch E.K., Nakano H., Shivji M.S. (2006). Global estimates of shark catches using trade records from commercial markets. *Ecology Letters*. Volume 9, Issue 10 October 2006 Pages 1115–1126.

Compagno, L.J.V. (1984) FAO species catalogue. Vol. 4. 1984 Sharks of the world. An annotated and illustrated catalogue of shark species known to date. Part 2. Carcharhiniformes. FAO Fish.Synop., (125)Vol.4,Pt.2:251-655.

Costa, D. P., Breed, G. A., & Robinson, P. W. (2012). New Insights into Pelagic Migrations: Implications for Ecology and Conservation. *Annual Review of Ecology, Evolution, and Systematics*, *43*(1), 73–96.

De Metrio G., Petrosino G., Montanaro C., Matarrese A., Lenti M. and Cecere E. (1984). Survey on summer-autumn population of *Prionace glauca* L. (Pisces, Chondricthyes) during the four-year period 1978-1981 and its incidence on swordfish (*Xiphias gladius* L.) and albacore (*Thunnus alalunga* (Bonn)) fishing. *Oebalia*, 1984, 10: 105-116.

Dray S & Dufour AB. 2007 The ade4 package: implementing the duality diagram for ecologists. *J STAT SOFTW* **22**, 1-20.

Drummond A. J., Rambaut A. (2007). Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 2007, **7**:214.

Drummond A. J., Suchard M.A. Dong Xie, Rambaut A. (2012). Bayesian phylogenetics with BEAUti and BEAST 1.7. *Mol. Biol. Evol.* 29(8):1969–1973.

ETS/STE 104 – Bern Convention / Convention de Berne (Appendix/Annexe III), 19.IX.1979

Excoffier L. & Lischer H.E. (2010). Arlequin suite ver3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* (2010) 10, 564-567.

Felsenstein, J. "PHYLIP (phylogeny inference package) Distributed by the author." *Department of Genome Sciences, University of Washington, Seattle), Version* 3 (2005).

Ferretti, F., Myers, R. A., Serena, F., &Lotze, H. K. (2008). Loss of Large Predatory Sharks from the Mediterranean Sea. *Conservation Biology*, *22*(4), 952–964.

Filanti T., Megalofonou P., Petrosino G. and De Metrio G. (1986). Incidenza dei Selaci nella pesca del pesce spada con long-line nel Golfo di Taranto. *Nova Thalassia* 8.Suppl 3 (1986): 667-669.

Fitzmaurice P., Green P., Keirse G., Kenny M., Clarke M. (2005). Stock discrimination of blue shark, based on Irish tagging data. International Commission for the Conservation of Atlantic Tunas, *Collective Volume of Scientific Papers* 58(3): 1171-1178.

Fitzpatrick S., Shijvi M., Chapman D., Prodöhl P. (2010). Population Genetics in the Open Ocean – A Global Assessment of Genetic Structuring in the Blue Shark (*Prionace glauca*). In: Programm Booklet of The 14th Annual Scientific Conference of the European Elasmobranch Association, Galway, 10-13 November 2010. Pp.16.

Green P, Sullivan DO, Roche W, Fitzmaurice P, Stokes D, Reilly SO, Kierse G, Kenny M, M. S., & M, C. (2009). Data on Blue Shark From the Irish Recreational Fishery. *Collective Volume of ScientificPapers, ICCAT*, *64*(5), 1522–1536.

Hazin, F. H., Boeckman, C. E., Leal, E. C., Lessa, R. P., Kihara, K., &Otsuka, K. (1994). Distribution and relative abundance of the blue shark, Prionace glauca, in the southwestern equatorial Atlantic Ocean. *Fishery Bulletin*, *92* (December 1987), 474–480.

Heessen, H. (2003). Development of elasmobranch assessments DELASS. *European Commission DG Fish Study Contract*.

Henderson, A. (2001). Observations on the biology and ecology of the blue shark in the North-east Atlantic. *Journal of Fish Biology*, *58*(5), 1347–1358.

Holsinger, K. E. Tajima's D, Fu's FS, Fay and Wu's H, and Zeng et al.'s E. (2013). http://darwin.eeb.uconn.edu/eeb348/lecturenotes/molevol-tajima.pdf

ICCAT. (2009). Report of the 2008 shark stock assessments meeting. *Collective Volume of Scientific Papers, ICCAT*, *64*(5), 1343–1491.

ICES WGEF Report 2007. Report of the Working Group on Elasmobranch Fishes (WGEF) 22 – 28 June 2007. Group, (June), 22–28.

Karleskint, G., Turner, R., Small, J.W. (2009). Marine fishes – Marine organisms. In *Introduction to Marine Biology*, pp. 262-295.

Kohler N., Turner P. (2008). Stock structure of the blue shark (*Prionace glauca*) in the North Atlantic Ocean based on tagging data. *Sharks of the Open Ocean: Biology, Fisheries and Conservation* (2008): 339-350.

Kohler, N. E., Casey, J. G., & Turner, P. a. (1998). NMFS Cooperative Shark Tagging Program, 1962-93: An Atlas of Shark Tag and Recapture Data. *Marine FisheriesReview*, *60*(2), 1–87.

Kohler, N. E., Turner, P. a, Hoey, J. J., Natanson, L. J., &Briggs, R. (2002). Tag and recapture data for three pelagic shark species: blue shark (*Prionace glauca*), shortfin mako (*Isurus oxyrinchus*) and porbeagle (*Lamna nasus*) in the North Atlantic Ocean. *International Commission for the Conservation of Atlantic Tunas, Collective Volume of Scientific Papers SCRS/2001/64*, *54*(4), 1231–1260.

Librado P. & Rozas J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451-1452.

Litvinov, F. F. (2006). On the role of dense aggregations of males and juveniles in the functional structure of the range of the blue shark *Prionace glauca*. *Journal of Ichthyology*, *46*(8), 613–624.

Megalofonou, P., Damalas, D., & de Metrio, G. (2009). Biological characteristics of blue shark, *Prionace glauca*, in the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom*, *89*(06), 1233.

Megalofonou, P., Damalas, D., & Yannopoulos, C. (2005). Composition and abundance of pelagic shark by-catch in the eastern Mediterranean Sea. *Cybium*, *29*(1), 135–140.

Megalofonou, P., Yannopoulos, C., Damalas, D., De Metrio, G., Deflorio, M., De La Serna, J. M., &Macias, D. (2005). Incidental catch and estimated discards of pelagic sharks from the swordfish and tuna fisheries in the Mediterranean Sea. *Fishery Bulletin*, *103*, 620–634.

Mejuto, J., & García-Cortés, B. (2005). Reproductive and distribution parameters of the blue shark *Prionace glauca*, on the basis of on-board observations at sea in the Atlantic, Indian and Pacific oceans. *Collective Volume of Scientific Papers, ICCAT, 58*(3), 951–973.

Myers R.A., Baum, J.K., Shepherd, T D., Powers, S P., Peterson, C. H. (2007) Cascading Effects of the Loss of Apex Predatory Sharks from a Coastal Ocean. Science 315 (5820): 1846-1850.

Nakano, H. (1994). Age reproduction and migration of blue shark in North Pacific Ocean. *Bulletin of the National Research Institute of Far Seas Fisheries*31 (1994): 141-256.

Nakano, H., Seki M.P. (2003). Synopsis of biological data on the blue shark, *Prionace glauca* Linnaeus. *BULLETIN-FISHERIES RESEARCH AGENCY JAPAN* (2003): 18-55.

Nakano, H., Stevens J.D. (2008). The biology and ecology of the blue shark, *Prionace glauca*. *Sharks of the Open Ocean: Biology, Fisheries and Conservation* (2008): 140-151.

Ovenden, J. R., Kashiwagi, T., Broderick, D., Giles, J., & Salini, J. (2009). The extent of population genetic subdivision differs among four co-distributed shark species in the Indo-Australian archipelago. *BMC Evolutionary Biology*, *9*, 40.

Paradis E, Claude J, Strimmer K. 2004 APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289–290.

Pardini, A.T., Jones, C.S., Noble, L.R., Kreiser, B., Malcolm, H., Bruce, B.D. Stevens, J.D., Cliff, G., Scholl, M.C., Francis, M., Duffy, C.A.J., Martin, A.P. (2001). Sex-biased dispersal of great white shark. *Nature* 412.6843 (2001): 139-140.

Pomi, C. (1997). Morphometric study of *Prionace glauca* (Linnaeus, 1758) in the Central and Northern Adriatic Sea. Quaderni della Civica Stazione Idrobiologica di Milano. 22, 159–161.

Portnoy, D. S., McDowell, J. R., Heist, E. J., Musick, J. A., &Graves, J. E. (2010). World phylogeography and male-mediated gene flow in the sandbar shark, *Carcharhinus plumbeus*. *Molecular Ecology*, *19*(10), 1994–2010.

Pratt, H. L. (1979). Reproduction in the Blue shark, *Prionace glauca*. *FisheryBulletin*, *77*(No 2), 445–470.

Psomadakis, P. N., Maio, N., &Vacchi, M. (2009). The chondrichthyan biodiversity in the Gulf of Naples (SW Italy, Tyrrhenian Sea): An historical overview. Cybium Paris, 33(3), 199–209. Retrieved from http://www.refdoc.fr/Detailnotice?idarticle=20634342

Queiroz, N. (2010). Diving behaviour, movement patterns and population structure of blue shark Prionace glauca (L. 1758) in the Northeast Atlantic. Diss. University of Aberdeen, 2010.

Queiroz, N., Humphries, N.E., Noble, L.R., Santos, A.M., Sims, D.W. (2010). Short-term movements and diving behaviour of satellite-tracked blue sharks *Prionace glauca* in the Northeastern Atlantic Ocean. *Marine Ecology Progress Series* 406 (2010): 265-279.

Queiroz, N., Lima, F.P., Maia, A., Ribeiro, P.A., Correia, J.P., Santos, A.M. (2005). Movement of blue shark, *Prionace glauca*, in the North-east Atlantic based on mark-recapture data. *ournal of the Marine Biological Association of the United Kingdom* 85.05 (2005): 1107-1112.

Quieroz, N., Humphries, N.E., Noble, L.R., Santos, A.M., Sims, D.W. (2012). Spatial dynamics and expanded vertical niche of blue sharks in oceanographic fronts reveal habitat targets for conservation. *PLoS One* 7.2 (2012): e32374.

R Development Core Team. 2013 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL https://www.r-project.org/

Robbins R.L. (2007). Environmental variables affecting the sexual segregation of great white shark *Carcharodon carcharias* at the Neptune islands South Australia. *Journal of Fish Biology* Volume 70, Issue 5 May 2007, pages 1350–1364.

Sampaio da Costa, I.R.F (2013). Genetic Diversity and Population Structure of the Cosmopolitan Blue Shark *Prionace glauca* (Linnaeus, 1758) in the Atlantic Ocean. MSc Thesis University of Porto - Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO) · Evolutionary Ecology and Genetics of Aquatic Organisms.

Silva C., Wilke C.G., Kerwath S.E., Meyer M., Lamberth S.J. (2010). First documented Southern Transatlantic Migration of a blue shark *Prionace glauca* tagged off South Africa. *African Journal of Marine Science* Volume 32, Issue 3, 2010

Simpfendorfer C.A., Hueter R.E., Bergman U., Connett S.M.H. (2002). Results of a fishery-independent survey for pelagic sharks in the western North Atlantic, 1997-1994. *Fisheries Research* Volume 55, Issues 1–3, March 2002, Pages 175–192

Skomal, G., &Natanson, L. (2003). Age and growth of the blue shark (*Prionace glauca*) in the North Atlantic Ocean. *Fishery Bulletin, 101*(3), 627–639.

Spear, N., Wells, R. J. D., & Kohin, S. (2012). Preliminary age validation of the blue shark (*Prionace glauca*) in the Eastern Pacific Ocean. ISC Shark Working Group Stock Assessment Workshop, Shimizu, Japan, ISC/12/SHARKWG-1/01.

Sperone, E., Parise, G., Leone, A., Milazzo, C., Circosta, V., Santoro, G., … Tripepi, S. (2012). Spatiotemporal patterns of distribution of large predatory sharks in Calabria (central Mediterranean, southern Italy). *Acta Adriatica*, *53*(1), 13–24.

Stevens J.D. (1990). Further results from a tagging study of pelagic sharks in the North-east Atlantic. *Journal of the Marine Biological Association of the United Kingdom* / Volume 70 / Issue 04 / November 1990, pp 707-720.

Stevens, J. 2009. *Prionace glauca*. The IUCN Red List of Threatened Species 2009: e.T39381A10222811.

Stevens, J. D., Bradford, R. W., & West, G. J. (2010). Satellite tagging of blue sharks (*Prionace glauca*) and other pelagic sharks off eastern Australia: depth behaviour, temperature experience and movements. *Marine Biology*, *157*(3), 575–591.

Taguchi, King J., Wetklo M., Withler M., Yokawa K. (2014) Population genetic structure and demographic history of Pacific blue sharks (Prionace glauca) inferred from mitochondrial DNA analysis. CSIRO publishing 2014, In press.

Tamura, K., Stecher, G., Peterson, D., Stecher, G., Filipski, A., Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution* (2013): mst197.

Tillett B.J., Meekan M.G., Field I.C., Thorburn D.C., Ovenden J.R. (2012). Evidence for reproductive philopatry in the bull shark Carcharhinus leucas. *Journal of Fish Biology* 80.6 (2012): 2140-2158.

UNEP MAP RAC/SPA. (2003). Action Plan for the Conservation of Cartilagenous Fishes (Chondrichthyans) in the Mediterranean Sea.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., &Rozen, S. G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), 1–12.

Ussami L.H.F., Mendonça F.F., Pereira L.H.G., Teixeira A.F., Oliveira C., Gadig O.B.F., Foresti F. (2011) Analysis of genetic population structure of the blue shark, Prionace glauca (Chondrichthyes, Carcharhinidae) in Brazilian coast using microsatellite markers. In: Resumos do 57º Congresso Brasileiro de Genética 30 agosto- 2 setembro 2011.

Valeiras, J. & Abad, E. (2009). Chapter 2.2.1.1. Blue Shark, ICCAT. 2006-2015. ICCAT Manual. International Commission for the Conservation of Atlantic Tuna. In: ICCAT Publication [on-line]. http://www.iccat.int/en/ICCATManual.asp, ISBN (Electronic Edition): 978-92-990055-0-7.

Vandeperre, F., Aires-da-Silva, A., Fontes, J., Santos, M., Serrão Santos, R., & Afonso, P. (2014). Movements of Blue Sharks (*Prionace glauca*) across Their Life History. *PLoS ONE*, *9*(8), e103538.

Verìssimo A., Sanpaio I., McDowell J. & Alexandrino P. (2013) Genetic population structure among Atlantic nurseries of the highly mobile oceanic Blue Shark, Prionace glauca. In: Programm Booklet of The 17th Annual Scientific Conference of the European Elasmobranch Association, Plymouth, UK, 1 – 3 November 2013. Pp.42.

Whelan K. (1991). The sea angler in Ireland. Dublin: Country House.

Appendix

Tab.4 Occurrence of haplotypes in each geographical sample for both CR and Cyt-b markers.

CR - Haplotye frequencies in populations

Haplotype	EATL	WMED	THYR	CADR	IONI	AEGE	MED	TOTAL
Hap_1	3	2	0	0	0	0	2	5
Hap_2	1	0	0	0	0	0	0	1
Hap_3	3	5	2	0	0	2	9	12
Hap_4	1	0	0	0	0	0	0	1
Hap_5	2	0	0	0	0	0	0	2
Hap_6	1	0	0	0	0	0	0	1
Hap_7	1	0	0	0	0	0	0	1
Hap_8	1	3	0	3	0	6	12	13
Hap_9	2	3	0	0	0	0	3	5
Hap_10	1	0	0	0	0	0	0	1
Hap_11	5	0	0	2	1	0	3	8
Hap_12	1	1	0	2	0	0	3	4
Hap_13	1	0	0	0	0	0	0	1
Hap_14	1	0	0	0	0	0	0	1
Hap_15	1	0	0	1	0	1	2	3
Hap_16	1	4	0	2	2	3	11	12
Hap_17	1	0	0	0	0	0	0	1
Hap_18	1	0	0	0	0	0	0	1
Hap_19	1	0	0	0	0	0	0	1
Hap_20	1	0	0	0	0	0	0	1
Hap_21	1	0	0	0	0	0	0	1
Hap_22	1	0	0	0	0	0	0	1
Hap_23	1	0	0	0	0	0	0	1
Hap_24	0	1	0	0	0	0	1	1
Hap_25	0	1	0	0	0	0	1	1
Hap_26	0	2	0	0	0	0	2	2
Hap_27	0	2	0	0	0	1	3	3
Hap_28	0	1	0	0	0	0	1	1
Hap_29	0	1	0	0	0	0	1	1
Hap_30	0	1	0	0	0	0	1	1
Hap_31	0	1	0	0	0	0	1	1
Hap_32	0	1	0	0	0	0	1	1
Hap_33	0	1	0	0	0	0	1	1
Hap_34	0	1	0	0	0	0	1	1
Hap_35	0	1	0	0	0	0	1	1
Hap_36	0	1	0	0	0	0	1	1
Hap_37	0	1	0	2	0	1	1	4
Hap_38	0	1	0	0	0	0	1	1
Hap_39	0	1	0	0	0	0	1	1
Hap_40	0	1	0	0	0	0	1	1
Hap_41	0	1	0	0	0	0	1	1
Hap_42	0	1	0	0	0	0	1	1
Hap_43	0	0	1	0	0	0	1	1
Hap_44	0	0	1	0	0	0	1	1
Hap_45	0	0	1	0	0	0	1	1
Hap_46	0	0	1	0	0	0	1	1
Hap_47	0	0	1	0	0	0	1	1
Hap_48	0	0	1	0	0	0	1	1
Hap_49	0	0	0	1	0	0	1	1
Hap_50	0	0	0	2	0	0	2	2
Hap_51	0	0	0	1	0	0	1	1
Hap_52	0	0	0	1	0	0	1	1
Hap_53	0	0	0	1	0	0	1	1
Hap_54	0	0	0	1	0	0	1	1
Hap_55	0	0	0	1	0	0	1	1
Hap_56	0	0	0	1	0	0	1	1
Hap_57	0	0	0	0	1	0	1	1
Hap_58	0	0	0	0	1	0	1	1
Hap_59	0	0	0	0	1	0	1	1
Hap_60	0	0	0	0	1	0	1	1
Hap_61	0	0	0	0	1	0	1	1
Hap_62	0	0	0	0	1	0	1	1
Hap_63	0	0	0	0	1	0	1	1
Hap_64	0	0	0	0	1	0	1	1
Hap_65	0	0	0	0	1	0	1	1
Hap_66	0	0	0	0	1	0	1	1
Hap_67	0	0	0	0	1	0	1	1
Hap_68	0	0	0	0	0	1	1	1
Hap_69	0	0	0	0	0	1	1	1
Hap_70	0	0	0	0	0	1	1	1
TOTAL	**33**	**39**	**8**	**21**	**14**	**17**	**96**	**132**

Cyt - b - Haplotye frequencies in populations

Haplotype	EATL	WMED	THYR	CADR	IONI	AEGE	MED	TOTAL
Hap_1	1	0	0	0	0	0	0	1
Hap_2	9	12	4	5	5	9	35	44
Hap_3	18	12	3	7	2	2	26	44
Hap_4	3	0	0	0	1	0	1	4
Hap_5	1	2	0	0	0	0	2	3
Hap_6	0	2	0	0	0	0	2	2
Hap_7	0	2	0	0	0	0	2	2
Hap_8	0	1	0	0	0	0	1	1
Hap_9	0	2	1	0	0	0	3	3
Hap_10	0	2	0	2	1	0	5	5
Hap_11	0	2	0	0	1	0	3	3
Hap_12	0	2	0	1	1	0	4	4
Hap_13	0	0	1	3	1	1	6	6
Hap_14	0	0	1	1	2	1	5	5
Hap_15	0	0	0	1	0	0	1	1
Hap_16	0	0	0	1	0	2	3	3
Hap_17	0	0	0	0	1	0	1	1
Hap_18	0	0	0	0	0	1	1	1
Hap_19	0	0	0	0	0	1	1	1
Hap_20	0	0	0	0	0	1	1	1
Hap_21	0	0	0	0	0	1	1	1
TOTAL	**32**	**39**	**10**	**21**	**15**	**19**	**104**	**136**

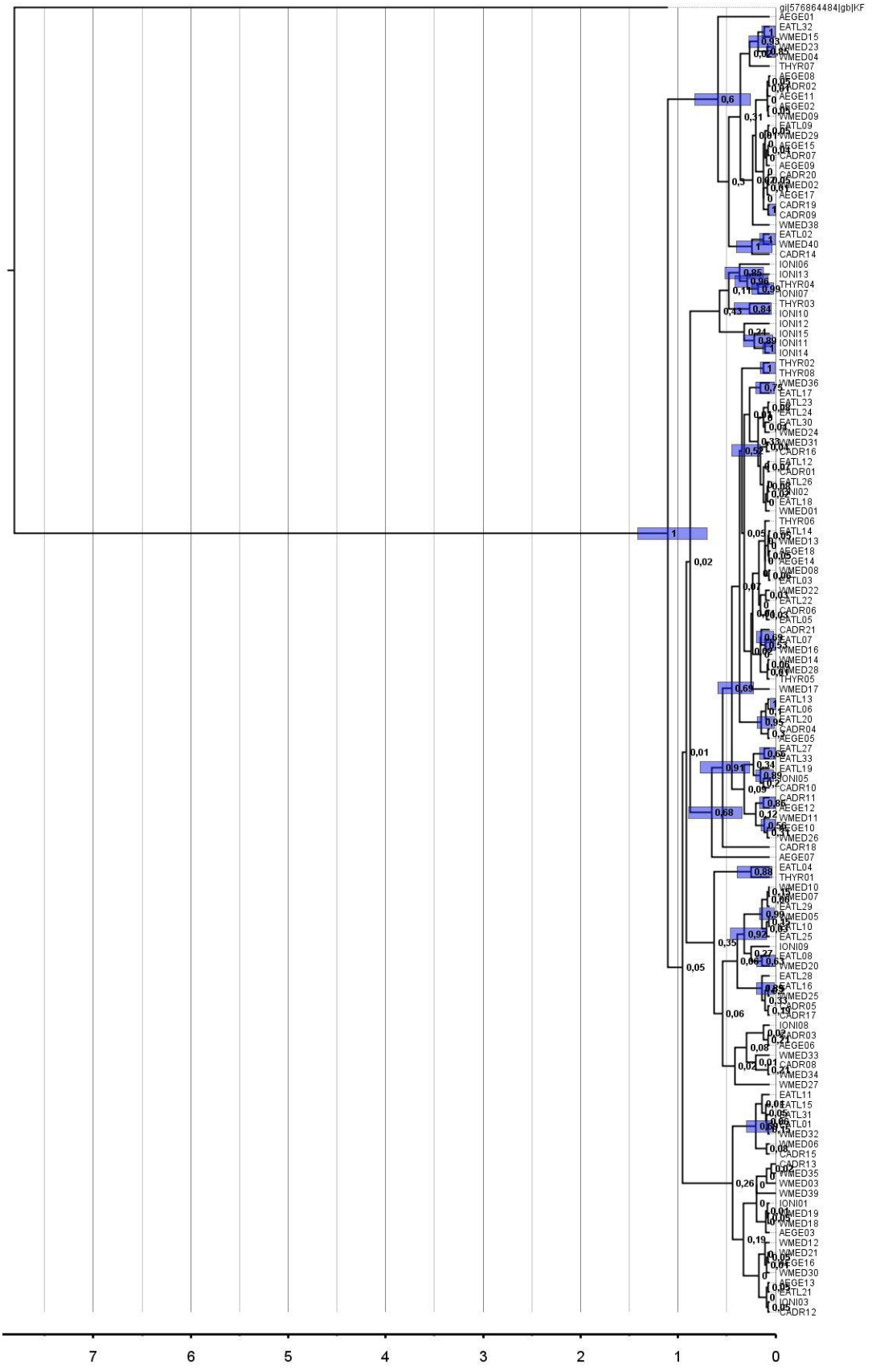

Fig.14 Divergence tree for CR marker, performed with software BEAST v. 1.8.2. Supported separation between the two major clades is dated 1,2/1,3 Mya.

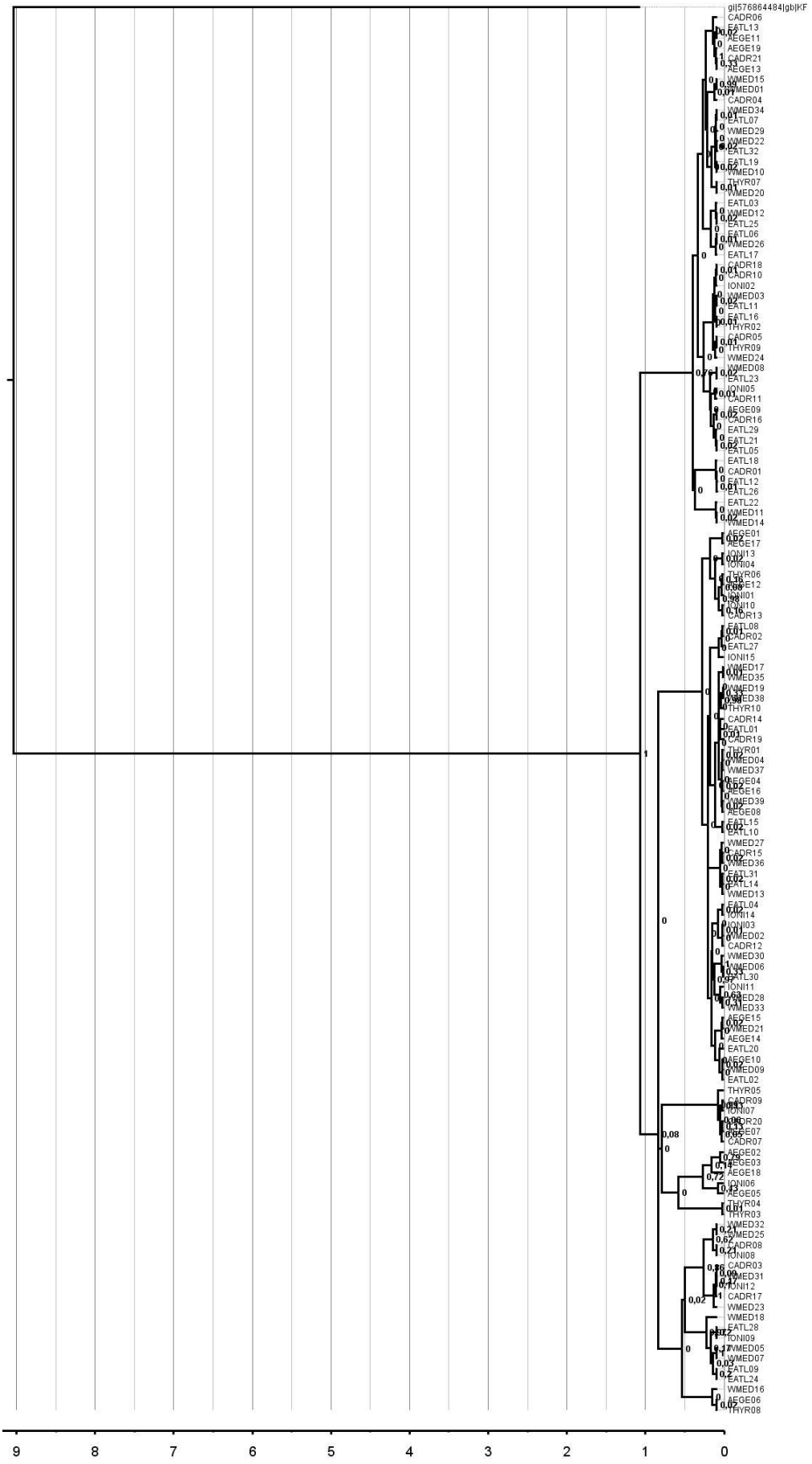

Fig.15 Divergence tree for Cyt-b marker, performed with software BEAST v. 1.8.2. Supported separation between the two major clades is dated 1,2/1,3 Mya.