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Genomic applications in fish traceability and fishery stock management: phylogeography and population structure of the Mediterranean-Atlantic blue shark, *Prionace glauca*

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"Genomic applications in fish traceability and fishery stock management: phylogeography and population structure of the Mediterranean-Atlantic blue shark, *Prionace glauca*"

Doctoral Thesis

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Thesis Abstract

The abundance of blue sharks, *Prionace glauca*, Linnaeus 1758 (BS), has dramatically declined over the last century in the Mediterranean Sea (a reduction of 75% over the last 30 years). In this study, the combination of a near-capillary sampling strategy of BS in several areas of the basin together with the development and use of genomic markers - that can represent both neutral and selective markers - offers the opportunity to assess and visualize for the first time for this poorly studied and vulnerable species population structure and potentially local adaptation.

The phylogeography between Mediterranean Sea and North-Eastern Atlantic Ocean, inferred using mtDNA (control region, Cytb), highlighted no obvious haplotypic pattern of geographical differentiation, while Φst analyses indicated significant genetic structure among four geographical groups.

Successively, spatial genetic differentiation of 203 Mediterranean and North-eastern Atlantic BS was estimated using 3,451 species specific SNP loci, developed using the ddRAD technology.

Neutral SNPs differentiation was null or very low (pairwise Fst ranged from 0.2 to 0.5%) indicating a near or fully-panmictic population. A subpanel of potentially under-selection loci revealed significant higher Fst values (pairwise Fst ranging from 0.01 to 0.09), clustering analyses failed to evidence strong signals of differentiation among areas, probably due to the combination of migrants-mediated gene flow and the big population size.

Based on these results, combined with the biology of this species, such the extreme vagility e complex population dynamics, it seems fair to suggest that the Mediterranean BS population could represent a meta-population, with spatially separated populations which interact through migrant specimens. This work has advanced knowledge on Mediterranean Blue Shark population biology and ecology relating ex-novo the Mediterranean BS population and the fishery stock to the Atlantic biological and management units, being the Mediterranean BS often not considered in the review of data and ecological relationships.

“Cum caniculis atrox dimicatio”
Pliny the Elder

Preface

The Mediterranean population of *Prionace glauca* (blue shark, BS) has been categorized by IUCN as "Critically Endangered" (Sims, et al. 2016), and according to Ferretti et al. (2008), Mediterranean BS population have been subjected to a 96% decline in abundance and 99% in biomass over the last 50 years. Tagging experiments carried out outside the Mediterranean demonstrated extensive individual movements, considering BS among the most high-migratory shark species. BS distribution in the Mediterranean Sea seems to be related to biological drivers, such as sex and size (Megalofonou, et al. 2009). So far genetic studies carried out in the world oceans revealed an interoceanic genetic structuring with gene-flow generally restricted within ocean. The connectivity of the Mediterranean BS with adjacent and other global populations still remains unclear since there are no data from tagging and/or genetic studies. Conservation and management of this resource in the Mediterranean requires a deeper knowledge in order to promote appropriate conservation policies.

In general, the IUCN classification is particularly challenging when a combination of issues occurs, such as: (1) trend data available only for parts of the geographic range of the species; (2) the species is moderately productive; (3) trend data or stock assessments at regional scale are uncertain; (4) the species is subject to management in specific areas and not in others, (5) lack of data for some regional populations (Dulvy et al., 2008) This is particularly true for BS, which faces all of these issues. In fact, despite the species is one of the most common fished sharks in the Mediterranean (Fawler, et al. 2005), the current genetic structure of BS across Mediterranean-North East Atlantic Ocean is unknown. Such knowledge gap in the status of the Mediterranean BS population highlights the necessity of improving the knowledge of this population through analyses robust for sampling design and methods used. This knowledge is absolutely required in order to produce reliable stock assessments. Given the extraordinary vagility of the species, there is a need for more discriminant and powerful genetic markers, still poorly known and widely neglected for this species, thus also affecting the accuracy of the assessments and the possibility to develop more realist stock management and conservation strategies.

AIMS

The primary aims of this PhD project are:

- to test the null hypothesis of panmixia between North Atlantic and Mediterranean BS, by comparing the mtDNA genetic variation
- to develop more powerful genomic resources for the investigation of the potential panmixia between Mediterranean Sea and Atlantic Ocean
- to test the null hypothesis of panmixia between the Mediterranean and the Atlantic Ocean using novel genomic markers, such the Single Nucleotide Polymorphisms (SNPs).
- to compare the results from genetic markers (using both matrilineal and uni-parental inheritance signals) with those from tagging.
- to develop genetic markers that could help the scientific community to correctly assess the Mediterranean population of blue shark, in terms of stock identification and management and contributing to the knowledge of BS from Mediterranean Sea and the adjacent North Eastern Atlantic Ocean, helping to develop a better fitting stock assessment and a sustainable management of the species.

In order to better frame the species, the **Capther 1** is dedicated to the general introduction to the species, with relevant biological, ecological, behavioral information as well as on the state-of-art on fisheries, genetic stock structure and connectivity.

Furthermore, highlighting the importance of the involvement of the international organizations, such the European Union, which acts in making decisions on exploitation and conservation policies, the MedBluesGen project is introduced.

Since the sharks are usually sex-biased dispersed (Pardini, et al. 2001) and philopatric (Mouriers, et al. 2013), in the **Chapter 2** we investigated the phylogeography and the matrilinean genetic structure of the Mediterranean/North Eastern Atlantic BS. The results revealed a complex phylogeographic structure, which appears to reject the assumption of panmixia across the study area, but also supports a certain degree of population connectivity across the Strait of Gibraltar, despite the lack of evidence of migratory movements observed by tagging data.

The **Chapter 3** is focused on the issues of doing genomics and bioinformatics analyses on non-model fish species with a large genome size, such the BS. In this chapter, the development of novel BS genomic tools (dd-RAD generated SNPs) and the optimization of the assembly and SNPs calling are discussed. Finally, in the **Chapter 4**, these BS SNPs were used to investigate the genetic diversity and differentiation of the Mediterrnanean and North Eastern Atlantic adjacent

populations. Furthermore, the issues of working on big vagile species and the difficulties of develop genomic markers for the traceability and its management were discussed.

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1 CHAPTER 1

Integrating information on the biology, ecology, fisheries, stock structure and management of the blue shark (*Prionace glauca*), and introduction to the MedBluesGen project

1.1 Introduction

The blue shark, *Prionace glauca*, L. 1758, BS henceforth, is one of the most abundant carcharhinid shark in the world (Compagno, 1984). The blue sharks (**Fig. 1.1**) are extremely vagile, and they can grow up to 334 cm fork length (Megalofonou et al., 2009). Morphologically, the BS, is characterized by a long, narrow body with a slight snout and large eyes, absence of spiracles, presence of papillose gillrakers on internal gills, weak lateral keels on the caudal fin, and back position of dorsal fin near the pelvic than fins (Compagno, 1984; Nakano & Seki, 2003). These characteristics enable the BS to continuously swimming with little effort, even with strong currents (Sims, 2010).

A BS adaptation to the open ocean habitat is the countershading coloration, from which the name "blue shark", ranging from a blue/cobalt on the dorsal side to a bright white on the ventral side (Nakano & Seki, 2003). This feature, that is common in most of the pelagic sharks, is bring to the extreme in this species, enabling a perfect camouflage, depending on the side you look at it; the blue back matches the dark blue surrounding the water column if seen from above, and the white belly matches the light of the sun on surface if seen from below (Karleskint et al., 2009).

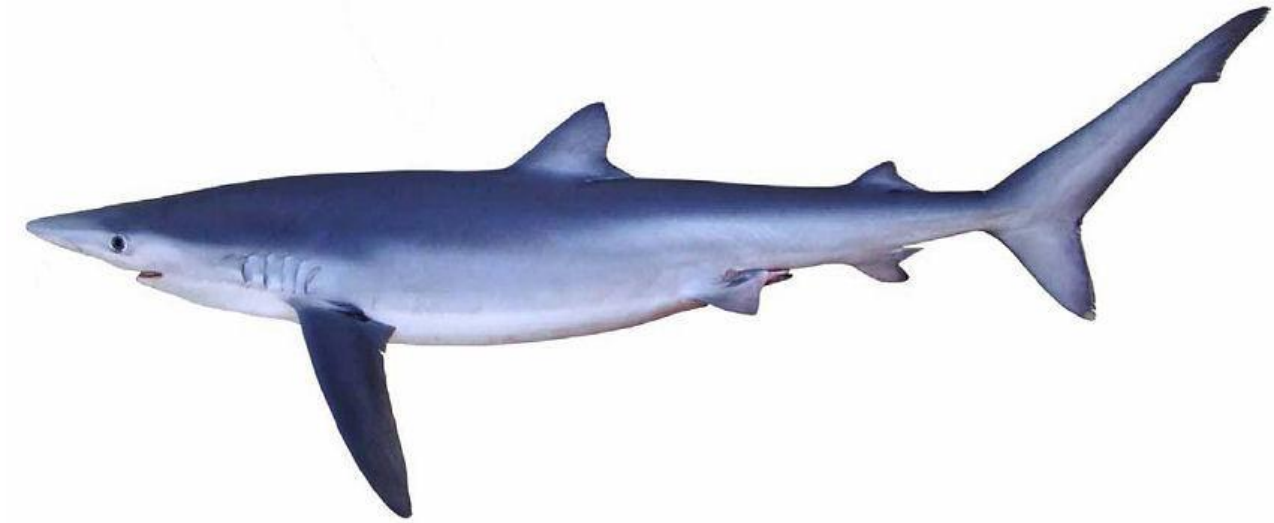


Fig. 1.1: Specimen of blue shark, *Prionace glauca*, L. 1758. Photo credit: Wikimedia Common

1.1.1 Systematics and Taxonomy

The BS is the only representative of the genus *Prionace* and it is closely related to species of the genus *Carcharhinus*. The monophyly of *Carcharhinus* is challenged by the inclusion of the three monotypic genera: *Nasolamia*, *Prionace*, and *Triaenodon* (Naylor, et al. 2012). From the analysis of

the mitochondrial DNA from 595 species of sharks, is evident that the species phylogenetically closest to the BS is the silky shark, *Carcharhinus falciformis* (Naylor, et al. 2012).

The current taxonomy for the BS is:

Kingdom: Animalia;

Phylum: Chordata;

Class: Chondrichthyes;

Order: Carcharhiniformes;

Family: Carcharhinidae;

Genus: *Prionace* (Cantor, 1849);

Species: *Prionace glauca* (Linnaeus, 1758).

1.1.2 Ecology, distribution and habitat

The BS is cosmopolite in all oceans from 60°N to 50°S (**Fig. 1.2**). In the Atlantic, BS is distributed from Canada to Argentina in the western side and from Norway to South Africa in the eastern side, including the Mediterranean Sea while it lacks in the Black Sea (Compagno, 1984).

Fig. 1.2: Distribution range of blue shark. Photo Credit: The IUCN Red List of Threatened Species. Version 2017-2. <www.iucnredlist.org>. Downloaded on 15 October 2017.

The population structure and dynamics of the Atlantic BS is still poorly known despite several and long-term tagging studies, which revealed extensive movements of BS tagged in the western side of the North Atlantic (henceforth NA) with well documented eastward trans-Atlantic migrations (Kohler et al. 1998, 2002; Kohler & Turner, 2008; Vandeperre, et al. 2014) between populations that are heavily by-catched by industrial tuna long-lining fisheries (Stevens, 1984; Casey, et al. 1985; Diaz & Serafy, 2004; Queiroz, et al. 2012). In this region, BS oceanic movements were found to be different based on stage and maturity of sharks, as well as for distribution of prey, suggesting a complex reproductive cycle with oceanic migrations associated to mating and pupping areas (Pratt, 1979; Casey, 1985; Stevens, 1990). BS spatial habitat heterogeneity and the overlapping of migratory routes with longline fisheries area might be a driver of the decline of BS populations accounted for a CPUE reduction of approximately 30% in the western North Atlantic from 1957 to 2000 (Silva, 2008).

As many chondrichthyans, the BS is characterized by low fecundity, with maturity size at ~220cm and a maximum size of 380cm. Being a K-selected species, BS shows low fertility and long ontogenetic development cycles, making it a species vulnerable to overexploitation (Pratt, 1979). Compared to the oceans, inshore BS records, including juvenile specimens, are not rare in the Mediterranean during spring and summer, in agreement with birth period. Large adult specimens are closely related to pelagic environments. Due to this feature, BSs are highly sensitive to direct fishing and by-catch. Juveniles are subject to inshore sport fishing and small commercial fishing, especially in summer, while adults are susceptible to the big professional fisheries in pelagic environments and often victims of by-catch in tuna and swordfish long-line fisheries, with whom they share their prey (Megalofonou, et al. 2005; Storai, et al. 2011). Since adults are charecterized by long gestation periods, the capture of even a few adults leads to the death of more embryos, greatly amplifying the damage caused by the removal of this species in the Mediterranean basin (Relini, et al. 2010).

Still few and unclear, tagging data of Pacific BS were obtained from longline and drift-net fisheries, and modelling analysis suggests a stock decline starting from 1980s followed by recovery (Kleiber, et al. 2009). However, the limited fishery assessments carried out, did not show evidence of catch rate declines of BS in the Atlantic or Indian Oceans. Since the BS is not a target species, the biggest issue is that most of BS catches are unreported, and therefore declining pattern and stock reduction could be strongly underestimated. No extensive tagging or genetic surveys were carried out on the Mediterranean BS. Nevertheless, detailed modelling analyses and catch rates showed

that BS distribution in the Mediterranean could be related to environmental cues, such as temperature, bottom topography and lunar cycle (Megalofonou, et al. 2009; Damalas & Megalofonou, 2010). A strong longitudinal constituent in the presence of BS with the occurrence probability increasing from east to west, has been confirmed throughout the Mediterranean. Availability of food and increased productivity/abundance of living resources may be a key factor in these differences (Megalofonou, et al. 2005).

Sexual segregation contributes to the spatial heterogeneity of BS distribution. In the NA, mature females concentrated in more temperate waters of the northernmost area while immature were predominant in the southernmost NA (Sampaio da Costa, 2013). Mature BS of both sexes seems to be distributed in the southern part of NA, while immature individuals of both sexes and sub-adult females are usually distributed in the northern areas (Kohler, et al. 2002). A prevalent occurrence of immature juveniles is reported in the Mediterranean Sea (Megalofonou, et al. 2009; Kohler, et al. 2002). In the NA, sub adult females (Total Length, TL, <180 cm) migrate from coastal to oceanic areas to form mating clubs with males (Simpfendorfer, et al. 2002; Nakano & Stevens, 2008). After mating, adult females usually leave the mating clubs, while some of them may remain there until close to parturition, which occurs closer to the shore (Litvinov, 2006; Tavares, et al. 2012; Vögler, et al. 2012).

1.1.3 Reproduction

BS, like other carcharinid sharks, is a placental viviparous shark, with embryos developing inside the female's uterus with a gestation period of 9 - 12 months. The generation time of the species is 8.2 and 9.8 years for South African and North Atlantic populations, respectively, which means that BS takes around 8.2/9.8 years between two consecutive generations in the lineages of its population (Cortès, et al. 2015).

BS mating and fertilization occur in early summer and the birth (with mean size-at-birth of about 35 cm TL) occurs often from March to June (Pratt, 1979; Castro & Mejuto, 1995; Nakano, 1994). The number of pups for each gestation range from 4 to 135, with an average number of 50-60 pups (Nakano, 1994).

Unlikely other relative sharks, the BS are moderately productive and characterized by a rapid growth. Male specimens reach sexual maturity at an average of 6 years, at approximately 183 cm of TL. Usually, males smaller than 125 cm TL were immature with non-calcified claspers that do not reach the posterior end of the pelvic fins (Pratt, 1979; Nakano & Stevens, 2008; Megalofonou,

et al. 2009; Jolly, et al. 2013). Females are sexually mature at about 8 years, when they are considered sub-adult. During this period the females are still developing sexual organs needed for gestation. Observations of the reproductive organs in relation to body length revealed that females smaller than 120 cm TL still have immature ovaries with no maturing oocytes, while female specimens larger than 203 cm TL showed mature ovaries with visible oocytes (Megalofonou, et al. 2009). The females are sexually mature at about 185 cm TL, after 4-5 years of age. The longevity of this species was estimated at 20 - 23 years (Cailliet, et al. 1983; Manning & Francis, 2005; Romanov, et al. 2011; Stevens, 2009). In the Mediterranean Sea, according to what observed by Megalofonou et al. (2009), the sex ratio is skewed in favor of males and this bias was similar to that reported in the BS inhabiting the adjacent Eastern NA areas and the Strait of Gibraltar. Nevertheless, a strongly biased sex ratio toward females was observed in the BS inhabiting the Western areas of NA (Pratt, 1979) and Celtic Sea (Buencuerpo, et al. 1998; Megalofonou, et al. 2009; Stevens, 1976; Henderson, et al. 2001).

The observations of gravid BS females in the Eastern Mediterranean support the hypothesis of the Mediterranean as reproductive area. Gravid females were observed in the Adriatic and Ionian Sea, suggesting that the region is a nursery area (Bianchi, et al. 1997; Pomi, 1997).

1.2 Population structure: tagging and genetic studies

Given its high vagility, BSs are able of great transoceanic movements, travelling between northern and southern hemispheres, overcoming 5,000 km of distances, with a record of distance travelled by a single BS specimen of 7,176 km (Kohler, et al. 2002; Quieroz, et al. 2010; Costa, et al. 2012).

In the Atlantic Ocean, the structure of BS populations is still poor known despite the long-term tagging studies, that revealed extensive movements in the NA, with numerous eastward trans-Atlantic migrations (Kohler, et al. 1998; Kohler, 2002; Kohler & Turner, 2008; Vandeperre, et al. 2014).

During these long distance movements, BS showed a site-fidelity behaviour to specific habitats, such as oceanic seamounts and continental shelves due to the potential high nutrient concentrations supplied by thermal front boundaries which provide high primary productivity, and consequently, more trophic resources (Bigelow, et al. 1999; Litvinov, et al. 2006; Quieroz, et al. 2012).

In the North Pacific Ocean, Nakano (1994) proposed a model of BS migration differentiated by sex, where the mating ground was restricted between 20°N and 30°N, and the parturition ground confined above 40°N. In this model, juvenile and subadult males and females were distributed in nursery areas located south and north of the mating ground respectively, while adult specimens were usually more common in subtropical and tropical waters (Nakano & Seki, 2003).

Population genetic studies carried out using microsatellite and mitochondrial Cyt-b markers failed to detect any genetic structure in the North Pacific and in the whole Pacific Ocean, respectively, suggesting a unique panmictic population of Pacific BS (Li, et al. 2016; King, et al. 2015). This lack of population structure in the Pacific Ocean may be the result of the combination of extreme vagility and lack of effective barrier to gene flow for this species.

The biggest BS tag-and-release efforts were carried out from 1962 to 2000 in the Atlantic Ocean by the National Marine Fisheries Service (NMFS). In the framework of the Cooperative Shark Tagging Program (CSTP), 91,450 BS were tagged in the NA and 570 in the Mediterranean Sea, of which just a total of 5,410 (5.9%) were recaptured. The maximum distance traveled was 3,740 nautical miles (6,926 Km), with a mean distance traveled of 463 nautical miles (857 Km), while the maximum time at liberty was 9.1 years, with a mean time at liberty of just 0.9 years (Kohler, et al. 1998; Green, et al. 2009; Kohler, 2002; Kohler & Turner, 2008). Despite the impressive number of BS specimens tagged and released in the NA, only one BS was recorded in the Mediterranean Sea, while of the sharks tagged in the Mediterranean just one subadult female moved a short distance to the North East Atlantic, supporting the idea of a separation between the Atlantic and the Mediterranean BS stocks (Kohler, et al. 2002).

Despite these large tagging programs and several studies were carried out in order to define the patterns of BS movements and connectivity in the NA, the BS population structure is still uncertain and, specifically for the Mediterranean Sea, it is still unknown because of the lacking of data (Kohler, et al. 1998; Fitzmaurice, et al. 2005; Mejuto, et al. 2005; Green, et al. 2009).

The NA showed a high occurrence of juvenile BS (<150 cm TL) off mainland Portugal, off the Azores and off western South Africa (Kohler, et al. 2002; Silva, et al. 2010; Quieroz, et al. 2012; Sampaio da Costa, 2013; Verissimo, et al. 2017). These areas were described as nursery areas for the species in the NA. Both juvenile males and females tended to remain for extended period within an area delimited by the Azores, the Atlantis – Great Meteor seamount complex and the Mid-Atlantic Ridge (Vandeperre, et al. 2014). Sub-adult females dominated summer catches off South-West England , while during the winter season, a bigger presence of female specimens was

recorded off coasts of Portugal and around the Azores (Pratt, 1979; Simpfendorfer, et al. 2002; Quieroz, et al. 2005; Campana, et al. 2006).

Although these long-term studies on blue shark movement using the capture and recapture method are an important source of information about the life history traits of the BS (ICCAT, 2013), there are limitations of these methods for the study of elasmobranchs population structure (Queiroz, 2010), such the incidental loss of tags, the undeclared catches or the erroneous assignment of the shark movement behavior to the correspondent release-recatch trait (Begg & Waldman, 1999; Queiroz, 2010).

Since there are no behavioral records during the span of time between the tagging and the recapture, the movements interpretations must be done with the right caution, especially when the estimated movement of the specimens are limited in space close to potential geographical barriers (e.g. specimens captured by a fleet in the Atlantic, but declared in the place of origin of the fleet, which may be the Mediterranean). Despite recent advances in satellite tagging technologies have provided scientists growing opportunities to resolve previously unknown spatial ecology, minimizing the biases attributable to the problems of conventional tagging with plastic tabs (Hammerschlag, et al. 2011), no satellite tagging data are available for BS between the North Eastern Atlantic and the Mediterranean Sea.

Regardless the advantages and limitations of the various methods used for the study of population dynamics, especially when investigating endangered and/or economically important species, integrated and interdisciplinary study is fundamental for a robust population structure study (Coyle, 1998).

Using mitochondrial and nuclear markers, the presence of a significant genetic heterogeneity among potential blue shark nurseries from the Atlantic Ocean, Portugal, Azores and South Africa was detected by Sampaio da Costa (2013), suggesting a deeper separation between nurseries of the northern (Portugal and Azores) and southern areas (South Africa) and supporting a male philopatry behavior to mating areas exclusively contributing to a single nursery ground.

The results of the DELASS project (Development of Elasmobranch Assessment – Heessen, 2003), which aims to improve the scientific basis for the management of fisheries affecting elasmobranch species, supported the presence of only one stock of BS in the NA (Fitzmaurice, et al. 2005), and the most probable division between North and South Atlantic BS stocks would be located near the 5°N parallel. This hypothesis was supported also by the ICCAT Shark Assessment Working Group (ICCAT, 2009). At least three stocks are identified in the Atlantic Ocean (i.e. the Mediterranean,

the North Atlantic and the South Atlantic BS stocks (ICCAT, 2009). However, tagging data from the Mediterranean Sea, combined with genetic data, are still lacking for this area, making difficult to define whether the area is really characterized by a different stock. Previous tagging projects highlighted how, in the NA BS stock, specimens would probably undertake cyclical up and down migration movements between 30-50°N, while it seems there are limited movements of BS between the NA and the Mediterranean Sea. (Kohler, et al. 2002; Skomal & Natanson, 2002; Fitzmaurice, et al. 2005), suggesting two separate stocks. Overall, it has to be noted that most of the data supporting the present BS stock structure are mainly related to geographic range, movements and migrations, while the genetic data are still poor and insufficient to investigate the potential separation suggested for NA and Mediterranean populations of BS.

Genetic studies have been carried out on worldwide populations using neutral microsatellite loci. Fitzpatrick et al. (2010) analyzed approximately 1,000 individuals collected worldwide at 16 neutral microsatellite loci. This work revealed an inter-oceanic genetic structuring with a gene-flow restricted within ocean, while a study on the population genetic of Brazilian BS populations (Ussami, et al. 2011), using the same markers, highlighted a moderate population structure among samples of Rio Grande do Norte, São Paulo and Rio Grande do Sul.

Verissimo, et al. (2017) carried out a population genetic analysis targeting three reported BS nursery areas in the Atlantic (namely, western Iberia, Azores, and South Africa) over two time periods (2002–2008 and 2012–2015). The results, based on the variation of mtDNA and neutral microsatellite markers revealed a temporally stable genetic homogeneity among the three Atlantic nurseries at both types of markers, suggesting a basin-wide panmixia. However, the analysis of molecular variance highlighted a weak but significant differentiation of the Brazilian BS from those of the Atlantic nurseries, at both mtDNA and microsatellites. In addition, a comparison of the mitochondrial DNA (control region) sequences between Atlantic and Indo-Pacific locations indicated genetic homogeneity and unrestricted female-mediated gene flow between the two oceans.

Summarizing the results from previous works and the state of art, in the Pacific and NA Ocean a poor, or lack, genetic structure was observed, even at a broad geographic and oceanic scale (Ovenden, et al. 2009; Sampaio da Costa, 2013, Verissimo, et al. 2017).

In a recent work carried out on global distributed BS, using both mitochondrial and nuclear (microsatellites) markers, Bailleul, et al. (2018), explaine this apparent global panmixia, by a genetic lag-time effect and demographic changes that were not detectable using conventional

genetic analysis before a long transitional phase nicknamed as the “population grey zone.” The authors argued that these results can thus encompass distinct explanatory scenarios, such single demographic population or several independent populations. Finally, the authors, surged that this phenomenon prevents any possibilities of genetic-based delineation of stocks, highlighting the importance of use more powerful genomewide markers to access more effective genetic information in order to discriminate potential different populations (Baileul, et al. 2018).

To date, just one genetic (using few specimens from the Adriatic Sea) and no genomic investigations were carried out on Mediterranean BS, highlighting the extreme importance to assess correctly this population (namely, based on a clear and reliable structure either within the basin or between the Mediterranean BS and those inhabiting the adjacent eastern areas of the NA) for its conservation (Ferretti, et al. 2008). Kohler et al. (2002) suggested that the Mediterranean BS are most likely local residents rather than occasional visitors, but to date there is a lack of knowledge about the real genetic structure and phylogeographic characteristics of these still poor known population.

1.3 Fishery, current status and management

The BS is rarely targeted by commercial fisheries, but it's a common species of by-catch of longline and driftnet fisheries, particularly in the areas with a massive use of high-seas fleets. Unfortunately, the majority of this by-catch is not recorded, and much of it, is likely to be valued and retained as 'by-product' and selled in the fish markets at low price or as shark fin (Fields, et al. 2017).

A recent study revealed how the BS fins are the most prevalent in the Hong Kong shark fin market (Fields, et al. 2017) and represent the majority of the global market. It has been estimated that 10.7 million of BS individuals (0.36 million tonnes) are killed for the global fin trade each year (Clarke, et al. 2006a,b). Just in the Hong Kong market, from 34.1 to 64.2% of shark fins were from BS (Fields, et al. 2017). With the economic crisis, this species has increased in commercial value and incidental catches are now rarely discarded. The BS meat is marketed in Greece, Italy, and Spain fish markets, and fins exported to Asia as luxurious food (Megalofonou, et al. 2005a).

In the Mediterranean, there are currently no BS catch limits or other regulation for protection, and this growing BS fin and meat trade is not monitored, despite the species' prominence in international trade (Rose, 1996; Clarke, et al. 2006a,b) and BS trans-oceanic migrations that

strongly support arguments for regional and global management (Camhi, et al. 2009). In 2013, the European Union (EU) banned removal of shark fins on board vessels (Regulation No. 605/2013), following the advice from the International Union for the Conservation of Nature (IUCN)'s Shark Specialist Group, in order to enhance the enforcement of the 2003 EU ban on shark finning (Regulation No. 1185/2003), improving the collection of fishery data for BS.

Overall, the trend data are available only for some BS populations and stock assessments are highly uncertain (Dulvy, et al. 2014, Coelho, et al. 2017); due to the huge amount of by-caught BS, the species has being categorized worldwide as "Near Threatened" by the IUCN Red List (Stevens, 2009).

In order to address conservation issues, the EU has promoted an actions of conservation based on the International Action plan for the Conservation and Management of Sharks (IPOA SHARKS) adopted by the FAO in 1998 (FAO, 1998). The EU Action plan for the Conservation and Management of Sharks aims to i) improve the knowledge on shark fisheries and ecosystem role , ii) regulate by-catching enhancing sustainable fisheries of sharks and iii) encourage a coherent approach between the internal and external EU fishery policy for shark fisheries.

This EU Action plan is particularly important for the Mediterranean BS, in fact, according to recreational and professional fishery data, BS was more abundant in the Mediterranean Sea, while in the last 30 years there was a drastic decline of its estimated abundance (Ferretti et al., 2008; Damalas & Megalofonou, 2012). Given this huge loss of estimated abundance and the high fishing pressure to which BS is subjected in the Mediterranean Sea, the Mediterranean BS is categorized as "Critically Endangered" by the IUCN Red List (Sims, et al. 2016). Most of the BS caught in this area (99% and 98% for males and females, respectively) were immature, indicating that the Mediterranean BS stock consists primarily of small immature BS of both sexes, with a sex-ratio skewed toward females or males, depending on different geographical areas (Kohler, et al. 2002, Megalofonou, et al. 2009).

A high number of pregnant females was observed in the Adriatic, North Ionian Sea and Ligurian Sea, suggesting potential nursery grounds for BS (Megalofonou, et al. 2009; Garibaldi, 2017 pers. comm.). On the other hand, the adjacent BS inhabiting the South-Eastern area of the NA was prevalently composed by primarily mature individuals of both sexes with male-based sex ratio.

The biological concept of population is of extreme importance in both types of definition to fishery management; the ecological and the evolutionary. The ecological definition of population, rely on the temporal-spatial co-occurence of individuals from a group and their demographic interections,

whereas the evolutionary definition of population rely on genetic structure due to reproductive interactions among individuals (Waples and Gaggiotti, 2006).

Is well documented that the fisheries management and stock assessments are manly based on the ecological definition of populations, whereas the main aims of a sustainability of fish stock and the conservation of their genetic diversity are manly based, contrarily, to the evolutionary definition of populations.

Reiss, et al. (2009) observed this mismatch in several ecologically different, and commercially important, fish species of the North Atlantic Ocean. Such discrepancy results in a biased and potentially ineffective management of fishery resources. In fact, delineation of management areas for a particular fish species, does not necessarily delineate biological meaningful entities, such a population (Carvalho and Hauser, 1994; Waples and Gaggiotti, 2006), highlithing the need for an integration of genetic and ecological data into stock management strategies.

Integrating ecological and genetic data in a fishery management strategy is beyond the scope of this thesis, however, it is very interesting to observe that the stock units delineation and the management of the blue shark stocks between the Mediterranean Sea and the Atlantic Ocean are solely based on ecological data (e.g. mark tagging, release, recapture and fishery data) (Kohler, et al. 1998; 2002; ICCAT; 2015).

These data led the scientists to consider the Mediterranean BS population as a separate stock (Fitzmaurice, et al. 2005), and the data collected in the following decade, have not shown results contrasting this hypothesis (Kohler, et al., 2002; ICCAT, 2015).

In a perspective of application of genetic methods to fisheries management, it's of crucial importance the assessment of the divergence among potentially different populations, and the amount of divergence needed to justify a separation in the management of fishery resources of the target species, taking into account also the management and the conservation objectives for that species (Waples, et al. 2008; Palsbøll, et al. 2007).

1.4 Genetic techniques for stock identification

The use of molecular genetic techniques for stocks identification has heavily increased over the past years due to the increased availability of new methodologies, ranging from the allozymes in the past decades, to mitochondrial DNA (mtDNA), microsatellites (from nuclear DNA), and finally, single nucelotide polimorphisms (SNPs) with the advent of Next Generation Sequencing Technologies (Koljonen & Wilmot, 2004; Magoulas, 2004; Kumar & Kocour, 2016).

1.4.1 Allozymes

Allozymes are the electrophoretic expression of alternative gene forms (alleles) of enzymes produced by a specific gene locus. The stock identification using allozymes is based on genotypes of individuals, that are the result of the combination of two alleles of a particular gene locus for that individual (Koljonen & Wilmot, 2004).

Looking at the genotypic composition over many loci of a group of individuals, of a possible population to investigate, we can observe a multilocus genetic mark that may be useful to discriminate different populations (Pella & Milner, 1987).

As usual for different genetic markers, allozymes data have bot advantages and the limitation. Looking at the advantages, the cost of analysis is extremely low compared to other methodologies, and since laboratory analysis is simple, a large amount of samples can be analyzed in short time. The allozymes data are discrete characteristics and its interpretation is relatively unambiguous. The genetic differences are usually stable from generation to generation, and in several cases, loci can be assumed to be not correlated variables, simplifying the data analyses (Waples, 1990).

Otherwise, looking at the limitations of allozymes markers in stock identification, there are several aspect that can affect the discriminant power of these marker, making difficult to correctly to delineate fishery stocks. A sufficient reproductive isolation among potentially different stocks for temporally stable genetic differences in allele frequencies is crucial to arise a sufficient discriminatory power in stocks delineation. In fact, differences in allele frequencies may occur, but they may be too small for justify a stock separation (Koljonen & Wilmot, 2004).

Allozymes are valid markers for that species where clearly different reproduction units were observed, but usually this is not the case of large pelagic species, such the BS. In these case, allozymes information alone is not sufficient for accurate stock identification, and more powerful methods and/or additional information is necessary for a correct sotcks delineation of the species (Koljonen & Wilmot, 2004).

1.4.2 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is a small size (usually around 16,000 base pairs (bp) long, in fishes), double-stranded circular DNA molecule observed in multiple copies in the mitochondria.

This mitochondrial genome (mitogenome) usually contains thirteen protein coding genes, two coding genes for ribosomal RNAs (12S and 16S rRNA), twentytwo coding genes for transfer RNAs

(tRNAs), and one noncoding control region and its d-loop, that occur during DNA replication and reparation (Chen & Butow, 2005).

The mtDNA evolves rapidly and the rate of mutation events of species that shared a common ancestor is estimated to be 2% per million years (Moritz et al., 1987). The non-coding portion of the mtDNA, such the control region (and d-loop) evolve very rapidly, making it extremely useful for high-resolution analysis of intraspecific population structure, while The slow evolving coding genes, such the CO I, II, III (coding for the Cytochrome Oxidase subunit), and the Cytochrome b are more suitable for interspecific analysis and species delineation (Hebert, et al. 2003).

Mitogenome is, generally, nonrecombining, and usually no novel genotypes will be generated because the two recombining sequence should be identical even in case of recombination, due to homoplasmy, typical in mtDNA (Rokas et al., 2003) The inheritence of mtDNA is exclusively marternal, and despite recent reports of cases of paternal mtDNA inheritance (Rokas et al., 2003; Passamonti, et al., 2011), the inheritance is predominant maternal. These mtDNA properties materialize in the creation of linear evolutionary history of maternal inheritance (matriarchal phylogeny) (Magoulas, 2004).

There a two main methods for the Analysis of mtDNA: a) restriction analysis, b) polymerase chain reaction (PCR) analysis.

Restriction endonucleases (RE) are enzymes that are able to recognize specific target sequences, from 4 to 6bp long, on the mtDNA and cleave the DNA in a specific position within the recognition sequence, with a process called restriction or digestion. With this process, the mtDNA can be cutted in as many multiple fragments as there are recognition sites for the endonucleases enzyme. The lenght in base pairs of the fragments produced are determined by the position of these recognition sites on the DNA.

In a RE analysis, the number and the size of these fragments can be detected after separation on a gel matrix of an electrophoresis. The key of this method is that for each mtDNA molecule from each individual, a restriction profile is produced, enabling the comparison of the restriction fragment profiles of several individuals as a representation of the nucleotide variation of their whole mitogenome. This analysis is also known as RFLP (restriction fragment length polymorphism) analysis (Botstein, et al. 1980).

The Polymerase Chain Reaction (PCR) (Mullis, et al. 1987) uses a thermostable enzyme for the DNA synsthesis, extracted from the thermophilic bacteria, *Thermus aquaticus*; the Taq polymerase.

This enzyme is used to replicate a strand of DNA starting from small amounts of DNA samples using appropriate primers, that are short fragment of single-stranded oligonucleotides (around 20bp in length), complementary to the regions flanking the target segment. The number of DNA strands copies produced during each cycle of PCR follow an exponential increasing.

Among the big advantages of the PCR technique, there are the rapidity of the method, the small amount of starting DNA needed for the process and the affordability.

Before a PCR analysis, is fundamental to choose the appropriate region of mtDNA to be amplified. For example, for population structure analysis the control region (or the d-loop) is usually a powerful gene because its variability, able to show a good amount of intraspecific variation. Neverthless, even stable protein-coding genes, such the cytochrome b, tend to show intraspecific variation, and it was used fish stocks delineation (Meyer, 1993).

The advantage of the PCR analysis over the RFLP analysis alone, is that the use of PCR-based analyses are usually more sensitive indicator of intraspecific differentiation than allozyme analysis (Avise, 1987).

Looking specifically at large pelagic marine species, ecologically more similar to the BS, several studies were carried out using mitochondrial DNA variation with different results.

The yellowfin tuna, *Thunnus albacores*, have shown little divergence among populations between ocean, and larger mtDNA differentiation within oceans (Scoles and Graves, 1993; Ward, et al. 1994). On the contrary, in two different tuna species, the albacore tuna, *Thunnus alalunga*, and the bigeye tuna, *Thunnus obesus*, differentiation between oceans (Atlantic vs. Pacific) was more evident (Chow and Ushiama, 1995; Alvarado Bremer, et al. 1998; Grewe and Hampton, 1998).

In the swordfish, *Xiphias gladius*, discrete Mediterranean and southern-eastern (Gulf of Guinea) Atlantic stocks were observed using RFLP analysis of mtDNA (Kotoulas, et al. 1995), subsequently confirmed using mtDNA sequences variation analysis (Chow & Takeyama, 2000) highlighting also the existence of a third stock in the Indo-Pacific. Finally, using a combined RFLP- DNA sequencing technique, Alvarado Bremer et al. (1996) found four different swordfish stocks in the Pacific, in the North Atlantic, in the South Atlantic and in the Mediterranean Sea, while Reeb et al. (2000), detected population structure in the Pacific Ocean, highlighting that northern and southern Pacific populations were significantly divergent.

As other genetic techniques for stocks delineation, also the mitochondrial DNA analyses are not immune of some defects. The main defect stemming principally from the uniparental inheritance of the mtDNA (in most of the cases).

If analysing the mtDNA in a continuosly distributed species, a geographically break is found, it is used to think that two different stocks have been genetically isolated for long time, but the same genetic signal can be observed in a species with limited dispersal capability and gene flow, suggesting caution when interpreting such geographical mtDNA break as evidence for different fishery stocks (Neigel and Avise, 1986). A good approach with this kind of potential bias could be the integration of non-genetic factors, such the life history and environmental carachteristics that may have played a role as barriers to gene flow, prior to decide if these stocks are real or not.

Since mtDNA is usually maternally inherited and usually it is not influenced by recombination, without the contribution of additional mutations, all offspring and their mothers will exhibit identical mtDNA haplotypes.

Furthermore, since the mtDNA is haploid, its effective population size (Ne) is a quarter of the Ne exhibited by autosomal sequences in nuclear DNA (nDNA) (Hare, 2001).

1.4.3 Nuclear DNA

The nuclear DNA (nDNA) is composed of more than 3 billion base pairs (bp), and it provides more powerful diagnostic markers, rather than the mtDNA alone, useful for stocks discrimination, offering a huge amount of DNA sequences variation that can be explored.

Nuclear DNA contain regions which have different functions (protein coding, noncoding and regulation of gene expression) .

Because of these different functions, the nDNA is composed by both highly conserved regions and more variable regions. The genes that encode for proteins are usually in single copy (except for polyploid genomes), and these coding regions have two regions within genes named exons and introns. The exons cointain the information that is transcribed into mRNA and encode for proteins, tRNA, and rRNA, and for these reason their sequences are under tight selective restraints, except for sites at the third nucleotide position within codons at which mutations doesn't results in amino acid changes for that protein.

Within the areas among genes of the nuclear genome, and in internal noncoding regions of genes, there are loci with show highly repetitive sequences called microsatellites and minisatellites. These loci are comprised of extremely short sequences in tandem repeat sequences. Microsatellites are comprised of 1–6bp of repeated sequences, while the minisatellites are longer, with usually 10 bp to 40 bp in length.

Since these loci are carachterized by high levels of allelic diversity and heterozygosity, and high level of polymorphisms, microsatellite are extremely useful for stock structure analyses.

One of the reasons why the microsatellites are powerful and resolutive markers for population genetics analyses is that microsatellite loci present higher levels of genetic diversity than in the other types of DNA typically analyzed, especially for fish (Neff and Gross, 2001; Zane, et al. 2003). Mutation rates for microsatellites can vary considerably among species (Zhang and Hewitt, 2003).

Stocks delineation using genetic markers is highly dependent on the amount of gene flow among stocks, the size of populations and their evolutionary history. In this sense, marine species can be very challenging cause the large population size of many species, their relatively hgh gene flow (especially for pelagic species and for species with a high larval dispersal) and the recent evolutionary history of many populations, dated back to post-Pleistocene colonization (Pita, et al. 2010; Cuéllar-Pinzón, et al. 2016; Pita, et al. 2016).

Among the firsts successfull applications of microsatellite analyses for stocks identification, there are the Atlantic cod study case. Canadian researchers were able to isolate 64 microsatellite loci, carachterizing the genetics diversity among Canadian Atlantic cod stocks (Brooker, et al. 1994) Using selected hypervariable loci, the researchers were able to discriminate adult northern cod aggregations on offshore banks known as Flemish Cap, a second northern aggregation on Funk Island Banks and a southern aggregation on Grand Banks (Bentzen, et al. 1996; Ruzzante, et al. 1998). Despite the impressive decline of cod stocks at spawning banks off Newfoundland and Labrador, a long-term stability was observed over 30 years as revealed by microsatellite analyses (Ruzzante, et al. 2001). In fact, no significant allelic differences were observed between contemporary samples (dated back to 1990s) and archived otoliths sampled in the 1960s. Similar results would not be observable using mtDNA alone.

The BS is carachterized by an incredibly vagility, high gene flow, large population size compared to others shark species, but a fewer offspring (Pratt, 1979; Verissimo, et al. 2017). Given its high vagility and high level of gene flow, find a genetic structure, even at interoceanic level, is challenging, and also hypervariable microsatellites failed in finding a clear genetic structure (Verissimo, et al. 2017), suggesting that an integrative approach with more molecular markers should be used, rather that use just a single approach.

1.5 Conservation genomics of sharks and traceability: the impact of philopatry and the importance of a mtDNA/nDNA investigation approach

The constantly decreasing costs and rapid advancements of next-generation sequencing (NGS) technologies have revolutionized the field of population genomics of non-model species offering a paradigm shift from gene to genome-wide research in the field of fisheries and aquaculture (Kumar & Kocour, 2016).

The use of this genome-wide markers has proved to be very useful both for the traceability and for the stocks delineation of various marine species of commercial importance (Nielsen, et al. 2012), and these markers can be useful for highly vagile species, such the BS.

In 25 years, various genetic tools were used to address ecological and evolutionary questions in elasmobranch studies, and these tools were rapidly evolved in the last decade (Dudgeon, et al. 2012), however, still few genomic markers were applied to address these biological questions.

Among the reasons why of this scarce use of genomic markers on sharks, there are the low commercial value compared to more commercialized species, which has led to give precedence in economical funds to the latter and the scarcity of shark genomic references. In fact, to date, just few reference genome are available for chondrichthyes, such the elephant shark (that is a chimaera and not a shark, with big differences in genome size and, potentially, structure); the whale shark, that despite it is a charismatic species and its conservation is fundamental, is a phylogenetically distant and taxonomically different order of shark (Orectolobiformes) compared to carcarinid sharks, such the BS; and the little skate (Wang, et al. 2012; Venkatesh, et al. 2014; Read, et al. 2017).

Fortunately, despite the lack of necessary reference genomes, the use of new genomic technologies has made it possible to study single nucleotide polymorphisms makers (SNPs) at genome-wide level with a *denovo* approach, enabling the genomic study of non-model species, such the BS, and improving the conventional traceability methodology, using thousands of both neutral and potentially under selection markers (Davey & Blaxter, 2010).

Despite the advent of new single nucleotide markers polymorphisms (SNPs), it is useful to maintain a multiple approach, analyzing both mitochondrial and nuclear DNA. The analysis of both matrilineal and uni-parentl inheritance markers are indispensable for a correct populations

delineation, cause shark species are usually characterized by a marked philopatry. In fact, evidence for reproductive philopatry and/or sex-biased dispersal, has been observed for the bull shark, *Carcharhinus leucas*, the great hammerhead, *Sphyrna mokarran*, the white shark, *Carcharodon carcharias*, and several other species of shark (Pardini, et al. 2001; Jorgensen, et al. 2010; Tillet, et al. 2012; Guttridge, et al. 2017).

Reproductive philopatry can be problematic for conventional concepts of population structure as genetic differences can occur between regions in the absence of physical barriers to movement (Dudgeone, et al. 2012). This behaviour highlight the complex patterns of habitat use in elasmobranchs, that are not solely influenced by the presence or absence of environmental barriers, but the structure of their populations could be behaviuor-related (Feldheim, et al. 2001; Pardini, et al. 2001; Hueter, et al. 2005).

These complex behaviour in shark species, highlight the need for a mtDNA/nDNA integrating approach, in order to investigate both matrilinean and bi-parental inheritance genetic information for a correct populations delineation in shark.

1.6 Conclusions and future actions

The BS of the Mediterranean Sea have faced a decline over the last century (Ferretti, et al. 2008; Damalas & Megalofonou, 2012) , and a deeper understanding of spatio-temporal population dynamics is necessary and fundamental in order to better manage and conserve this functionally important ecosystem biodiversity. This 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 cascade effect (Myers, et al. 2007). Surface drifting long-line fisheries is considered the principal source of BS fishing mortality all over the Mediterranean Sea (Megalofonou, et al. 2000). A comparison between historical BS catch rates recorded in the North Ionian Sea in the 1980s (De Metrio, et al. 1984; Filanti, et al. 1986) with those recorded 20 years later (Megalofonou et al., 2005) revealed a decrease of 38.5%. According to Ferretti et al. (2008), the Mediterranean BS populations have been subjected to a 75%-90% decline in abundance over the last 30 years, particularly in the Ionian Sea.

Modelling studies apart, there are no studies of connectivity and dispersal in Mediterranean BS populations and the poor info and data on the ecology and population dynamics of this species derive mainly from the Atlantic and Indo-Pacific populations.

Transoceanic migrations of BS in the NA are well known, while movements in the Mediterranean are not yet well known. These BS oceanic movements overlap with industrial long-line fishery areas (Stevens, 1976; Casey, et al. 1985; Diaz & Serafy, 2005; Queiroz, et al. 2012) and they were found to be different for stage and maturity of individuals as well as for distribution of prey, suggesting a complex reproductive cycle with oceanic migrations associated to mating and pupping areas (Pratt 1979, Casey 1985, Stevens 1990). BS spatial habitat heterogeneity and the overlapping of migratory routes with longline fisheries might be correlated with a decline of BS populations of approximately 30% of CPUE in the western North Atlantic from 1957 to 2000 (Aires-da-Silva, et al. 2008).

Still few and unclear, tagging data of Pacific BS populations obtained from longline and drift-net fisheries allowed to conduct modelling analysis that suggested decline pattern starting from 1980s followed by recovery (Kleiber, et al. 2009). However, the limited fishery assessments carried out, shown no evidence of catch rate declines of BS in the Atlantic or Indian Oceans. The biggest issue is that most of catches, since it is not a target species, are undeclared, and therefore similar results could be strongly underestimated. Furthermore, specifically for Mediterranean area, very poor tagging and no robust genetic data were reported.

With the advent of Next Generation Sequencing technology it is now possible to apply the resources of population genomics (i.e. several hundreds-thousands of marker loci; potentially adaptive loci) to resolve the connectivity and structure dynamics of BS populations. Modern population genetics is offering powerful tools to identify connectivity and structure of marine populations, which might escape direct observation. The multivariate analysis of genetic data is particularly crucial when dealing with relatively weak genetic differences, as commonly detected in high-dispersal marine species. Multidisciplinary seascape genetics (sensu Selkoe, et al. 2008; Hemmer-Hansen, 2007) addressed important issues in the spatial ecology of marine populations combining genetic and oceanographic data under ecological modeling (Gerlach, et al 2007; Riccioni, et al. 2013).

The rationale of this thesis and of the tendered BlueSMedGen project underlies on the ecology of the target species *Prionace glauca* (Blue Shark; BS). The combination of a near-capillary sampling strategy and the development and use of genomic markers - that can represent both neutral and selective markers – offers the opportunity to assess and visualize for the first time for this poorly studied and vulnerable species population structure and local adaptation, and to help the scientific community to develop genome-wide markers useful for the species traceability.

1.7 The MedBluesGen Project

1.7.1 Introduction to the project

This thesis was developed in the context of the EU funded project MedBluesGen.
The project aims to undertake the first genetic and genomic survey of the Mediterranean BS, with a special focus on its connectivity with the adjacent Atlantic Ocean.
Many international institutions collaborated within the MedBluesGen project:

1) The Alma Mater Studiorum - University of Bologna (UNIBO) - Leading Partner;
2) The National and Kapodistrian University of Athens (NKUA);
3) The University of Padova (UNIPD);
4) The Institute Español de Oceanografia (IEO);
5) The University of Calabria (UNICAL);
6) Queen's University Belfast (QUEEN'S);
7) University of Genova (UNIGE), and
8) The Joint Research Center (JRC), European Commission.

The Joint Research Centre (JRC) operates a network of National Contact Points (NCPs) which was created under the Seventh Framework Programme for Research and Technological Development for 2007-2013 and is still going on under the Horizon 2020 Framework Programme (2014-2020).
The mission of the JRC NCPs is to act as intermediaries and operational contact points between the JRC and the relevant stakeholders from the scientific community, industry and public authorities of the EU Member States and Associated Countries.
In this context, the JRC had a key role in the communication among the research groups coordinated by the UNIBO team, as leading partner of the MedBlueSGen.
Following the mission of the JRC and in order to render the data public, a complete and coincise presentation of the project is available at the official website: https://fishreg.jrc.ec.europa.eu/web/medbluesgen/

1.7.2 MedBluesGen aims

The main idea behind this initiative is to contribute new unprecedented knowledge on Mediterranean BS, by creating a robust baseline of data describing the genetic stratification of the BS in the Mediterranean Sea. The approach would shed light on aspects related to their population structure, the connection to non-Mediterranean populations, such the NA population and its nursery areas, and help to design management schemes in order to strengthen conservation efforts for this highly by-catched species of shark. The key objective is to scrutinize the prevailing assumption that Mediterranean BS form only one population, or one potential "stock".

This is of extremely importance since the huge loss in biomass of BS in the Mediterranean Sea (Ferretti, et al. 2008). Obviously, the findings will be of use to all scientific and management bodies (ICCAT, GFCM/FAO, IUC, ICES, STECF), in that they will help to tailor current management schemes through the assessment of the true status of the population stock(s), helping to revise and enhance conservation actions.

The tendered MedBlueSGen project aims to:

- Build up an unprecedented archive of BS tissue sample associated with biological and fisheries data
- Develop novel genomic resources and assess genetic diversity and spatial population structure of BS
-Apply population genetic analysis for visualizing genetic break and barriers to gene flow among subpopulations
-Create genomic resources that can be useful for the traceability of BS products at global level.

1.7.3 Sampling design

The sampling strategy of the project is based on the sampling design targeting Western/Central Western and Central Eastern/Eastern Mediterranean Sea as two main areas as suggested in previous work on distribution and potential segregation of BS in the Mediterranean Sea (Megalofonou, et al. 2009). A third area included in the sampling design is the North-Eastern Atlantic (**Fig 2.1**), divided in South North Eastern Atlantic (off the Atlantic coasts of Spain and Portugal, and in North North Eastern Atlantic (off the coasts of South England and Celtic Sea).

Sampling of BS in the target areas and seas was carried out during the first six months of the project by Team partners UNIBO, NKUA, UNICAL, IEO and QUEENS, excluding the tissues which have already been made available by project collaborators as archived samples. Sampling was carried out between 2003 and 2016 and tissue specimens and individual data were collected by means of contracted commercial fishermen and scientific surveys.

During the tissues collection, muscle or skin tissue from by-catched specimens of BS from both NA and Mediterranean Sea were stored in 96% ethanol.

1.7.4 Public database and tool

Since the BS specimens came from accidentally catched fishes, in most of the cases, biological data for each individual, such size (in cm), sex (female/male) and fishery data such capture date and geographical coordinates (longitude/latitude) were collected. These data were further used into a GIS-interfaced database to geo-represent collected samples and genetic variation of BS at the seascape level (**Tab.2.1**).

Trying to promote the transparency of the results and the workplan, as part of the JRC mission, a unique webtool for the consultation and the download of these data was created in addition with the graphic presentation of the sampling design, accompanied by all the biological characteristics of the sampled sharks.

This free webtool is available for consulting and data download visiting the project web site (https://fishreg.jrc.ec.europa.eu/web/medbluesgen/sampling-data).

1.7.5 Preliminary results from biological data

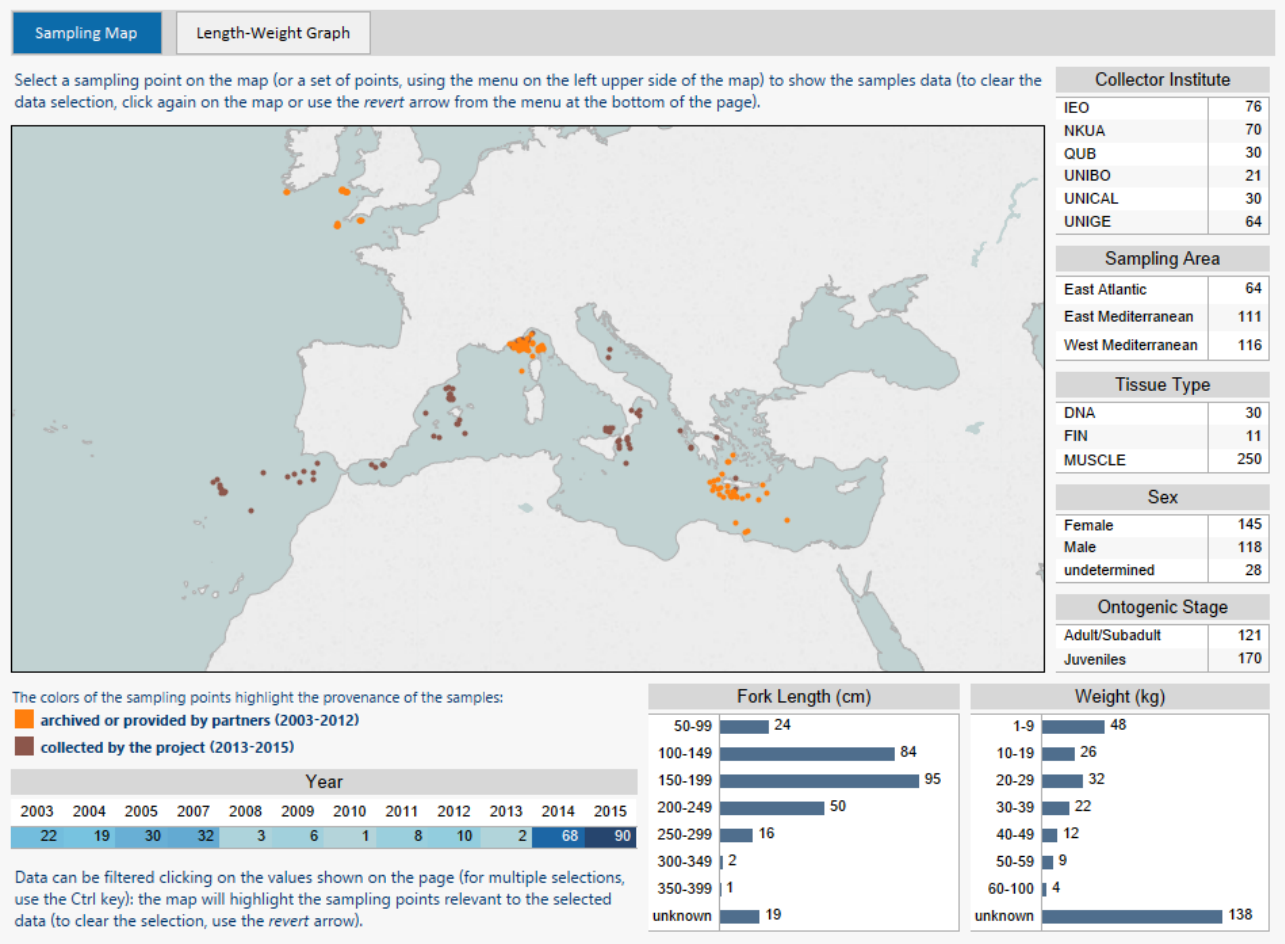
A total of 291 Blue Shark samples (**Tab 2.1**) were obtained from the Eastern Mediterranean (EMED: N = 111), Western Mediterranean (WMED: N = 116) and adjacent North-eastern Atlantic waters from Gibraltar to Azores (Southern North-eastern Atlantic, SEATL: N = 34) and from Southern Ireland and Great Britain (Northern North-eastern Atlantic, NEATL: N = 30).

Among collected BS, 118 males and 145 females were sexed while 28 individuals remained unsexed. Of these sexed individuals (N = 263), BS females outnumbered significantly males in the North-eastern Atlantic population samples (sex-ratio = 0.26, χ^2-test: 10.256 P2tail = 0.001; P1tail = 0, d.f. 1) while in the two Mediterranean BS groups a weak and not significant predominance of

males was observed (WMED: 1.08, χ^2-test: 0.786 P2tail = 0.375; P1tail = 0.188, d.f. 1; EMED: 1.07, χ^2test: 0.087 P2tail = 0.768; P1tail = 0.384, d.f. 1) (**Tab. 2.2**).

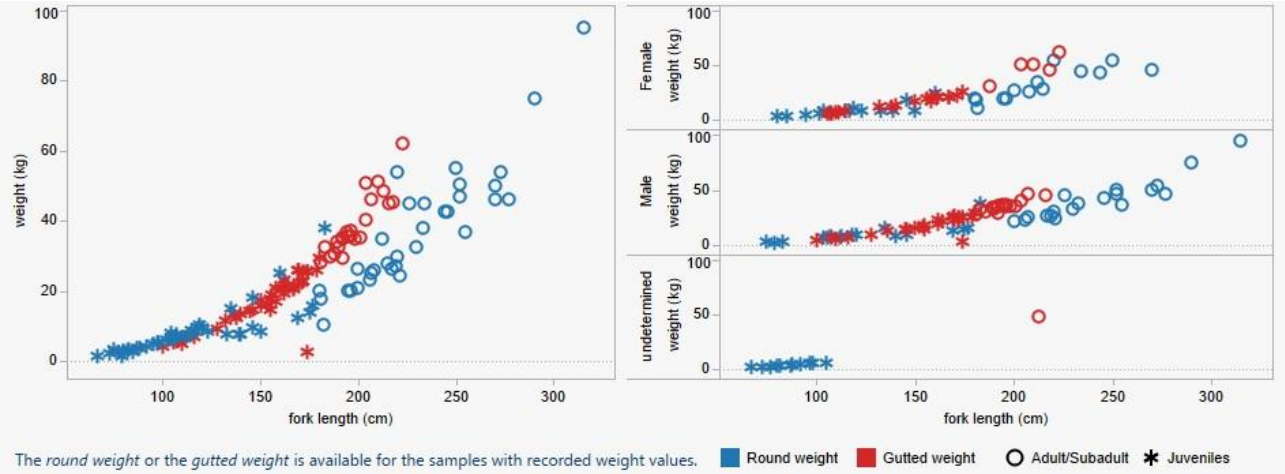
The BS individuals were grouped according the Total Length (TL) in three size categories (Vandeperre, et al. 2014). Overall, juveniles (FL < 180 cm) predominated over the large sub-adult and adult (FL > 180 cm) individuals (J: N = 170; L: N = 121). In the population samples collected from North-eastern Atlantic, large sub-adult and adult BS are more abundant than juveniles (J: N = 28; L: N = 36). In the WMED juveniles predominated over large sub-adult and adult BS (J: N = 86; L: N = 30), while in the EMED any predominance was observed (J: N = 56; L: N = 55) (**Tab 2.1**).

Fig. 2.1: Sampling design of the blue shark samples by the MedBlueSGen project and the already archived BS specimens, previous collected by collaborators of the project. The image is freely available at the project website, and synthesizes biological and fishery data of the samples.



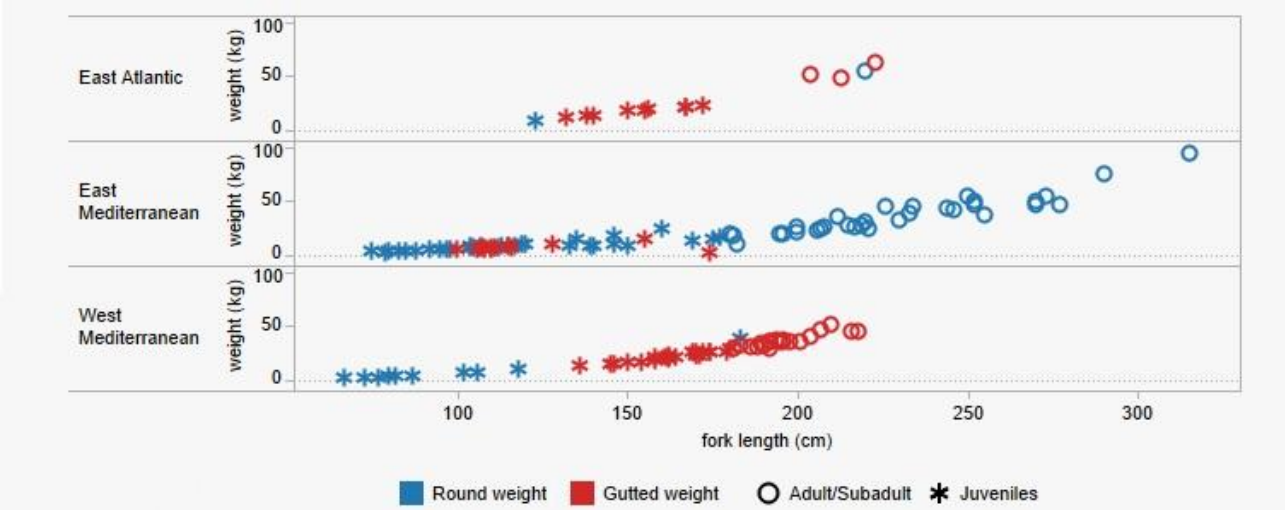
The sampled BS length ranging from <1 m to > 3 m, with the males that usually reach the bigger size (between 2 and 3 meters long) (**Fig. 2.2**).

Fig. 2.2: Weight and fork length distribution along the overall BS collected and along the sexed specimens. This image is freely available to download from the official project web site.



The bigger BS are usually located in the Eastern Mediterranean, however, sexually mature BS were observed at both sides of the Mediterranean, with a major concentration in the Eastern Mediterranean, and at both sides of Gibraltar (**Fig. 2.3**).

Fig. 2.3: Weight and fork length distribution along the BS collected, splitted in three different macroareas, suche the East Atlantic, the East Mediterranean and the West Mediterranean. This image is freely available to download from the official project web site.



Despite the low sample size, these preliminary results from biological data, suggests a potential segregation by sex and stage of BS within the Mediterranean basin, as noted in previous work, using bigger sample size, on Atlantic and Pacific Oceans (Nakano, 1994; Kohler et al. 1998, 2002; Nakano & Seki, 2003; Kohler & Turner, 2008; Vandeperre, et al. 2014). A Bigger sample size will be useful, in the future, for better understand these distribution patterns.

Specifically for the Mediterranean Sea, this observation was possible just splitting the Eastern and Western Mediterranean in six sampling location depending on their geographical position between basins and seas within the Mediterranean.

These separations in the sampled BS showed a significant deviation from the 1:1 sex ratio in the areas of the Balearic Sea and the Ligurian Sea (**Table 2.2**), with a strong and significant deviation in favor of females in the Ligurian Sea, and in favor of males in the Balearic Sea.

Tab. 2.2: Sex counts in BS collected and organized in different areas, and related sex ratio test

Location	M	F	na	d.f.	chi-square	P-value (two-tailed)	P-value (one-tailed)
WMED	57	53	6	1	0,145	0,703	0,351
EMED	49	46	16	1	0,216	0,642	0,321
SNEATL	9	19	6	1	3,571	0,059	0,029
NNEATL	3	27	0	1	19,2	0	0
Location	**M**	**F**	**na**	**d.f.**	**chi-square**	**P-value (two-tailed)**	**P-value (one-tailed)**
TYRR	3	1	6	1	1	0,317	0,159
LIGU	22	42	0	1	6,25	0,012	0,006
BALE	32	10	0	1	11,524	0,001	0
CADR	10	11	0	1	0,048	0,827	0,414
IONI	7	7	6	1	0	1	0,5
AEGE	32	28	10	1	0,267	0,606	0,303
SNEATL	9	19	6	1	3,571	0,059	0,029
NNEATL	3	27	0	1	19,2	0	0

The significant deviations from sex ratio 1:1 observed in the Ligurian Sea and in the Balearic Sea could be evidence that this area is an area with a key role in the mating processes between BS of the Eastern Atlantic and BS from the Mediterranean Sea. In fact, the low number of tagged BS observed to cross the Gibraltar Strait with an Eastward migration from the Eastern Atlantic to the Mediterranean were adult males (Kohler et al., 2002). The males BS samples in the Balearic Sea could come from the Eastern NA, and they could mate with the high number of females observed in the near region of Ligurian Sea. The biological and tagging data are unfortunately too lacking, but if this pattern will be confirmed in the future, it will be the confirmation that this area turns out to be a mating area for the BS, while the Eastern Mediterranean could represent a relatively safe port for newborn and juvenile BS individuals. Despite the low sample size, the predominance of female specimens observed in the Nort North Eastern Atlantic, is in line with the sex-based segregation observed in the NA in previous works with a big sample size (Kohler & Turner, 2008; Kohler, et al. 2002).

Tab. 2.1: List of the blue shark specimens collected within the MedBlueSGen project, and related biological and fishery data.

SPECIMEN CODE	BIOLOGICAL DATA					FISHERY DATA			
MEDBLUESGEN CODE	FL (cm)	ROUND WEIGHT (kg)	GUTTED WEIGHT (kg)	SEX	LOCATION	LATITUDE	LONGITUDE	DATE	GEAR
BSH_WMED_IEO_2014_J_001	134			F	North Eastern Atlantic	39,607	0,628	06/02/14	LLHB
BSH_EATL_IEO_2014_L_002	220	54		F	North Eastern Atlantic	35,319	-9,109	15/02/14	LLAM
BSH_EATL_IEO_2014_J_003	123	8,5		F	North Eastern Atlantic	36,515	-7,797	23/03/14	LLAM
BSH_WMED_IEO_2014_L_004	195		35,5	M	North Eastern Atlantic	39,183	3,222	29/05/14	LLSP
BSH_WMED_IEO_2014_L_005	198		35	M	North Eastern Atlantic	38,392	3,612	18/06/14	LLSP
BSH_WMED_IEO_2014_L_006	210		51	F	North Eastern Atlantic	38,901	3,028	21/06/14	LLSP
BSH_WMED_IEO_2014_L_007	207		46	M	North Eastern Atlantic	38,944	3,146	26/06/14	LLSP
BSH_WMED_IEO_2014_L_008	188		30,5	F	North Eastern Atlantic	38,237	1,264	19/07/14	LLSP
BSH_WMED_IEO_2014_L_009	218		45,5	F	North Eastern Atlantic	38,089	1,673	06/08/14	LLSP
BSH_WMED_IEO_2014_J_010	162		19,5	M	North Eastern Atlantic	36,456	-2,599	10/09/14	LLSP
BSH_WMED_IEO_2014_J_011	170		26	M	North Eastern Atlantic	36,436	-2,678	12/09/14	LLSP
BSH_WMED_IEO_2014_J_012	164		21,5	M	North Eastern Atlantic	36,436	-2,678	12/09/14	LLSP
BSH_WMED_IEO_2014_J_013	146		14,5	M	North Eastern Atlantic	36,445	-2,607	14/09/14	LLSP
BSH_WMED_IEO_2014_J_014	161		21	F	North Eastern Atlantic	36,445	-2,607	14/09/14	LLSP
BSH_WMED_IEO_2014_J_015	158		21	F	North Eastern Atlantic	36,416	-2,735	16/09/14	LLSP
BSH_WMED_IEO_2014_L_016	186		30	M	North Eastern Atlantic	36,421	-3,544	28/09/14	LLSP
BSH_WMED_IEO_2014_J_017	162		20,5	F	North Eastern Atlantic	36,303	-3,266	30/09/14	LLSP
BSH_EATL_IEO_2014_J_018	172		23	M	North Eastern Atlantic	35,989	-11,92	02/11/14	LLAM
BSH_EATL_IEO_2014_J_019	150		17,5	F	North Eastern Atlantic	35,999	-8,842	17/11/14	LLAM
BSH_EATL_IEO_2014_J_020	167		20,5	F	North Eastern Atlantic	35,717	-10,065	26/11/14	LLAM
BSH_EATL_IEO_2014_J_021	140		13,5	F	North Eastern Atlantic	35,826	-9,541	12/12/14	LLAM
BSH_EATL_IEO_2014_J_022	167		21	F	North Eastern Atlantic	35,826	-9,541	12/12/14	LLAM
BSH_EATL_IEO_2014_J_023	132		11,5	F	North Eastern Atlantic	35,954	-8,056	18/12/14	LLAM
BSH_EATL_IEO_2014_J_024	138		12,5	F	North Eastern Atlantic	35,954	-8,056	18/12/14	LLAM
BSH_EATL_IEO_2014_J_025	156		19	F	North Eastern Atlantic	35,557	-8,06	19/12/14	LLAM
BSH_WMED_IEO_2014_L_026	192		29,4	M	North Eastern Atlantic	41,012	2,660	11/07/14	LLSP
BSH_WMED_IEO_2014_L_027	194		36,8	M	North Eastern Atlantic	40,691	2,460	16/07/14	LLSP
BSH_WMED_IEO_2014_J_028	171		22,4	F	North Eastern Atlantic	40,661	2,424	17/07/14	LLSP
BSH_WMED_IEO_2014_J_029	158		17,2	F	North Eastern Atlantic	40,738	2,496	06/08/14	LLSP
BSH_WMED_IEO_2014_L_030	216		45	M	North Eastern Atlantic	40,738	2,496	07/08/14	LLSP
BSH_WMED_IEO_2014_J_031	172		25,6	M	North Eastern Atlantic	40,738	2,496	07/08/14	LLSP
BSH_WMED_IEO_2014_J_032	174		25,6	F	North Eastern Atlantic	40,738	2,496	07/08/14	LLSP

BSH_WMED_IEO_2014_J_033	150		16	M	North Eastern Atlantic	41,050	2,169	07/08/14	LLSP
BSH_WMED_IEO_2014_L_034	189		34,1	M	North Eastern Atlantic	41,050	2,169	08/08/14	LLSP
BSH_WMED_IEO_2014_L_035	192		35,2	M	North Eastern Atlantic	41,050	2,169	08/08/14	LLSP
BSH_WMED_IEO_2014_L_036	196		35,6	M	North Eastern Atlantic	41,050	2,169	08/08/14	LLSP
BSH_WMED_IEO_2014_L_037	183		32,4	M	North Eastern Atlantic	41,050	2,169	08/08/14	LLSP
BSH_WMED_IEO_2014_L_038	181		28,4	M	North Eastern Atlantic	40,446	2,322	09/08/14	LLSP
BSH_WMED_IEO_2014_J_039	162		23,2	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_040	136		13,4	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_041	154		16,2	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_042	145		14,2	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_L_043	192		35	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_044	174		25,4	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_045	180		29,4	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_L_046	190		32,6	M	North Eastern Atlantic	41,070	2,379	10/08/14	LLSP
BSH_WMED_IEO_2014_J_047	169		25,8	M	North Eastern Atlantic	40,457	2,546	21/08/14	LLSP
BSH_WMED_IEO_2014_L_048	204		40,4	M	North Eastern Atlantic	40,369	2,439	22/08/14	LLSP
BSH_WMED_IEO_2014_L_049	196		37	M	North Eastern Atlantic	40,449	2,665	28/08/14	LLSP
BSH_WMED_IEO_2014_J_050	170		22,4	M	North Eastern Atlantic	40,395	2,644	06/09/14	LLSP
BSH_WMED_IEO_2014_J_051	179		25,8	M	North Eastern Atlantic	40,395	2,644	06/09/14	LLSP
BSH_WMED_IEO_2014_L_052	201		35,2	M	North Eastern Atlantic	40,395	2,644	06/09/14	LLSP
BSH_EATL_IEO_2015_L_053	223		62	F	North Eastern Atlantic	33,531	-12,886	13/02/15	LLAM
BSH_EATL_IEO_2015_L_054	204		50,75	F	North Eastern Atlantic	33,531	-12,886	13/02/15	LLAM
BSH_EATL_IEO_2015_L_055	213		48,5	na	North Eastern Atlantic	33,531	-12,886	13/02/15	LLAM
BSH_EATL_IEO_2015_J_056	155		17,65	M	North Eastern Atlantic	33,531	-12,886	13/02/15	LLAM
BSH_EATL_IEO_2015_J_057	111			F	North Eastern Atlantic	35,144	-15,276	26/02/15	LLAM
BSH_EATL_IEO_2015_J_058	126			M	North Eastern Atlantic	34,940	-15,224	27/02/15	LLAM
BSH_EATL_IEO_2015_J_059	159			F	North Eastern Atlantic	34,940	-15,224	27/02/15	LLAM
BSH_EATL_IEO_2015_L_060	217			M	North Eastern Atlantic	34,940	-15,224	27/02/15	LLAM
BSH_EATL_IEO_2015_J_061	113			M	North Eastern Atlantic	35,051	-15,267	28/02/15	LLAM
BSH_EATL_IEO_2015_J_062	155			F	North Eastern Atlantic	35,051	-15,267	28/02/15	LLAM
BSH_EATL_IEO_2015_J_063	125			F	North Eastern Atlantic	34,756	-14,948	01/03/15	LLAM
BSH_EATL_IEO_2015_L_064	195			M	North Eastern Atlantic	34,756	-14,948	01/03/15	LLAM
BSH_EATL_IEO_2015_J_065	108			M	North Eastern Atlantic	34,672	-14,985	02/03/15	LLAM
BSH_EATL_IEO_2015_J_066	141			F	North Eastern Atlantic	34,672	-14,985	02/03/15	LLAM
BSH_EATL_IEO_2015_J_067	129			F	North Eastern Atlantic	34,651	-15,219	03/03/15	LLAM
BSH_EATL_IEO_2015_J_068	110			F	North Eastern Atlantic	34,651	-15,219	03/03/15	LLAM
BSH_EATL_IEO_2015_J_069	118			F	North Eastern Atlantic	34,791	-14,865	04/03/15	LLAM
BSH_EATL_IEO_2015_J_070	112			na	North Eastern Atlantic	34,791	-14,865	04/03/15	LLAM

BSH_EATL_IEO_2015_J_071	146			na	North Eastern Atlantic	35,500	-15,546	05/03/15	LLAM
BSH_EATL_IEO_2015_J_072	151			na	North Eastern Atlantic	35,500	-15,546	05/03/15	LLAM
BSH_EATL_IEO_2015_J_073	143			na	North Eastern Atlantic	35,364	-15,841	06/03/15	LLAM
BSH_EATL_IEO_2015_J_074	109			M	North Eastern Atlantic	35,364	-15,841	06/03/15	LLAM
BSH_EATL_IEO_2015_J_075	145			M	North Eastern Atlantic	34,716	-15,129	07/03/15	LLAM
BSH_EATL_IEO_2015_J_076	107			na	North Eastern Atlantic	34,716	-15,129	07/03/15	LLAM
BSH_EMED_UNIBO_2015_J_001	106		5,33	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_002	111		6,594	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_003	110		6,698	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_004	108		5,69	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_005	116		6,84	M	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_006	115		7,862	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_007	100		4,362	M	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_008	107		6,174	F	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_009	108		6,198	M	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_010	128		9,434	M	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_011	110		4,906	M	Central Adriatic	42,788	14,6976	11/07/15	LL
BSH_EMED_UNIBO_2015_J_012	117	8,604		F	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_013	104	8,136		F	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_014	104	6,862		M	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_015	119	10,336		F	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_016	115	8,718		F	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_017	107	7,818		M	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_018	95	4,958		F	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_019	120	9,508		M	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_020	113	7,798		M	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_EMED_UNIBO_2015_J_021	112	6,948		M	Central Adriatic	43,238	14,7649	25/07/15	LL
BSH_WMED_UNICAL_2015_J_001	183	38		M	Tyrrenian Sea	38,668	14,512	06/06/15	LL
BSH_WMED_UNICAL_2015_J_002	106	6,9		M	Tyrrenian Sea	38,666	14,792	14/06/15	LL
BSH_WMED_UNICAL_2015_J_003	118	9,6		M	Tyrrenian Sea	38,513	14,672	22/07/15	LL
BSH_WMED_UNICAL_2015_J_004	102	6,1		F	Tyrrenian Sea	38,499	14,528	16/09/15	LL
BSH_WMED_UNICAL_2015_J_005	87	3,7		na	Tyrrenian Sea	38,496	14,697	21/09/15	LL
BSH_WMED_UNICAL_2015_J_006	82	3,1		na	Tyrrenian Sea	38,471	14,811	26/09/15	LL
BSH_WMED_UNICAL_2015_J_007	73	2,1		na	Tyrrenian Sea	38,501	14,740	15/10/15	LL
BSH_WMED_UNICAL_2015_J_008	67	1,6		na	Tyrrenian Sea	38,501	14,740	15/10/15	LL
BSH_WMED_UNICAL_2015_J_009	77	2,5		na	Tyrrenian Sea	38,501	14,740	15/10/15	LL
BSH_WMED_UNICAL_2015_J_010	80	2,8		na	Tyrrenian Sea	38,687	14,995	17/10/15	LL
BSH_EMED_UNICAL_2015_J_011	107	7,1		F	Ionian Sea		16,311	14/05/15	LL

						37,689			
BSH_EMED_UNICAL_2015_J_012	88	3,8		na	Ionian Sea	37,436	16,405	04/07/15	LL
BSH_EMED_UNICAL_2015_J_013	92	4,4		na	Ionian Sea	36,580	16,062	08/08/15	LL
BSH_EMED_UNICAL_2015_J_014	97	5,2		na	Ionian Sea	37,649	15,482	16/08/15	LL
BSH_EMED_UNICAL_2015_J_015	160	25		F	Ionian Sea	37,481	15,557	04/09/15	LL
BSH_EMED_UNICAL_2015_J_016	83	3,3		M	Ionian Sea	37,853	15,469	13/10/15	LL
BSH_EMED_UNICAL_2015_J_017	135	15		M	Ionian Sea	37,853	15,469	13/10/15	LL
BSH_EMED_UNICAL_2015_J_018	146	18		F	Ionian Sea	37,957	15,480	14/10/15	LL
BSH_EMED_UNICAL_2015_J_019	105	6		na	Ionian Sea	37,957	15,480	14/10/15	LL
BSH_EMED_UNICAL_2015_J_020	98	5,3		na	Ionian Sea	37,957	15,480	14/10/15	LL
BSH_EMED_UNICAL_2012_J_021	79	1,5		M	Ionian Sea			03/08/12	LL
BSH_EMED_UNICAL_2014_J_022	80	3		F	Ionian Sea	39,735	17,103	20/07/14	LL
BSH_EMED_UNICAL_2014_J_023	75	3,5		M	Ionian Sea	39,543	16,863	23/07/14	ST
BSH_EMED_UNICAL_2013_L_024	268			M	Ionian Sea	39,383	17,143	13/08/13	ST
BSH_EMED_UNICAL_2014_L_025	226	45		M	Ionian Sea	38,043	16,143	21/07/14	LL
BSH_EMED_UNICAL_2014_L_026				na	Ionian Sea			04/08/14	
BSH_EMED_UNICAL_2015_L_027	370			F	Ionian Sea	37,94	16,15	15/07/15	LL
BSH_EMED_UNICAL_2015_J_028	85	2,8		F	Ionian Sea	39,75	16,52	12/09/15	RE
BSH_EMED_UNICAL_2015_J_029	90			F	Ionian Sea	38,14	16,18	12/07/15	RE
BSH_EMED_UNICAL_2015_J_030	87			M	Ionian Sea	38,14	16,18	12/07/15	RE
BSH_EMED_NKUA_2015_J_001	110			M	Ionian	37,546	21,107	02/04/15	LL
BSH_EMED_NKUA_2015_J_002	109			M	Ionian	37,546	21,107	02/04/15	LL
BSH_EMED_NKUA_2015_J_003	108			M	Ionian	37,546	21,107	02/04/15	LL
BSH_EMED_NKUA_2015_J_004	110			F	Ionian	37,546	21,107	02/04/15	LL
BSH_EMED_NKUA_2015_L_005	219			na	Ionian	37,546	21,107	02/04/15	LL
BSH_EMED_NKUA_2015_L_006	310			F	Corinthian	38,09	23,1	11/02/15	LL
BSH_EMED_NKUA_2015_L_007	230			F	Ionian	38,546	20,231	13/06/15	LL
BSH_EMED_NKUA_2015_L_008	218			F	Ionian	38,546	20,231	13/06/15	LL
BSH_EMED_NKUA_2015_L_009	242			na	Creete	35,584	24,56	17/07/15	LL
BSH_EMED_NKUA_2015_L_010	184			na	Creete	35,584	24,56	17/07/15	LL
BSH_EMED_NKUA_2015_L_011	196			na	Creete	35,584	24,56	17/07/15	LL
BSH_EMED_NKUA_2015_L_012	221			F	Creete	34,902	24,516	30/07/15	LL
BSH_EMED_NKUA_2015_J_013	155		14.5	M	Ionian	37,455	21,023	22/09/15	LL
BSH_EMED_NKUA_2015_J_014	174		23.5	M	Ionian	37,455	21,023	22/09/15	LL
BSH_EMED_NKUA_2003_L_001	273	54		M	Gavdos	32,16	25,26	12/05/03	LL
BSH_EMED_NKUA_2005_L_002	208	26		F	Egypt	32,26	25,42	27/05/05	LL
BSH_EMED_NKUA_2005_J_003		13		na	Liviko	32,80	24,47	18/05/05	LL

BSH_EMED_NKUA_2005_L_004	315	95		M	Libyan Sea	32,95	28,47	18/06/05	LL
BSH_EMED_NKUA_2003_L_005	277	46		M	Libyan Sea	34,25	26,33	13/05/03	LL
BSH_EMED_NKUA_2005_L_006	244	42,8		F	Gavdos-Liviko	34,31	25,02	27/03/05	LL
BSH_EMED_NKUA_2005_L_007		50		F	Gavdos	34,41	23,60	17/06/05	LL
BSH_EMED_NKUA_2003_L_008	215	28		F	Gavdos	34,42	24,21	23/06/03	LL
BSH_EMED_NKUA_2004_L_009	250	55		F	Paximadia	34,45	24,58	08/03/04	LL
BSH_EMED_NKUA_2003_L_010	290	75		M	Libyan Sea	34,47	24,47	19/07/03	LL
BSH_EMED_NKUA_2004_L_011			30	F	Gaidouronisi	34,50	25,50	13/03/04	LL
BSH_EMED_NKUA_2004_L_012	252	47		M	Gavdos	34,50	24,05	22/08/04	LL
BSH_EMED_NKUA_2005_L_013		53		F	Gavdos	34,53	24,38	18/06/05	LL
BSH_EMED_NKUA_2005_L_014	284			F	Gavdos	34,58	24,35	18/03/05	LL
BSH_EMED_NKUA_2005_L_015		36		na	Gavdos	34,60	23,26	16/06/05	LL
BSH_EMED_NKUA_2003_J_016			9	na	Libyan Sea	34,67	26,93	14/07/03	LL
BSH_EMED_NKUA_2003_L_017	270	46		F	Gavdos	34,73	23,86	21/06/03	LL
BSH_EMED_NKUA_2003_L_018	219	27		M	Gavdos	34,77	24,35	12/05/03	LL
BSH_EMED_NKUA_2003_J_019	175	14		M	Gavdos	34,80	24,37	24/06/03	LL
BSH_EMED_NKUA_2003_L_020	220			F	Gavdos-W.Crete	34,87	22,72	12/07/03	LL
BSH_EMED_NKUA_2003_L_021	255	36,7		M	Gavdos-W.Crete	34,87	22,72	15/06/03	LL
BSH_EMED_NKUA_2005_L_022		68		na	Gavdos-W.Crete	34,87	22,72	20/06/05	LL
BSH_EMED_NKUA_2004_L_023	181	17,7		F	W. Crete	34,91	23,12	23/05/04	LL
BSH_EMED_NKUA_2005_J_024		16		M	Gavdos	35,01	23,39	15/06/05	LL
BSH_EMED_NKUA_2004_L_025	233	38		M	W. Crete	35,06	22,81	22/05/04	LL
BSH_EMED_NKUA_2003_L_026	246	42,5		M	Gavdos	35,08	23,85	23/06/03	LL
BSH_EMED_NKUA_2003_J_027			14,5	na	Libyan Sea	35,22	26,57	13/07/03	LL
BSH_EMED_NKUA_2003_L_028	226			F	Gavdos-W.Crete	35,37	22,58	12/07/03	LL
BSH_EMED_NKUA_2003_L_029	230	32,7		M	Gavdos-W.Crete	35,37	22,58	15/06/03	LL
BSH_EMED_NKUA_2005_J_030		16		na	Gavdos-W.Crete	35,37	22,58	19/06/05	LL
BSH_EMED_NKUA_2003_L_031	234	45		F	Antikythera	35,40	22,80	24/06/03	LL
BSH_EMED_NKUA_2003_L_032	195	20		F	Antikythera	35,53	23,20	07/09/03	LL
BSH_EMED_NKUA_2004_L_033	196	20		F	Antikythera	35,83	23,50	19/08/04	LL
BSH_EMED_NKUA_2004_L_034	207	25		M	Milos	36,63	23,91	22/08/04	LL
BSH_EMED_NKUA_2004_L_035	252	50,5		M	Milos	36,64	23,99	22/08/04	LL
BSH_EMED_NKUA_2004_L_036	180	20		F	Milos	37,05	24,35	21/08/04	LL
BSH_EMED_NKUA_2003_L_037	182	10,4		F	Levantine			15/06/03	LL
BSH_EMED_NKUA_2003_J_038	139	7,7		F	Levantine			15/06/03	LL
BSH_EMED_NKUA_2003_L_039	245			F	Levantine			15/06/03	LL
BSH_EMED_NKUA_2003_J_040	133	7,8		F	Levantine			15/06/03	LL

BSH_EMED_NKUA_2004_L_041	200	26,5		F	East Mediterranean			01/04/04	LL
BSH_EMED_NKUA_2004_L_042	212	35		F	East Mediterranean			01/05/04	LL
BSH_EMED_NKUA_2004_J_043	150	8,4		F	East Mediterranean			16/07/04	LL
BSH_EMED_NKUA_2003_L_044	262			M	Levantine			15/06/03	LL
BSH_EMED_NKUA_2003_J_045	146	9,5		M	Levantine			15/06/03	LL
BSH_EMED_NKUA_2004_J_046	140	7,6		M	East Mediterranean			13/03/04	LL
BSH_EMED_NKUA_2004_L_047	283			M	Libyan Sea			01/03/04	LL
BSH_EMED_NKUA_2004_L_048			36,5	M	East Mediterranean			01/04/04	LL
BSH_EMED_NKUA_2004_J_049	177	16		M	East Mediterranean			01/04/04	LL
BSH_EMED_NKUA_2004_J_050	169	12,5		M	East Mediterranean			01/04/04	LL
BSH_EMED_NKUA_2004_L_051	221	24,5		M	East Mediterranean			16/07/04	LL
BSH_EMED_NKUA_2004_L_052	206	23,4		M	East Mediterranean			16/07/04	LL
BSH_EMED_NKUA_2005_L_053	217	26,5		M	East Mediterranean			June 2005	LL
BSH_EMED_NKUA_2005_L_054	220	30		M	East Mediterranean			June 2005	LL
BSH_EMED_NKUA_2005_L_055	200	21		M	East Mediterranean			June 2005	LL
BSH_EMED_NKUA_2005_L_056	270	50	25	M	East Mediterranean			June 2005	LL
BSH_EATL_QUB_2007_L_001	195			F	South England/Celtic Sea	51,433	-5,583		
BSH_EATL_QUB_2007_L_002				F	South England/Celtic Sea	51,450	-5,533		
BSH_EATL_QUB_2007_L_003				F	South England/Celtic Sea	51,450	-5,616		
BSH_EATL_QUB_2007_L_004	193			F	South England/Celtic Sea	51,466	-10,150		
BSH_EATL_QUB_2007_L_005	196			F	South England/Celtic Sea	51,450	-10,150		
BSH_EATL_QUB_2007_L_006	198			F	South England/Celtic Sea	51,450	-10,150		
BSH_EATL_QUB_2007_L_007				F	South England/Celtic Sea	51,433	-5,466		
BSH_EATL_QUB_2007_L_008	190			F	South England/Celtic Sea	51,550	-5,833		
BSH_EATL_QUB_2007_L_009				F	South England/Celtic Sea	51,416	-5,516		
BSH_EATL_QUB_2007_L_010				F	South England/Celtic Sea	51,483	-5,916		
BSH_EATL_QUB_2007_L_011				M	South England/Celtic Sea	51,516	-5,900		
BSH_EATL_QUB_2007_L_012	196			F	South England/Celtic Sea	51,516	-5,866		
BSH_EATL_QUB_2007_L_013				F	South England/Celtic Sea	51,600	-5,850		
BSH_EATL_QUB_2007_L_014	200			F	South England/Celtic Sea	51,450	-5,433		
BSH_EATL_QUB_2007_L_015	190			F	South England/Celtic Sea	51,416	-5,483		
BSH_EATL_QUB_2007_L_016	200			F	South England/Celtic Sea	51,450	-5,566		
BSH_EATL_QUB_2007_L_017	205			F	South England/Celtic Sea	49,816	-6,233		
BSH_EATL_QUB_2007_L_018	150			M	South England/Celtic Sea	49,816	-6,166		
BSH_EATL_QUB_2007_L_019	207			F	South England/Celtic Sea	49,916	-6,200		
BSH_EATL_QUB_2007_L_020	135			M	South England/Celtic Sea	49,816	-6,300		
BSH_EATL_QUB_2007_L_021	213			F	South England/Celtic Sea	49,816	-6,216		
BSH_EATL_QUB_2007_L_022	182			F	South England/Celtic Sea	49,833	-6,183		

BSH_EATL_QUB_2007_L_023	185			F	South England/Celtic Sea	49,816	-6,233		
BSH_EATL_QUB_2007_L_024	175			F	South England/Celtic Sea	50,066	-4,466		
BSH_EATL_QUB_2007_L_025	215			F	South England/Celtic Sea	50,050	-4,433		
BSH_EATL_QUB_2007_L_026	200			F	South England/Celtic Sea	50,066	-4,333		
BSH_EATL_QUB_2007_L_027	200			F	South England/Celtic Sea	50,083	-4,500		
BSH_EATL_QUB_2007_L_028	193			F	South England/Celtic Sea	50,066	-4,400		
BSH_EATL_QUB_2007_L_029	216			F	South England/Celtic Sea	51,466	-10,116		
BSH_EATL_QUB_2007_L_030	175			F	South England/Celtic Sea	51,466	-10,116		
BSH_WMED_UNIGE_2015_J_001	71			F	Ligurian sea	43,781	7,888	11/08/15	LL
BSH_WMED_UNIGE_2015_J_002	121			M	Ligurian sea	43,598	8,151	04/07/15	LL
BSH_WMED_UNIGE_2015_L_003	221			M	Ligurian sea	43,448	7,631	28/06/15	LL
BSH_WMED_UNIGE_2015_L_004	308			M	Ligurian sea	43,689	8,428	25/06/15	LL
BSH_WMED_UNIGE_2015_J_005	122			F	Ligurian sea	43,803	8,446	22/06/15	LL
BSH_WMED_UNIGE_2015_L_006	198			F	Ligurian sea	44,167	8,811	12/04/15	LL
BSH_WMED_UNIGE_2015_J_007	167			F	Ligurian sea	43,729	7,715	10/04/15	LL
BSH_WMED_UNIGE_2014_L_008	254			F	Ligurian sea	43,486	7,887	18/10/14	LL
BSH_WMED_UNIGE_2014_J_009	192			F	Ligurian sea	43,731	8,394	17/09/14	LL
BSH_WMED_UNIGE_2014_J_010	98			F	Ligurian sea	43,863	8,504	17/09/14	LL
BSH_WMED_UNIGE_2014_J_011	75			F	Ligurian sea	43,550	7,943	09/09/14	LL
BSH_WMED_UNIGE_2014_L_012	214			F	Ligurian sea	43,727	7,730	07/09/14	LL
BSH_WMED_UNIGE_2014_J_013	78			M	Ligurian sea	43,554	7,992	02/09/14	LL
BSH_WMED_UNIGE_2014_L_014	262			M	Ligurian sea	43,653	8,031	19/07/14	LL
BSH_WMED_UNIGE_2014_J_015	180			F	Ligurian sea	43,700	8,433	16/07/14	LL
BSH_WMED_UNIGE_2014_J_016	176			F	Ligurian sea	43,599	7,971	13/07/14	LL
BSH_WMED_UNIGE_2014_J_017	136			F	Ligurian sea	43,407	7,290	13/07/14	LL
BSH_WMED_UNIGE_2014_J_018	126			M	Ligurian sea	43,509	7,714	30/06/14	LL
BSH_WMED_UNIGE_2014_J_019	169			F	Ligurian sea	43,800	8,060	19/06/14	LL
BSH_WMED_UNIGE_2013_J_020	146			F	Ligurian sea	43,782	7,954	09/08/13	LL
BSH_WMED_UNIGE_2012_J_021	161			M	Ligurian sea	43,624	8,148	25/07/12	LL
BSH_WMED_UNIGE_2012_J_022	148			F	Ligurian sea	43,507	7,704	10/07/12	LL
BSH_WMED_UNIGE_2012_J_023	150			M	Ligurian sea	43,599	7,971	08/07/12	LL
BSH_WMED_UNIGE_2012_J_024	195			F	Ligurian sea	43,599	7,971	08/07/12	LL
BSH_WMED_UNIGE_2012_J_025	144			F	Ligurian sea	43,626	8,052	08/07/12	LL
BSH_WMED_UNIGE_2012_L_026	244			M	Ligurian sea	43,648	8,274	04/07/12	LL
BSH_WMED_UNIGE_2012_L_027	206			F	Ligurian sea	43,499	8,101	27/06/12	LL
BSH_WMED_UNIGE_2012_J_028	187			F	Ligurian sea	43,294	7,776	20/06/12	LL
BSH_WMED_UNIGE_2012_J_029	131			F	Ligurian sea	43,561	8,820	25/05/12	LL
BSH_WMED_UNIGE_2011_J_030	159			F	Ligurian sea	43,458	7,862	24/08/11	LL

BSH_WMED_UNIGE_2011_J_031	167			M	Ligurian sea	43,345	7,742	18/08/11	LL
BSH_WMED_UNIGE_2011_J_032	192			F	Ligurian sea	43,539	7,095	14/08/11	LL
BSH_WMED_UNIGE_2011_J_033	154			M	Ligurian sea	43,539	7,428	13/08/11	LL
BSH_WMED_UNIGE_2011_J_034	147			M	Ligurian sea	43,342	8,102	06/08/11	LL
BSH_WMED_UNIGE_2011_J_035	161			M	Ligurian sea	43,657	8,830	03/08/11	LL
BSH_WMED_UNIGE_2011_L_036	200			F	Ligurian sea	44,077	8,720	09/06/11	LL
BSH_WMED_UNIGE_2011_L_037	199			F	Ligurian sea	43,268	8,365	09/06/11	LL
BSH_WMED_UNIGE_2010_J_038	62			M	Ligurian sea	43,158	7,828	06/07/10	LL
BSH_WMED_UNIGE_2009_J_039	151			F	Ligurian sea	43,771	7,766	02/07/09	LL
BSH_WMED_UNIGE_2009_J_040	162			F	Ligurian sea	43,333	7,416	30/06/09	LL
BSH_WMED_UNIGE_2009_J_041	138			M	Ligurian sea	43,249	7,960	18/06/09	LL
BSH_WMED_UNIGE_2009_J_042	113			M	Ligurian sea	43,289	8,377	18/06/09	LL
BSH_WMED_UNIGE_2009_J_043	132			F	Ligurian sea	43,850	8,427	18/06/09	LL
BSH_WMED_UNIGE_2009_J_044	133			M	Ligurian sea	43,473	8,441	18/06/09	LL
BSH_WMED_UNIGE_2008_J_045	136			M	Ligurian sea	43,461	7,868	26/07/08	LL
BSH_WMED_UNIGE_2008_J_046	141			F	Ligurian sea	43,474	7,887	10/07/08	LL
BSH_WMED_UNIGE_2008_J_047	156			F	Ligurian sea	43,438	8,075	24/06/08	LL
BSH_WMED_UNIGE_2007_J_048	166			F	Ligurian sea	43,269	8,457	26/08/07	LL
BSH_WMED_UNIGE_2007_J_049	59			F	Ligurian sea	43,168	8,497	05/08/07	LL
BSH_WMED_UNIGE_2005_J_050	171			F	Ligurian sea	42,918	8,867	14/07/05	LL
BSH_WMED_UNIGE_2005_J_051	175			F	Ligurian sea	42,064	7,993	14/07/05	LL
BSH_WMED_UNIGE_2016_J_052	111			M	Ligurian sea	43,375	9,361	21/01/16	LL
BSH_WMED_UNIGE_2016_J_053	120			F	Ligurian sea	43,287	9,323	21/01/16	LL
BSH_WMED_UNIGE_2016_J_054	124			F	Ligurian sea	43,299	9,462	21/01/16	LL
BSH_WMED_UNIGE_2016_J_055	125			F	Ligurian sea	43,302	9,482	21/01/16	LL
BSH_WMED_UNIGE_2016_J_056	113			M	Ligurian sea	43,196	9,290	21/01/16	LL
BSH_WMED_UNIGE_2016_J_057	128			M	Ligurian sea	43,339	9,639	21/01/16	LL
BSH_WMED_UNIGE_2016_J_058	109			F	Ligurian sea	43,259	9,687	21/01/16	LL
BSH_WMED_UNIGE_2016_J_059	153			F	Ligurian sea	43,365	9,561	21/01/16	LL
BSH_WMED_UNIGE_2016_J_060	151			F	Ligurian sea	43,470	9,593	27/01/16	LL
BSH_WMED_UNIGE_2016_J_061	126			F	Ligurian sea	43,402	9,478	27/01/16	LL
BSH_WMED_UNIGE_2016_J_062	106			F	Ligurian sea	43,384	9,448	27/01/16	LL
BSH_WMED_UNIGE_2016_J_063	113			M	Ligurian sea	43,311	9,434	27/01/16	LL
BSH_WMED_UNIGE_2016_J_064	193			F	Ligurian sea	43,369	9,209	27/01/16	LL

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2 CHAPTER 2

Genetic differentiation and phylogeography of Mediterranean-North Eastern Atlantic blue shark (*Prionace glauca*, L. 1758) using mitochondrial DNA: panmixia or complex stock structure?

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Title: Genetic differentiation and phylogeography of Mediterranean-North Eastern Atlantic blue shark (*Prionace glauca*, L. 1758) using mitochondrial DNA: panmixia or complex stock structure?

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Abstract

Background. The blue shark (*Prionace glauca*, Linnaeus 1758) is one of the most abundant epipelagic shark inhabiting all the oceans except the poles, including the Mediterranean Sea, but genetic structure has not been confirmed at basin and interoceanic distances. Past tagging programs in the Atlantic Ocean failed to find evidence of migration of blue sharks between the Mediterranean and the adjacent Atlantic, despite the extreme vagility of the species. Despite the high rate of by-catch in the Mediterranean basin, to date no genetic study on Mediterranean blue shark was carried out, which constitutes a significant knowledge gap, considering that this population is classified as "Critically Endangered", unlike its open-ocean counterpart.

Methods. Blue shark phylogeography and demography in the Mediterranean Sea and North-Eastern Atlantic Ocean were inferred using two mitochondrial genes (Cytb and control region) amplified from 207 and 170 individuals respectively, collected from six localities across the Mediterranean and two from the North-Eastern Atlantic.

Results. Although no obvious pattern of geographical differentiation was apparent from the haplotype network, Φst analyses indicated significant genetic structure among four geographical groups. Demographic analyses suggest that these populations have experienced a constant population expansion in the last 0.4-0.1 million of years.

Discussion. The weak, but significant, differences in Mediterranean and adjacent North-eastern Atlantic blue sharks revealed a complex phylogeographic structure, which appears to reject the assumption of panmixia across the study area, but also supports a certain degree of population connectivity across the Strait of Gibraltar, despite the lack of evidence of migratory movements observed by tagging data. Analyses of spatial genetic structure in relation to sex-ratio and size could indicate some level of sex/stage biased migratory behaviour.

2.1 Introduction

The blue shark (*Prionace glauca*, Linnaeus 1758; BS henceforth) is one of the most abundant epipelagic sharks that is found in all oceans from 60°N to 50°S (Compagno, 1984). Blue sharks are rarely targeted by commercial fishing, but feature prominently as by-catch of fisheries targeting large pelagic fish, especially swordfish and tuna longlines (Fowler et al., 2005). BS populations trend data are available only for a part of the geographic range and stock assessments are highly uncertain (Dulvy et al., 2014; Coelho et al., 2017); due to the huge amount of by-caught BS (approx. 20 million per annum, Stevens et al., 2009), the species has being categorized worldwide as "Near Threatened" in the IUCN Red List (Stevens, 2009). Based on recent assessment (ICCAT, 2015), the North Atlantic stock is unlikely to be currently overfished. The Mediterranean BS, on the other hand, is estimated to have undergone a 90% decline over three generations, primarily due to overfishing (Ferretti et al., 2008), and is now categorized as "Critically Endangered" (Sims et al., 2016). Given the vast amount of poorly reported by-catch, the increasing commercial value of the species (Megalofonou et al., 2005) and the persistent issue of the global trade in shark fin products, of which BS is the main component (Clarke et al., 2006), a more explicit management is needed for this species, which should be underpinned by robust knowledge of its population structure.

In the Atlantic, BS is distributed from Canada to Argentina, on the western side, and from Norway to South Africa on the eastern side, including the Mediterranean Sea (Compagno, 1984). The population structure and dynamics of Atlantic BS is still poorly known, despite several long-term tagging studies, which revealed extensive movements of BS tagged in the western side of the North Atlantic (henceforth NA), with well documented eastward trans-Atlantic migrations (Kohler et al. 1998, 2002; Kohler & Turner, 2008; Vandeperre et al., 2014). Sexual segregation was also evident, with a concentration of mature females in more temperate waters of the northernmost NA, and immature males predominant in the southernmost NA (Sampaio da Costa, 2013). Mature BS of both sexes seemed to be distributed in the southern part of NA, while immature individuals of both sexes and sub-adult females are usually distributed in the northern areas (Kohler et al., 2002). Conversely, a prevalent occurrence of immature juveniles is reported in the Mediterranean Sea (Megalofonou et al., 2009; Kohler et al., 2002). A significant genetic heterogeneity among potential BS nurseries from the Atlantic Ocean (Portugal and Azores) and those from South Africa was detected by Sampaio da Costa (2013) from mitochondrial and nuclear marker variation. Their

finding indicated a deeper separation between the northern and the southern NA nurseries and supported a male philopatry behaviour to mating areas exclusively contributing to a single nursery ground. Contradictorily, a recent genetic survey (Verissimo et al., 2017) carried out on the same dataset (i.e., young-of-year and <2 years juveniles) collected from the same nurseries, enriched with more samples from different areas (i.e. coasts of Brazil), and using the same type of markers, showed a lack of spatio-temporal genetic differentiation, suggesting the presence of a panmictic population in the whole Atlantic.

To date, no genetic data are available for the Mediterranean BS population and population structure and dynamics of BS in the Mediterranean are presently inferred only by Atlantic-Mediterranean integrated tagging studies and fishing data assessments (Kohler et al., 1998, 2002; Ferretti et al., 2008; Kohler & Turner, 2008; Megalofonou et al., 2009).

Irrespective of the small recapture rate (out of the 91,450 BS specimens tagged in the north western Atlantic, only 5.9% were recaptured), extensive tag-recapture surveys carried out from 1962 to 2000, indicated that North Atlantic BS form a single stock and that trans-Atlantic migratory movements were quite frequent, likely favoured by the oceanic current system (Kohler et al., 2002). Focusing on the Atlantic-Mediterranean connectivity, the reproductive migratory movements of Atlantic BS towards Mediterranean and the degree of population connectivity between the two areas are still unknown, because only one adult BS male tagged in the north-western Atlantic and one sub adult female tagged in the North-Eastern Atlantic were recaptured in the Mediterranean (Kohler et al., 2002). The large majority of BS tagged in the Mediterranean Sea were immature and remained in the tagging area, with the only exception of a subadult female that moved a short distance to the adjacent north-eastern Atlantic area. Most of the BS caught in the Mediterranean (99% and 98% for males and females, respectively) are immature, indicating that the Mediterranean BS stock consists primarily of small immature BS of both sexes, with a sex-ratio skewed toward females or males, depending on different geographical areas (Kohler et al., 2002; Megalofonou et al., 2009). A high number of pregnant females was observed in the Adriatic, North Ionian Sea and Ligurian Sea, suggesting potential nursery grounds for BS (Megalofonou et al., 2009; Garibaldi, 2017 pers.comm.). On the other hand, the adjacent South-Eastern North Atlantic BS was prevalently composed by primarily mature individuals of both sexes with male-based sex ratio.

The primary aims of this study is to test the null hypothesis of panmixia between North Atlantic and Mediterranean BS, by comparing the mtDNA genetic variation of two gene regions, the

control region (CR) and the Cytochrome b (Cytb) among four population samples collected in the North-Eastern and South-Eastern North Atlantic and in the Western and Eastern Mediterranean. Given the female philopatry observed in other carcharhiniformes (Mourier & Planes, 2013; Tillet et al, 2012), mtDNA markers are likely to be useful to spot localised groups due to site-fidelity. Accordingly, this work aims to provide further and needed data on matrilineal genetic structure, female philopatry and demography of Mediterranean BS. These, previously lacking, data will contribute to a better understanding and inclusion of the Mediterranean BS dynamics in the wider North Atlantic population model, to improve assessment and management of BS stocks in the area.

2.2 Materials and Methods

2.2.1 Blue shark sampling

Sampling was carried out between 2003 and 2016 and tissue specimens and individual data were collected by means of commercial fishermen and scientific surveys. BS were collected from multiple locations in the Eastern (Central Adriatic, CADR, 21; Ionian Sea, IONI, 15; Aegean Sea and Levantine Sea, AEGE, 20) and Western areas (South Tyrrhenian, TYRR, 10; Ligurian Sea, LIGU, 57; Balearic Islands, BALE, 42). North Atlantic BS were caught from the North Eastern Atlantic Ocean off the coasts of Portugal (SNEATL, 33) and Celtic Sea (NNEATL, 16) (**Fig. 2.1**; **Tab. S 2.1**)

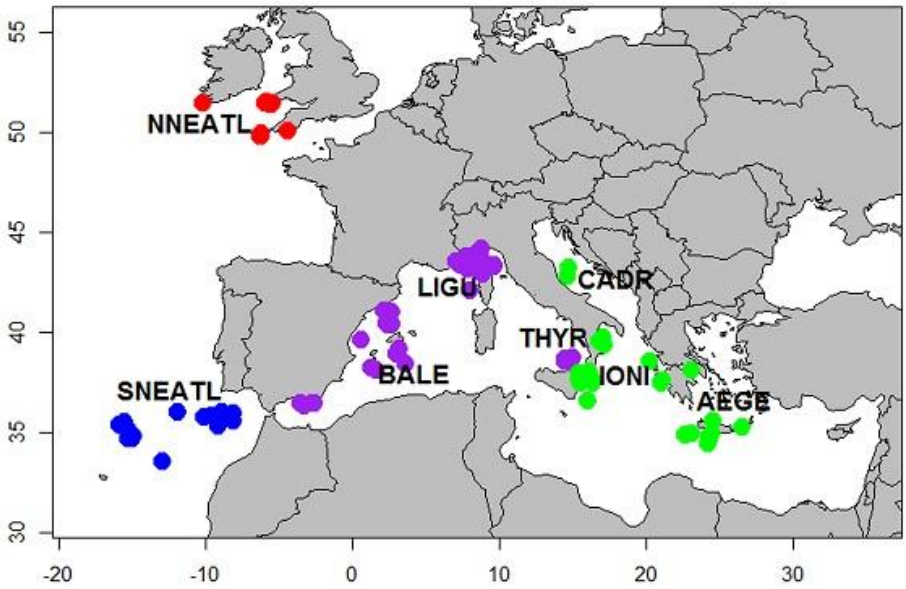

Fig. 2.1: Sampling sites of Mediterranean and North Eastern Atlantic Blue Sharks. North North-Eastern Atlantic (NNEATL, red, N=16), South North-Eastern Atlantic (SNEATL, blue, N=33), Western Mediterranean (WMED, purple,

N=109) and Eastern Mediterranean (EMED, greenN=56). The map was created using R version 3.4.1 (R Core Team, 2016; Becker, Wilks & Brownrigg, 2017).

The BS individuals were grouped according the Total Length (TL) in three size categories (Pratt, 1979; Vandeperre et al., 2014): juveniles (J, TL ≤ 120 cm), young (Y, TL = 120 - 180 cm) and large (L, TL ≥ 180 cm).

A unique and transparent sampling documentation tool was developed within the project, in order to render data public. This tool can be used by everyone as an interactive map visiting the website https://fishreg.jrc.ec.europa.eu/web/medbluesgen/sampling-data

2.2.2 Molecular methods

Individual fin clips or skeletal muscle tissue samples were collected and preserved in 96% ethanol and kept at -20°C until laboratory analyses. DNA extraction was carried out using the Invisorb® Spin Tissue Kit, Invitek (© STRATEC Molecular) and the Wizard® Genomic DNA Purification Kit, Promega kits, following the manufacturers' protocols.

Species-specific primer pairs for the amplification of the mitochondrial control region (CR) and cytochrome b (Cytb) genes were designed. Homologous complete CR and Cytb sequences of *Prionace glauca* available in GenBank were retrieved and aligned using ClusterW algorithm implemented in MEGA ver.7.0 (Tamura et al., 2013). Primer pairs were designed using the online software PRIMER3 (ver.0.4.0) (Untergasser et al., 2012), minimizing the propensity of oligos to form hairpins or dimers or to hybridize or prime from unintended sites in the full mitochondrial BS genome (Acc. Num. NC_022819, Chen et al., 2013).

The designed primer pairs (control region: CR-Blues-F 5'AAACACATCAGGGGAAGGAG3', CR-Blues-R 5'CATCTTAGCATCTTCAGTGCC3'; Cytochrome-b: Cytb-Blues-F 5' TCCTCACAGGACTCTTCCTAGC3', Cytb-Blues-R 5'GTCGAAAGATGGTGCTTCGT3') were tested using a temperature gradient to identify the most suitable melting temperatures (T_m = from 50°C to 60°C) according to PCR cycling conditions described by Ovenden et al. (2009).

Once the optimal melting temperature was identified, the PCR thermal profile was adjusted and the PCR reactions were performed for both markers in a final volume of 50 µL containing 31.75 µL of distilled sterile H_2O, 8 µL of Buffer 10x (Tris-HCl; final 1X), 3 µL of $MgCl_2$ (25mM; final 1.5 mM), 2 µL of dNTPs (10mM; final 0.37 mM), 2.5 µL (10µM; final 0.46 µM) of each primer, 0.25 µL (5U/µL; final 1.5U) of *Taq* polymerase and 2 µL of template DNA(10-20ng). 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 30 s, elongation at 72°C for 30 s and a final elongation step at 72°C for 5 min. PCR amplicons were sequenced using the external service provider MACROGEN® Europe.

2.2.3 Data analysis

The CR and Cytb nucleotide sequences obtained were validated with the homologous gene sequences deposited in the GenBank with the BLASTn search implemented in the NCBI website (Altschul et al., 1990), and aligned using the ClusterW algorithm implemented in MEGA ver.7.0 (Tamura et al., 2013). When aligned to the complete BS mitochondrial genome, Cytb sequences mapped from nucleotide position 14,530 to 15,291 and CR from 15,651 to 16,397.

Given the high potential of geographical dispersal of the species, sequence data were grouped according to the four geographical areas: EMED, WMED, SNEATL and NNEATL (**Fig. 2.1**). The software DNAsp v.5.10.01 (Librado & Rozas, 2009) was used to assess the genetic diversity parameters at both markers: the number of haplotypes (*Nh*), the number of polymorphic sites (*S*), the haplotype (*h*) and nucleotide diversity (□) with associated standard deviation (stdev).

Haplotype relationships were inferred using the dnaml program of the PHYLIP package version 3.6 (Felsenstein, 1989; Felsenstein, 2005) implemented in the software program HaploViewer (http://www.cibiv.at/~greg/haploviewer).

Partition of molecular variance and its significance was estimated with the AMOVA (Excoffier et al., 1992) implemented in Arlequin ver 3.5.2.2 software (Excoffier & Lischer, 2010), testing four alternative groupings of geographical sampling locations (1: no groups; 2: NNEATL+SNEATL vs WMED+EMED; 3: NNEATL+SNEATL vs WMED vs EMED; 4: NNEATL vs SNEATL vs WMED vs EMED). Haplotype frequencies and pairwise Φ_{ST} with the associated p-values were calculated using the software Arlequin ver 3.5.2.2 (Excoffier & Lischer, 2010) after 20,000 permutations, setting up a □ = 0.05 significance threshold level.

Demographic history was investigated using the mismatch distribution as implemented in the DNAsp software (Librado & Rozas, 2009).

Furthermore, historical demographic trend of the four groups was investigated using Bayesian Skyline Plot (BSP) analysis implemented in the software BEAST v.1.8.2 (Drummond et al., 2005; 2012), using the best evolutionary models for both Cytb and CR markers inferred using JModelTest 2.1.1 (Darriba et al., 2012), and the average mutation rate for sharks, 0.62% and 0.31% for CR and Cytb respectively (Martin and Palumbi, 1993; Galván-Tiradoa et al., 2013). The same software and

parameters, with associate software TreeAnnotator and FigTree, were used to define the phylogeny of the Mediterranean and Eastern Atlantic BS populations.

2.3 Results

Among sexed individuals (N = 192; Table S1), BS females significantly outnumbered males in the NA samples (sex-ratio 0.34, χ^2 test: 10.256 P_{2tail} = 0.001; P_{1tail} = 0, d.f. 1) while in the two Mediterranean BS groups a weak and not significant predominance of males was observed (WMED: 1.19, χ^2 test: 0.786 P_{2tail} = 0.375; P_{1tail} = 0.188, d.f. 1; EMED: 1.09, χ^2 test: 0.087 P_{2tail} = 0.768; P_{1tail} = 0.384, d.f. 1). Sized BS (N = 209) were composed by 63 juvenile, 82 young and 64 large individuals (Table S1). In the NA and WMED the young BS (TL = 120 – 180 cm; 48% and 50%, respectively) were predominant, while in the EMED a large predominance of juveniles was observed (TL ≤ 120 cm; 63%). Noticeably 67% of the BS sampled in the Ionian Sea and 95% of those sampled in the Adriatic Sea were juveniles. Large BS are similarly represented in the geographical groups with percentages varying from 25% (EMED) to 34% (NA), full details presented in **Table S 2.1**.

A total of 207 and 170 BS individuals were sequenced for Cytb (762bp) and CR (747bp), respectively. Haplotype sequences (Cytb, N = 23 and CR, N = 55) were deposited in GenBank under the Accession Numbers MG515900-MG516106 and MG545732-MG545901 for Cytb and control region, respectively.

The Cytb sequence dataset exhibited 16 polymorphic segregating sites while CR dataset showed 27 polymorphic segregating sites. The Cytb haplotype diversity ranged from 0.784 to 0.835, and that of the CR from 0.932 to 1.000. The Cytb nucleotide diversity ranged from 0.001 to 0.002, and that of the CR from 0.004 to 0.008. Detailed genetic diversity of BS samples collected from the four macro areas and all sampling locations is presented in **Table 2.1** and **Table S 2.2**, respectively.

The Cytb and CR haplotype networks highlighted the distribution of haplotypes irrespective of the geographical origin of BS samples, indicating the lack of phylogeographical structure in the Mediterranean and adjacent North Atlantic BS (see **Fig. 2.2**, **Fig. S 2.1**). In the Cytb network, the four main frequent haplotypes were shared by BS from all the four geographical areas, except for the most frequent haplotype which was shared by BS from the three geographical areas, SNEATL, WMED and EMED. In the CR network, six most frequent haplotypes (N° individuals ≥ 10) were observed. Although these six haplotypes were shared by all geographical areas, three of them

were shared by Mediterranean and SNEATL, one by Mediterranean and NNEATL, and two within the Mediterranean. In both networks, most of the NNEATL haplotypes were singletons (**Fig. 2.2**).

The AMOVA (**Tab. 2.2**) revealed a significant overall Φ_{ST} among population samples for both markers.

Tab. 2.1: Mitochondrial gene polymorphism of Prionace glauca population samples subdivided according to the four macro areas. N, number of individuals; Nh, number of haplotypes; S, Number of segregating informative sites; h, haplotype diversity and associate standard deviation; π, nucleotide diversity and associate standard deviation; NNEATL, North North-Eastern Atlantic; SNEATL, South North-Eastern Atlantic; WMED, Western Mediterranean; EMED, Eastern Mediterranean.

Cytb

POP	*N*	*Nh*	*S*	*h*	*stdev h*	π	*stdev* π
NNEATL	14	9	6	0.835	0.010	0.00231	0.00046
SNEATL	33	8	10	0.822	0.034	0.00200	0.00038
WMED	105	13	6	0.801	0.023	0.00167	0.00011
EMED	55	10	6	0.784	0.033	0.00151	0.00013
TOTAL	207	23	16	0.821	0.013	0.00184	0.00010

CR

POP	*N*	*Nh*	*S*	*h*	*stdev h*	π	*stdev* π
NNEATL	6	6	15	1.000	0.093	0.00812	0.00106
SNEATL	33	17	13	0.932	0.026	0.00424	0.00038
WMED	79	34	18	0.949	0.011	0.00418	0.00019
EMED	52	19	12	0.894	0.028	0.00382	0.00031
TOTAL	170	55	27	0.951	0.006	0.00453	0.00014

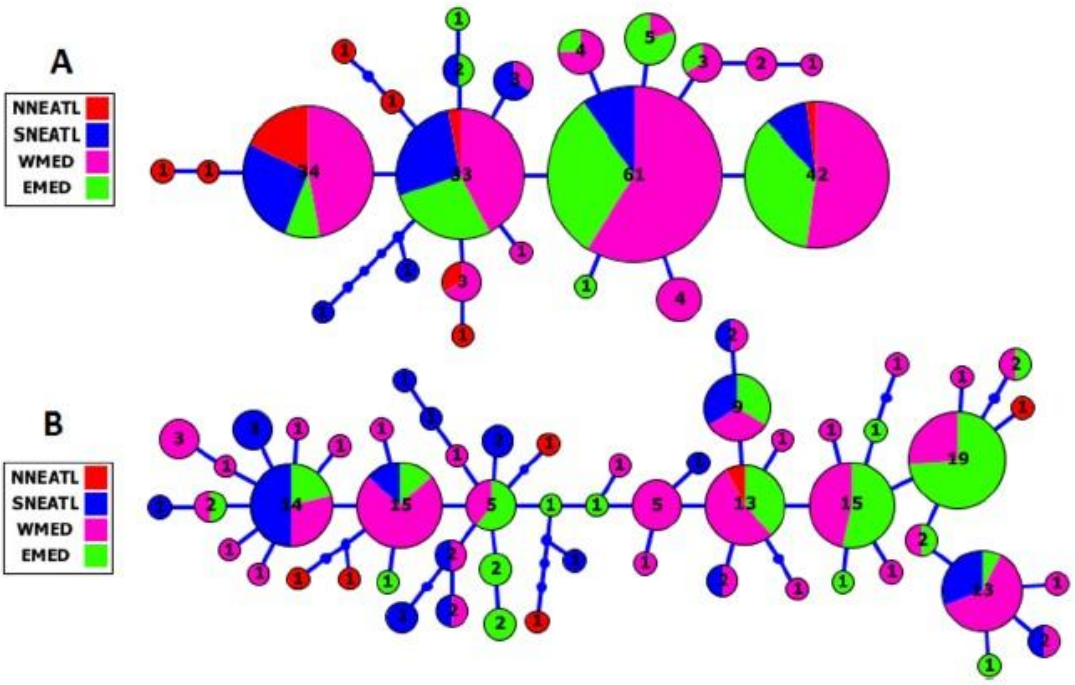

Fig.2.2: Cytochrome-b (A) and Control Region (B) Maximum Likelihood Haplotype Network of Mediterranean/North East Atlantic Blue Shark collected from the four geographical areas. NNEATL: North North-Eastern Atlantic; SNEATL: South North-Eastern Atlantic; WMED: Western Mediterranean; EMED: Eastern Mediterranean.

Significant partition of molecular variance among areas was observed when BS sampling locations were grouped according to the four geographical areas in both markers (AMOVA4), according to three areas (NEATL (NNEATL+SNEATL) vs WMED vs EMED; AMOVA3), and according to two areas (NEATL (NNEATL+SNEATL) vs MED (WMED+EMED), for both dataset. However, the grouping that best described the partitioning of genetic variance is when the different sampling locations are subdivided into four areas showing the lowest partition of molecular variance among populations within group.

Tab. 2.2: Analysis of molecular variance (AMOVA) of Cytochrome b (Cytb) and Control Region (CR) of the Mediterranean and North-Eastern Atlantic Blue Sharks (Prionace glauca).

	Cytb			CR		
	% variation	Φ-Statistics	p	% variation	Φ-Statistics	p
AMOVA1: Overall (all population samples)						
Among populations	8.20			11.25		
Within populations	91.80	ST = 0.0819	0.00000	88.75	ST = 0.11249	0.00000
AMOVA2: 2 groups: (NNEATL+SNEATL vs WMED+EMED)						
Among groups	12.39	CT = 0.1239	0.03496	7.89	CT = 0.0788	0.03471
Among pops within group	2.40	SC = 0.0273	0.02287	7.41	SC = = 0.0804	0.00005
Within populations	92.68	ST = 0.1479	0.00000	84.70	ST = 0.1529	0.00000
AMOVA3: 3 groups: (NNEATL+SNEATL vs WMED vs EMED)						
Among groups	7.01	CT = 0.0701	0.02188	5.68	CT = 0.0568	0.03656
Among pops within group	2.84	SC = 0.0305	0.02397	6.78	SC = 0.0719	0.00075
Within populations	90.15	ST = 0.0985	0.00000	87.54	ST = 0.1246	0.00000
AMOVA4: 4 groups: (NNEATL vs SNEATL vs WMED vs EMED)						
Among groups	8.87	CT = 0.0887	0.02073	7.93	CT = 0.0793	0.03726
Among pops within group	1.20	SC = 0.0132	0.13076	4.89	SC = 0.0531	0.00649
Within populations	89.92	ST = 0.1007	0.00000	87.18	ST = 0.1282	0.00000

With the Cytb sequence data, all pairwise Φ_{ST} values among the four geographical areas were significant except that between the two Atlantic groups (Φ_{ST} = 0.1152; p = 0.019) that became non-significant after the Bonferroni correction for multiple tests (Martin & Douglas, 1995) (Table 3). Unlike the CR dataset, only the pairwise Φ_{ST} values between SNEATL and the two Mediterranean areas and between WMED and EMED remained significant after the Bonferroni correction for multiple tests (**Table 2.3**).

AMOVA and pairwise Φ_{ST} analyses were performed on a reduced dataset, selecting only juvenile and immature specimens from each sampling site. Despite the reduced sample sizes and the

complete absence of data from the site NNEATL, the results obtained are in agreement with the values observed with the complete dataset (**Tab. S 2.3**; **S 2.4**).

The Cytb distribution of sequence mismatch pairwise differences showed a skewed unimodal distribution in all four BS macro areas suggesting a recent bottleneck or sudden population expansion (**Fig. S 2.2**). A unimodal mismatch distribution was obtained with CR dataset in the NNEATL BS. The CR mismatch distribution of EMED, SNEATL and NNEATL BS resulted to a slightly ragged pattern (**Fig. S 2.2**) that could suggest a more constant population size of the Mediterranean BS over generations.

Tab: 2.3: Pairwise 8st values (below the diagonal) and associated p-values (above the diagonal) among the blue sharks of the four geographical areas.

Cytb	NNEATL	SNEATL	WMED	EMED
NNEATL		0.01868*	0.00000	0.00000
SNEATL	0.08167*		0.00055	0.00015
WMED	0.23969	0.08633		0.20052
EMED	0.29481	0.12441	0.00658	

CR	NNEATL	SNEATL	WMED	EMED
NNEATL		0.0097*	0.0482*	0.0187*
SNEATL	0.1649*		0.0003	0.0000
WMED	0.1061*	0.1049		0.0072
EMED	0.1620*	0.2188	0.0463	

Both BSP analyses suggested a constant population size increase of Mediterranean and North-eastern Atlantic BS, starting more recently in the Mediterranean than in the North-eastern Atlantic (~0.02-0.15 Mya vs 0.15-0.4 Mya**; Fig. 2.3**). Divergence time analysis based on both markers (**Fig. S 2.3**) highlights a similar pattern of separation between two main groups, composed by BS from all regions, without any evidence of separation between defined geographic areas. The separation between the two clades, which is strongly supported of Posterior Probability (PP = 1.0) in both markers, is dated back to 1.24 Mya and 0.94 Mya using Cytb and control region, respectively.

2.4 Discussion and Conclusion

The BS is probably the most mobile shark species in the world (Stevens, 1990) and past research works, using both mitochondrial and nuclear markers, have struggled to find genetic structure at

interoceanic scale (Sampaio da Costa, 2013; King et al., 2015; Li et al., 2016; Veríssimo et al., 2017). This high level of gene flow make it difficult to define clear BS population units. In the Pacific Ocean, the lack of structure may be the result of the combination of high potential of migration and the lack of effective barriers to gene flow (Veríssimo et al., 2017). The BS is probably the most mobile shark species in the world (Stevens, 1990) and past research works, using both mitochondrial and nuclear markers, have struggled to find genetic structure at interoceanic scale (Sampaio da Costa, 2013; King et al., 2015; Li et al., 2016; Veríssimo et al., 2017). This high level of gene flow make it difficult to define clear BS population units. In the Pacific Ocean, the lack of structure may be the result of the combination of high potential of migration and the lack of effective barriers to gene flow (Veríssimo et al., 2017).

Experimental data have indicated that no significant genetic structure is detected in spatially distant BS samples (King et al., 2015; Li et al., 2016; Veríssimo et al., 2017). Our results revealed significant signals of geographical structuring for Mediterranean and adjacent Atlantic BS, with several frequent mtDNA haplotypes that are exclusive of the Mediterranean BS and other that are shared with the Atlantic population samples.

While both haplotype networks failed to evidence a clear geographical structure, either between Mediterranean and North Atlantic BS or within the Mediterranean, the results of AMOVA revealed a significant partition of molecular variance among all population samples and when they were grouped according to the four geographical areas with both mitochondrial markers (8.87% for Cytb and 7.93% for CR). Previous studies carrying out AMOVA on the Atlantic BS using the control region variation, showed a significance variance among groups formed by the North Atlantic BS collected from Portugal and Azores and by the South African (See Table 7 of Sampaio da Costa, 2013) or Brazilian BS (Veríssimo et al., 2017).

On the contrary, the global population genetics carried out by Fitzpatrick (2012), using concatenated fragments from: 16S, tRNA, COII, ATPase and control region genes, showed no significance variation among oceans, based upon comparisons between North Atlantic and all sampling locations combined (See Table 5.7 of Fitzpatrick, 2012). Although BS exhibits high potential of dispersal and migration, our results seem to reject an absence of geographical structure in the Mediterranean and adjacent North-eastern Atlantic BS. The pairwise Φst analysis revealed a geographical structuring between the two Mediterranean groups and Southern North-eastern Atlantic BS, with a closer genetic similarity of the Southern North-eastern Atlantic with the Western Mediterranean BS rather than with the Eastern Mediterranean BS.

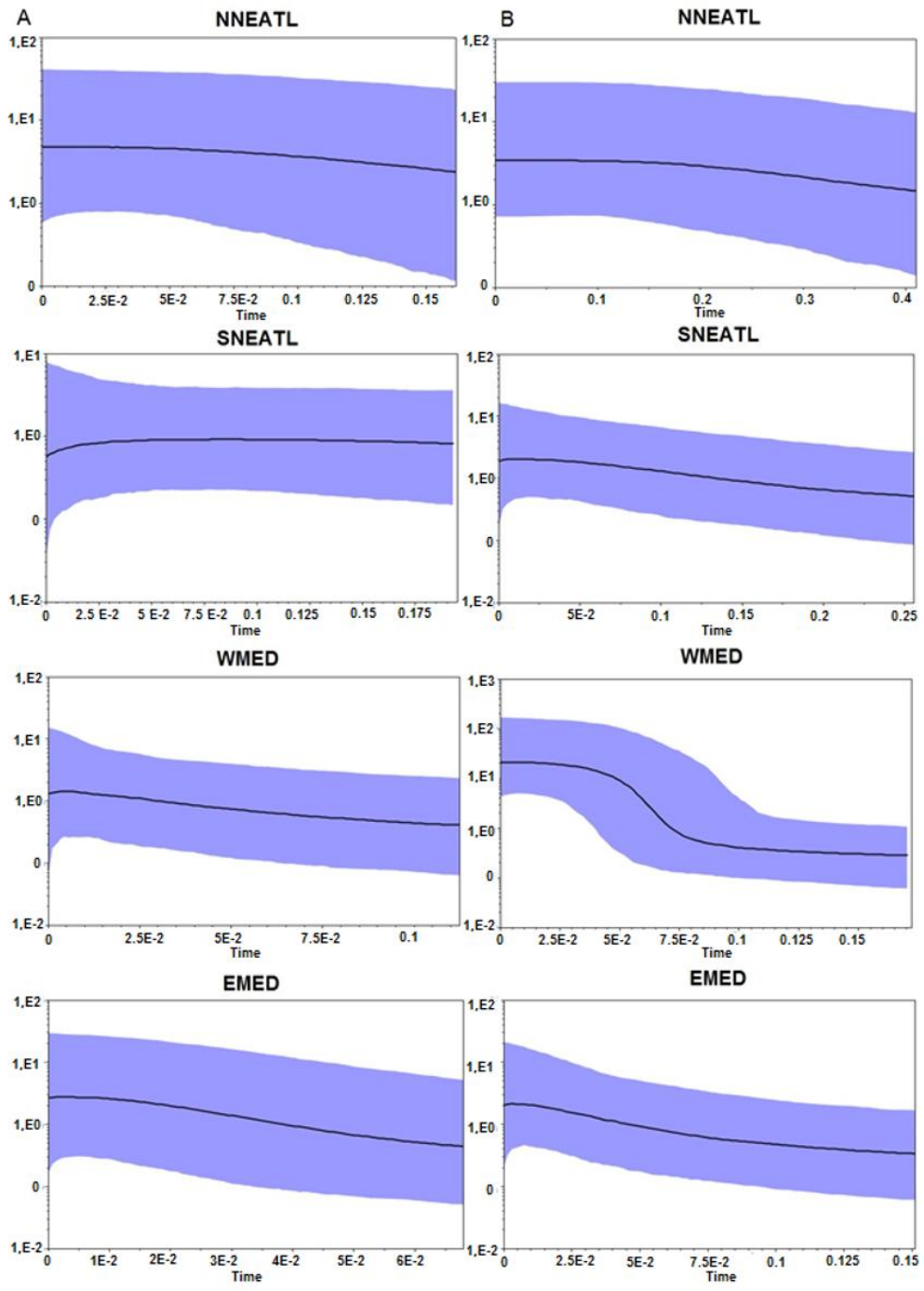

Fig. 2.3: Bayesian Skyline Plot from the Cytb, A-D, and control region, E-H, of the four different geographical areas. NNEATL: North North eastern Atlantic; SNEATL: South North-Eastern Atlantic; WMED: Western Mediterranean; EMED: Eastern Mediterranean. The Y –axis indicates effective population size (Ne) x generation time, while the X-axis indicates mean time in million of years before present. The thick line represents the average, while the blue band represents 95% highest posterior density (HPD) intervals.

This pattern of differentiation seems to suggest that reproductive movements, such as female philopatry, may occur between the Western Mediterranean and the Southern North-eastern Atlantic BS. In addition, pairwise Φst values highlighted that the EMED BS are the more divergent from the NATL BS Given that SNNEATL specimens are from a previously identified nursery site (Veríssimo et al., 2017), the pairwise Φ_{ST} values could suggest that specimens from WMED can be

reproductively related to the SNNEATL, while EMED could represent a nursery site in itself (Megalofonou et al., 2009).

Our sampling work has also preliminarily revealed significant differences between North-eastern Atlantic and Mediterranean BS by sex-ratio and size. This pattern could be the result of a sex-biased reproductive migratory behaviour that could contribute to explain the significant phylogeographical structure. Similarly, size differences were observed between WMED and EMED BS, with the large and sexually mature individuals abundant in the easternmost Mediterranean sampling location (Aegean Sea) while the sub-adult and juvenile BS frequent in the Adriatic and Ionian Seas. The great abundance of juvenile BS in the Adriatic Sea seemed to confirm the nursery role of this area for BS (Megalofonou et al., 2009).

The biological data reported in Megalofonou and colleagues (2009) describe a larger amount of big female in the easternmost Mediterranean (e.g. Aegen Sea) which is in agreement with the pattern inferred from our dataset. Conversely, using data on size and maturity stages, Kohler and colleagues (2002) observe that the majority of sharks from the Mediterranean Sea were juvenile and immature (99% of males and 98% of females; mean = 65 cm of fork length). The difference may be related to the different sampling design and fishing gear used in the studies. In fact, the majority of the data collected by Kohler and colleagues (2002) came from volunteer recreational fishermen, while the individuals from Megalofonou et al. (2009) and from this work, originated principally as by-catch from commercial fisheries, such tuna and swordfish longline.

Overall, Mediterranean and adjacent North-eastern Atlantic BS displayed a complex geographical structure in which weak but significant differences proved that a certain degree of population connectivity across the Strait of Gibraltar occurred. These results are in contrast with those obtained by tagging data in the past (Kohler et al., 1998; 2002; 2008; Poisson et al., 2015). Similar findings of genetic differences were observed in other shark species, more related to a benthic environment, such the small-spotted catshark, *Scyliorhinus canicula*, and the velvet belly lanternshark, *Etmopterus spinax* (Gubili et al., 2014;2016; Kousteni et al., 2015). The reported evidence of genetic structure in the blue shark analyzed in this study are associated with geographical differences in sex-ratio and size. Our results suggest BS in the NE Atlantic and the Mediterranean are not panmictic. There is still no direct observations of mating events take place in the Eastern Mediterranean, but the biological data analysis results support the Eastern Mediterranean as an important nursery area for this species (Megalofonou et al., 2009). Such microevolutionary pattern of differentiation of Mediterranean and North-eastern Atlantic BS

prompt the need for a deeper population genetic analysis on the same population samples with more powerful markers for investigating potential subtle structure of BS populations (e.g. microsatellites or SNPs) to provide robust data on BS population structure that are of priority for the BS stock management. High genetic diversity values are usually related to large population size (Frankham, 1996), and the high genetic diversity showed by both Mediterranean and North-eastern Atlantic BS at the two mitochondrial makers advocates in favour of a large size of these populations. Mediterranean and North-eastern Atlantic BS showed higher Cytb gene polymorphism than Pacific BS (Mediterranean and North-eastern Atlantic: h = 0.777 - 0.814; π = 0.002 - 0.004; Pacific: h = 0.517 - 0.768; π = 0.0007 - 0.0011, Li et al., 2016).

Based on nuclear markers, similar values of observed heterozygosity were detected between Pacific and North Atlantic BS (Sampaio da Costa, 2013; King et al., 2015; Veríssimo et al., 2017). High genetic diversity in abundant species is likely due to a combination of demographic factors, such as local population sizes, fast generation times and high rates of gene flow with other populations (Hague & Routman, 2016). The high genetic diversity shown by Mediterranean and North-eastern Atlantic BS could be a consequence of the short time elapsed, in proportion to the relative generation time, since the population started to suffer overexploitation. In fact, the abundance of the Mediterranean BS has declined by ~78–90% over the past 30 years (Ferretti et al., 2008), approximately corresponding to three generations; the BS generation time was estimated at 8.2 and 9.8 years for South African and North Atlantic populations, respectively (Cortès et al., 2015). Furthermore, biological characters such as the large size of litters, the low nucleotide substitution rate compared to other vertebrates (Martin et al., 1992), the high potential of migration and the high gene flow between geographical distant populations, may have affected the relationship between genetic diversity and population size, masking the sudden potential population bottleneck of the last three decades, without genetic erosion.

Otherwise, the mismatch distributions of the different macro areas appear to be slightly skewed unimodal, related to a recent bottleneck or a sudden population expansion (**Fig. S 2.2**), and given the Bayesian skyline plots (**Fig. 2.3**), there is overwhelming evidence that the Mediterranean and North East Atlantic populations have undergone a constant population expansion during the last 400-200 Kya, especially within the Mediterranean samples.

The data we show here represent a novelty for the knowledge of Mediterranean blue shark, and our findings highlight the importance of the Mediterranean Sea as nursery area for this species, with direct implication to specific conservation measures for the species.

This work sheds new light on the understudied BS of the Mediterranean Sea, and emphasizes the need of conducting further population genetic surveys on this population. With ongoing efforts, (i.e. https://fishreg.jrc.ec.europa.eu/web/medbluesgen/) a greater understanding of the genetic diversity, spatial population structure and gene flow in this species will be achieved, which will enable us to devise more effective strategies for the management of this increasingly exploited ocean predator.

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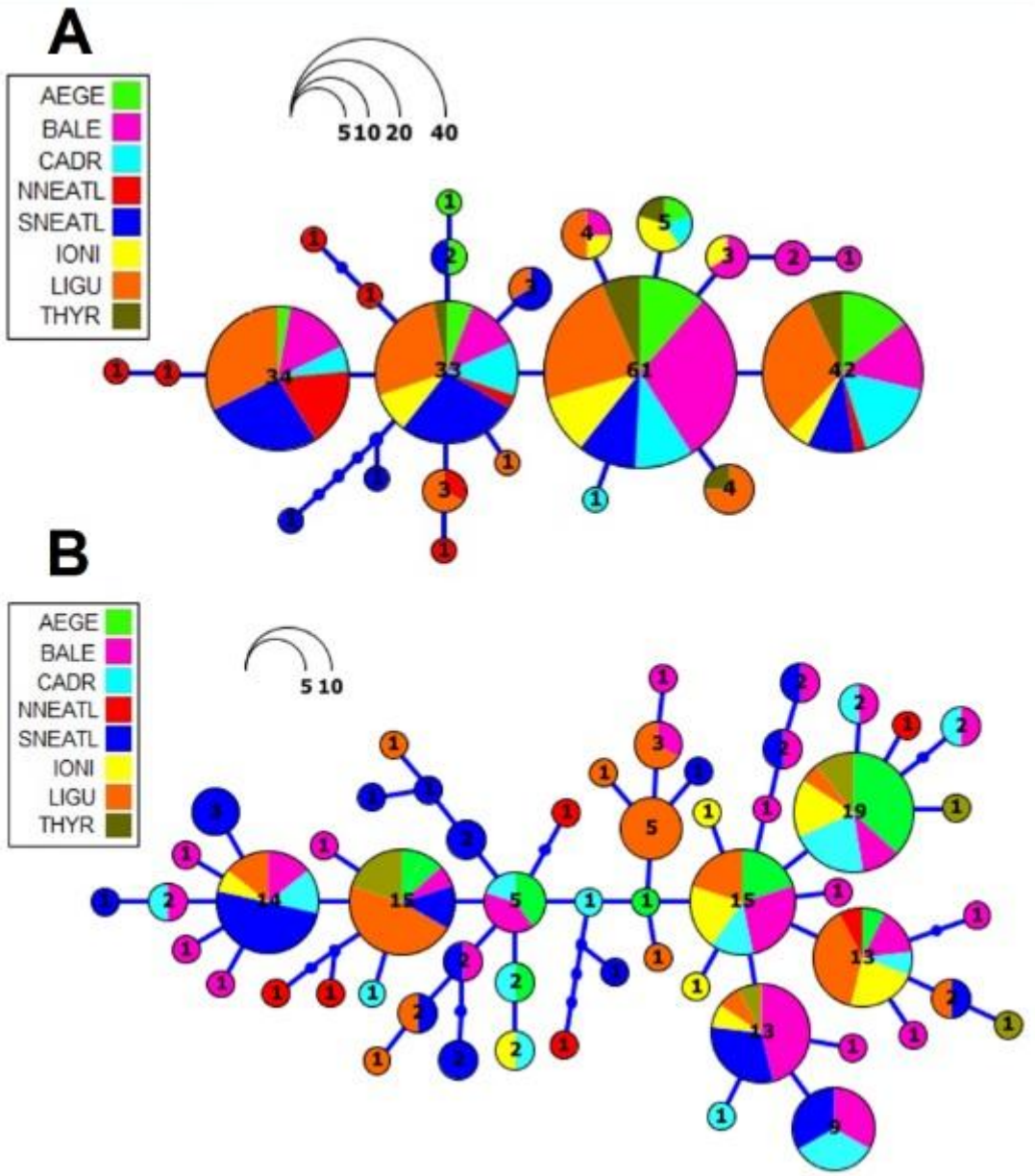

Fig. S2.1: Cytochrome-b (Cytb) and Control Region (CR) Maximum Likelihood Haplotype Network of Mediterranean and North Atlantic Blue Shark populations detailed per sampling locations

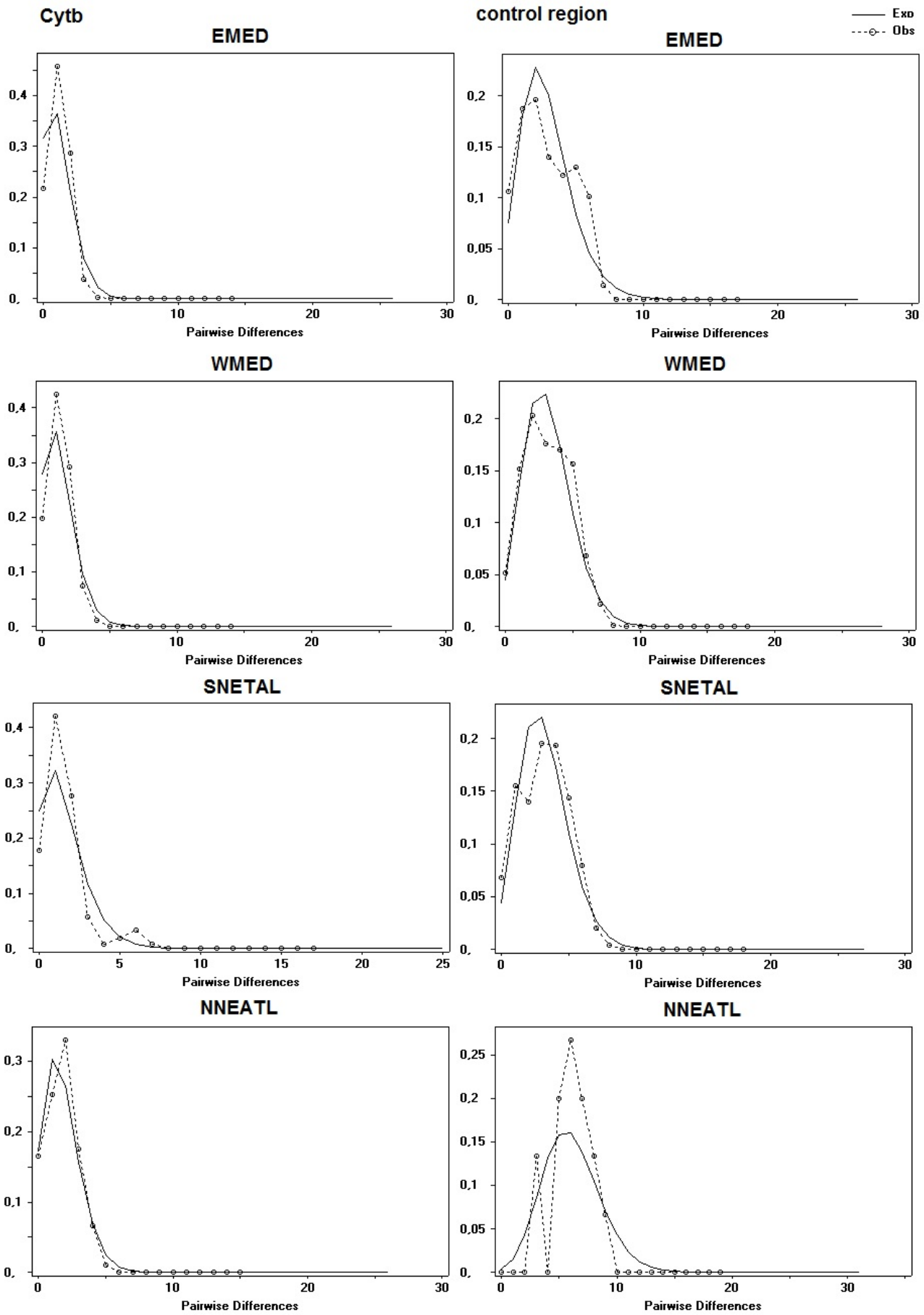

Fig. S2.2: Mismatch Distribution for the four different blue sharks geographical groups for both Cytochrome-b (Cytb) and Control Region (CR) mitochondrial markers

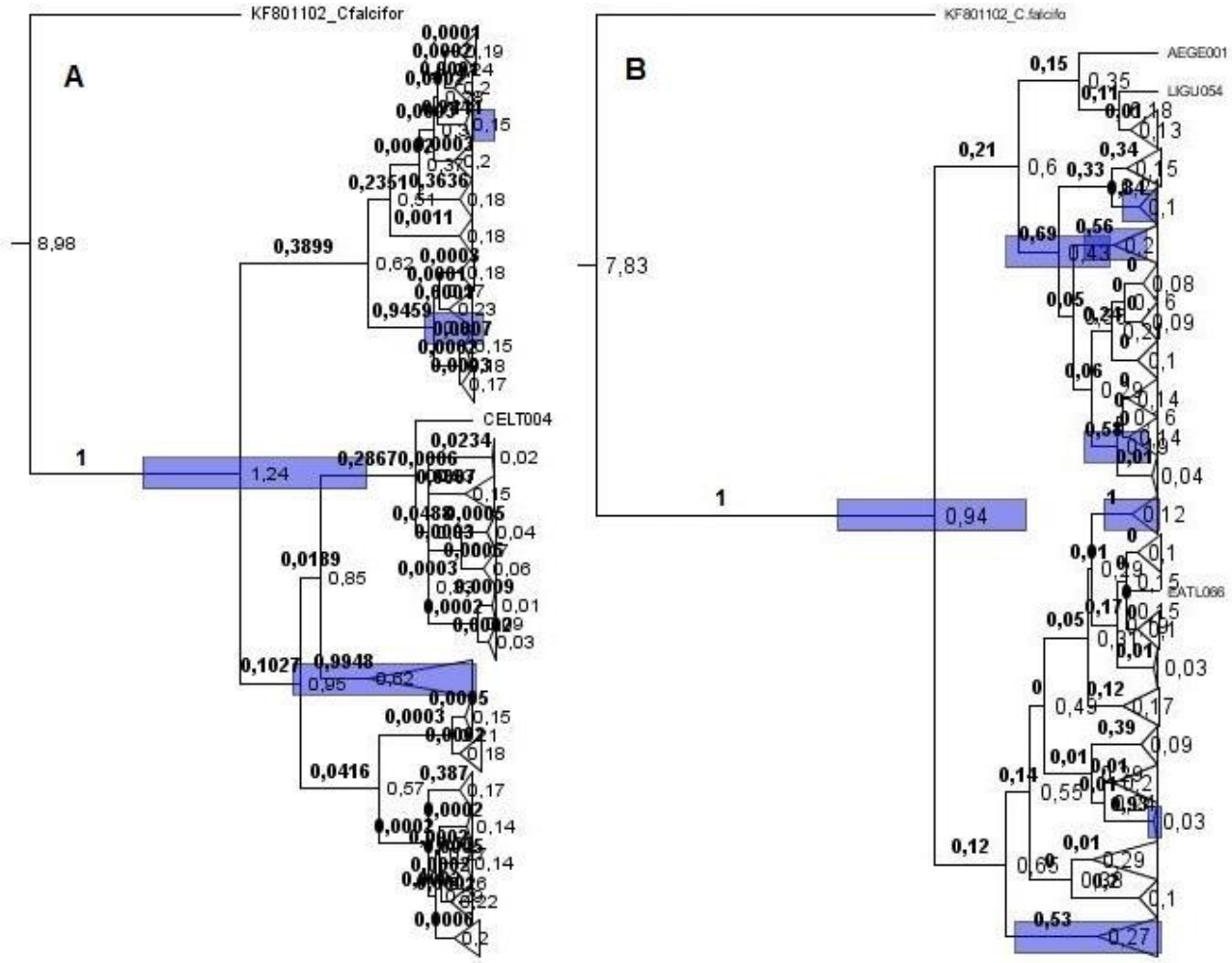

Fig. S2.3: Cytochrome-b (Cytb), A, and Control Region (CR), B, divergence time analysis of the total BS dataset. Node ages on the node labels. Posterior probability on the branches. 95% HPD bars showed for nodes with Posterior > 0.5. In this picture, is evident the separation of two main haplogoup at 1.24 Ma and 9.94 Ma for control region and Cytb, respectively

Tab. S2.1: Collected BS individuals categorized by sex and size across sampling areas and regions

	NNEATL	SNEATL	BALE	LIGU	THYR	IONI	AEGE	CADR	NATL	WMED	EMED
M	2	9	32	21	3	6	8	10	11	56	24
F	14	18	10	36	1	4	7	11	32	47	22
na	0	6	0	0	6	5	5	0	6	6	10
Total	16	33	42	57	10	15	20	21	49	109	56
Total sexed	16	27	42	57	4	10	15	21	43	103	46
%M	13%	33%	76%	37%	75%	60%	53%	48%	26%	54%	52%
%F	88%	67%	24%	63%	25%	40%	47%	52%	74%	46%	48%
Sex-ratio	0.14	0.50	3.20	0.58	3.00	1.50	1.14	0.91	0.34	1.19	1.09
J	0	8	0	11	9	10	5	20	8	20	35
Y	2	19	21	33	0	3	3	1	21	54	7
L	9	6	21	13	1	2	12	0	15	35	14
Total sized	11	33	42	57	10	15	20	21	44	109	56
%J	0%	24%	0%	19%	90%	67%	25%	95%	18%	18%	63%
%Y	18%	58%	50%	58%	0%	20%	15%	5%	48%	50%	13%
%L	82%	18%	50%	23%	10%	13%	60%	0%	34%	32%	25%

Tab. S2.2: Cytb, A, and control region, B, genetic diversity data from the eight sub-populations

A

POP	*N*	*Nh*	*S*	*h*	*stdev h*	π	*stdev* π
NNEATL	14	9	6	0.835	0.101	0.00231	0.00046
SNEATL	33	8	10	0.822	0.034	0.00200	0.00038
BALE	39	8	5	0.749	0.059	0.00162	0.00022
LIGU	56	9	5	0.828	0.022	0.00171	0.00011
THYR	10	5	4	0.800	0.100	0.00140	0.00030
IONI	15	6	5	0.810	0.078	0.00145	0.00025
AEGE	19	7	3	0.784	0.067	0.00158	0.00024
CADR	21	6	4	0.795	0.051	0.00149	0.00017
TOTAL	207	23	16	0.821	0.013	0.00184	0.00010

B

POP	*N*	*Nh*	*S*	*h*	*stdev h*	π	*stdev* π
NNEATL	6	6	15	1.000	0.096	0.00812	0.00106
SNEATL	33	17	13	0.932	0.026	0.00424	0.00038
BALE	39	25	16	0.962	0.017	0.00444	0.00033
LIGU	32	14	9	0.907	0.029	0.00323	0.00028
THYR	8	5	8	0.857	0.108	0.00488	0.00062
IONI	14	8	9	0.901	0.052	0.00319	0.00078
AEGE	17	7	6	0.809	0.079	0.00309	0.00047
CADR	21	14	10	0.948	0.031	0.00463	0.00037
TOTAL	170	55	27	0.951	0.006	0.00453	0.00014

Tab. S2.3: Analysis of molecular variance (AMOVA) of Cytochrome b (Cytb) and Control Region (CR) of the Juvenile and Immature Mediterranean and North-eastern Atlantic Blue Sharks (*Prionace glauca*)

	Cytb			CR		
	% variation	Φ-Statistics	p	% variation	Φ-Statistics	p
AMOVA1: Overall (all population samples)						
Among populations	4.81			11.30		
Within populations	95.19	ST = 0.0481	0.0079	88.70	ST = 0.11302	0.00000
AMOVA2: 2 groups: (SNEATL vs WMED+EMED)						
Among groups	10.69	CT = 0.1069	0.1403	11.87	CT = 0.1187	0.14035
Among pops within group	0.58	SC = 0.0065	0.3115	5.72	SC = 0.0649	0.00489
Within populations	88.72	ST = 0.1128	0.0084	82.41	ST = 0.1759	0.00000
AMOVA3: 3 groups: (SNEATL vs WMED vs EMED)						
Among groups	5.06	CT = 0.0506	0.2095	9.87	CT = 0.0987	0.02912
Among pops within group	0.97	SC = 0.0103	0.2666	3.48	SC = 0.0386	0.05584
Within populations	93.97	ST = 0.0603	0.0072	86.65	ST = 0.1335	0.00000

Cytb	SNEATL	WMED	EMED
SNEATL		0.0025	0.0004
WMED	0.0912		0.4341
EMED	0.1402	-0.0019	

CR	SNEATL	WMED	EMED
SNEATL		0.0004	0.0000
WMED	0.1202		0.0066
EMED	0.2170	0.0605	

Tab. S2.4: Pairwise Φst values (below the diagonal) and associated p-values (above the diagonal) among the juvenile and immature blue sharks of the three geographical areas estimated at the two mitochondrial markers (Cytochrome b, Cytb; Control Region, CR). *Values that resulted not significant after the Bonferroni correction for multiple tests (a-level of significance after Bonferonni correction: p = 0.0166).

3 CHAPTER 3

Genomics and bioinformatics of the blue sharks: optimization of the Reads Assembly, Mapping and SNPs Calling

3.1 Introduction

The genetic information of an organism, in the form of genetic code, is stored in its genome.

This genome can be found either in specific organelles, such the mitochondria, and in the nucleus, where the genome is organized in discrete chromosomes (Futuyma, 1998). The size of an entire genome is usually measured in picograms (pg) of DNA per haploid nucleus and it is directly proportional to the total number of base pairs in an entire organisms' genome. The genome size can vary heavily in different taxa. The genome size of the Elasmobranchs can vary largely in relation to the Superorder: Batoidea, which includes rays and skates, and Selachimorpha, which includes sharks such the blue shark, *Prionace glauca* are characterized by very different genome size. The shark genome size range from 3 to 34 pg (Stingo and Rocco, 2001), and with the exception of lungfishes and salamanders, it is the largest genome in vertebrates.

This value is easily convertible in bp with the formula: N° bp = mass in pg x 0.978 x 10^9, or simply, 1pg = 978 Mbp, so 3 pg = 2.9 Gbp and 34 pg = 33.2 Gbp.

This incredible variation in the shark genome size is not related to a primitive/derived evolutionary trend, and the advent of new sequencing technologies is allowing a deeper study of the genomic architecture of these taxa (Schwartz and Maddock, 2002).

DNA sequencing technologies are quickly improving, in particular with the advent of the Next Generation Sequencing (NGS) and the constant decrease of the high throughput sequencing costs (Goodwin et al., 2016). This technological innovation allowed the researchers to identify co-dominant single nucleotide polymorphisms (SNPs) at a genome-wide scale (Davey et al., 2011), boosting the discriminant power of the analyses to distinguish among weakly structured populations, that is quite common in pelagic marine species (Allendorf et al., 2010; Davey et al., 2011; Narum et al., 2013; Andrews and Luikart 2014; Bekkevold, et al. 2015).

Usually, the conventional methods for population genetics use just few of the thousands of discriminant markers available in a genome (Zhang, et al. 2011) but the NGS advent is representing a game changing approach in the study of the genetic variation of several species.

The SNPs are useful in deeply scan the genome variation for population genetics purposes. Despite the fact the biallelic SNPs are less polymorphic than other nuclear markers, such the microsatellite loci, their lower discriminant power is outweighed by their abundance across the entire genome (Baird et al., 2008).

These novel opportunities to detect weak genetic differentiation, play a fundamental role in the molecular ecology and conservation, and in the integration of genetic data into the management of marine resources (Waples, et al. 2008).

The study of the genetic variation at genome-wide level was possible, in the past, when huge economical resources were available for massive sequencing, assembly and annotation of entire genomes, and this can be very difficult for non-model organisms (Glenn, 2011; Grabherr, et al. 2011), such the sharks. Furthermore, the sequencing of large genomes, such a shark's genome, can represent a very difficult technical challenge, due to the potential presence of many repetitive regions and paralogs as expected in a large genome (Glasauer & Neuhauss, 2014).

Up to date, the only draft genomes available for a shark species is that of the whale shark, *Rhincodon typus*, which measures 3.4 Gbp, fully similar in size to the human genome (3.4 Gbp) (Read, et al. 2017). Unfortunately, there are no reference genome for carcharinid sharks, such the BS, which has a genome size of 4.3 pg corresponding to 4.2 Gbp (Hinegardner, 1976; Asahida and Ida, 1995).

Since the great evolutionary distance between the BS (Carcharhiniformes) and the whale shark (Orectolobiformes), it is impractical to use the whale shark genome as a backbone information, or reference genome, to map DNA reads for such different species of sharks.

With the advent of NGS methodologies, numerous techniques for the reduction of complexity of genomes came to light, such as the Restriction-site Associated DNA sequencing (RAD). This method permits to sample a reduced complexity across an entire genome, enabling the production of high resolution population genomic data for any organism at very low costs (Davey and Blaxter, 2010).

Just in the last years, RAD sequencing technologies were used on non-model species such sharks, for which there are no reference genome (Portnoy et al., 2015; Maisano Delser, et al. 2016). The bioinformatic pipelines dedicated to the analysis of DNA reads data are able to implement a "*denovo*" assembly module, in order to produce a backbone reference using the sequences produced during the library sequencing, and then map the same DNA reads, produced during the library preparation, for variant detection and SNPs calling (Portnoy, et al., 2010; Bernal, et al. 2014; Maisano Delser, et al. 2016; Anderson, et al. 2017).

Within the scope of this PhD thesis and the MedBlueSGen project, here we applied the double-digest RAD Genotyping-By-Sequencing (GBS) technique (dd-RAD; Peterson, et al., 2012) with slight modifications following Brown, et al. (2016), in order to test its potential as genomic approach to a

large genome and highly mobile shark species, investigating its population genetic structure. Like other RAD approaches, this genomic tool is based on the sequencing of reduced-representation libraries produced by a double-restriction enzyme (RE) digestion (i.e., a restriction digest with two enzymes simultaneously). This double digestion determines an at least five-fold reduction in library production cost, combined with precise size selection by sequence-specific fragmentation.

In this technique the entire genomic DNA is digested by two restriction enzymes (usually a combination of a low-frequency cutter and a high-frequency cutter enzyme) (**Fig. 3.1**). Barcoded P1 adapters (with an overhang end matching the first restriction site) and P2 adapters (with an overhang end matching the second restriction site) are ligated onto digested DNA fragments by a single sticky-end ligation. The samples are pooled and selected by size with gel electrophoresis and then, the libraries are enriched using PCR where a second barcode in the form of an Illumina index are included, increasing multiplexing potential (Peterson, et al. 2012).

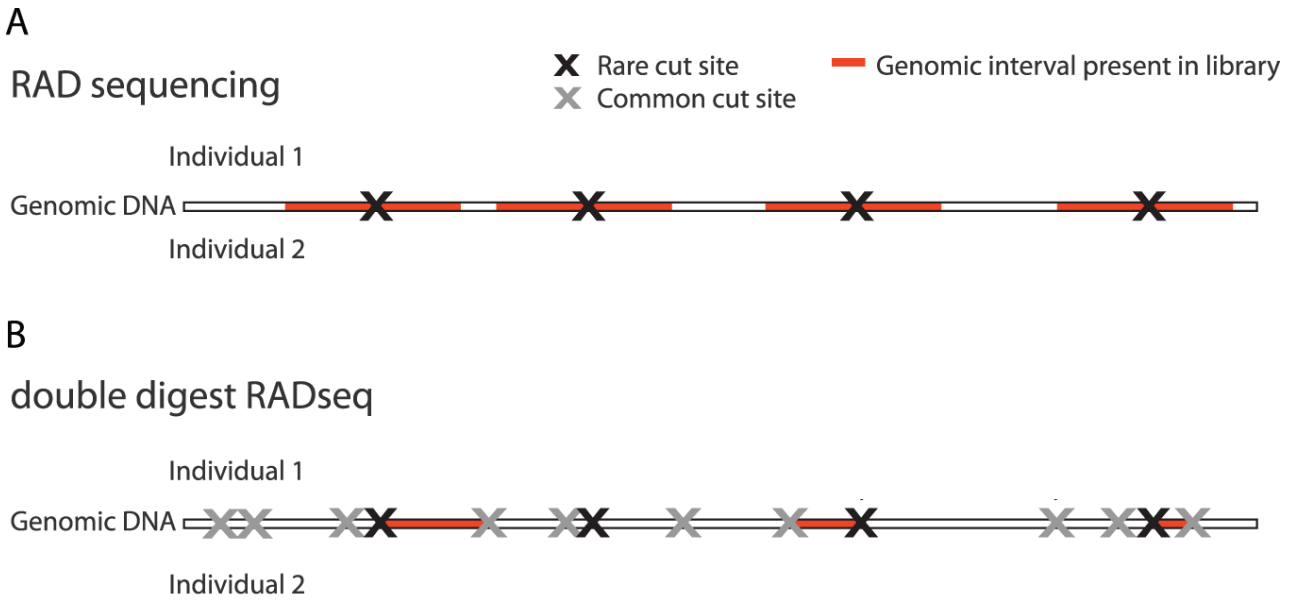

Fig. 3.3: Double digest RAD approach. (A) Traditional Restriction-Site Associated DNA sequencing (RADseq) characterized by the use of asingle restriction enzyme (RE) and digest coupled with secondary random fragmentation and broad size selection to generate reduced representation libraries consisting genomic regions adjacent to the enzyme cut site (red). (B) Double digest RAD sequencing (ddRADseq), characterize by the use two enzyme that double digest the DNA, prior to precise size selection that excludes regions flanked very close and very distant RE cut sites. The last approach recover a library consisting of only fragments close to the target size (red). Modified image from Peterson et al. (2012).

This technique produces sequencing libraries consisting of only the subset of genomic restricted fragments generated by both enzyme cuts which fall within the size-selection window, generating tags of uniform length suitable for NGS platforms and subsampling large genomes with great reproducibility (Peterson et al., 2012; Puritz et al., 2014a).

The main differences between the conventional Genotyping by Sequencing approach the the RADseq are the lack of the random shear, the size selection (for the selection of length fixed sequences) and the second ligate adaptors steps (**Fig. 3.2**).

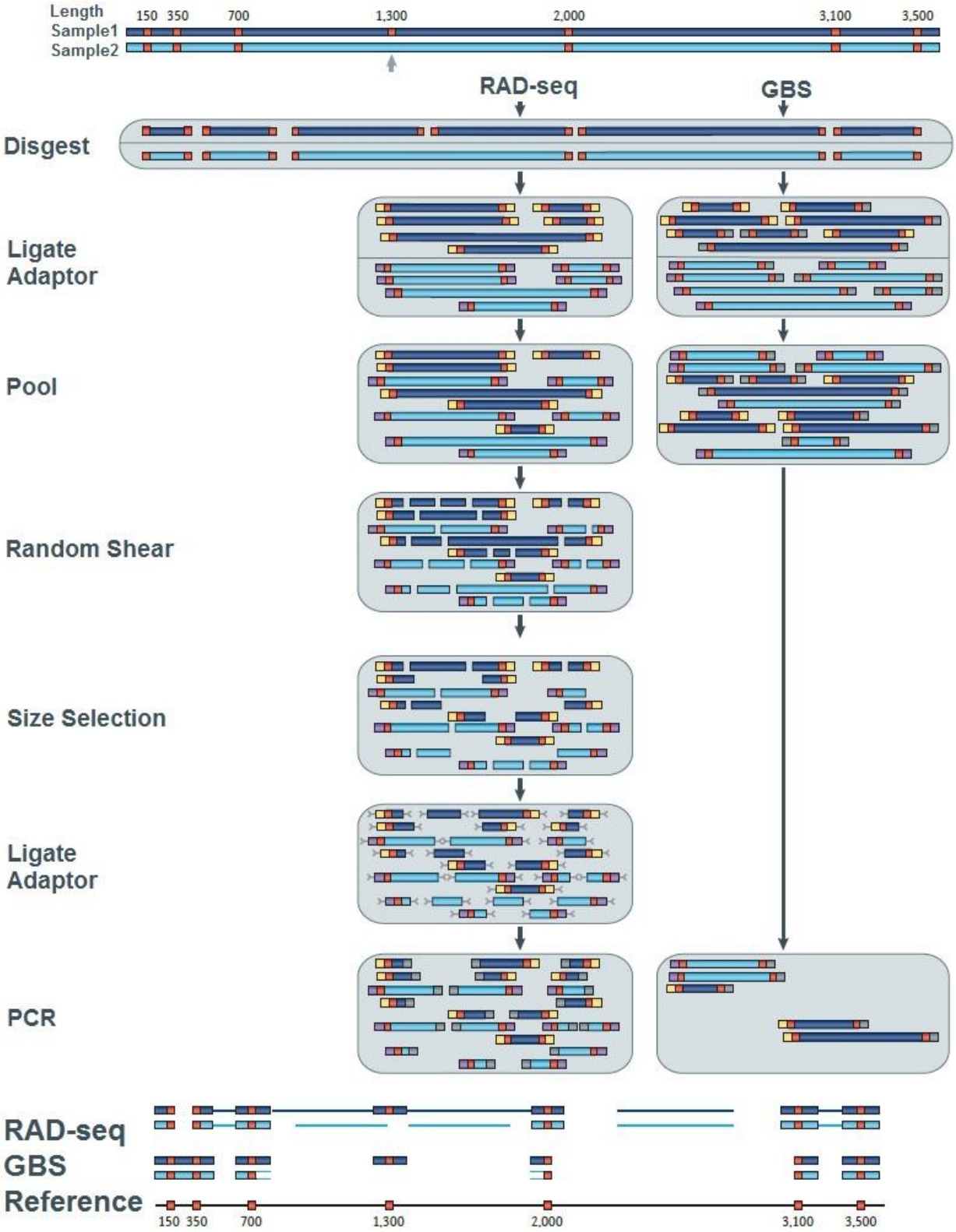

Fig. 3.2: Differences, in steps, between the Genotyping by Sequencin (GBS) and the RAD approach. Modified Image from Davey et al. (2011).

In this work, we: i) first examine the utility of the ddRAD approach in genotyping the genome of BS; ii) we use the pipeline dDocent (Puritz et al., 2014b) to assess the correct reads assembly, mapping and SNPs calling in BS; iii) optimize the reads assembly of BS; and iv) finally assess the applicability of ddRAD approach for future investigations in this highly migratory species.

3.2 Material and Methods

3.2.1 Libraries Preparation and Sequencing

Genomic DNA (gDNA) was extracted using the SSTNE buffer, a modified TNE buffer added of spermidine and spermine (Pardo et al., 2005). A modified double digest restriction-site associated DNA (ddRAD) protocol (Peterson et al., 2012) was used to simultaneously discover and genotype individuals at thousands of SNPs, following the procedure first described by Peterson et al. (2012) with slight modifications (Brown et al., 2016) (**Fig. 3.3**). After a gDNA quality check carried out measuring the absorbance ratio 260/230 and 260/280 with a Microdrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) , 30 ng of each individual gDNA were digested with 0.43U each of *Sbf*I and *Sph*I (New England Biolabs). P1 and P2 barcoded adapters, compatible with *Sbf*I and *Sph*I overhangs, respectively, were added together with T4 DNA ligase.

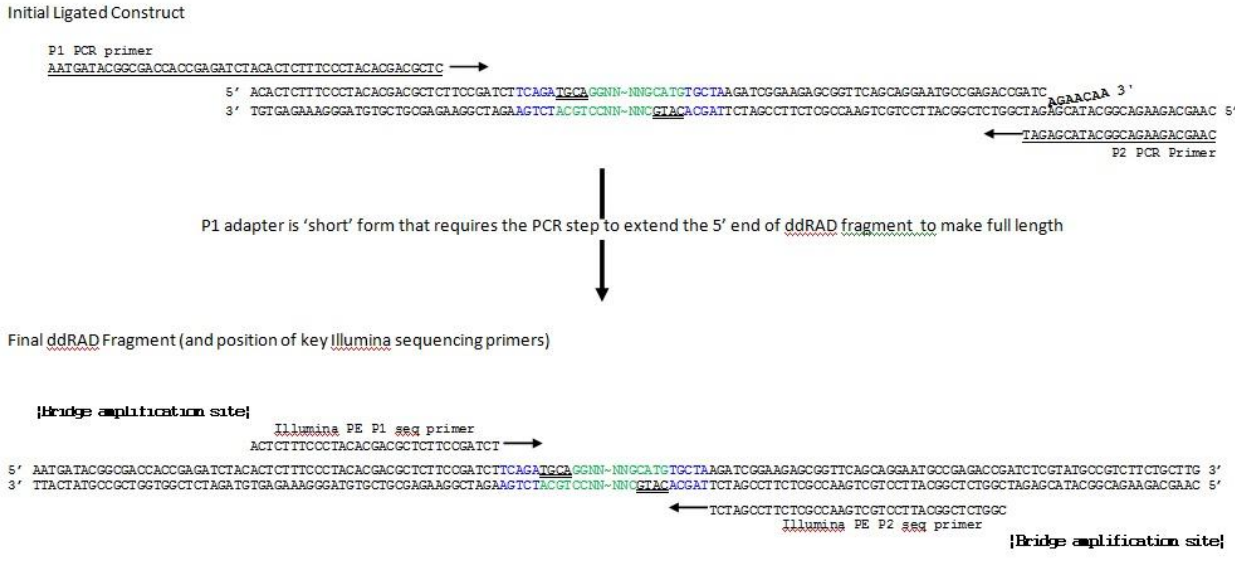

Fig. 3.3: Architecture of a ddRAD library fragment formed by initial ligation of an SbfI P1 adapter (barcode TCAGA) and a SphI P2 adapter (barcode: TAGCA). Blue (barcode); Double underline (3' RE overhang on adapter); Green (genomic DNA); Single underline (PCR primers).

After enzyme heat inactivation, restricted individual samples were pooled and cleaned up with MinElute PCR Clean Up Kit (Qiagen, Venlo, Netherlands). The library was run on an agarose gel

(1.1%), to size select fragments of 200-300 bp. Size-selected DNAs were eluted from gel slice. Eluted fragment library were PCR amplified with generic P1 and P2 primers after having optimized PCR conditions. Amplified libraries were purified using AMPure XP Magnetic Beads (Beckman Coulter, Pasadena, California, USA). The obtained ddRAD libraries were paired-end sequenced using a HiSeq 4000 150 bp PE method at an external sequencing service provider (UC Davis). A test for consistency of results and data obtained across each library was carried out by constructing and sequencing independently two libraries from two individuals. BS individuals from the different geographical areas were distributed across each library to avoid batch effect in processing of samples during library construction and sequencing that could introduce artefacts in the subsequent data analysis and could bias inferred results. The composition of each library is reported in **Table 3.1**, for a total of 212 BS specimens subdivided in three ddRAD BS libraries.

ddRAD library	**Area**				**Total**
	NEATL	**SEATL**	**WMED**	**EMED**	
Pg_ddRAD_01		28	15	29	**72**
Pg_ddRAD_02	20	5	33	12	**70**
Pg_ddRAD_03	10		41	19	**70**
Total	**30**	**33**	**89**	**60**	**212**

Tab. 3.1: Composition of the three ddRAD Blue Shark libraries in terms of geographical distribution of individuals.

3.2.2 Reference assembly optimization and reads mapping

After the HiSeq 4000 150 bp PE sequencing, the samples were demultiplexed using the program process_radtags of the pipeline STACKS (Catchen, et al. 2011, 2013), avoiding the use of the parameters -c, to clean data, remove any read with an uncalled base, and -q, to discard reads with low quality scores. These filtration steps are included in the dDocent pipeline (Puritz et al. 2014b) used subsequently. This step is used to retrieve pooled sequences using the DNA barcodes (**Tab. S 3.1**).

After the demultiplexing , the samples with <500k reads were discarded. The resulting Fastq files were checked with the FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/) that provides a report for Quality Check (QC) and highlights any potential bias in the raw data that may affect the downstream analysis, such a low quality base calling (Del Fabbro et al., 2013). A

phred score of 20, that is equal to 1 error in 100bp, or 99% of correct base calling, has been used as limit "threshold" of good quality base calling.

Then, in the dDocent environment, samples underwent to simultaneous de-novo SNP genotyping in a third-party software on a Unix platform.

The dDocent pipeline employs a series of data reduction techniques, alignment based clustering, and, for PairedEnd assembly, a specialized RAD assembly software. This combination, according to the dDocent authors, allows for accurate and efficient de novo assembly increase accuracy with respect to the other available pipeline (i.e. STACKS and PyRAD, http://ddocent.com/why/). dDocent has been successfully employed in several recent works analyzing elasmobranch species (Dimens, 2016; Manuzzi, 2016; Portnoy et al. 2015) and in other marine fish species which are generally characterized by high diversity and low differentiation (Hollenbeck et al. 2017; Puritz et al. 2016). However, utilizing RADseq data without a reference genome can lead to a series of challenges. One the major challenge is the trade-off between splitting alleles at a single locus into separate clusters (loci), creating inflated homozygosity, and lumping multiple loci into a single contig (locus), creating artefacts and inflated heterozygosity (Willis, et al. 2017). This issue has been addressed primarily through the use of similarity cut-offs in sequence clustering. Most of the published works however coupled the dDocent pipeline with customized script to mitigate the high levels of repeats and duplications expected in large genomes, as we expected also in BS.

To create a representative reference assembly, and to choose the optimal parameters in the construction of the reference assembly, at first, trimmed reads from 52 BS individuals (**Tab. S 3.2**), with coverage within 0.5 standard deviations of the mean coverage of the total dataset, representative of all sampling areas, size, date of capture of the entire dataset were used to construct a reference assembly through the program CD-HIT (Fu et al., 2012). This subset of BS individuals was used to avoid a site-based bias during the reference library construction.

During the reference assembly process, particular attention should be given to the main parameters for cluster similarity (C): the number of contigs within individuals (K1) and the number of contigs between individuals (K2).

The custom bash scripts ReferenceOpt.sh and RefMapOpt.sh (https://github.com/jpuritz/dDocent/tree/master/scripts) were used to choose the best assembly parameters (C,K1,K2) for the reference assembly constructed using the subset of 52 BS individuals.

After the identification of the optimal parameters (see Results) for the reference assembly, dDocent was then run again, and the program BWA (Li & Durbin, 2009) was used to map paired-end reads to the reference assembly using the matching score parameter (A), the mismatching score (B), and the gap penalty (O), set to 1, 3, and 5, respectively, which are the default values of BWA.

Subsequently, the program FreeBayes (Garrison & Marth, 2012) was used in the SNP discovery process, and the SNPs present among 97.5% of 52 BS individuals, as expected in a condition of Hardy-Weinberg equilibrium (HWE) within samples, with a minor allele frequency MAF > 5% were retained for further analyses, as suggested by the dDocent pipeline (Portnoy et al., 2015).

3.2.3 SNPs filtering

After the reads mapping and SNPs calling, the initial raw SNPs dataset consisted of 56,004 variants. Subsequently, the raw dataset underwent to several filtering steps following, as a mold, the steps by Puritz et al. (2016) (details below).

Initially, all genotypes with < 5 reads, and all variants with MAF < 1% and not called in at least 50% of samples were filtered out with a custom bash script (filter_missing_ind.sh).

Then, the same missing values (NA) filter was used at population level with the script pop_missing_filter.sh. Subsequently, a Vcftools v. 0.1.14 (Danecek et al., 2011) was used to filter out any sites < 90% overall call rate.

The dDocent_filters bash script was used to filter loci not in a properly paired status. This script filters out 1) loci that in average have heterzygote genotypes with < 0.28 of allele balance between reads from each allele, 2) loci that have reads from both strands, with some leeway for a bad individual or two, and 3) loci above the mean depth +1 of standard deviation that have quality scores < 2X depth. Then, it calculates a mean depth cut-off to use for filtering (sigma, σ, cutoff, ~90% of the data) (**Fig. 3.4**).

The indels were removed from the dataset and the loci that do not respect the HWE in more than half the population samples were removed using the perl script filter_hwe_by_pop.pl written by Chris Hollenbeck, restricting the SNPs dataset only to loci with 2 alleles using Vcftools v. 0.1.14.

Finally, SNPs were organized with the perl script rad_haplotyper.pl by Chris Hollenbeck, that use called genotypes and aligned reads to make haplotype calls across RAD loci using both F and R reads. This step is useful for detection and filtering of the potential paralogs, which are quite expected in a large genome.

In order to assess the potential contamination due to the presence of mtDNA in the data, both complete and final and filtered contigs were mapped against a BS mitogenome. Each filtering steps and scripts used are shown in **Supplementary Text 3.1**.

3.3 Results and Discussion

Among the 291 BS gDNAs, 212 BS met the minimum quality requirements for the library preparation and were successfully used for the construction of the libraries using the ddRAD protocol designed by Peterson et al. (2012) and modified by Brown et al. (2016).

After the paired-end sequencing and demultiplexing, excluding samples with a number of reads < 500k, 207 BS individuals met the required characteristics for the downstream analyses.

The diagnostic script ReferenceOpt.sh identified the best cluster similarity threshold for the reference assembly at 0.9 (90%) testing different combinations of K1 and K2 (all possible combination of K1K2 from 1 to 17) (**Fig. 3.5**).

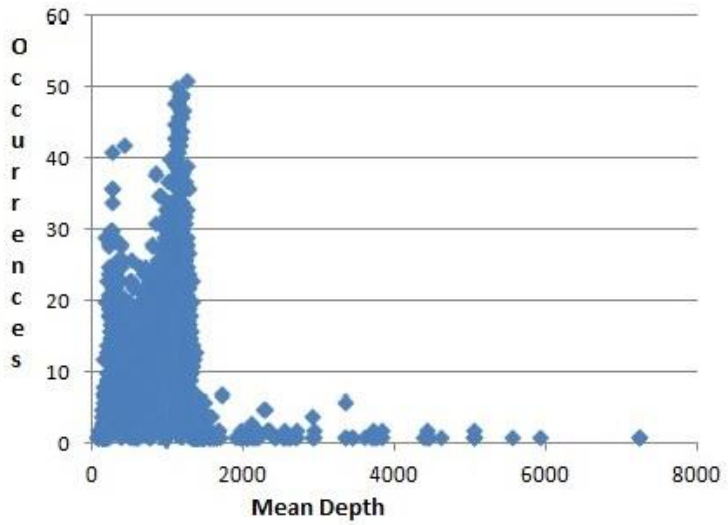

Fig. 3.4: Plot of the mean depth across loci.

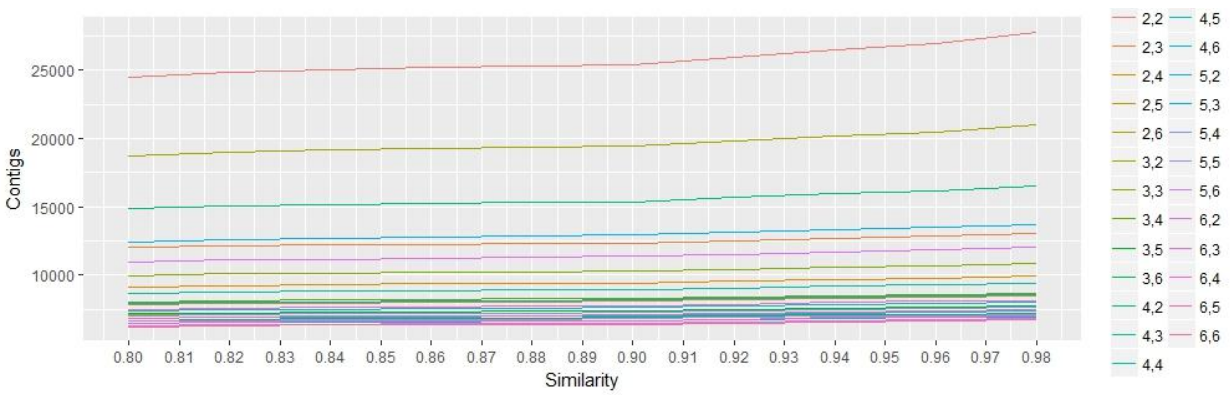

Fig. 3.5: Best cluster similarity value, C, identified using the diagnostic script ReferenceOpt.sh and different combination of K1 and K2. The best threshold is identified at the point of inflection on the curve.

Then, testing again different K1 and K2 combinations, the diagnostic script RefMapOpt.sh, identified the best value for the number of unique sequences with more than X coverage (counted within individuals) and the best value for the number of number of unique sequences present in more than X individuals as K1=3 and K2=6, respectively. These K1-K2 cut-off values maximized the number of properly paired reads mapped and their coverage while minimizing the number of mismatched reads (improperly mapped) (**Fig. 3.6**).

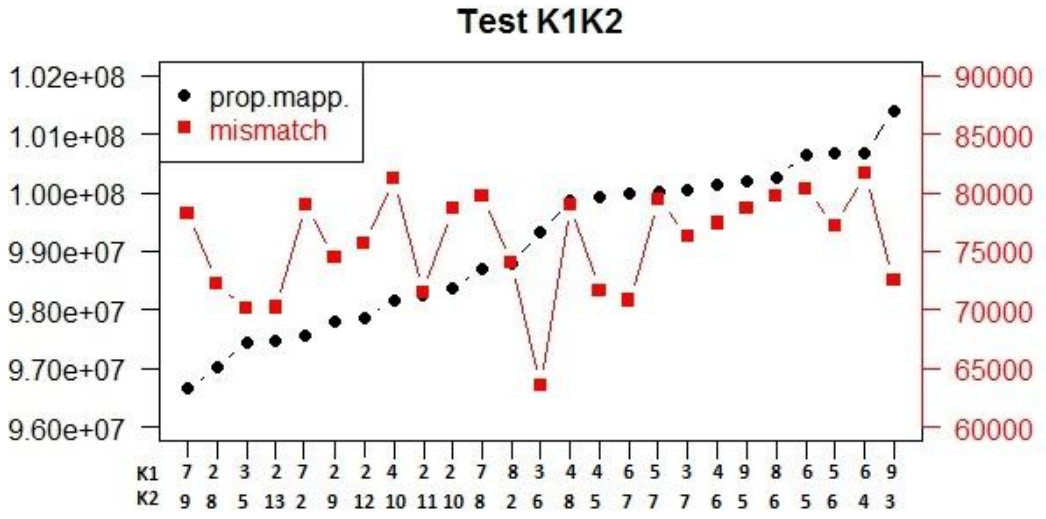

Fig. 4.2: Best K1K2 combination identified using the diagnostic script RefMapOpt.sh

These diagnostic scripts were extremely useful in the optimization of the parameters to be used in the construction of the reference library on which mapping the paired reads and perform the SNPs calling. Since there is no reference genome for the BS, this reference library can be useful to map reads from RAD techniques carried out on Mediterranean and North Eastern Atlantic BS, having this reference library, covered in a representative way, the genetic variability present in the target areas.

The total raw dataset, derived from the reads mapping and SNPs calling steps, consisted of 56,004 loci. After removal of genotypes with < 5 reads and of variants with MAF < 1% and that are not called in at least 50% of individuals with the custom bash script filter_missing_ind.sh, 27,863 out of 56,004 sites were retained. The same filtering applied at the population level retained 23,638 candidate loci out of 27,863. Further filter for MAF = 0.01 and any sites with less than 90% overall call rate, as suggested by the pipeline author (Puritz, et al. 2016), filtered out 978 sites, leaving 22,660 candidate loci. The dDocent_filters bash script, used to filters the loci on the base if their

paired status, allele balance and mean depth (resulted 1300) removed 5,578 sites, retaining 17,082 candidate loci.

The filtering for indels and loci not on HWE and subsequent selection of only bi-allelic loci, retained 16,775 loci. Finally, the construction and filtering of haplotypes from genotypes using the perl script rad_haplotyper.pl removed 13,324 loci, creating a final dataset of 3,451 loci.

The analysis of the relatedness among individuals revealed that the replicated individuals genotypes are identical, demonstrating the reliability and reproducibility of the method.

After retrieved list of contig with selected (retained) SNP from the final variant calling file (vcf), and after the elimination of duplicates contigs from the list (more contig with the same name, because they have more SNP), an approx number of 1000 contigs were retained and splitted in forward and reverse sequences. Once mapped these sequences on a BS mitogenome, using BLAST with relaxed arameters,none of these sequences matched with the mtDNA, suggesting a complete absence of mtDNA contamination. Furthermore, using the entire number of contigs (6432), the blast matched with a portion of the mitochondrial gene COI. The *in silico* analysis identified 1 SbfI recognition site and 4 SphI recognition sites: one of the latter is 258 bp far from the SbfI site and then, combined with adapters. It was selected on gel (size 300-550), sequenced and identified as contig 6432.

3.4 Conclusions

In conclusion, the dDocent pipeline, and all its implemented programs, was successfully used for assembling a reference library and mapping paired reads from ddRAD libraries of a large genome species such as it is the blue shark.

The data reduction approach used by dDocent, in combination with the optimal use of the paired-end data and the fact that the pipeline was ideated specifically for marine organisms (Purtiz et al., 2014b), allows the study of non-model species, with a high intrinsic complexity at the genome level, such as numerous repetitive regions and paralogs.

Of extreme importance for a correct use of the pipeline, minimizing potential bias due to a biased representativity of the assembly on which the reads will be mapped, is the selection of specimens reads that are representative of all the possible genetic variation observed in the study case.

The choice of a ddRAD approach, combined with the use of a bioinformatic pipeline specifically designed for the analysis of paired-end data, was a correct choice for the study case "blueshark",

allowing the calibration of the optimal parameters to use in the assembly and consequent SNPs calling.

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Tab. S 3.1: List of the library samples sequenced, barcodes, total number of reads, no° of tags and number of retained reads after demultiplexing with process_radtags program

Filename	P1 (SbfI) barcode	P2 (SphI) barcode	Total	No RadTag	Retained
EATL_001	TCAGA	GTCAAGT	12802440	893177	11909263
EATL_002	GATCG	GTCAAGT	4622146	110482	4511664
EATL_003	CATGA	GTCAAGT	2393206	220921	2172285
EATL_004	ATCGA	GTCAAGT	725800	37746	688054
EATL_005	TCGAG	GTCAAGT	2539692	71867	2467825
EMED_001	GTCAC	GTCAAGT	3668678	272490	3396188
EMED_002	GCATT	GTCAAGT	4357448	214988	4142460
EMED_003	CGATA	GTCAAGT	4543580	242340	4301240
WMED_001	TGCAACA	GTCAAGT	14668972	551241	14117731
WMED_002	CGTATCA	GTCAAGT	14739996	908213	13831783
WMED_003	CACAGAC	GTCAAGT	9463064	254926	9208138
EATL_006	ACTGCAC	GTCAAGT	2128576	21981	2106595
EATL_007	TCTCTCA	GTCAAGT	11031258	318374	10712884
EATL_008	GTACACA	GTCAAGT	8855758	482179	8373579
EATL_009	CTCTTCA	GTCAAGT	9509902	414534	9095368
EATL_010	CTAGGAC	GTCAAGT	9444760	158298	9286462
EATL_011	ACGTA	GTCAAGT	16030844	178367	15852477
EATL_012	AGAGT	GTCAAGT	5418162	429342	4988820
EATL_013	ATGCT	GTCAAGT	10486756	214997	10271759
EATL_014	GACTA	GTCAAGT	2697956	52324	2645632
EATL_015	CAGTCAC	GTCAAGT	6342318	116102	6226216
EATL_016	GCTAACA	GTCAAGT	2190134	75946	2114188
EATL_017	ACACGAG	GTCAAGT	3413168	65051	3348117
EATL_018	AGGACAC	GTCAAGT	2404150	30307	2373843
EATL_019	TCAGA	GAAGC	13033328	491489	12541839
EATL_020	GATCG	GAAGC	11303212	189781	11113431
EATL_021	CATGA	GAAGC	11714962	406862	11308100
EATL_022	ATCGA	GAAGC	2311082	99215	2211867
EATL_023	TCGAG	GAAGC	19816782	342944	19473838
EATL_024	GTCAC	GAAGC	11182702	316236	10866466
EATL_025	GCATT	GAAGC	8150616	315921	7834695
EATL_026	CGATA	GAAGC	7074316	250348	6823968
EATL_027	TGCAACA	GAAGC	21234088	448072	20786016
EATL_028	CGTATCA	GAAGC	12416066	307651	12108415
EMED_004	CACAGAC	GAAGC	5592196	549719	5042477
EMED_005	ACTGCAC	GAAGC	8629172	134961	8494211
EMED_006	TCTCTCA	GAAGC	19017238	543954	18473284

EMED_007	GTACACA	GAAGC	7066796	261639	6805157
EMED_008	CTCTTCA	GAAGC	10623714	408663	10215051
EMED_009	CTAGGAC	GAAGC	10479344	235926	10243418
EMED_010	ACGTA	GAAGC	9155988	202943	8953045
EMED_011	AGAGT	GAAGC	5192834	574955	4617879
EMED_012	ATGCT	GAAGC	10235598	243490	9992108
EMED_013	GACTA	GAAGC	13848952	188249	13660703
EMED_014	CAGTCAC	GAAGC	4640092	226791	4413301
EMED_015	GCTAACA	GAAGC	1088010	33077	1054933
EMED_016	ACACGAG	GAAGC	943690	19830	923860
EMED_017	AGGACAC	GAAGC	3604630	332706	3271924
EMED_018	TCAGA	ATACGGT	6328012	458361	5869651
EMED_019	GATCG	ATACGGT	3979182	114736	3864446
EMED_020	CATGA	ATACGGT	7907012	467724	7439288
EMED_021	ATCGA	ATACGGT	1827096	90174	1736922
EMED_022	TCGAG	ATACGGT	3839532	163475	3676057
EMED_023	GTCAC	ATACGGT	8249942	285716	7964226
EMED_024	GCATT	ATACGGT	4530784	204812	4325972
EMED_025	CGATA	ATACGGT	7274874	149823	7125051
EMED_026	TGCAACA	ATACGGT	7332068	244082	7087986
EMED_027	CGTATCA	ATACGGT	11126516	613441	10513075
EMED_028	CACAGAC	ATACGGT	8630320	135168	8495152
EMED_029	ACTGCAC	ATACGGT	10428412	93498	10334914
WMED_004	TCTCTCA	ATACGGT	1563932	207682	1356250
WMED_005	GTACACA	ATACGGT	5595986	438651	5157335
WMED_006	CTCTTCA	ATACGGT	3241734	152034	3089700
WMED_007	CTAGGAC	ATACGGT	10076964	197986	9878978
WMED_008	ACGTA	ATACGGT	10727022	221507	10505515
WMED_009	AGAGT	ATACGGT	8778192	427478	8350714
WMED_010	ATGCT	ATACGGT	5078358	172373	4905985
WMED_011	GACTA	ATACGGT	10606506	140963	10465543
WMED_012	CAGTCAC	ATACGGT	6007130	68815	5938315
WMED_013	GCTAACA	ATACGGT	5153088	73132	5079956
WMED_014	ACACGAG	ATACGGT	1242774	15327	1227447
WMED_015	AGGACAC	ATACGGT	13710186	122650	13587536
EATL_015	CATGA	ATACGGT	10320360	306098	10014262
EMED_024	ATCGA	ATACGGT	11147812	179485	43228625
EATL_029	TCAGA	GTCAAGT	18040044	1064902	10014262
EATL_030	GATCG	GTCAAGT	14806070	150475	14655595
EATL_031	CATGA	GTCAAGT	16222702	522474	15700228
EATL_032	ATCGA	GTCAAGT	12203648	217276	11986372
EATL_033	TCGAG	GTCAAGT	43840440	611815	43228625
EMED_030	GTCAC	GTCAAGT	2966044	297193	2668851
EMED_031	GCATT	GTCAAGT	15141986	428784	14713202
EMED_032	CGATA	GTCAAGT	17059120	515941	16543179

EMED_033	TGCAACA	GTCAAGT	17366120	448851	16917269
EMED_034	CGTATCA	GTCAAGT	16058070	640371	15417699
EMED_035	CACAGAC	GTCAAGT	14711946	236536	14475410
EMED_036	ACTGCAC	GTCAAGT	9815670	229787	9585883
EMED_037	TCTCTCA	GTCAAGT	18405568	401180	18004388
EMED_038	GTACACA	GTCAAGT	14527538	474046	14053492
EMED_039	CTCTTCA	GTCAAGT	19366612	418988	18947624
EMED_040	CTAGGAC	GTCAAGT	6867102	80379	6786723
EMED_041	ACGTA	GTCAAGT	14373630	159111	14214519
NATL_001	CTAGGAC	ATACGGT	2358590	31137	2327453
NATL_002	AGGACAC	ATACGGT	1736884	13334	1723550
NATL_003	CTCTTCA	ATACGGT	4014966	134219	3880747
NATL_004	ACACGAG	ATACGGT	3986094	25220	3960874
NATL_005	GTACACA	ATACGGT	1873932	295897	1578035
NATL_006	GCTAACA	ATACGGT	4078606	64381	4014225
NATL_007	TCTCTCA	ATACGGT	3989056	203981	3785075
NATL_008	CAGTCAC	ATACGGT	3649176	23981	3625195
NATL_009	ACTGCAC	ATACGGT	1327606	15913	1311693
NATL_010	GACTA	ATACGGT	2183182	22383	2160799
NATL_011	CGATA	ATACGGT	1081980	30453	1051527
NATL_012	CACAGAC	ATACGGT	1748752	21445	1727307
NATL_013	ATGCT	ATACGGT	1702788	132842	1569946
NATL_014	GCATT	ATACGGT	7064106	106003	6958103
NATL_015	CGTATCA	ATACGGT	927472	192122	735350
NATL_016	AGAGT	ATACGGT	2185030	59656	2125374
NATL_017	GTCAC	ATACGGT	2938868	53010	2885858
NATL_018	TGCAACA	ATACGGT	3344036	184662	3159374
NATL_019	ACGTA	ATACGGT	1922158	23014	1899144
NATL_020	TCGAG	ATACGGT	1787316	100226	1687090
WMED_016	AGAGT	GTCAAGT	12724046	971898	11752148
WMED_017	ATGCT	GTCAAGT	5117588	380673	4736915
WMED_018	GACTA	GTCAAGT	4222474	119101	4103373
WMED_019	CAGTCAC	GTCAAGT	17686236	372454	17313782
WMED_020	GCTAACA	GTCAAGT	14903892	240275	14663617
WMED_021	ACACGAG	GTCAAGT	8684364	173984	8510380
WMED_022	AGGACAC	GTCAAGT	5463126	111994	5351132
WMED_023	TCAGA	GAAGC	4347722	240296	4107426
WMED_024	GATCG	GAAGC	6255606	100380	6155226
WMED_025	CATGA	GAAGC	7182242	276225	6906017
WMED_026	ATCGA	GAAGC	7380626	141617	7239009
WMED_027	TCGAG	GAAGC	6527742	164179	6363563
WMED_028	GTCAC	GAAGC	9560986	253872	9307114
WMED_029	GCATT	GAAGC	10417620	354672	10062948
WMED_030	CGATA	GAAGC	8512070	507243	8004827
WMED_031	TGCAACA	GAAGC	7151562	106514	7045048

WMED_032	CGTATCA	GAAGC	23068244	276587	22791657
WMED_033	CACAGAC	GAAGC	6127192	320590	5806602
WMED_034	ACTGCAC	GAAGC	17166002	155809	17010193
WMED_035	TCTCTCA	GAAGC	8855724	129918	8725806
WMED_036	GTACACA	GAAGC	9132840	196674	8936166
WMED_037	CTCTTCA	GAAGC	17203478	677827	16525651
WMED_038	CTAGGAC	GAAGC	6532312	179321	6352991
WMED_039	ACGTA	GAAGC	14915564	192211	14723353
WMED_040	AGAGT	GAAGC	13679788	289618	13390170
WMED_041	ATGCT	GAAGC	10793152	289784	10503368
WMED_042	GACTA	GAAGC	7641882	125036	7516846
WMED_043	CAGTCAC	GAAGC	8466804	292044	8174760
WMED_044	GCTAACA	GAAGC	16630898	149632	16481266
WMED_045	ACACGAG	GAAGC	10384296	376660	10007636
WMED_046	AGGACAC	GAAGC	4416036	191186	4224850
WMED_047	TCAGA	ATACGGT	3600968	136408	3464560
WMED_048	GATCG	ATACGGT	3634402	100798	3533604
EMED_042	CTAGGAC	ATACGGT	111886	9833	102053
EMED_043	ACGTA	ATACGGT	1466754	106964	1359790
EMED_044	AGAGT	ATACGGT	3516712	537028	2979684
EMED_045	ATGCT	ATACGGT	3003628	181359	2822269
EMED_046	GACTA	ATACGGT	2332022	104320	2227702
EMED_047	CAGTCAC	ATACGGT	79738	9633	70105
EMED_048	GCTAACA	ATACGGT	419658	48699	370959
EMED_049	ACACGAG	ATACGGT	269150	21277	247873
EMED_050	AGGACAC	ATACGGT	42640	6651	35989
EMED_051	TCAGA	GTCAAGT	15061136	450778	14610358
EMED_052	GATCG	GTCAAGT	9153014	74003	9079011
EMED_053	CATGA	GTCAAGT	1602362	181113	1421249
EMED_054	ATCGA	GTCAAGT	11078656	353040	10725616
EMED_055	TCGAG	GTCAAGT	7082424	218361	6864063
EMED_056	GTCAC	GTCAAGT	22659804	288559	22371245
EMED_057	GCATT	GTCAAGT	7315814	464644	6851170
EMED_058	CGATA	GTCAAGT	3834214	198583	3635631
EMED_059	TGCAACA	GTCAAGT	13556652	174308	13382344
EMED_060	CGTATCA	GTCAAGT	11731042	132453	11598589
NATL_021	ATCGA	ATACGGT	1362266	31341	1330925
NATL_022	CATGA	ATACGGT	4176366	134678	4041688
NATL_023	GATCG	ATACGGT	2360208	23103	2337105
NATL_024	AGGACAC	GAAGC	5085546	71610	5013936
NATL_025	TCAGA	ATACGGT	1743098	293999	1449099
NATL_026	ACACGAG	GAAGC	7356326	35372	7320954
NATL_027	GCTAACA	GAAGC	6316368	34420	6281948
NATL_028	CAGTCAC	GAAGC	4124574	43066	4081508
NATL_029	GACTA	GAAGC	4633252	70241	4563011

NATL_030	ATGCT	GAAGC	19593546	422155	19171391
WMED_049	TCGAG	ATACGGT	21833512	260713	21572799
WMED_050	GCATT	ATACGGT	33063712	426352	32637360
WMED_051	CGATA	ATACGGT	1720780	103021	1617759
WMED_052	CGTATCA	ATACGGT	10783986	160051	10623935
WMED_053	TCTCTCA	ATACGGT	1990976	49966	1941010
WMED_054	GTACACA	ATACGGT	3257020	130995	3126025
WMED_055	CACAGAC	GTCAAGT	2725034	208716	2516318
WMED_056	ACTGCAC	GTCAAGT	8112382	273666	7838716
WMED_057	TCTCTCA	GTCAAGT	7288836	142128	7146708
WMED_058	GTACACA	GTCAAGT	7428400	207330	7221070
WMED_059	CTCTTCA	GTCAAGT	15170602	530337	14640265
WMED_060	CTAGGAC	GTCAAGT	15448908	627491	14821417
WMED_061	ACGTA	GTCAAGT	6331500	327229	6004271
WMED_062	AGAGT	GTCAAGT	2327022	285115	2041907
WMED_063	ATGCT	GTCAAGT	23566110	456493	23109617
WMED_064	GACTA	GTCAAGT	5349148	60205	5288943
WMED_065	CAGTCAC	GTCAAGT	2319856	62658	2257198
WMED_066	GCTAACA	GTCAAGT	5457290	76954	5380336
WMED_067	ACACGAG	GTCAAGT	3889930	44860	3845070
WMED_068	GTCAC	ATACGGT	4513772	312404	4201368
WMED_069	TGCAACA	ATACGGT	2769304	50037	2719267
WMED_070	CACAGAC	ATACGGT	18630162	308226	18321936
WMED_071	ACTGCAC	ATACGGT	2974760	49172	2925588
WMED_072	CTCTTCA	ATACGGT	11121528	178547	10942981
WMED_073	AGGACAC	GTCAAGT	26154	4971	21183
WMED_074	TCAGA	GAAGC	7122358	258705	6863653
WMED_075	GATCG	GAAGC	10296280	115902	10180378
WMED_076	CATGA	GAAGC	5268384	155985	5112399
WMED_077	ATCGA	GAAGC	66933466	444516	66488950
WMED_078	TCGAG	GAAGC	12097518	529420	11568098
WMED_079	GTCAC	GAAGC	47718536	475845	47242691
WMED_080	GCATT	GAAGC	5566136	720674	4845462
WMED_081	CGATA	GAAGC	49340414	352796	48987618
WMED_082	TGCAACA	GAAGC	3985712	54197	3931515
WMED_083	CGTATCA	GAAGC	3009266	88735	2920531
WMED_084	CACAGAC	GAAGC	1910664	101870	1808794
WMED_085	ACTGCAC	GAAGC	4225400	51060	4174340
WMED_086	TCTCTCA	GAAGC	2664958	42252	2622706
WMED_087	GTACACA	GAAGC	6353172	125386	6227786
WMED_088	CTCTTCA	GAAGC	4036822	63445	3973377
WMED_089	CTAGGAC	GAAGC	3408382	53771	3354611
EATL_015	ACGTA	GAAGC	3399854	82558	3317296
EMED_024	AGAGT	GAAGC	9912358	297353	9615005

Tab. S 3.2: List of samples selected as representatives of the total variation in the dataset. AREAS: NNEATL: North North Eastern Atlantic; SNEATL: South North Eastern Atlantic; WMED: Western Mediterranean; EMED: Eastern Mediterranean.

MEDBLUESGEN CODE	Filename	AREA	Reads
BSH_EATL_IEO_2014_J_024	EATL_002	SNEATL	2202294
BSH_EATLA_IEO_15_J_057	EATL_012	SNEATL	2287612
BSH_EATLA_IEO_15_J_063	EATL_015	SNEATL	3056520
BSH_EATLA_IEO_15_L_053	EATL_025	SNEATL	3764378
BSH_EATLA_IEO_15_L_054	EATL_026	SNEATL	3290910
BSH_EATLA_IEO_14_J_023	EATL_010	SNEATL	4566158
BSH_EMED_NKUA_2003_J_038	EMED_030	AEGE	1189129
BSH_EMED_NKUA_2004_L_011	EMED_045	AEGE	1324312
BSH_EMED_NKUA_15_J_001	EMED_004	AEGE	2260818
BSH_EMED_NKUA_15_J_002	EMED_005	AEGE	4181482
BSH_EMED_NKUA_15_J_014	EMED_007	AEGE	3279191
BSH_EMED_NKUA_15_L_007	EMED_010	AEGE	4377882
BSH_EMED_NKUA_2015_L_009	EMED_052	AEGE	4504215
BSH_EMED_NKUA_15_L_010	EMED_012	AEGE	4882867
BSH_EMED_UNIBO_15_J_005	EMED_018	CADR	2739238
BSH_EMED_UNIBO_15_J_010	EMED_023	CADR	3844219
BSH_EMED_UNIBO_15_J_012	EMED_025	CADR	3489608
BSH_EMED_UNIBO_15_J_013	EMED_026	CADR	3425321
BSH_EMED_UNIBO_2015_J_011	EMED_099	CADR	4679476
BSH_EMED_UNIBO_15_J_007	EMED_020	CADR	3507540
BSH_EMED_UNICAL_2014_J_022	EMED_002	IONI	1966252
BSH_EMED_UNICAL_2014_J_023	EMED_036	IONI	4680418
BSH_EMED_UNICAL_2014_L_025	EMED_003	IONI	2034179
BSH_EMED_UNICAL_2015_J_014	EMED_040	IONI	3355180
BSH_EMED_UNICAL_2015_J_018	EMED_058	IONI	1721091
BSH_EMED_UNICAL_2015_J_015	EMED_055	IONI	3326261
BSH_EATL_QUB_2007_L_003	NATL_003	NNEATL	1874876
BSH_EATL_QUB_2007_L_004	NATL_004	NNEATL	1969517
BSH_EATL_QUB_2007_L_022	NATL_022	NNEATL	1956903
BSH_EATL_QUB_2007_L_006	NATL_006	NNEATL	1975839
BSH_EATL_QUB_2007_L_028	NATL_028	NNEATL	2021651
BSH_EATL_QUB_2007_L_008	NATL_008	NNEATL	1801276
BSH_EATL_QUB_2007_L_029	NATL_029	NNEATL	2259362
BSH_WMED_IEO_2014_J_029	WMED_022	BALE	2621086
BSH_WMED_IEO_2014_J_040	WMED_026	BALE	3557127
BSH_WMED_IEO_2014_L_009	WMED_058	BALE	3509306
BSH_WMED_IEO_2014_L_035	WMED_064	BALE	2615914
BSH_WMED_IEO_2014_L_037	WMED_066	BALE	2652290

BSH_WMED_IEO_2014_J_039	WMED_025	BALE	3332249
BSH_WMED_UNICAL_15_J_002	WMED_005	TYRR	2366209
BSH_WMED_UNICAL_15_J_005	WMED_007	TYRR	4843687
BSH_WMED_UNICAL_2015_J_003	WMED_030	TYRR	3762281
BSH_WMED_UNICAL_2015_J_006	WMED_031	TYRR	3472639
BSH_WMED_UNICAL_2015_J_009	WMED_074	TYRR	3321733
BSH_WMED_UNICAL_2015_J_010	WMED_075	TYRR	5041349
BSH_WMED_UNIGE_15_J_005	WMED_013	LIGU	2504439
BSH_WMED_UNIGE_2008_J_047	WMED_033	LIGU	2750852
BSH_WMED_UNIGE_2012_L_026	WMED_076	LIGU	2502172
BSH_WMED_UNIGE_2014_J_019	WMED_080	LIGU	2081629
BSH_WMED_UNIGE_2016_J_060	WMED_085	LIGU	2063341
BSH_WMED_UNIGE_2014_J_015	WMED_038	LIGU	3090239
BSH_WMED_UNIGE_15_J_001	WMED_012	LIGU	2935667

Supplementary Text 3.1: SNPs filtering codes (in bold) and outputs

1) vcftools --vcf TotalRawSNPs.vcf --recode-INFO-all --minDP 5 --out BSdp5 –recode

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf TotalRawSNPs.vcf
--recode-INFO-all
--minDP 5
--out BSdp5
--recode

After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 56004 out of a possible 56004 Sites
Run Time = 49.00 seconds

2) vcftools --vcf BSdp5.recode.vcf --recode-INFO-all --maf 0.01 -- max-missing 0.5 --out BSdp5g5 –recode

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009
Parameters as interpreted:
--vcf BSdp5.recode.vcf
--recode-INFO-all
--maf 0.01
--max-missing 0.5
--out BSdp5g5
--recode
After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 27863 out of a possible 56004 Sites
Run Time = 26.00 seconds

- Now use a custom script called filter_missing_ind.sh to filter out bad individuals (there were a lot in this data set).

3) bash filter_missing_ind.sh BSdp5g5.recode.vcf BSdp5MI

VCFtools - 0.1.14

(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5g5.recode.vcf
--exclude lowDP.indv
--recode-INFO-all
--out BSdp5MI
--recode

Excluding individuals in 'exclude' list
After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 27863 out of a possible 27863 Sites
Run Time = 23.00 seconds

- Now use a second custom script pop_missing_filter.sh to filter loci that have high missing data values in a single population. You will need a file that maps individuals to populations popmap

4) ./pop_missing_filter.sh BSdp5MI.recode.vcf popmap 0.25 0 BSdp5MIp25

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MI.recode.vcf
--keep keep.EATL
--out EATL
--missing-site

Keeping individuals in 'keep' list
After filtering, kept 35 out of 207 Individuals
Outputting Site Missingness
After filtering, kept 27863 out of a possible 27863 Sites
Run Time = 1.00 seconds

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MI.recode.vcf
--keep keep.EMED
--out EMED
--missing-site

Keeping individuals in 'keep' list
After filtering, kept 57 out of 207 Individuals
Outputting Site Missingness
After filtering, kept 27863 out of a possible 27863 Sites
Run Time = 1.00 seconds

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MI.recode.vcf
--keep keep.NATL
--out NATL
--missing-site

Keeping individuals in 'keep' list
After filtering, kept 30 out of 207 Individuals
Outputting Site Missingness
After filtering, kept 27863 out of a possible 27863 Sites
Run Time = 1.00 seconds

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MI.recode.vcf
--keep keep.WMED
--out WMED
--missing-site

Keeping individuals in 'keep' list
After filtering, kept 88 out of 207 Individuals
Outputting Site Missingness
After filtering, kept 27863 out of a possible 27863 Sites
Run Time = 1.00 seconds

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MI.recode.vcf
--exclude-positions loci.to.remove
--recode-INFO-all
--out BSdp5MIp25
--recode

After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 23638 out of a possible 27863 Sites

- Next, filter sites again my MAF, and filter out any sites with less than 90% overall call rate

5) vcftools --vcf BSdp5MIp25.recode.vcf --recode-INFO-all --maf 0.01 --geno 0.9 --out BSdp5MIp25g9 –recode

6) vcftools --vcf BSdp5MIp25.recode.vcf --recode-INFO-all --maf 0.01 --max-missing 0.9 --out BSdp5MIp25g9 –recode

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MIp25.recode.vcf
--recode-INFO-all
--maf 0.01
--max-missing 0.9
--out BSdp5MIp25g9
--recode

After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 22660 out of a possible 23638 Sites
Run Time = 19.00 seconds

- Next, use a third custom filter script dDocent_filters

7) ./dDocent_filters.sh BSdp5MIp25g9.recode.vcf BSdp5MIp25g9

Below is the included output:
Docen This script will automatically filter a FreeBayes generated VCF file using criteria related to site depth,
quality versus depth, strand representation, allelic balance at heterzygous individuals, and paired read representation.
The script assumes that loci and individuals with low call rates (or depth) have already been removed.
Contact Jon Puritz (jpuritz@gmail.com) for questions and see script comments for more details on particular filters
Number of sites filtered based on allele balance at heterozygous loci, locus quality, and mapping quality / Depth
2535 of 22660
Number of additional sites filtered based on overlapping forward and reverse reads
1855 of 20125
Is this from a mixture of SE and PE libraries? Enter yes or no.
no

Number of additional sites filtered based on properly paired status
211 of 18270
Number of sites filtered based on high depth and lower than 2*DEPTH quality score
976 of 18059

The 95% cutoff would be 1255
Would you like to use a different maximum mean depth cutoff than 1255, yes or no
no
Number of sites filtered based on maximum mean depth
975 of 18059
Total number of sites filtered
5578 of 22660
Remaining sites
17082
Filtered VCF file is called Output_prefix.FIL.recode.vcf
Filter stats stored in BSdp5MIp25g9.filterstats

- Now, we need to break complex mutational events (combinations of SNPs and INDELs) into sepearte SNP and INDEL calls, and then remove INDELs.

8) vcfallelicprimitives -k -g BSdp5MIp25g9.FIL.recode.vcf | sed 's:\.|\.:\.\/\.:g' > BSdp5MIp25g9.prim

9) vcftools --vcf BSdp5MIp25g9.prim --recode-INFO-all --recode --out SNP.BSdp5MIp25g9 --remove-indels

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf BSdp5MIp25g9.prim
--recode-INFO-all
--out SNP.BSdp5MIp25g9
--recode
--remove-indels

After filtering, kept 207 out of 207 Individuals
Outputting VCF file...
After filtering, kept 16964 out of a possible 18102 Sites
Run Time = 15.00 seconds

- Next, filter out loci that are out of HWE in more than half the populations, using filter_hwe_by_pop.pl written by Chris Hollenbeck

10) perl filter_hwe_by_pop.pl -v SNP.BSdp5MIp25g9.recode.vcf -p popmap -c 0.5 -o SNP.BSdp5MIp25g9HWE

Processing population: EATL (35 inds)
Processing population: EMED (57 inds)
Processing population: NATL (30 inds)
Processing population: WMED (88 inds)
Outputting results of HWE test for filtered loci to 'filtered.hwe'
Kept 16872 of a possible 16964 loci (filtered 92 loci)

- Restrict SNPs to loci only with 2 alleles.

11) vcftools --vcf SNP.BSdp5MIp25g9HWE.recode.vcf --recode-INFO-all --out SNP.BSdp5MIp25g9HWE2a --recode --max-alleles 2

VCFtools - 0.1.14
(C) Adam Auton and Anthony Marcketta 2009

Parameters as interpreted:
--vcf SNP.BSdp5MIp25g9HWE.recode.vcf
--recode-INFO-all
--max-alleles 2
--out SNP.BSdp5MIp25g9HWE2a
--recode

After filtering, kept 207 out of 207 Individuals

Outputting VCF file...
After filtering, kept 16775 out of a possible 16872 Sites

- Use rad_haplotyper written by Chris Hollenbeck

12) perl rad_haplotyper.pl -v SNP.BSdp5MIp25g9HWE2a.recode.vcf -p popmap -r reference.fasta -x 10 -mp 5

Filtered 1635 loci below missing data cutoff
Filtered 192 possible paralogs
Filtered 0 loci with low coverage or genotyping errors
Filtered 0 loci with an excess of haplotypes
This script uses called genotypes and aligned reads to make haplotype calls across RAD loci using both F and R reads.

- Move output and create a list of files that had high levels of missing data and potential paralogs

```
cp stats.out stats.out.HF
mawk '/Missi/' stats.out.HF | mawk '$9 > 30' > HF.missing
mawk '/para/' stats.out.HF > HF.para
cat HF.para HF.missing > HF.loci.tofilter
```

- Remove these loci entirely from the data set

13) ./remove.bad.hap.loci.sh HF.loci.tofilter SNP.BSdp5MIp25g9HWE.recode.vcf

To see how many loci were retained:

```
mawk '!/#/' SNP.BSdp5MIp25g9HWE.filtered.vcf | wc –l
3451
```

4 CHAPTER 4

Population genomics of Mediterranean and North Eastern Atlantic blue shark, *Prionace glauca*, and management implications

Publication note:

An improved version of this chapter is in submission to a journal with the potential title: First population genomic study on Mediterranean-North Eastern Atlantic blue shark revealed: implication for stock identification and conservation

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Abstract

Globally the abundance of blue sharks (*Prionace glauca*, L. 1758, BS) has dramatically declined over the last 30 years, which is why, in some areas, this species has been categorized as "Critically Endangered" by IUCN. Conservation and management of Mediterranean blue sharks (BS) suffers from a lack of knowledge about the population structure inhabiting the epipelagic ecosystem of the basin and of the adjacent North-eastern Atlantic. We filled in this knowledge gap by measuring genetic diversity and differentiation of BS population samples (N = 212) from Western and Eastern Mediterranean and adjacent Northern and Southern areas of the North-eastern Atlantic based on novel genomic resources developed throughout a RAD sequencing technology. The ddRAD allowed the development of 3,451 species-specific SNP loci of which 63 constitute candidate outliers. The SNP variation revealed a complex genetic structure of the Mediterranean BS with subtle but significant genetic differences between Western and Eastern Mediterranean BS as detected by the pairwise F_{ST}s and AMOVA. Bayesian and spatial clustering in contrast failed to reveal genetic structure indicating the occurrence of a near- or fully-panmictic population of BS in the North-eastern Atlantic and Mediterranean with high reproductive connectivity between the two areas. Such evidence corroborates the role of the Mediterranean as nursery area of the North-Atlantic BS population with small significant divergence of the peripheral local subpopulations. Therefore, the Mediterranean BS population can be considered an extension of the North-eastern Atlantic population, and this is in contrast with the current stock delineation and management. The developed SNPs can be exploited for the traceability of BS products globally, helping to tackle the major threat to BS populations, caused by the great portion of unrecorded BS by-catches, which enter the global fin trade each year (more than 10 millions of individuals).

4.1 Introduction

According to the International Union for Conservation of Nature (IUCN) the Mediterranean region has the highest percentage of threatened sharks and rays in the world: 42% of the species are threatened with extinction. Overfishing, including by-catch (non-target species caught incidentally), is the main cause of decline. Since sharks and rays are top predators, this decline can greatly affect the marine ecosystem including lower trophic levels (Myers et al., 2007). The general vulnerable state of cartilaginous fishes has also been acknowledged by the European Union and an

Action Plan for the Conservation and Management of Sharks has been implemented from several years by the European Community (European Commission, 2009). The EU Action Plan is based on the international Action plan for the Conservation and Management of Sharks (IPOA SHARKS) adopted by the FAO in 1999. The objective of the Action Plan is to guarantee the conservation and management of sharks by improving the knowledge of fisheries and shark species, as well as their role in the ecosystem and by ensuring a sustainable exploitation of shark stocks.

With regards to this generally observed trend, blue sharks (*Prionace glauca*, L. 1758; BS henceforth) are no exception in that their abundance has dramatically declined over the last century in the Mediterranean Sea with a peak in reduction of ~78–90% over the last 30 years (Ferretti et al., 2008). The BS is one of the most commonly found epipelagics shark occuring in all oceans from 60°N to 50°S. Rarely being targeted by commercial fishing, it is a major by-catch of longline and driftnet fisheries (Megalofonou et al., 2000), and considered one of the most overfished shark species (Fowler et al., 2005). This led worldwide to the categorization “Near Threatened” in the IUCN Red List (Stevens, 2009), while, given the high fishing pressure to which BS is subjected in and the huge loss of estimated abundance in last decades (Ferretti et al., 2008), Mediterranean BS is classified as "Critically Endangered"(Sims et al., 2016).

The conservation and management of BS require sound knowledge population structure, that can underpin effective policies. It is still unclear whether Atlantic and Mediterranean populations are reproductively interacting, and there are no statistically significant data from tagging and/or genetic study to support genetic connectivity. The population structure of Mediterranean BS remains unknown and since the Mediterranean Sea ecosystem is sensitive and responds quickly to environmental changes and disturbances, it is necessary to reveal the population structure of Mediterranean BS to predict if it may rely on external reinforcements from the BS inhabiting the adjacent North-eastern Atlantic Ocean.

Blue shark displays low fecundity, with maturity size at approximately 220 cm and a maximum Total Length (TL) of 380 cm. Being a K-selected species, BS shows low fertility (relatively low number of offspring) and long ontogenetic development cycles. BS is a viviparous shark, usually producing 15-30 litters with a gestation of 9-12 months (Pratt 1979). Males reach sexual maturity at about 4-6 years and females at 5-7 years. In the Mediterranean Sea, the BS length at 50% of maturity (L50) was estimated to be 202.9 cm TL for males and 214.7 cm TL for females (Megalofonou et al., 2009a). Based on the age–length relationship estimated for Mediterranean BS, the age of specimens range from 2 to over 20 years (101– 387 cm TL), while age at 50%

maturity was estimated at 4.9 years for males and 5.5 years for females (Megalofonou et al., 2009a). With an average age of about 20 years, the BS generation time is approximately 12 years (Serena, 2005). Inshore BS records (especially juveniles) are not rare in the Mediterranean during spring and summer, in agreement with birth period; on the contrary, large adults are closely related to pelagic environments. Due to these features, BS is highly sensitive to fishing activities. Juveniles are subject to inshore recreational fishing and small-scale commercial fishing, especially in summer, while adults caught mostly by commercial pelagic fisheries often as by-catch in tuna and swordfish long-line fisheries (Megalofonou et al., 2005; Storai et al., 2011).

Most of the BS caught in the Mediterranean (99% and 98% for males and females, respectively) were immature, indicating that the Mediterranean BS stock consists primarily of small immature BS of both sexes, with a sex-ratio skewed toward females (Kohler et al., 2002). A high number of pregnant females was observed in the Adriatic and North Ionian areas, suggesting they serve as BS nurseries (Megalofonou et al., 2009a). In contrast, the adjacent south-eastern North Atlantic BS was primarily composed of mature individuals of both sexes with male-based sex ratio.

In the Atlantic, BS is distributed from Canada to Argentina in the Western side and from Norway to South Africa in the eastern side, including the Mediterranean Sea (Compagno, 1984). The population structure and dynamics of Atlantic BS is still poorly known despite several long-term tagging studies, which revealed extensive movements of BS tagged in the western side of the North Atlantic (NA) with well documented eastward trans-Atlantic migrations (Kohler et al., 1998, 2002; Kohler and Turner, 2008; Vandeperre et al., 2014). Sexual segregation was also evident, with a concentration of mature females in more temperate waters of the Northern NA, and immature males predominant in the Southern NA (Sampaio da Costa, 2013). Mature BS of both sexes seemed to be distributed in the southern NA, while immature individuals of both sexes and sub-adult females are usually distributed in the northern areas (Kohler et al., 2002). Conversely, a prevalent occurrence of immature juveniles is reported for the Mediterranean Sea (Kohler et al., 2002; Megalofonou et al., 2009a).

In the NA, juvenile females (TL < 180 cm) migrate from coastal to oceanic regions where they form mating aggregations with males (Simpfendorfer et al., 2002; Nakano and Stevens, 2008). After mating, females usually move out from the mating aggregations, but some of them may remain there until close to parturition, which occurs closer to the shore (Litvinov 2006; Tavares et al., 2012; Vögler et al., 2012).

Despite the poor recapture rate (from 91,450 BS specimens being tagged in the north western Atlantic, only 5.9% were recaptured), extensive tag-recapture surveys carried out from 1962 to 2000 suggest that NA BS form a single stock and that trans-Atlantic migratory movements are quite frequent, probably driven by the oceanic current system (Kohler et al., 2002). The reproductive migratory movements of NA BS into the Mediterranean and the degree of connectivity between the two populations are still unknown.

Release-recapture data of 16,804 BS from Irish recreational fishery in 1970-2006 showed that only one adult male of 813 recaptured BS crossed the Gibraltar Strait and none of the BS tagged in the other NA areas was recaptured in the Mediterranean (Kohler et al., 2002; Green et al., 2009). These observations support the hypothesis of very limited reproductive interactions between North Atlantic and Mediterranean BS (Kohler et al., 2002).

To date, no population genetic data are available for the Mediterranean BS. Population structure and dynamics of BS in the Mediterranean are presently inferred only by Atlantic-Mediterranean integrated tagging studies and the analysis of fisheries data (Kohler et al., 1998, 2002; Kohler and Turner 2008; Ferretti et al., 2008; Megalofonou et al., 2009b). The large majority of BS tagged in the Mediterranean Sea were immature and remained in the tagging area, without significant migration movements towards the adjacent southern areas of the North-east Atlantic (Kohler et al., 2002). The only exception was a subadult female that moved a short distance to the adjacent north-eastern Atlantic area.

Detailed analyses modelling catches rates showed that BS distribution in the Mediterranean Sea is significantly related to environmental cues, such as temperature, bottom topography and lunar cycle (Megalofonou et al., 2009b; Damalas and Megalofonou 2010). A strong longitudinal constituent in the presence of BS, the probability increasing from east to west, has been confirmed throughout the Mediterranean. Availability of food and increased productivity/abundance of living resources may be a key factor in these differences (Megalofonou et al., 2005).

Genetic studies have been carried out on the Atlantic and Pacific BS populations using microsatellites. Fitzpatrick et al., (2010) analysed approximately 1,000 individuals collected worldwide at 16 microsatellite loci revealing an interoceanic genetic structuring with gene-flow generally restricted within ocean. Within the Atlantic 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). 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 and supported philopatric behaviour of males to mating areas exclusively contributing to a single nursery ground (Veríssimo et al., 2013; Sampaio da Costa, 2013). On the contrary, a recent genetic survey (Veríssimo et al., 2017) carried out with the same type of markers on less mobile BS stages (i.e., young-of-year and <2 years juveniles) collected from the same nurseries showed the spatio-temporal lack of genetic differentiation, suggesting the presence of a panmictic population in the whole Atlantic.

This work aims to fill the prevailing knowledge gap on the BS Mediterranean population genetic structure by assessing genetic diversity and differentiation of BS population samples collected from Western and Eastern Mediterranean and adjacent Northern and Southern areas of the North-eastern Atlantic based on novel genomic resources developed with the RAD sequencing technology. In addition, since an estimated 10.7 million individuals are killed for the global fin trade each year (Clarke et al., 2006) and BS products are the most prevalent shark product on the market (Fields et al., 2017), the discriminatory capacity of the developed genomic resources will be discussed as an asset for shark product traceability supporting the fight against Illegal fishing, food substitution and mislabelling fraud.

4.2 Materials and methods

The sampling of 173 BS was carried out in the Mediterranean and adjacent North-eastern Atlantic from 2014 to 2016 from longline fishery (**Fig. 4.1**). In addition, 118 archived BS tissue specimens from previous collections and programs (2003-2013) were provided by P. Megalofonou, F. Garibaldi and P. Prodhol. A total of 291 individual BS from a total of 179 hauls were collected (**Fig. 4.1; Chapter1 for details**). Sampling data and locations of BS in the Mediterranean and adjacent North-Eastern Atlantic can be visualized through a GIS-interfaced database available at the MEDBLUESGEN project web site (https://fishreg.jrc.ec.europa.eu/web/medbluesgen/sampling-data).

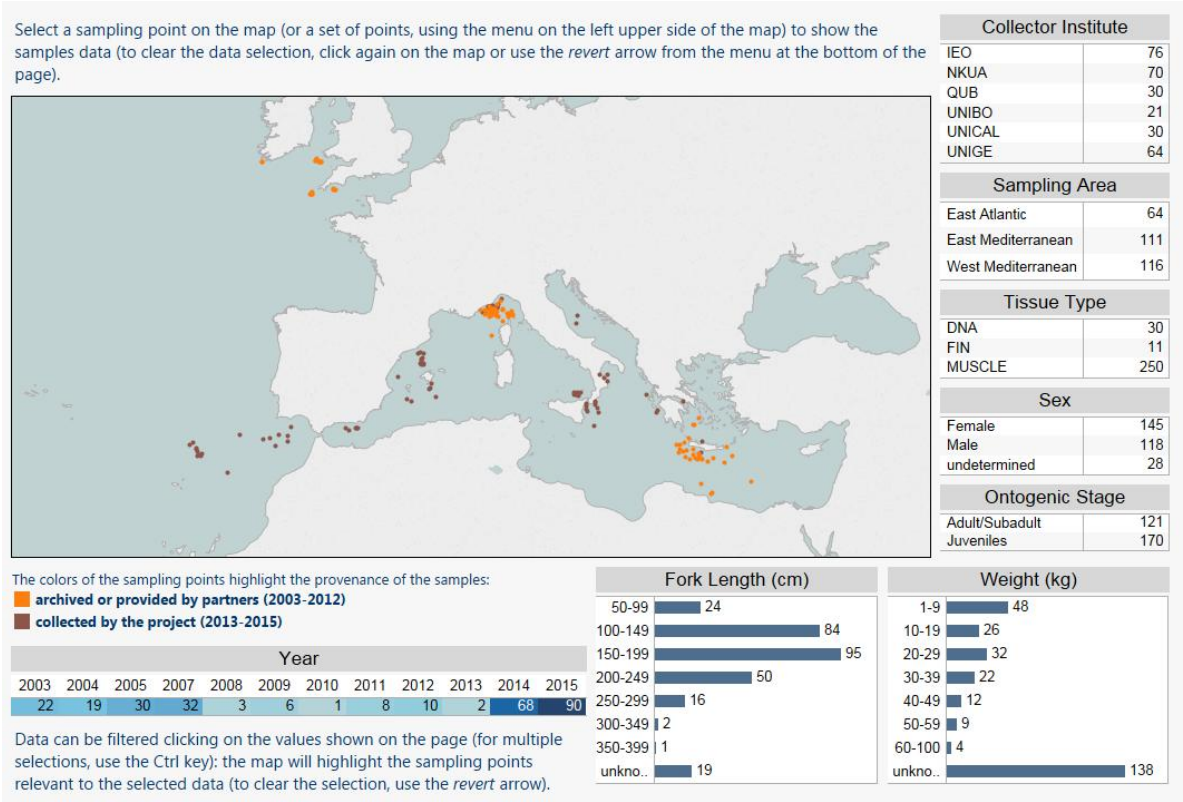

Fig. 4.1: Maps of sampling locations of Blue shark in the Mediterranean and North-eastern Atlantic

Muscle or skin tissue specimens (ca 0.1-0.2 g) were collected using cleaned, sterile scissors or tweezers and stored in 96% ethanol at -20 °C until use. Biological data as Fork Length (in cm) and sex (female/male) as well as fishery data as collecting date, geographical coordinates (longitude/latitude) and depth (in m) were collected whenever possible and are documented in the public MEDBLUESGEN sampling map.

Genomic DNA (gDNA) was extracted using the SSTNE buffer, a modified TNE buffer to which spermidine and spermine is added (Pardo et al., 2005). A modified double digest Restriction-site Associated DNA (dd-RAD) protocol was used to simultaneously discover and genotype individuals at thousands of SNPs, following the procedure first described by Peterson et al., (2012) with slight modifications (Brown et al., 2016). In brief, for each individual, 30 ng gDNA was digested with *Sbf*I and *Sph*I (0.43 U of each, New England Biolabs). P1 and P2 barcoded adapters, compatible with the *Sbf*I and *Sph*I overhang respectively, were mixed with T4 *ligase*. After enzyme heat inactivation, individual samples were pooled and cleaned up with MinElute PCR Clean Up Kit (Qiagen, Hilden, Germany). The library was run on an agarose gel (1.1%), to size select fragments of 200-300 bp. Size-selected DNA was eluted from the gel. The eluted library was PCR amplified with generic P1 and P2 primers after having optimized the PCR conditions. The amplified library

was purified using AMPure XP Magnetic Beads (Beckman Coulter, Pasadena, California, USA). The same two individuals were replicated across each library to assess consistency of data and results. The obtained ddRAD libraries were paired-end sequenced by an external sequencing service provider (UC Davis) using a HiSeq 4000 150 bp PE method.

Individuals from the different geographical areas were distributed across three different libraries (**Tab. S 4.1**) to avoid a batch effect in processing of samples during library construction and sequencing that could introduce artefacts in the subsequent data analysis and biased inferred results.

Reads were demultiplexed and filtered with the program 'process_radtags version 1.42' from the Stacks package (http://creskolab.uoregon.edu/stacks/; Catchen et al., 2011, 2013). For each library, data has been separated according to inline barcodes and specifying the restriction site (*Sbf*I and *Sph*I, respectively for reads in Pool_R1_001.fasq.gz and in Pool_R2_001.fasq.gz). The dDocent pipeline (www.ddocent.com; Puritz et al., 2014a, b) was used for reference construction, mapping reads and SNP calling. dDocent has been successfully employed in several recent studies, analyzing elasmobranch species (Portnoy et al., 2015; Dimens, 2016; Manuzzi, 2016; Baker et al., 2017) and in other marine fish species which are generally characterized by high diversity and low differentiation (Puritz et al., 2016; Hollenbeck et al., 2017). Utilizing genotype data in the absence of a reference genome, however, presents a number of challenges. One major challenge is the trade-off between splitting alleles at a single locus into separate clusters (loci), creating inflated homozygosity, and lumping multiple loci into a single contig (locus), creating artefacts and inflated heterozygosity. This issue has been addressed primarily through the use of similarity cut-offs in sequence clustering. Most of the published studies, however, coupled the dDocent pipeline with customized script to mitigate the high levels of repeats and duplications expected in large genomes, as we expected also in BS. Recently the script and the approach that was used, was formalized and published (Willis et al., 2017) and we followed the recommended filtering to the dataset of candidate informative variants identified by dDocent.

Detailed assembly, SNPs calling and filtering steps are described in the **Chapter 3**. A catalogue of SNP loci with relative genotypes was produced at the end of the filtering procedure (see **Chapter 3**). From such a catalogue, genomic data was converted to the appropriate file format for subsequent genetic analysis with PDGSpider (Lischer and Excoffier, 2012).

Basic statistics of genetic diversity were computed using the software Arlequin (Excoffier and Lischer, 2010), GenAlEx (Peakall and Smouse, 2006, 2012) and GenePop web version (Rousset,

2008; Raymond and Rousset, 1995). Genetic differentiation and population structure were inferred using multiple approaches. Discriminant Analysis of Principal Components (DAPC), Principal Components Analysis (PCA) and assignment probability were performed using the R package Adegenet (Jombart, 2008; Jombart et al., 2010; Jombart and Ahmed, 2011; R version 3.1.2, R Development Core Team, 2014; http://www.r-project.org). Bayesian clustering was inferred using fastSTRUCTURE (Raj et al., 2014), a modified version of the software STRUCTURE to analyse large SNP datasets and a model developed by Hubisz et al., (2009). Partition of molecular variance and its significance was estimated with the AMOVA (Excoffier et al., 1992) implemented in Arlequin v.3.5.2.2 software (Excoffier and Lischer, 2010), testing four alternative groupings of geographical sampling locations (AMOVA1: no groups; AMOVA2: NNEATL+SNEATL vs WMED+EMED; AMOVA3: NNEATL vs SNEATL + WMED vs EMED; AMVOVA4: NNEATL vs SNEATL + WMED vs EMED). The AMOVA and pairwise F statistics were computed with Arlequin using 20,000 permutations and 0.01 significance threshold as settings.

4.3 Results

A total of 291 BS individuals were obtained from the Mediterranean (East Mediterranean, EMED: N = 111; West Mediterranean, WMED: N = 116) and adjacent North-eastern Atlantic areas from Gibraltar to Azores (Southern North-eastern Atlantic, SNEATL: N = 34) and from Southern Ireland and Great Britain (Northern North-eastern Atlantic, NNEATL: N = 30). Each individual BS was geo-referenced and geo-visualized with a GIS-interfaced database implemented by the JRC on the MEDBLUESGEN project web site. Among collected BS, the sex of 263 animals (118 males and 145 females) was determined, while that of 28 individuals remained unknown. Among the 263 individuals, females outnumbered males significantly in the North-eastern Atlantic population samples (sex-ratio = 0.26, χ^2-test: 10.256, p2tail = 0.001; p1tail = 0, d.f. 1) while in the two Mediterranean BS groups a weak and not significant predominance of males was observed (WMED: 1.08, χ^2-test: 0.786 p2tail = 0.375; p1tail = 0.188, d.f. 1; EMED: 1.07, χ^2test: 0.087; p2tail = 0.768; p1tail = 0.384, d.f. 1).

Overall, juveniles (TL < 180 cm) predominated over the large sub-adult and adult (TL > 180 cm) individuals (J: N = 170; L: N = 121). In the population samples collected from North-eastern Atlantic, large sub-adult and adult BS are more abundant than juveniles (J: N = 28; L: N = 36). In the

WMED, juveniles predominated over large sub-adult and adult BS (J: N = 86; L: N = 30), while in the EMED any predominance was observed (J: N = 56; L: N = 55) (**see Chapter 1 & 2 for details**).

More than 1800 million reads were obtained for 216 BS individuals (including 4 replicates). Sequencing failed for six samples, which were eliminated. The dDocent analysis on 210 samples identified 6432 unique contigs and 56,004 candidate variants. After processing and filtering (**Chapter 3**) the resulting dataset consisted of 3,451 SNPs and 203 BS individuals, distributed in four geographical areas and across different years (**Tab. S 4.2)**.

A significant deficit of heterozygous genotypes was observed both overall and in each population sample, with heterozygosity values similar among localities (**Tab. S 4.3**).

The overall genetic differentiation among the four geographical BS population samples estimated using the 3,451 SNP dataset was very low but significant (F_{ST} = 0.00284; p-value = 0.00055), an observation confirmed by pairwise F_{ST} values among BS of the four target areas. An exception to this observation arises from the comparison between NNEATL and WMED after Bonferroni correction for multiple tests (**Tab. S 4.4**), where the level of differentiation is significant, albeit very low, ranging from 0.2 to 0.5%. Multivariate PCA and DAPC analyses fails to hint at any structuring of samples (**Fig. S 4.1, S 4.2**). A Locus-by-Locus AMOVA was performed to assess the relative contribute of each locus to differentiation among samples. In this test, 283 SNPs showed a significant F_{ST} among the four BS population samples, with F_{ST} values ranging from 0% to 17%, two orders of magnitude higher than the overall value (**Fig. S 4.3)**. The great majority of loci had a negative F_{ST} value, as expected by the results of the previous analyses.

While the chooseK algorithm implemented in fastSTRUCTURE suggested K = 3 and K = 4 as the best number of clusters, the Bayesian clustering didn't find strong evidences of genetic clustering (**Fig. 4.2a**). The only apparent differentiation is that observed in the NNEATL group for the K=3 plot.

In order to identify loci with high F_{ST}, the whole dataset was screened for outliers with Lositan and BayeScan and only 1.8% of loci (63 out of the 3,451 SNPs) were identified as candidate outliers. Several of these 63 outlier loci exhibited significant and high single-locus F_{ST} values, which is why they were used to assess the genetic differentiation among BS population samples.

Using the 63 candidate outlier loci, the Bayesian clustering approach, implemented in fastSTRUCTURE, revealed a cluster pattern similar to that obtained using the 3,451 SNP dataset (**Fig. 4.2b**). Despite the chooseK program suggested K = 2, no strong evidence of geographical clustering was observed, except for the apparent differentiation of the NNEATL in the K=2 plot. Overall, also using this subset of selected outliers SNPs no strong evidences of clustering was

observed. While the overall genetic differentiation among the four population samples is low, it is significant and ten times higher than that obtained using the 3,451 SNP panel (F_{ST} = 0.03145; p-value < 0.0001). Also the pairwise F_{ST} values among BS population samples were higher and significant after Bonferroni correction for multiple tests (**Tab. 4.1**).

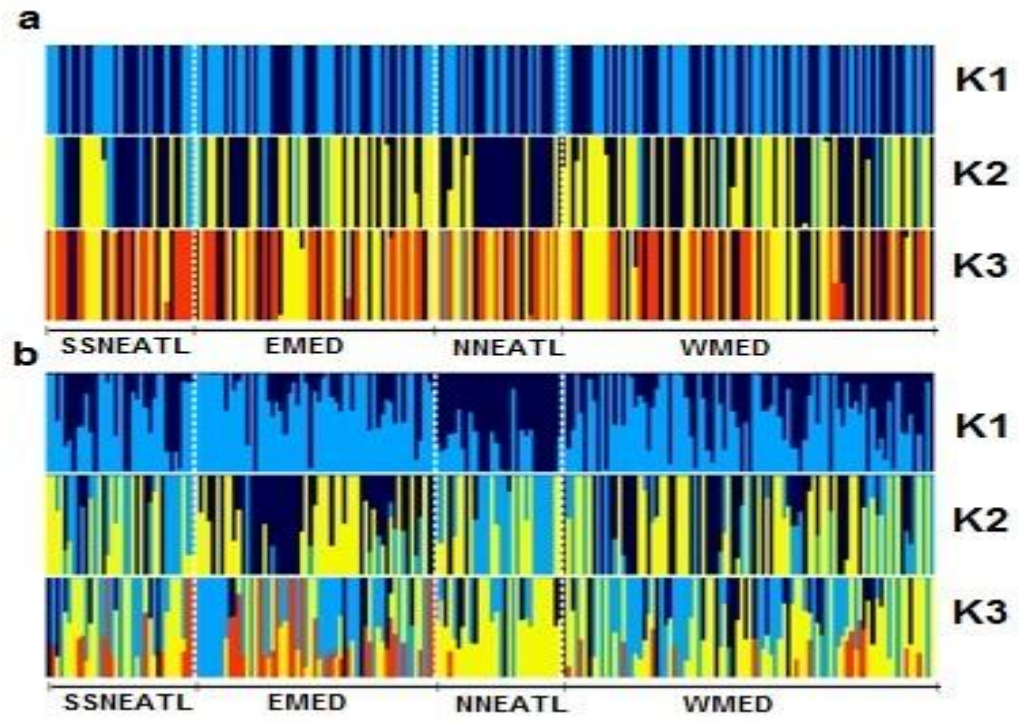

Fig. 4.2: Plots of the fastSTRUCTURE Bayesian clustering for K= 1-4 using the panel of 3,451 SNPs (a) and the panel of 63 outlier SNPs (b). In both clustering a logistic prior was used.

While any partitioning of molecular variance among groups of the BS population samples was significant in the AMOVA performed with 3,451 SNPs, the AMOVA performed with the 63 outlier loci (**Tab. 4.2**) revealed that the partitioning with the highest percentage of variation among groups was that grouping separately NNEATL from the SNEATL and the Mediterranean samples (4.73%), even if this variation was not significant (P = 0.12691). The only significant geographical structure detected was that imposed in the AMOVA4 with three groups formed by NNEATL, SNEATL+WMED and EMED (F_{CT} = 0.02425, p = 0.03516). The PCA analysis performed using the 63 outlier SNPs, appears to be more efficient in separating NNEATL apart from the other population samples, but it did not allow any apparent geographical clustering (**Fig. S4.4**). The majority of the specimens were caught in the time span 2013-2016, while few specimens (N = 5) from the AEGE are from 2003-2005, and few specimens from the LIGU site (N ~ 10) are from 2005-2009 (**Tab. S 4.2**). Unfortunately, our dataset is not homogeneous per years, season, size of the specimens. Given the different time ranges represented in our dataset and the fact that this time span is max

13 years, which is 1 generation (Generation time for North Atlantic BS stock = 9.8 years (Mejuto, J., & García-Cortés, B.2005). we avoided any temporal comparisons, due to not sufficient data that could lead to non-significant and biased results testing potential temporal variation.

On the contrary, the find.cluster and DAPC function results suggest the presence of four clusters, but they were formed by BS individual genotypes belonging to different population samples (**Fig. 4.3**).

Tab. 4.1: Pairwise F_{ST} values (below diagonal) and associated p-values (above diagonal) between BS population samples based on the 63 outlier SNPs. * indicates values significant after Bonferroni correction for multiple tests (p-value=0.008).

	NNEATL	SNEATL	WMED	EMED
NNEATL		<0.0001	<0.0001	<0.0001
SNEATL	0.09375*		<0.0001	<0.0001
WMED	0.04158*	0.04829*		<0.0001
EMED	0.08563*	0.03478*	0.01505*	

Tab. 4.2: Analysis of molecular variance (AMOVA) using the whole dataset (All loci; 3,541 SNPs) and the outlier dataset (Outliers; 63 SNPs) of the Mediterranean and North-eastern Atlantic Blue Sharks in four different arbitrary partitioning.

	All loci			Outliers		
	% variation	F-Statistics	p	% variation	F-Statistics	p
AMOVA1: Overall (all population samples)						
Among populations	0.28			3.14		
Within populations	99.72	ST = 0.00284	0.00055	96.86	ST = 0.03145	0.00000
AMOVA2: 2 groups: (NNEATL+SNEATL vs WMED+EMED)						
Among groups	0.03	CT = 0.00027	0.21396	1.18	CT = 0.01183	0.17735
Among pops within group	0.27	SC = 0.00270	0.00699	2.54	SC = 0.02573	0.00000
Within populations	99.70	ST = 0.00298	0.00070	96.27	ST = 0.03726	0.00000
AMOVA3: 2 groups: (NNEATL vs SNEATL +WMED + EMED)						
Among groups	0.08	CT = 0.00075	0.04320	4.73	CT = 0.04729	0.12691
Among pops within group	0.23	SC = 0.00235	0.03581	1.68	SC = 0.01765	0.00000
Within populations	99.69	ST = 0.00310	0.00095	93.59	ST = 0.06410	0.00000
AMOVA4: 3 groups: (NNEATL vs SNEATL + WMED vs EMED)						
Among groups	0.13	CT = 0.00133	0.24833	2.43	CT = 0.02425	0.03516
Among pops within group	0.25	SC = 0.00246	0.01748	1.53	SC = 0.01566	0.00035
Within populations	99.62	ST = 0.00379	0.00080	96.05	ST = 0.03953	0.00000

In fact, despite this evidence of discrete genetic clustering, the DAPC coordinates mapping each BS individual led to shown that these four clusters were composed by BS regardless their geographical origin (**Fig. S4.5**). Furthermore, if we use the DAPC to plot the assignment for each BS individuals to the 4 clusters inferred, this admixture pattern is evident (**Fig. 4.4**).

4.4 Discussion

This work has filled a prevailing gap on the knowledge about population genetic structure of the Mediterranean BS and of the genetic connectivity with BS inhabiting the adjacent North-eastern Atlantic Ocean. This has been achieved through 1) an extensive sampling effort across the whole Mediterranean and in the adjacent Southern and Northern areas of the North-eastern Atlantic areas that allowed for the creation of an unprecedented collection of Mediterranean BS samples (N = 173), to which N = 118 archived tissue specimens were added, 2) an innovative NGS based genomic approach (i.e. the ddRAD technology) that allowed the development of 3,451 species-specific SNP loci and of 63 candidate outlier loci and 3) a robust experimental analysis performed with several of high-performance statistical tests for genetic differentiation at the population taxonomic level, showing a complex genetic structure of the Mediterranean BS in which low but significant genetic differences between Western and Eastern Mediterranean BS were detected by only a few statistical tests using both the whole dataset of 3,451 SNPs and the outlier 63 SNP sub-panel (i.e. the pairwise F_{ST}s).

In parallel, a similar pattern of genetic divergences was observed in the comparison between the Mediterranean BS and those inhabiting the adjacent southern areas of the North-eastern Atlantic Ocean even if significant differences between Western Mediterranean and Southern North-Eastern Atlantic BS samples were provided only by pairwise F_{ST}s estimated by both whole and outlier datasets of SNP loci and not by the DAPC analysis.

A more marked differentiation of the BS inhabiting the Northern areas of the North-eastern Atlantic Ocean with respect to the Mediterranean BS was detected but this observation should ideally be confirmed by using a larger sample size derived from this area.

Overall, our study on the Mediterranean and adjacent North-eastern Atlantic BS did not reveal a strong genetic structure, either within the basin-scale level or between basins.

Our results indicate rather that a panmictic BS population inhabits the Mediterranean and interacts reproductively with the adjacent BS of the Southern North-eastern Atlantic Ocean.

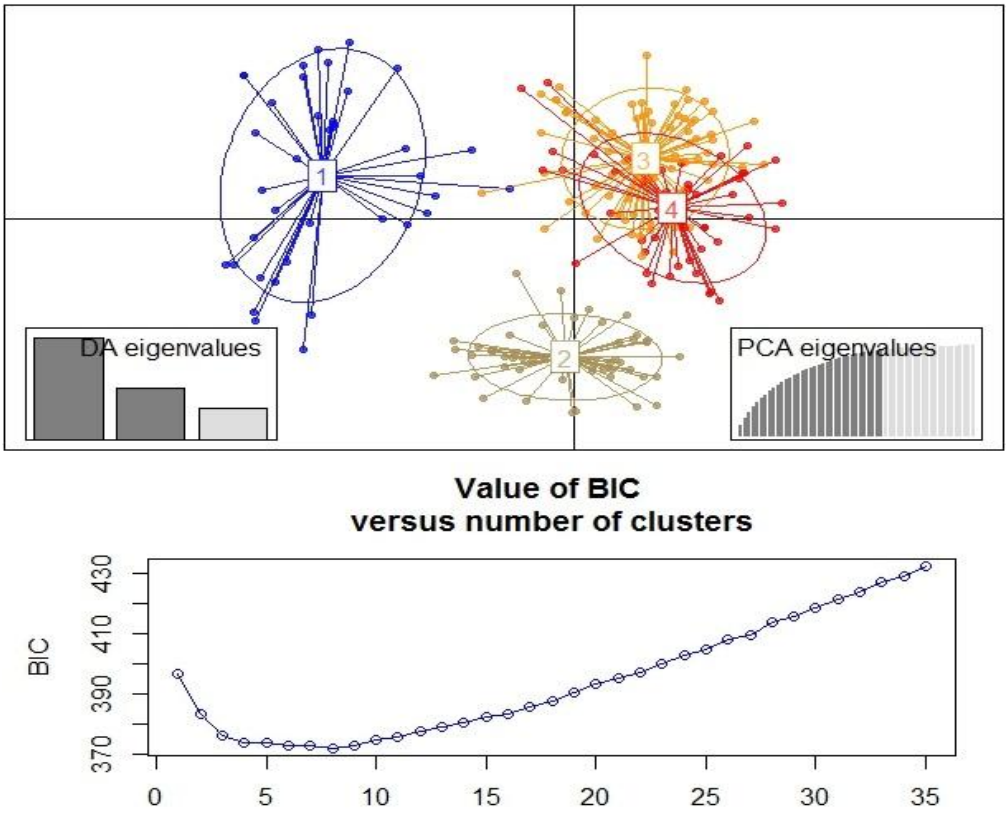

Fig. 4.3: Results from find.cluster function, using the Bayesian Information Criteria, and DAPC for the four different clusters inferred using the SNPs subset (63).

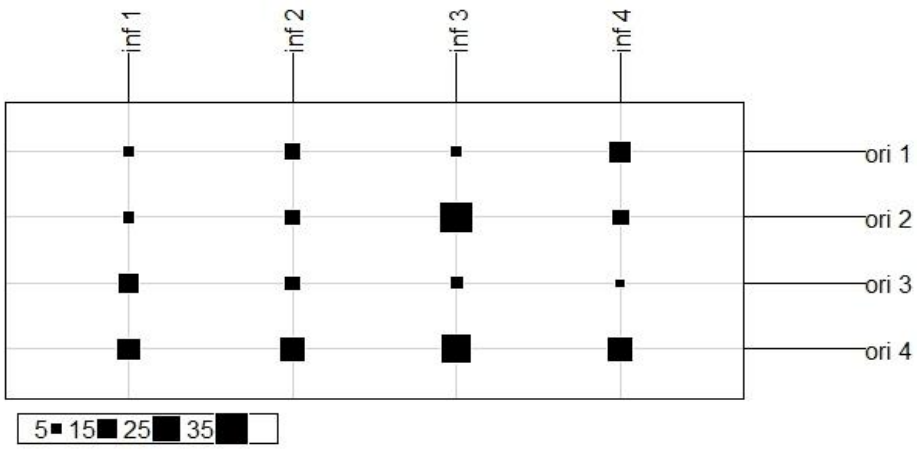

Fig. 4.4: Assignment plot of BS individuals from the four geographical areas (ori 1: NNEATL; ori 2: SNEATL; ori 3: WMED; ori 4: EMED) to the four inferred clusters (inf1-4).

This observed pattern could be explained by a metapopulation model first proposed by Kritzer and Sale (2004), in which genetic drift and gene flow determine "the dynamics of local populations

strongly dependent upon local demographic processes, but also influenced by a nontrivial element of external replenishment” .

4.4.1 The performance of developed genomic tools

The efficacy of the ddRAD sequencing technology to discover and develop species-specific genetic markers renders this technology a potent genomic tool that can readiliy be applied to marine fish and other marine natural living resources. The reliability and value of this genomic technique results from the fact that, like other RADseq techniques, a number of quality checks are applied in both during the analytical steps in the lab and during data processing steps, in order to exclude problematic samples with low DNA concentration, low coverage and potential PCR artefacts (Puritz et al., 2014a, b).

In our study, the reliability of the approach was further demonstrated by the comparable number of reads obtained after sequencing per each pool. The mean number of reads obtained from each BS individual sample was roughly equal to 4,000K reads, ensuring a high depth of coverage per each individual. Furthermore, the analyses of the identical genotypes from replicated individuals highlighted the affordability of the method.

To our knowledge this newly developed panel of SNPs is the first genome wide resource for BS, and we suggest that is a readily available asset for future investigations at global level, for evolutionary studies, as well as for conservation and management measures of this species. In fact, to date, stock assessments on this species have considered the Mediterranean as a separate and independent population, although the need for further study has always been underlined to address the huge lack of data from this area of extreme interest (ICCAT, 2015). Our work shows the need to critically review the notion of independent populations, which will help to rationalise future sampling schemes and stock assessment approaches.

The developed panel of 63 outlier SNPs, i.e. SNPs that are presumably under directional selection, should help to further look into the lack of differentiation between Mediterranean and North Atlantic, as it allows for a more in-depth analysis of the genetic structure among groups. Looking at our results, it appears that the potential discrimination between Mediterranean BS and specimen originating from the North-Eastern Atlantic is particularly challenging. Overall our newly developed genomic markers represent an important tool for the study and the discrimination of

discrete stock units of BS at global level (e.g. North Western Atlantic vs North Eastern Atlantic and Mediterranean populations/metapopulations, or Pacific population vs Atlantic population).

4.4.2 Genetic diversity and population structure

When assessing genetic diversity parameters in the Mediterranean and North eastern Atlantic BS a diffuse genetic disequilibrium and low levels of observed heterozygosity in all population samples was detected, as well as at loci level. Spatial genetic differentiation of Mediterranean and North-eastern Atlantic BS estimated using the whole panel of 3,451 SNPs was null or very low (overall F_{ST} = 0.003) and the pairwise F_{ST} comparison, although significant, resulted extremely low (ranging from 0.2 to 0.5%) indicating a near-panmictic population. On the contrary, the 63 candidate outlier loci selected by Lositan and BayeScan, were characterized by higher and significant F_{ST} values when the different areas were compared (pairwise F_{ST} values ranging from 0.01 to 0.09).

The AMOVA analyses using the whole SNPs dataset highlighted very low percentage of variation among groups. Despite significant pairwise F_{ST}s, the percentage of variation among groups of BS samples was low and does therefore not suggest a significant geographical structure of Mediterranean and North East Atlantic BS. Again, in contrast to the slight and <5%-significant partitioning of the BS molecular variance detected by the whole SNP dataset, the panel of the 63 outlier SNPs showed a weakly-significant partition separating the BS inhabiting the Northern areas of the North-eastern Atlantic from those spreading around the Gibraltar Strait (a group formed by the SNEATL and WMED population samples) and in the Eastern Mediterranean. It is noteworthy that the highest, yet not significant, value of genetic difference among groups was obtained with a geographical structure in which the BS inhabiting the Northern areas of the North-eastern Atlantic were separated from those of all the other Southern North-eastern Atlantic and Mediterranean areas.

Although the Bayesian clustering implemented in fastSTRUCTURE (Raj et al., 2014) didn't revealed strong genetic clustering using the whole 3,451 loci dataset, except for the only apparent differentiation of the NNEATL in the K=3 plot, the percentage of variation detected by the AMOVA with the related imposed geographical grouping (i.e. NNEATL vs SNEATL + WMED + EMED) was low and insignificant, suggesting that structuring between the NNEATL and the other areas does not strongly occur. Congruently, the pairwise F_{ST} values involving the NNEATL, with the exception of the comparison NNEATL-WMED, were not significant also after Bonferroni correction for

multiple tests. As expected, all the AMOVAs performed, using the panel formed by the 63 outlier SNPs showed higher level of genetic variation among groups with respect to the differentiation detected by the dataset with the 3,451 SNP loci. However, such variation was insignificant regardless the geographical groupings arbitrarily imposed, with the exception of the three-group structure in which the NNEATL, SNEATL+WMED and EMED were separated.

Basically, these results, combined with the fact that clustering methodologies such the PCA and the DAPC failed to find signals of significant clustering on this dataset. This apparent lack of genetic structure speaks in favour of a near- or fully-panmictic population of BS in the North-eastern Atlantic and Mediterranean and high reproductive connectivity between the stocks. Such evidence accounts for and corroborates the hypothesis that the Mediterranean Sea serves as nursery area of the North-Atlantic BS population with small significant divergence of the peripheral local subpopulations. This leads to the assertion that the Mediterranean BS population is an extension of the North-eastern Atlantic BS population, being in contradiction to the stock delineation and management implemented during the last decades (ICCAT, 2015). This scenario is concordant with a separation in three main groups of which one group representing an admixture area between BS from SNEATL and WMED, suggesting that this area, even using markers under selection, does not represent a geographical barrier between the Mediterranean and the North Eastern Atlantic BS.

4.4.3 Outliers SNPs selection and their use to detect genetic structure

The detection of genetic structure in fish populations, and the use of this structure for the delineation of fishery stocks, is extremely crucial for developing and implementing effective management strategies for fishery resources (Kritzer and Sale, 2004). However, it is rather common, in population genetics studies on marine epipelagic fish, to fail when looking for genetic structure or trying to detect even only subtle and insignificant differences. That is true even for apparently isolated populations, such as those occurring in the Mediterranean (Waples, 1998; Riccioni et al., 2010, 2013, 2017). Sometimes, however, this failure is misleading, and due to the large population size and the high level of gene flow among different stocks, combined with high levels of reproduction (relative high of pups in BS compared to other species). In fact, even a low rate of exchange among groups with large population size will be sufficient to delete the genetic signals of differentiation at neutral loci (Pampoulie et al., 2006).

The utility of markers presumably under directional selection, such the outlier SNPs found in our study, should help to address this issue, allowing a more in-depth analysis of the genetic structure among groups, investigating loci potentially under selection for certain environmental features, such temperature and salinity (Nielsen et al., 2012; Milano et al., 2011). In our study though, while the outlier data sub-set outperformed the entire dataset in assessing genetic differences among population samples and helped to reveal the occurrence of genetic clusters, neither the Bayesian and PCA clustering nor the DAPC clustering of BS genotypes irrespective of their geographic origin hinted at a robust geographical genetic structure of Mediterranean and North-eastern Atlantic BS.

These results fit into a scenario of weak but significant differentiation among groups, with BS undergoing extensive migrations covering the whole study area with gene flow depending on the distance between the areas, and confirming that the Strait of Gibraltar does not constitute a geographical barrier for this BS. In fact, the area between SNEATL and the WMED is an area of mixing for BS. These findings lend support to the hypothesis that the Mediterranean BS population could be a metapopulation, spatially separated from those of Atlantic, with which interacts through migrant specimens.

Even if the BS from the Mediterranean area are usually characterized by individuals not sexually mature (Kohler et al., 2002; Megalofonou et al., 2005, 2009a), a reasonable good number of BS from the area of Balearic Sea, and the area of Ligurian Sea in the WMED (~30 individuals), are large sexually mature specimens (especially males), suggesting that this mixing area could play a fundamental role as mating area between BS coming from North East Atlantic and Mediterranean Sea. This hypothesis, combined with the nursery role of the Mediterranean Sea for the species (Kohler et al., 2002; Megalofonou et al., 2005, 2009a), leads the Mediterranean to have an even more fundamental role for the conservation of the species in the whole study area. However, further and robust tagging studies in the Mediterranean Sea would be essential to better understand the reproductive and nursery role played by the Mediterranean Sea.

4.4.4 A perspective of blue shark traceability

Many marine species are widely distributed in space, and overexploited populations may well intermingle with abundant ones, meaning that accurate forecasting in fisheries management relies on identifying populations of origin (Bernatchez et al., 2017).

Population genomic approaches to support marine living resource management should also extend to the management and conservation of sharks. One of the still few cases is the genomic management of two hybrid species of blacktip shark, *Carcharhinus tilstoni* and *C. limbatus* (Morgan et al., 2012). Thousands of SNPs were used to identify different classes of hybrids between these two co-occurring blacktip shark species in Australia providing accurate tools to assess the population status of these sharks.

In the case of seafood, traceability of marine resources and seafood throughout the food chain is crucial for their sustainable utilisation, conservation of marine stocks, and to prevent food fraud, such the illegal substitution and the mislabelling (Stawitz et al., 2016). Generally, the species may be recognized by morphological traits, however, phenotypic traits becomes useless once the fish has entered into the processing chain, and in this case the only useful tools are the molecular approaches. Genetic and genomic resources provide powerful and reliable tools to identify and to trace seafood products (Martinsohn et al., 2011). The genetic traceability of a seafood product can be applied on three multiple taxonomic levels: species, population and individual level. To date, the species and population levels have been explored using either genetic or genomic methodologies (Morgan et al., 2017; Martinsohn et al., 2011).

Since the BS are widely subject to by-catch, especially in swordfish and tuna longlines (Gallagher et al., 2014), with the complicity of the economic crisis, the cheap meat of BS became of huge economic interests in Europe (Clarke et al., 2006). The management approach to which the Atlantic population of BS was subject, considering the Mediterranean population as separate stock (Kohler et al., 2002; ICCAT, 2015), may had led to an incorrect management of the BS population in the entire region.

Despite the need to developing genetic markers suitable for discriminating spatially close populations, such as those of the Mediterranean and the North-eastern Atlantic, the genomic markers developed and selected here have not proved to be conclusive. Even though a low but significant differentiation was detected by the analysis of molecular variance and pairwise F_{ST}, as suggested by clustering analyses, such as PCA, differentiation is insufficient to develop a panel of SNPs useful to discriminate specimen from these areas. This is likely due to a high rate of gene flow among groups that delete any signals of differentiation. The assignment of BS individuals to the four different areas highlights this mixing nature, even if the assignment of individuals to the

inferred clusters highlights how in specific areas, the individuals seem to be weakly but well assigned to the correct EMED and EATL clusters.

Although the developed markers are not conclusive on the short-distance traceability, given the extremely migratory nature of the species, they could be applied for traceability of BS meat products globally, allowing the differentiation of BS populations and BS products of the Mediterranean/Atlantic from those of the Pacific Ocean. This appears to be particularly relevant given the different status assessed for the Mediterranean and Pacific stocks, - Critically Endangered and Near Threatened, respectively (Stevens, 2009; Sims et al., 2016).

4.4.5 Implications for stock management

The genetic homogeneity across the whole study areas (Mediterranean and North East Atlantic) contradicts the currently assumed distinction of the two populations, considered to be different stocks. Indeed, the evidence gathered thus far indicates that the BS exhibits huge dispersal capacity with gene flow over very large distances (Veríssimo et al., 2017). Nevertheless, this high potential of dispersal, combined with the fact that large sized oceanic stocks exhibit similar allele frequencies among distant areas, that can be maintained, over long distances, by a few migrants per generation preventing the genetic drift of populations (Palsbøll et al., 2007). Practically, the gene flow among large population may mask the genetic signals useful to distinguish different populations and different stocks.

The F_{ST} values observed in the present study are quite low (relatively small considering only selected SNPs), and in these cases, the gene flow among areas are often difficult to estimate when F_{ST}s are very low (Waples, 1998; Lowe and Allendorf, 2010). However, in cases of big effective populations sizes (e.g. *Ne* =~1,000-100,000), the genetic divergence rates associated with migration rates which could lead to demographic connectivity may be difficult to detect (Hastings, 1993). The BS appears to perfectly match this description, as it displays a large population size, high migration rates, and low F_{ST}s but significant that could be indicative of different discrete subpopulations.

Given our results, and given the biological characteristics of the BS, we would suggest to apply a precautionary approach when implementing conservation and management measures for this species This includes the recommendation to regard the North Eastern Atlantic and the

Mediterranean as separated nursery areas, subject to potentially independent demographic dynamics (Sims et al, 2016), and subject to different fishing pressure. A closer look to the data available, from both genetics/genomics and tagging studies, indicates that the most probable scenario is that of, at least, one single metapopulation along the Mediterranean-North Eastern Atlantic gradient. The migration rate between these two areas remains unknown, but if the only exchange is represented by a few migrant individuals per generation among nurseries, the recruit survival will mostly represent the actual maintenance of these stocks rather than on immigrants from adjacent stocks.

This scenario is implying a challenging management scheme for the BS in the Atlantic, which is rendered difficult due to the extensive migratorion behaviour and complex movement dynamics of BS (Kohler and Turner, 2008; Queiroz et al., 2012, 2016; Leone et al., 2017). Perhaps, competent management bodies, such as ICCAT, should start to consider the incomplete reproductive separation of the Atlantic and Mediterranean BS populations. Additional information on connectivity among nursery areas (e.g., those obtained from long-term tagging studies) need to be obtained and to be combined with population genetic data, in order to better define the real rate of exchange between the two areas, and therefore, to better define the fishery stocks.

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Supplementary Tables

Tab. S 4.1 Geographical composition of the three ddRAD libraries of blue shark

ddRAD library	Area				Total
	NEATL	SEATL	WMED	EMED	
Pg_ddRAD_01		28	15	29	**72**
Pg_ddRAD_02	20	5	33	12	**70**
Pg_ddRAD_03	10		41	19	**70**
Total	**30**	**33**	**89**	**60**	**212**

Tab. S 4.2 Temporal and geographical composition of the blue shark dataset after filtering of ddRAD data.

AREA	UNKNOWN	2003	2004	2008	2009	2012	2013	2014	2015	2016	Total
NEATL	28										28
SEATL								9	24		33
EMED		2	3			1		3	45		54
WMED				1	1	4	1	53	15	13	88
Total	**28**	**2**	**3**	**1**	**1**	**5**	**1**	**65**	**84**	**13**	**203**

Tab. S4.3 ***Observed (Ho) and Expected (He) Heterozygosity in the BS geographical samples and overall the dataset, with associated percentage of missing value (%NA), percentage of polymorphic loci (P%) and significance of the test for Hardy-Weinberg Equilibrium (HWE).***

	Ho	He	%NA	HWE	%P
NNEATL	0.13	0.15	1%	*p value* = 0	96.11%
SNEATL	0.12	0.14	0.73%	*p value* = 0	97.79%
WMED	0.14	0.15	0.75%	*p value* = 0	100.00%
EMED	0.14	0.15	1.20%	*p value* = 0	99.38%
Overall	0.13	0.15	0.92%	*p value* = 0	100.00%

Table S 4.4 ***Pairwise F_{ST} values between geographical BS samples. Lower diagonal F_{ST} values, upper diagonal associated p-values; * indicates values significant after Bonferroni correction for multiple tests (p-value=0.008).***

	NNEATL	SNEATL	WMED	EMED
NNEATL		0.00290	0.01059	0.00055
SNEATL	0.00516*		0.00020	0.00395
WMED	0.00245	0.00328*		0.00474
EMED	0.00439*	0.00332*	0.00474*	

Supplementary Figures

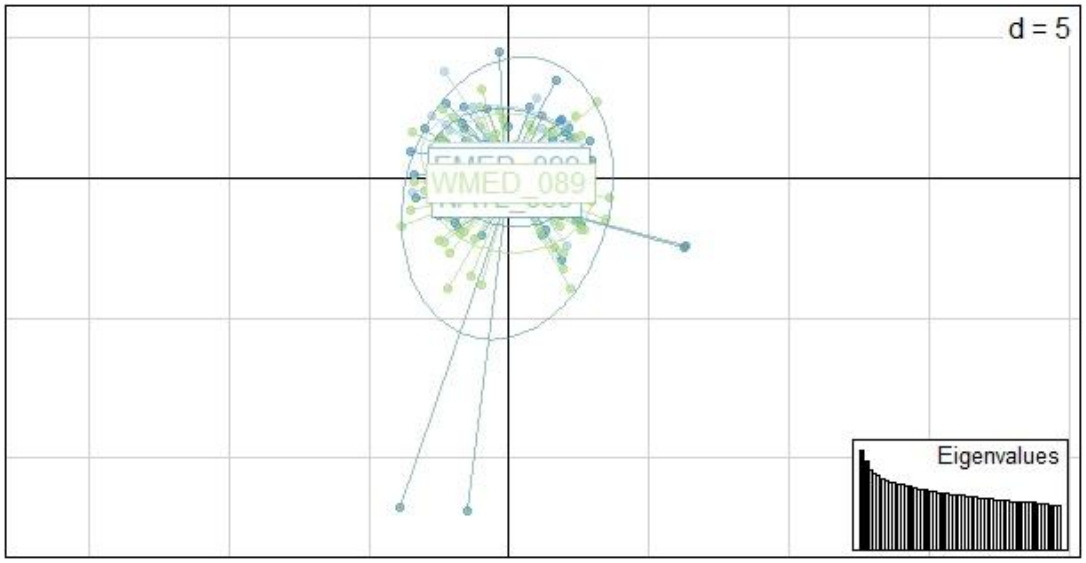

Fig. S 4.1: ***Blue Shark Principal Component Analysis plot (with related Eigenvalues) of BS genotypes based on the whole SNPs dataset.***

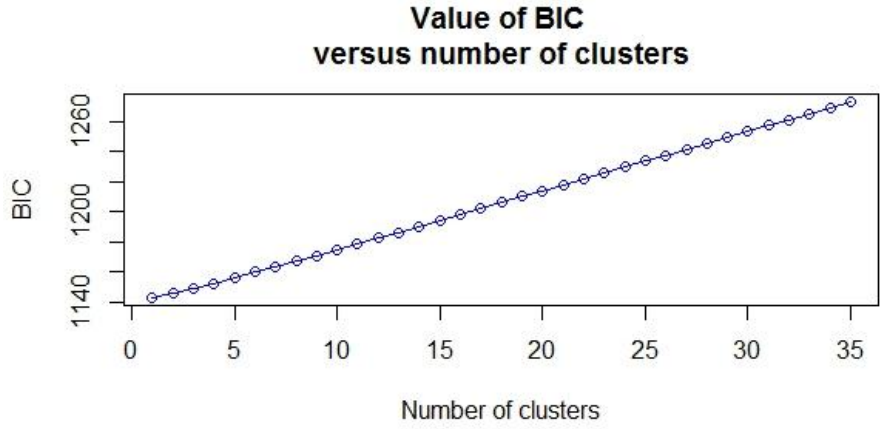

Fig. S 4.2: Pre-DAPC find cluster analysis using Bayesian Information Criteria, on the whole SNPs dataset. No clusters were revealed.

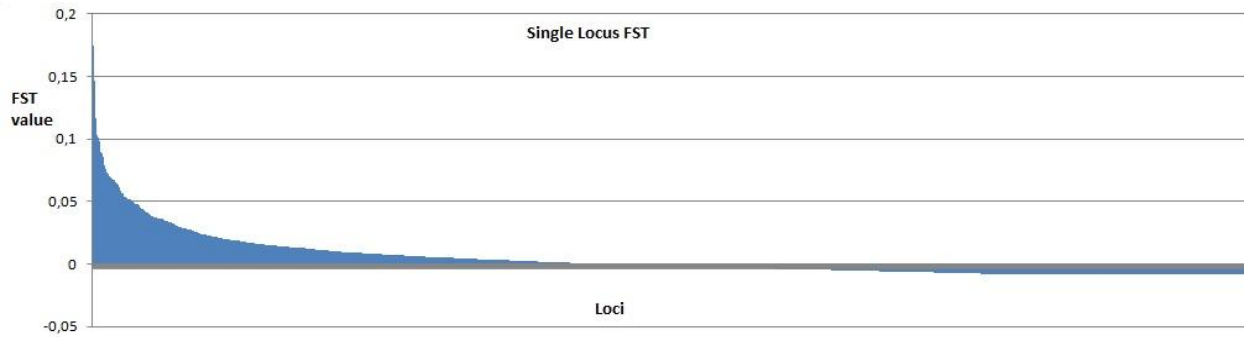

Fig. S 4.3 ***Single locus FST values among BS population samples obtained from the Locus-by-Locus AMOVA.***

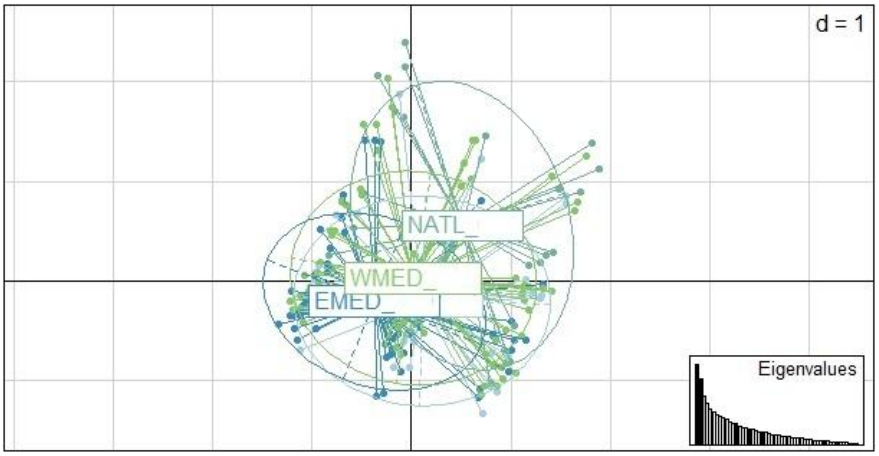

Fig. S 4.4: ***PCA plot and related Eigenvalues of the clustering of BS population samples obtained using the genetic variation outlier 63 SNPs dataset.***

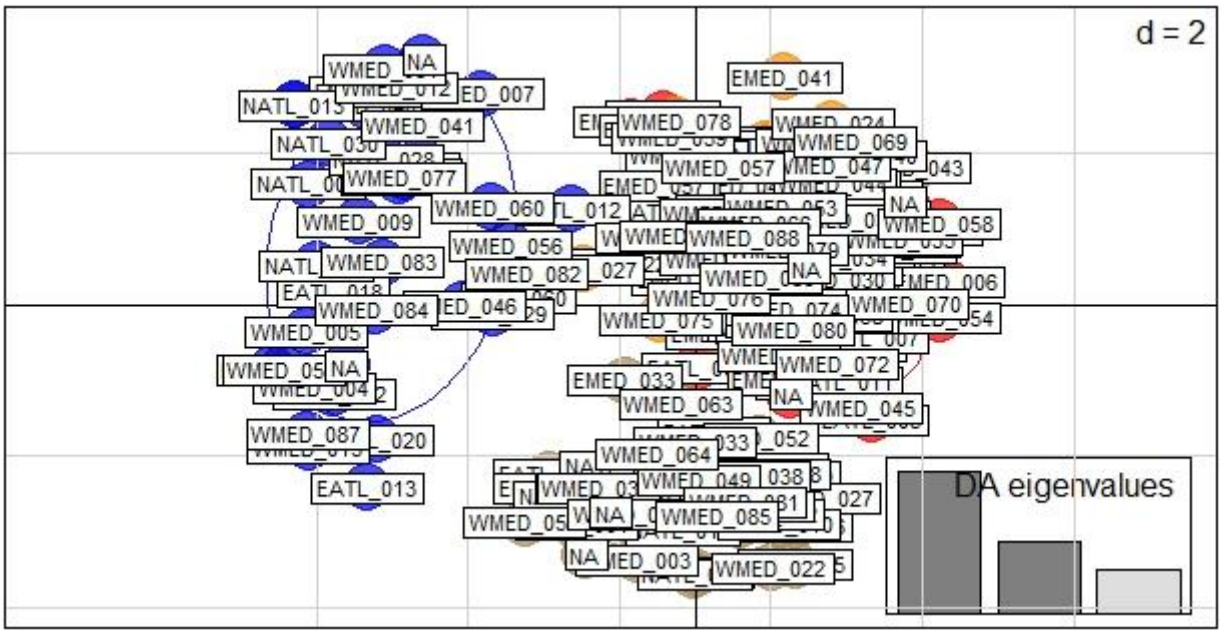

Fig. S 4.5: ***The DAPC plot based on the 63 outlier SNP dataset already shown in Figure 10 with individuals labelled by population samples.***