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Analisi degli stock di pescespada (*Xiphias gladius*, Linnaeus 1758) nel Mar Mediterraneo tramite l'utilizzo di marcatori molecolari: DNA mitocondriale (D-loop) e DNA microsatellite

Analysis of swordfish stocks (*Xiphias gladius*, Linnaeus 1758) in the Mediterranean Sea through the use of molecular markers: mitochondrial DNA (D-loop) and microsatellite DNA

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SOMMARIO

ABSTRACT

Il pesce spada (*Xiphias gladius*, Linnaeus 1758) è un grande pesce pelagico, appartenente alla famiglia Xiphiidae, che può superare i 4 metri di lunghezza e i 500 kg di peso. Ha una distribuzione cosmopolita, è diffuso tra i 45ºN e 45ºS in tutte le acque tropicali subtropicali e temperate degli oceani Atlantico, Indiano e Pacifico, inoltre popola anche le acque del Mar Mediterraneo, Mar Nero, Mare di Marmara e Mare di Azov.

Tra gli studi riguardanti la genetica di popolazione di questa specie è emerso come lo stock del Mediterraneo abbia caratteristiche genetiche uniche, dovute principalmente al ridotto flusso genico dall'Atlantico. Lo studio presentato in questa tesi si basa sull'analisi genetica dello stock di pesce spada presente in Mediterraneo, in quanto relativamente poco analizzato rispetto a quelli degli altri bacini. Le analisi sono state condotte utilizzando due diversi marcatori molecolari: DNA microsatellite (nucleare) e D-loop (mitocondriale), e sono state eseguite su 298 individui campionati nel Mar Mediterraneo e 25 in acque canadesi (Atlantico), per un totale di 323 individui. Per quanto riguarda i campioni Mediterranei, questi provengono rispettivamente da Adriatico Meridionale (62), bacino a sud-est della Grecia (20), zona delle Baleari (85), Mar Tirreno (16), coste siciliane (61) e Sardegna (54).

L’elaborazione statistica dei risultati riguardanti il DNA microsatellite, condotta tramite DAPC, ha evidenziato la presenza di 3 cluster genetici distinti. Individui riconducibili ai 3 cluster sono presenti in tutte le aree campionate: queste zone quindi potrebbero essere considerate aree di mixing utilizzate per scopi di feeding o migrazione, e la segregazione genetica in 3 cluster potrebbe essere attribuita ad un comportamento filopatrico verso le principali zone di spawning del Mediterraneo.

In contrasto con la debole struttura genetica osservata a livello dei microsatelliti, i risultati dell’analisi sul DNA mitocondriale non hanno evidenziato differenze statisticamente significative nella composizione aplotipica delle popolazioni esaminate. D’altra parte, questa discrepanza non è del tutto inaspettata, dal momento che è noto che i microsatelliti narrano la storia filogenetica nell’arco di decine di migliaia di anni mentre i marcatori mitocondriali sono in grado di svelare la storia filogenetica più profonda. (Emerson B.C., Hewitt G.M.,2005). Inoltre, il problema di fondo con i grandi pelagici è che sono caratterizzati da una bassa differenziazione genetica in quanto l’elevato flusso genetico dovuto alla loro considerevole capacità di dispersione favorisce una sostanziale omogeneità genetica su ampie distanze. Ciò rappresenta chiaramente un limite circa la possibilità di utilizzare i marcatori molecolari per delimitare popolazioni geneticamente suddivisibili da

utilizzare come unità gestionali (stock genetici) in specie sottoposte ad un elevato sforzo di pesca come il pesce spada.

1.INTRODUCTION

1.1 Biology and ecology of swordfish

Global distribution

Swordfish (*Xiphias gladius*, Linnaeus 1758) is a large pelagic fish worldwide distributed, from 45°N to 45°S, in tropical, subtropical and temperate waters (sometimes even in cold waters) of the Pacific, Indian and Atlantic Ocean and also in Mediterranean Sea, Sea of Marmara, Black Sea and Sea of Azov (Palko & Beardsley, 1981).

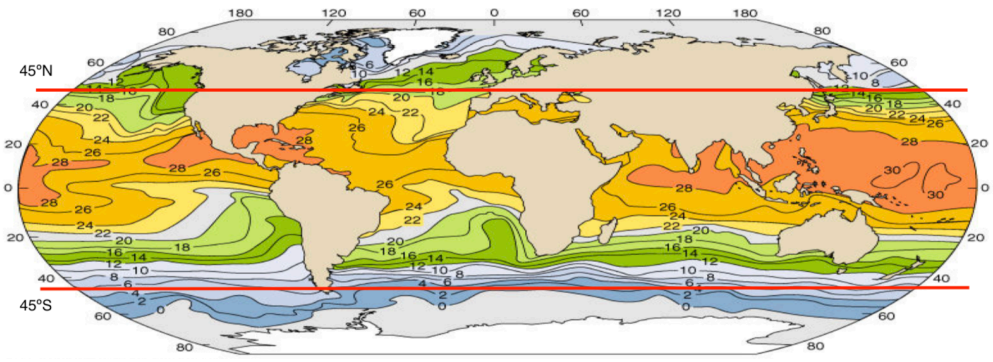
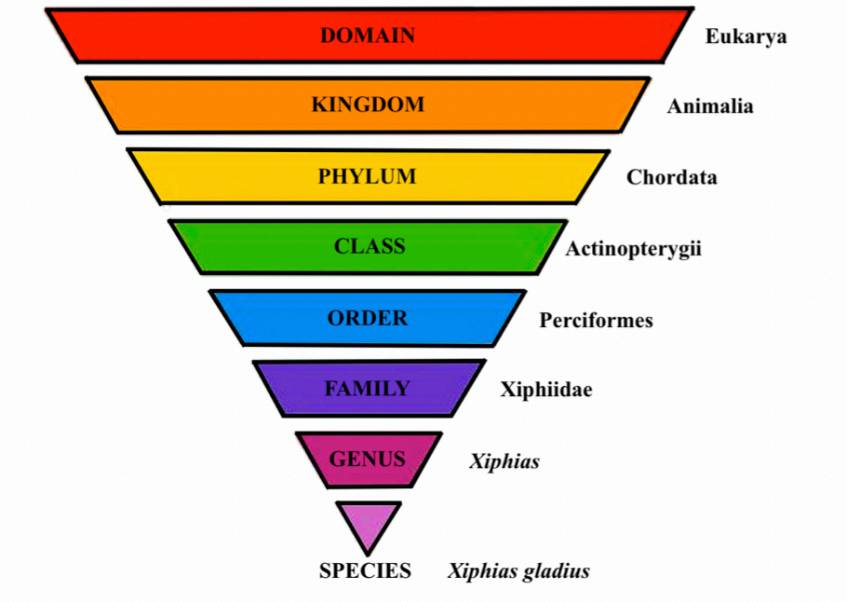


Fig. 1.1.: Global distribution of swordfish: the distribution range is included between the two red lines, approximately 45°N and 45°S.

Taxonomy

People might think fish like swordfish, marlin, sailfish and other billfishes are closely related. Yes, they are, but actually not so closely like the majority of people think. In fact, swordfish belong to the monotypic family of *Xiphiidae* while other billfishes belong to the *Istiophoridae* family.

Even without proceed in genetic analysis, external morphology comes to help us distinguish the two family: sword-like bill depressed in cross section, absence of jaw teeth and scales in adults, short based dorsal fin separated from the second one, absence of pelvic fins, all features present only in *Xiphias gladius*.



Morphology

As known by most, swordfish main feature is the elongated shape of the jaws. The upper one is elongated and flatten with sharp edges (bill), which is about a third of the length of the entire animal.

This species is characterized by a strong dimorphism by age, in fact gradual metamorphosis causes drastic changes in swordfish shape during growth from larval to adult stage.

Adult fish have long, heavy and round-shaped body without scales. Adults have two separated dorsal fins (have respectively 38-49 rays and 4-5 rays), two anal fins (12-16 rays in the first one, 3-4 in the second) and two pectoral fins, with 17-19 rays, each situated on the lower part of the two flanks. No pelvic fins are present. The caudal fin is croissant-shaped and there is a single pronounced side keel on each side of the caudal peduncle.

In young stage, instead, body is long, thin and snakelike with atypical scales. First dorsal fin is confluent with the second one, and the same is for anal fins (Palko & Beardsley, 1981). Moreover, not only the upper but both jaws are prolonged into long bills (Nakamura, 1975) and also small teeth are present, and disappear during growth.

Other featuring like color change in the maturation process: first stages of development are characterized by increasing numbers of chromatophores

(Arata, 1954). This trend leads adults to show a brown-black color on the back and a light brown on the ventral part, while dorsal fins are blackish brown and other fins are light brown.

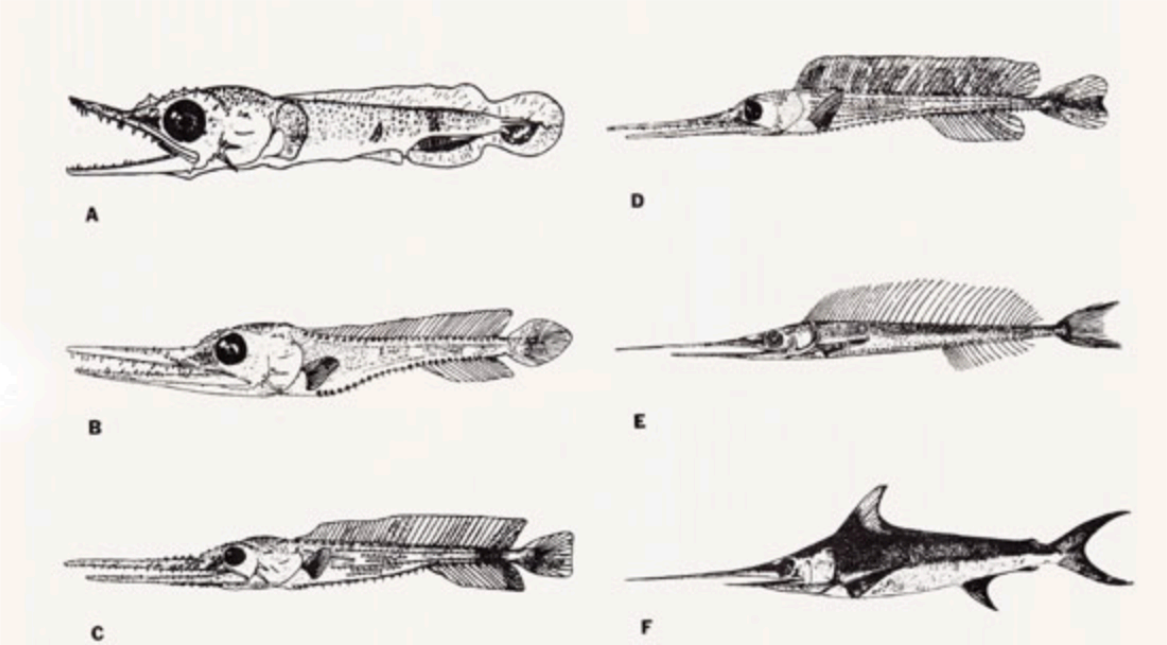


Fig.1.3: Swordfish larvae. A: 7.8mm TL (Total Length); B: 14.5mm TL; C: 27.2mm TL; D: 68.8mm TL; E: 252mm BL (from the posterior edge of orbit to base of the caudal fin); F: 580mm BL. (Nakamura et. al., 1951)

Growth

In swordfish growth is very rapid, especially during the first year of the lifecycle when, as said previously, individuals are involved into a strong morphological differentiation. After the first year growth rate slows down considerably (Ehrhardt, 1992).

Several studies investigate age and growth rate of swordfish basing mainly on spines from anal fins (Tserpes & Tsimenides, 1995; Ehrhardt, 1992) or otoliths

(Megalofonou, 1995) showing differential growth between sexes. Males showed shorter life spans, slower growth rate and reached smaller size than females (Berkeley & Houde 1984, Wilson 1984). In Mediterranean Sea, for example, sex ratio for swordfish bigger than 135 cm is in favor of females and reaches almost 100% in the range of 200-215 cm (Aliçli & Oray, 2012). Size differences have also been observed between unit stocks (see Table 1 for details).

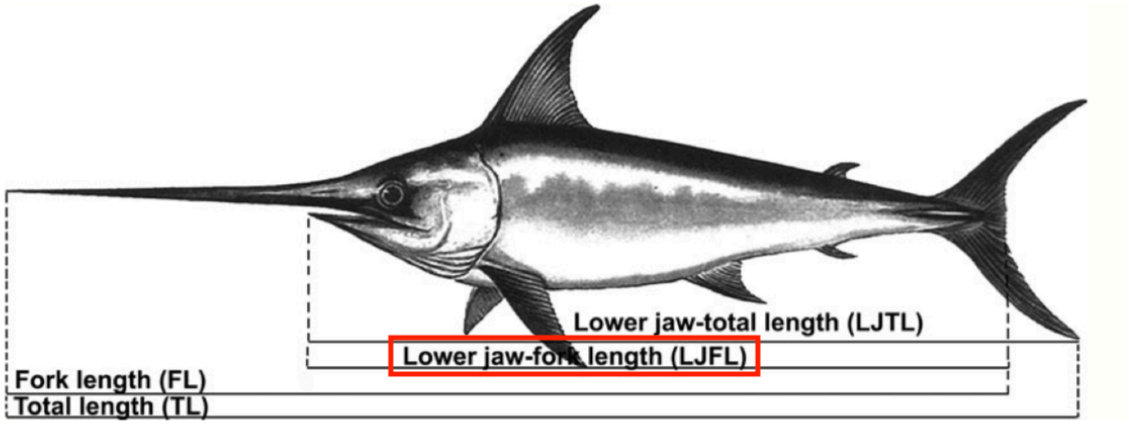


Fig. 1.4: Some unit of measurement to quantify swordfish length. LJFL is one of the most common

Reproduction

Swordfish is a gonochoric species but there are no external characters distinguish males from females, except the females are generally larger. Swordfish are solitary animals and rarely gather in schools, in fact they mate only in the spawning season and in specific areas. Fertilization is external and eggs are pelagic, so they are transparent with a large oil droplet (Sanzo, 1922), with a diameter around 1,6-1,8 mm (Palko & Beardsley, 1981). Age and length at first maturity are sex-dependent and also differ between unit stocks.

AREA	LJFL (cm)		ASM		L50(cm)		CIT.
	M	F	M	F	M	F	
MED.	82-105	106-135	2	2-3		135	DeMetrio, 1989
		110		2-3		142	De la Serna, 2002
		130-139		4		148	Orsi-Relini, 2003
N.ATL.	100	170	1	4		180	Taylor, 1992
			2-3	4-5			Wilson, 1984
		150		4-5		178	Arocha, 2007
S.ATL.	120-130	125			120	156	Hazin, 2002
		125				156	Arocha, 2007
PAC.		135		5		168	Wang, 2003
	116*	161*			117	162	DeMartini, 2000
IND.	101	127			119	170	Poisson, 2001

Tab. 1.1: Table shows values of length and age at first sex maturity, for each ocean basin: LJFM, length at first sex maturity; ASM, age at first sex maturity; L50: length at which 50% of individuals have reach sexual maturity. (Each value is reported for males and females). Values with the symbol "*" are converted from EFL using equation from Sun (2005).

From reported studies in Table 1.1 it is possible to see that generally males mature at a smaller size and younger age than females. In line with growth rate, age and size at first maturity estimated for swordfish differ between unit stocks: in the western north Atlantic, females were observed to mature at largest sizes and oldest ages while Mediterranean swordfish show the smallest of those ones observed for any other breeding unit in the world.

Spawning is closely related to the water surface temperature and generally occurs in tropical waters, where temperatures are about 20-22 ºC. This determines distinct temporal and spatial spawning patterns between the oceans and characterizes distinct breeding units.

In Atlantic Ocean Arocha (2007) has identified 3 reproductive unit stocks, separated into North Atlantic, South Atlantic and also including Mediterranean Sea.

North Atlantic stock area is also divided in temperate, subtropical and tropical: here the main spawning season was defined from December to June, and a possible spawning event in late summer (August). In western Atlantic the highest density of larvae occurs from the straits of Florida to Cape Hatteras and Virgin Islands area, suggesting Gulf of Mexico serves as a nursery ground for swordfish.

In South Atlantic female spawning year-round: in October-December on the east side and in April-June on the west one.

In Mediterranean spawning take place between June and August in three areas restricted between 35º and 40º N: in western basin, east of Gibraltar up to the Baleares Islands, in southern Tyrrhenian and Strait of Messina, and in eastern basin between Rhodes and Cyprus island (Arocha, 2007).

Finally, in eastern Pacific Ocean spawn appears to be most abundant in March-July in northern latitudes, and around January in southern latitudes (Kume & Joseph, 1969).

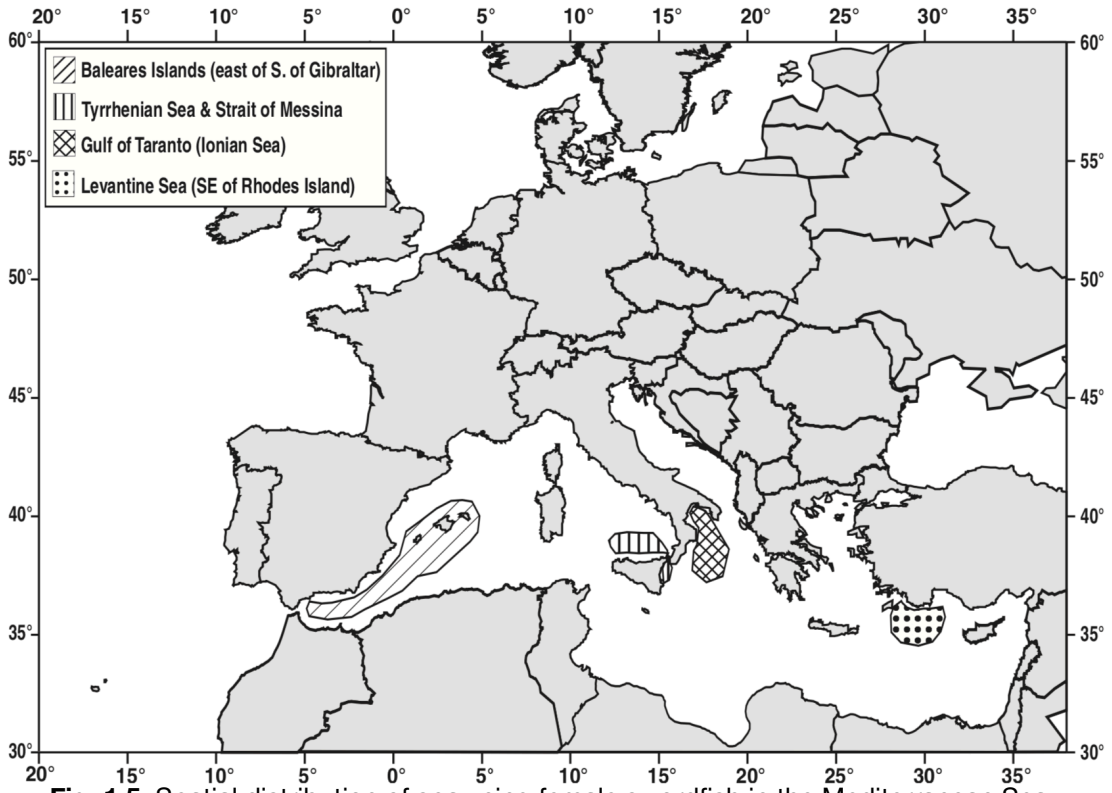


Fig. 1.5: Spatial distribution of spawning female swordfish in the Mediterranean Sea, showing the three main spawning grounds: one from East of Gibraltar to the Balearic Islands, one in the southern Tyrrhenian Sea and Strait of Sicily and one in the Levantine Sea (Arocha, 2007).

Diet and feeding

Studies performed mainly in Atlantic Ocean reveal swordfish is an opportunistic predator feeding in pelagic vertebrates and invertebrates. According with Chancollon et al. (2006), diet is mainly composed in fishes (40,5% of the contribution by mass) like *Symbolophorus veranyi* and *Notoscopelus kroeyeri*, and cephalopods (59,3% of the contribution by mass) like *Gonatus steenstrupi and Ommastrephes bartramii*. Also, a small amount of crustaceans (only 0,2% of the contribution by mass) is included in swordfish diet (Chancollon et al. 2006).

His opportunistic behavior allows him to adapt the diet between areas: in neritic zone swordfish are found near the bottom and pray upon benthic species during the day; at night they move offshore feeding mainly on squids.

In oceanic zone swordfish undergo in daily vertical migration, in fact, during daylight hours they can reach 600 m depths to feed, while at night ascend to shallow waters (Carey & Robinson, 1981).

To endure rapid change in temperature, caused by the significant vertical migration, swordfish has evolved a specialized muscle that function as a brain heater, essential to a predator that hunts on the cold side boundaries between ocean masses or at high depth (Ward et al., 2000). Diet changes not only related

to the area but also according with the moment of lifecycle: larvae feed on zooplancton, other fish larvae or Amphipoda; juvenile are already able to feed on squids, fishes and crustaceans (Arata, 1954).

State of swordfish stocks

Multi-disciplinary approach (molecular, morphological, tagging, etc.) have demonstrated that world population of swordfish does not represent a single stock but is made up of several population among and within oceans. Despite of the highly migratory behavior and the fecundities of several millions of eggs per female, which leads to a long-range dispersal, hypothesis of panmixia has been rejected. According with this assertion swordfish populations are usually studied separately in Atlantic and Pacific Ocean, and even dividing them in north and south basins.

Genetic evidence of inter-oceanic population differentiation between Atlantic, Mediterranean and Indo-Pacific swordfish populations has been detected by the heterogeneity distribution of mitochondrial D-loop region lineages (Alvarado Bremer *et al.*, 1995, 1996, 2005; Chow *et al.*,1997) and nuclear locus calmodulin (*CaM*) gene intron-4 alleles (Alvarado-Bremer 2007). Mitochondrial Clade II lineage and *CaM* allele B have not been detected in the Indo-Pacific, occur at a low frequency in the South Atlantic, increase in

abundance towards the North Atlantic, and reaching highest frequency in the Mediterranean Sea (Alvarado Bremer *et al.*, 1996; 1998; 2007; Rosel & Block, 1996; Reeb *et al.*, 2000).

In Indo-Pacific, analysis of mitochondrial control region show that the swordfish stock structure can be summarized in 3 groups: an area off northern Madagascar, the Bay of Bengal, and the rest of the Indian Ocean and western Pacific (Lu et al., 2006).

Within Atlantic Ocean significant genetic differentiation among Mediterranean, North Atlantic, and South Atlantic swordfish populations was confirmed by heterogeneous distribution of *Cam* alleles and the mtDNA Clade I haplotypes (Alvarado Bremer *et al.*, 1996, 1998, 2005), SNPs (Smith 2015) and other single nuclear loci as aldolase B (*ald*B) and lactate dehydrogenase A (*ldh*A) (Greig *et al.*, 1999; 2000).

Northwest Atlantic swordfish extends from the tropical spawning area, in northwestern Atlantic waters, to 50ºN; the South Atlantic population extends from 50ºS through the South Atlantic tropical and equatorial spawning areas; Mediterranean population extends past the Strait of Gibraltar, as far as 8ºW. (Smith, 2015). Tagging results have not observed trans-equatorial migrations (García-Cortés et al., 2003), corroborating intra-oceanic stocks segregation.

North and South Atlantic stocks are separated by an imaginary boundary located at 5ºN (Abid, 2006).

In Mediterranean Sea, both mitochondrial and nuclear markers (Smith, 2015; Kotoulas, 2007; Reeb, 2000) have identified a substantial genetic differentiation and the lower levels of genetic variation, compared to any other population worldwide (Alvarado Bremer *et al.*, 1995, 1996, 2005; Rosel & Block, 1996; Pujolar *et al.*, 2002). Currently, all Mediterranean swordfish are considered and managed as a single population (Anon, 2003), but despite an apparent genetic homogeneity, intra-clade genetic variation of the two mtDNA clades (Clade I and Clade II) suggest population subdivision between Western and the Eastern basins. Moreover, the presence of discrete spawning areas in each of two Mediterranean basins corroborates the hypothesis of population subdivision, and comparison among locations shows a decrease of haplotypic diversity from west to east (Vinas et al., 2010).

Two alternative hypotheses, based on present-life-history traits, may explain the observed heterogeneity within the two basins: first, larval retention mechanism, due to surface current in eastern basin, may prevent gene flow between populations (Hamad et al., 2005); alternatively, spawning site fidelity to discrete spawning grounds may produces genetic heterogeneity.

Due to the absence of physical barrier separating North Atlantic from South Atlantic populations, and North Atlantic from Mediterranean populations, admixture occurring over a considerably broader geographic area: Atlantic and Mediterranean population contacts are confined to the shallower waters of the northwest African shelf and the Gulf of Cadiz (Smith, 2015). Mediterranean individuals carrying out the trophic migration, moving toward the northeast Atlantic but remaining in the region between 15° W and Gibraltar (Pujolar et al., 2002). About North and South Atlantic, boundary area begins around Western Sahara and continues north to Morocco and the Iberian sea, extending west towards the central North Atlantic and then south towards the northern coast of Brazil. Despite admixture zone, very little gene flow occurs between swordfish populations, thanks the high philopatric behaviour through discrete spawning areas (Alvarado Bremer, 2005).

Fisheries and Management

Since 1966, the International Commission for Conservation of Atlantic Tuna (ICCAT) regulate the fisheries activity of tuna, swordfish and other pelagic fish stocks, in Atlantic Ocean and Mediterranean Sea, with the aim of maintain the population of these fishes “at levels which will permit the maximum sustainable catch for food and other purposes”. Basing on scientific evidences

and studies, ICCAT considers Atlantic and Mediterranean swordfish as different management units; moreover, it divides Atlantic group in North and South units, separating them around 5 ºN.

According with ICCAT reports, Mediterranean annual reported catches are similar to those from the North Atlantic but it should be considered that Mediterranean Sea is a much smaller body of water. Data from 2015 shown that North Atlantic total catches were around 11,000 t, while in Mediterranean Sea were almost 10,000 t (Figure 1.6). It has been suggested that this trend is mainly due to some ecologic parameters characterizing Mediterranean Sea like: higher recruitment levels, larger spawning areas in relation to the distribution area of the stock and lower abundance of large pelagic predators (e.g. sharks). So, while North Atlantic stock is stable to increasing in biomass, in the Mediterranean Sea, this species is currently overfished. Last ICCAT report (REPORT OF THE STANDING COMMITTEE ON RESEARCH AND STATISTICS (SCRS) (Madrid, Spain, 1 to 5 October 2018)) indicate that "recruitment shows a declining trend in the last decade, while stock biomass remains stable at low levels, that are about 1/3 of that in the mid-1980s. Low biomasses and abundances pull fisheries to increase catches of small individuals, many of which have probably never spawned.

IUCN indicates that Mediterranean swordfish is being fished between 13-40% over maximum sustainable yield (MSY) and around 28% worldwide, so it is listed into the extinction risk category “Least Concern” of the IUCN red list and population trend is constantly decreasing (https://www.iucnredlist.org/species/23148/9420041#assessment-information_30/5/2019).

Trying to protect Swordfish stock ICCAT imposes several management measures: driftnet ban, three month fishery closure, gear specifications (number and size of hooks and length of gear), minimum catching size (100 LJFL for Mediterranean sea), regulations, list of authorized vessels, fishing capacity restrictions, Total Annual Catch (10,500 t in 2017).

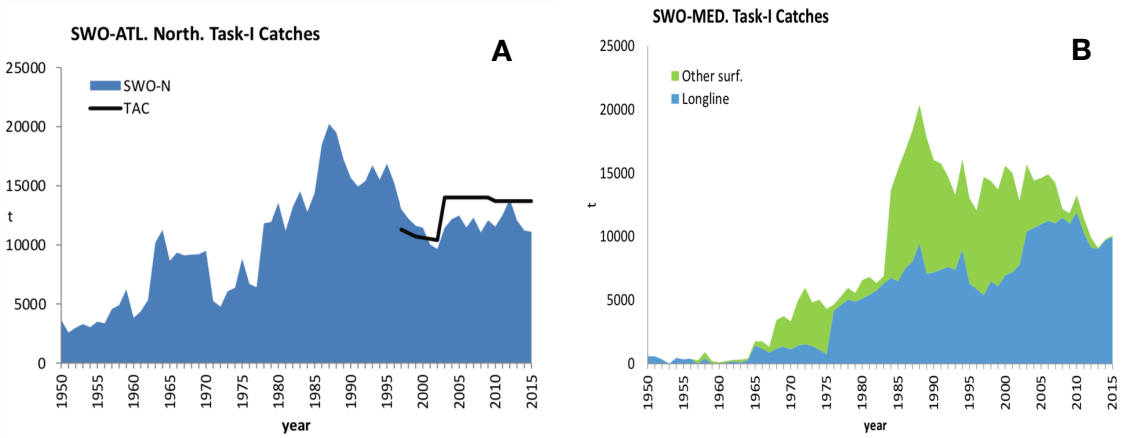


Fig. 1.6: A, North Atlantic catches in tons from 1950 to 2015; **B**, cumulative estimates of swordfish catches in tons in Mediterranean Sea by major gear types from 1950 to 2015. (ICCAT Report 2014-2015)

1.2. Genetic Analysis

The Stock Concept

A general definition for the term “stock” was proposed by Booke (1981) as a “group of fish that maintains and sustains itself over time in a definable area”. Actually, it is not so simple to define this concept, and its meaning may be different according to the scope that you are considering about. The concept of stock is fundamental in fisheries science or management and, according with Milton and Shaklee (1987), it is referred to any group of a fish species that was available for exploitation in a given area. From a biological point of view, instead, it can be considered as an arbitrary group of fishes large enough to be essentially self-reproducing, with members of each group having similar life history characteristics (Hilborn & Walters, 1992).

It is common to use the terms “stock” and “population” interchangeably, and it is not wrong to do, but usually the stock concept really has to do with interaction between a fish species and his management. The stock management, generally aims to achieve maximum sustainable production from fish stocks, usually defined on the basis of the same vital rate parameters (Begg, 1999). Swordfish is a prime example of fish stocks that need to be managed, because of its importance in fisheries market and also in marine ecosystem.

Identification and management of fish stocks may be highly technical, especially in situation where are involved large number of stocks, and where differences among them are slight (Begg, 1999). Genetic differences between individuals are one the most proper tool to investigate stocks characteristics like reproductive isolation, the fundamental mechanism structuring differences between stocks. Some of the main genetic techniques used in stock identification are the following: protein or enzyme variation, due to an indirect expression of nucleotide base differences between groups; however, the usefulness of this approach is undermined by the fact that same protein may be coded by multiple nucleotide sequences. Mitochondrial DNA (mtDNA), a molecule of about 16.000-20.000 base pairs, haploid and maternal inherited: different regions of mtDNA evolve at different rates (Mayer, 1993), with differences in base pairs used to separate stocks. Nuclear DNA (nDNA), that present a large number of potential markers; nDNA is diploid, and its recombination reflects characteristics of both sexes. Generally, is thought to evolve slower than mtDNA, but there are some noncoding regions, such as microsatellite, that may evolve even faster than mtDNA, so it can be used as molecular markers (Dowling *et. al.*, 1990).

Population Genetics

To clarify the state of the stocks of swordfish in Mediterranean Sea one of the most proper tool to be used is genetics applied on biological population: the so-called population genetics.

Population genetics seeks to understand how and why allelic and genetic frequencies may change over time between or within population and provides the clearest understanding of how evolutionary changes occurs (Clark, 2001). From genetics point of view a "population" is a group of organisms of one species that interbreed and live in the same place at the same time, this means every population has its genetic diversity, due to evolution and natural selection. Evolution can be separate in "microevolution" and "macroevolution": the first one indicate an adaptive modification within population (and requires a relative short amount of time), while the latter refers to the origin of new species with more complex adaptive modifications, so this mean a large amount of time is necessary (Reznick & Ricklefs, 2009).

Hardy-Weinberg law is at the base of population genetics, in fact it clarifies how the concept of segregation explain genotypic and phenotypic frequencies within one population: Hardy-Weinberg equilibrium is the principle stating that allelic frequencies in a population remain constant from one generation to the next in absence of disturbing factors.

There are five conditions for that to happen:

- Population may be almost numerically endless;
- There is not immigration or emigration within the population;
- Presence of panmixia (casual breeding);
- Absence of selection for a given genetic character;
- Absence of mutation.

Obviously, this is only theoretical, because in nature there is a combination of factors and events that avoid one or all these conditions, but exactly these factors and events permit the evolution and creation of new species.

Changes in Allelic Frequencies

• *Mutation*

Mutation is a permanent and hereditary random change of the DNA sequence thanks to which new alleles are created.

As mutation rates are very low, and its effect on alleles frequencies variations is usually considered negligible.

• *Random Genetic Drift*

Genetic drift refers to the component of the evolution of a population which is caused by random factors and determines a random modification on allele frequency. It is independent of adaptive forces and it is driven only by chance (Kimura, 1968).

Genetic drift has major impacts on the evolution of small populations in which the allele frequency is less stable in time and, after a few generations, its increase or decrease occurs in a random, unpredictable way. For instance, with a small number of individuals, the probability that a rare allele will be lost from that population is high, because it may not be passed from the parent generation to their offspring. In the long term, the main result of genetic drift is the loss of genetic variation, which may lead to negative outcomes, such as the loss of capability to adapt to environmental changes (Hartl et al., 1997). Consequently, in natural populations that for some reasons drastically reduce their demographic size, there will be the bottle-neck effect, with a subsequent deficit of heterozygotes (Nei, 1984) and so the reduction of genetic variability (Groombridge & Jenkins, 2000). Furthermore, genetic drift may genetically diversify one population from the others: this because different alleles will become, always by chance, more frequent or fixed, generating speciation events.

• *Migration*

Some populations are not completely isolated from other ones, so it is possible that a genes exchange takes place through migration of some individuals.

Migration may affect Hardy-Weinberg equilibrium because gene flow may introduce new alleles within the population, modifying original allelic frequencies. Exchange of alleles between distinct population results in the homogenization of genetic characteristic reducing differences between populations.

• *Inbreeding and Outbreeding*

Inbreeding is the preferential mating among relatives. the small populations suffer more from the effect of inbreeding because, although random, the mating occurs with a greater probability between relatives.

Inbreeding is estimated by Inbreeding coefficient "F_{IS}": the greater the F_{IS} value is, the greater is the reduction of heterozygosity compared to the expected value from Hardy-Weinberg equilibrium.

Outbreeding refers to mating between individuals from different populations or subspecies. Even outbreeding can result in fitness reduction known as outbreeding depression (less common than inbreeding), due to genetic incompatibility between genes from a different population.

Outbreeding depression is caused mainly by three mechanisms: chromosomal incompatibilities, adaptive differentiation between populations, and genetic drift (Ralls, Frankham, Ballou, 2013).

• *Natural Selection*

The natural selection is a force which leads an isolated population to genetically differentiate from the others. This adaptative process, contrary to genetic drift, is based on the differential reproductive success of the individuals within the populations: who is best to adapt to the environment and therefore has a higher fitness, survives and leaves through reproduction more descendants than others, so its alleles will increase in the population.

F-Statistics

Also known as fixation indices, F-statistics in population genetics describe the statistically expected degree of heterozygosity reduction in a population, when compared with Hardy-Weinberg equilibrium. This index was developed by Seawall Wright in 1940's as a tool to describe the partitioning of genetic diversity between and among populations, and how this diversity has a relationship with important evolutionary process like migration, genetic drift or mutation.

F-statistics include three fixation indices: F_{ST}, F_{IS} and F_{IT}.

- ***F_{ST}***: describe the correlation between gametes chosen randomly from the same subpopulation, relative to the entire population. If the index is small the allele frequencies among populations are similar; if it is large the allele frequencies are different.

$$F_{ST} = 1 - (H_s / H_T)$$

H_T is the expected Hardy-Weinberg heterozygosity if the entire population were panmictic, while H_s is the expected heterozygosity averaged over all subpopulation.

- ***F_{IS}***: is the correlation between gametes within an individual, relative to the subpopulation that which individual belongs. It may be positive in case of deficit of heterozygotes, or negative if there is an excess of heterozygotes.

$$F_{IS} = 1 - (H_0 / H_s)$$

H_0 is the observed heterozygosity averaged over all populations.

- ***F_{IT}***: correlation between gametes within an individual, relative to the entire population.

$$F_{IT} = 1 - (H_0 / H_T)$$

It is possible to relate these three indices with the following relationship:

$$F_{IT} = F_{IS} + F_{ST} - (F_{IS}) \times (F_{ST})$$

Molecular Markers

Molecular genetics is a useful tool to identify species, delineate geographic ranges when they feed or reproduce and, as noted above, manage stock or populations. The most important feature of genetic (or molecular) markers, DNA and/or RNA fragments, is that they comprise innate tags: in fact, all fishes are inherently marked.

The main features to identify a good molecular marker are: 1) Independent of environmental changes during an individual lifetime; 2) Composed of a discrete unit of information, so differences can be quantified; 3) Encoded in the universal language of DNA; 4) Measurable; 5) Analyzed with a statistical method to provide estimates of error (Antoniou & Magoulas, 2014).

At DNA level, a genetic marker is a short sequence of DNA, or a gene, with known location on chromosome and associated with a gene or trait (Al-Samarai & Al-Kazaz, 2015), used to identify species or find phylogenetic relationships between individuals.

This genes, or sequences, used as molecular markers may be distinguished in neutral or adaptive markers, relative to the effect they have on the survival of individual: a “neutral marker” is subjected to stochastic processes, like mutation, that have no effect on the survival of the organism who owns it; even “adaptive markers” may be suggested by stochastic events but, when this

happens, has an effect on the fitness of individuals (Mariani & Bekkevold, 2014). In the last two decades adaptive markers has acquired more attention than neutral ones, especially because they are involved in ecologic adaptation while other ones are poorly correlated with variation at ecologically relevant traits (Merila and Crnokrak, 2001).

Nevertheless, both neutral and adaptive markers have a significant role in stock identification and understanding mechanisms behind populations divergence (Utter & Seeb, 2010).

In this work, as molecular marker, we mainly consider Microsatellite DNA and Mitochondrial DNA. These markers differ each other in their mode of inheritance: microsatellite is nuclear DNA, so it is derived from both mother and father, and is subjected to recombination; mitochondrial DNA (mtDNA) is inherited only from mother, so there is no recombination.

Microsatellite DNA

These segments of nuclear DNA are also known as “Short Tandem Repeats” (STR) because of their characteristics: they are small (their length goes only from tens to hundreds base pairs), repetitive and non-coding DNA segments, and the repetitive region in constituted by two to six base pairs repeated. In genetic studies loci that are most commonly employed are the ones with two

(dinucleotides), three (trinucleotides), or four (tetranucleotides) base pair repeated. Number of repeats may be different between population but also within alleles of a single individual, because they are part of nuclear DNA so derived half from mother and half from father.

Microsatellite often evolve neutrally and accumulate mutation at higher rate than any other nuclear genomic region. This is the main reason, together with biparental inheritance and codominance, that made this little fragment of DNA a good marker of choice to investigate gene flow over short timescale.

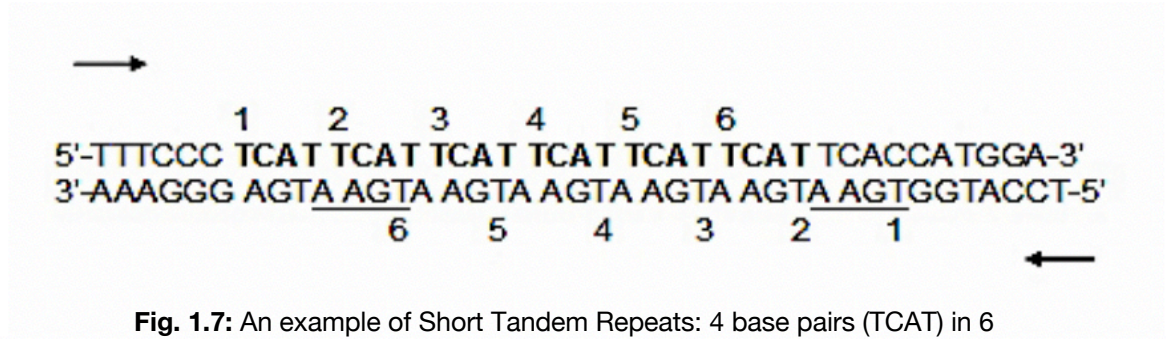


Fig. 1.7: An example of Short Tandem Repeats: 4 base pairs (TCAT) in 6 repeats. (Butler, 2005)

Thanks to modern molecular and bioinformatics technologies, analysis of STR it simple, and able to give vast and high-quality amount of information, isolating thousands of loci in a relative short amount of time.

Analysis begin with PCR amplification using a species-specific fluorescent primer, that sizes about 15-20 bp, annealed to the nonrepetitive region of the locus. After PCR, the obtained fragments are subjected to electrophoresis, and

their size is estimated using an automated sequencer: alleles with less tandem repeats are shorter, so migrate faster through the gel, the opposite is for alleles with high number of tandem repeats. Using bioinformatic programs it is possible to detect both the alleles (due to codominance): if the software shows only one peak it means the individual is homozygote for this locus; if two peaks are shown the individual is heterozygote instead.

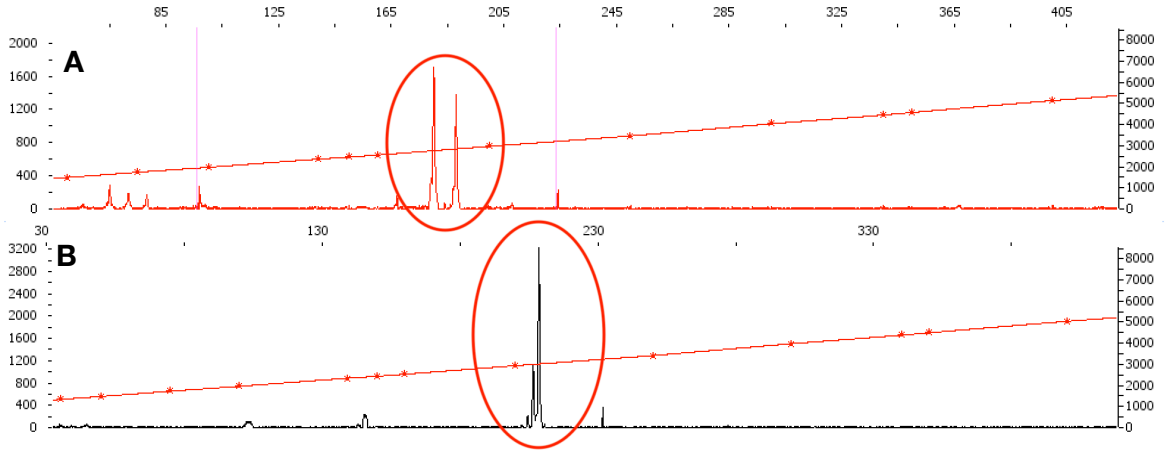


Fig. 1.8: example of result obtained with bioinformatic software PeakScanner: progressive numbers along the x-axis refer to the length of the fragments expressed in base pairs (bp), with the shorter fragments on the left and the longest to the right; Values on the y-axis express the amount of PCR product obtained for a given allele.
A, software results show the presence of two peaks, meaning that the locus is heterozygote; **B**, results show instead only one peak, so the locus is homozygote.

Even though the simplicity, this method can be affected by several errors and disturbances.

- Allele Stuttering: is the production of multiple peaks for the same alleles. Often it is the result of strand slippage during PCR. In case of homozygous the last peak generally corresponds to the true alleles, but heterozygotes can result difficult to score.
- Allele dropout: it occurs as a result of under-amplification of one allele (generally the larger of the two), due to PCR bias, low quality of template or low concentration of DNA.
- Null Alleles: due to the failed amplification of one allele, caused by failed amplification as result of mutation in primer region which prevent the primer to bind the site.
- Homoplasy: occurs when two alleles are identical not as a result of common descent but due to random mutation. This error may obscure the signal o population differentiation over long timescales, in loci with high mutation rates (Mariani & Bekkevold, 2014).

Mitocondrial DNA (mtDNA)

Mitochondrial DNA has been characterized as an “extreme example of genetic economy” (Attardi, 1985). This feature is due to the absence of introns, little repetitive DNA and no spacer sequences between genes. Moreover, the majority of gene product used by mitochondrion is encoded in nucleus.

The mtDNA in animals owns few stable genes: 13 genes coding for proteins, 2 genes for ribosomal RNAs (16S rRNA and 12S rRNA), 22 genes involved in transcription of transfer RNAs (tRNAs) and 1 non-coding control region (D-loop, in vertebrates, about 1000bp long) that includes the origin of mtDNA replication.

Only one type of mtDNA is included in animal cells, a condition called “homoplasmy”. Some cases of heteroplasmy are detected (more than only one type of mtDNAs), where the two mtDNAs differs in length as a result of tandem duplication. Mitochondrial DNA is only maternal inherited and characterized by a high mutation rate and high level of polymorphism and divergence, all features that give an important informative value for genetic population structure and inference of population history. This high mutation rate is a consequences of several factors including: the fact that mtDNA encodes few polypeptides or proteins involved in his replication transcription or translation, so it has less functional constrains; absence of histone proteins; subjected to a

strong oxidative stress and well provided in reparation system (Avise, 2009). This DNA continuously generates new alleles that persist mainly because of the low recombination rate.

Some regions, present in mtDNA, like cytochrome c oxidase subunit I or subunit II, *Cytb*, and other ones, are often involved in population genetic studies. In this work we focus on D-loop control region, in fact fragments like this are ones of the most effective tool to detect infraspecific genetic variation between stocks. The basic idea behind using mtDNA genes like D-loop for stock structure analysis is that if samples of individuals, taken from different areas or from the same area at different times, belong to the same stock, they will contain the same types of mtDNA molecules or haplotypes (mitotypes) in similar frequencies. Contrary, if substantial differences between samples are encountered, there is strong evidence that these samples belong to different stocks, with a certain degree of genetic isolation (Antoniou & Magoulas, 2014). One of the most used methodology to analyze mtDNA is RFLP (Restriction Fragment Length Polymorphism). It is based on amplification of DNA through PCR and then the digestion using specific endonuclease (restriction enzymes). These enzymes cut DNA at specific nucleotide regions called restriction sites.

Once the cut sequences are obtained it is possible to discriminate one type from each other through an electrophoretic run.

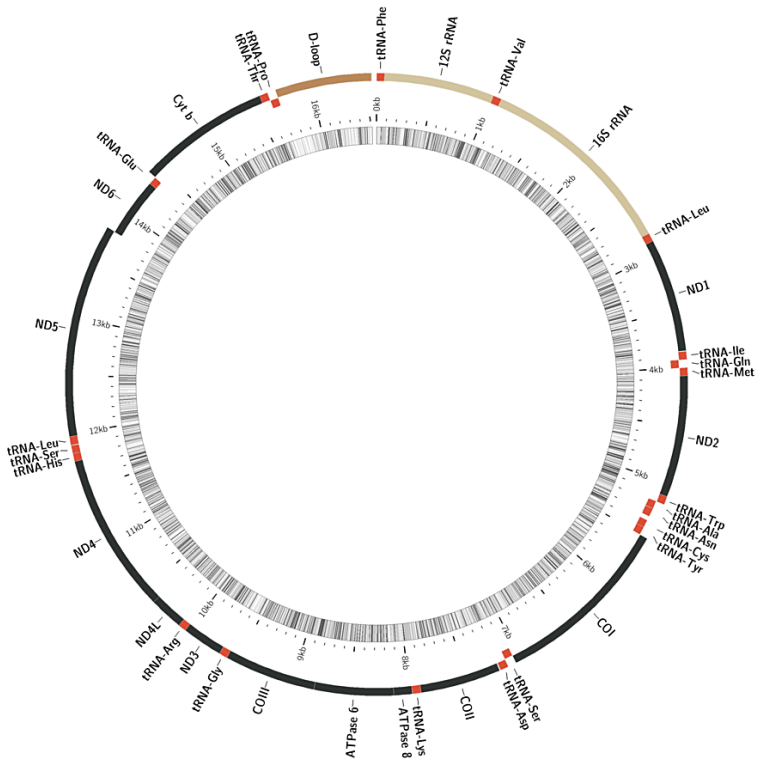


Fig.1.9: complete sequence of swordfish mtDNA. The D-loop is long about 1000 bp and cover the last fragment of the mitochondrial genome, including the origin of replication of mtDNA.

AIM OF THE STUDY

Swordfish is an important economical resource, primarily, but has also an important ecological value, that makes necessary doing more scientific studies about its biology distribution and exploitation, in order to best manage this resource and ensure its continuity over time.

The conservation status in Mediterranean Sea that, as already said, is more critical than in other ocean basins, together with a possible substructure of this population, as suggested by recent studies, may have important repercussions on its management and recovery from an over-exploited state.

Basing on this assumption, this study has the intent to evaluate the genetic variability of swordfish within Mediterranean Sea. Analysis based on molecular markers, nuclear DNA (Microsatellites) and mitochondrial DNA (D-loop), have been carried out to characterize the genetic structure of the species and evaluate the presence of possible stocks, with the aim of providing informations for the identification of correct management units and their conservation.

2.MATERIALS AND METHODS

Sampling

A total of 323 swordfish were collected during three years 2016-2018 from 6 Mediterranean locations and 1 Atlantic location (61 specimens from Sicily, 85 from Spain, 20 from Greece, 62 from the Adriatic Sea near "Fossa di Pomo", 16 from the Tyrrhenian Sea and, finally, 54 from Sardinia). Moreover 25 Atlantic swordfish were collected offshore Eastern Canadian coasts and including as a comparison. Sampling took place at the fishing landing of commercial longline or from trap by-catch (only in case of Sardinian samples). A piece of the caudal fin was collected from each specimen and was stored in ethanol absolute and kept at -20°C until DNA extraction.

DNA Extraction

Overnight digestion at 55ºC with enzyme proteinase K and a chaotropic salt was performed to lysis tissue cell and degrade proteins. Total genomic DNA was extracted using specific cartridge 401 in the *MagCore*® automated Nucleic Acid extractor (*MagCore* ®, *Genomic DNA Tissue Kit, n° 401*). This method uses cellulose coated magnetic beads to bind DNA. After washing off the contaminants, the purified DNA was eluted in 150 µl of pure water and stored

at -20ºC until quantification, performed with NanoDrop 2000 Spectrophotometer (ThermoScientific).

I. Microsatellite Analysis

After DNA extraction and quantification, twenty microsatellite loci were analyzed. In detail: D2A, D2B, C8 (Muths et al., 2009); XglSau-98R1, Xgl-14, Xgl-35, Xgl-65b, Xgl-74, Xgl-94, Xgl-106, Xgl-121, Xgl-148b, Xgl-523b, Xgl-561 (Kasapidis et al., 2009); Xg-56, Xg-66, Xg-144, Xg-166, Xg-394, Xg-402 (Reeb et al., 2003).

PCR amplification was performed combining the loci in multiplex reactions. A 5-plex with Xgl-65b, Xgl-74, Xgl-94, Xgl-121, Xgl-161; a 4-plex with XglSau-98R1, Xgl-523b, Xgl-148b, Xgl-14 and two duplex: the first with Xgl-35 and Xgl-106, and the latter with Xgl-394 and Xgl-402. The loci D2A, D2B, C8, Xgl-56, Xgl-66, Xgl-144, Xgl-166 were amplified singularly.

PCR reactions were performed in 10 µl volume for simplex and duplex and 15 µl for 4-plex and 5-plex; all these reactions contain genomic DNA (40 ng/µl), 3µl of 5X MyTaQ reaction buffer (BioLine), 0,8 µl of microsatellite primers (5 µM) and 0,5 µl of BIOTaQ DNA-polymerase (5 u/µl) (BioLine).

Forward primers were previously labelled with a different fluorescent dye (FAM, VIC, NED and PET) to distinguish different loci in the same capillary electrophoresis.

Locus	Repeat motif	Primer sequence (5'-3')	Fluorescent dye	T_a (°C)
Xgl-35	$(CA)_{13}$	F: TCAAGCCTTAGATGCAGCAG R: GTGATCCGCACACACTTCAG	NED	58
Xgl-121	$(GT)_6(GC)_5(GT)_6$	F: TCTTTAAGTCAGTGTAGCTGAGGAC R: TGGTAGAACAGCACGTAGCC	6-FAM	58
Xgl-561	$(CA)_6GA(CA)_7$	F: GCATGCTGGGAAGACAATTC R: AATCCTGCTCGTCTTTATAGG	VIC	58
Xgl-94	$(GGA)_8$	F: ATGACAGGCCTCCGATTAGC R: AATGCCAATGTCACCAATCC	6-FAM	58
Xgl-106	$(GA)_{10}$	F: GGAGAGGCAGACAGGAAATG R: TCCGGGTTGTATTTGAATCG	PET	58
Xgl-65b	$(CT)_{16}$	F: TGCACTGCATTCAACAGTGTC R: GCAAACCATCGTGTGAGTTG	6-FAM	58
Xgl-74	$(AGG)_7$	F: ATGATCACGTGTGGCTGATG R: GGATCCTCGCTCTCTGAGTC	VIC	58
Xg-Sau98R1	$(CA)_8$	F: CCACAATGTTTACGCTTCATC R: CGAATACTTTGTGTAGATGGCACA	NED	58
Xgl-523b	$(GA)_6AAGG$ $(GA)_6GC(GA)_8$	F: CAAACAAGAGAGCCGAGACAC R: TTTGGGAGGAGAGCTTAGTAGC	6-FAM	58
Xgl-14	$(CAT)_6CAC(CAT)_3$ $CAC(CAT)_4(CGT)_7$	F: AATGTCCGAGTTTGCCTCTC R: GACCCTTGGCTTCAATTCAC	6-FAM	58
Xg-148b	$(GGA)_8$	F: GACCCAGGGATTACTCAGACC R: TCCATCAACCTCTTCCTTGC	6-FAM	58
D2A	$(CCT)_6$	F: CAGTCGGGCGTCATCACTCAAACTGAGACTTTCCAAGTAATCCT R: GTTTCACTTCCAGCCAAACTCTTGTTCGT	NED	50
D2B	$(CAGT)_8$	F: CAGTCGGGCGTCATCAAGCAACAACATTGTCTTCTG R: GTTTCTGGCGTGAACGTGGCTCAATCC	PET	50
C8	$(CTAT)_{22}$	F: CAGTCGGGCGTCATCACCTTCAATGTAGGATGGCAGG R: GTTTCAAATGTCGGTGGAGCTGTGGACAGA	VIC	50
Xg-394	$(TCC)_9$	F: AGCGACAAACAGACCTGCCA R: GAGGAAACCGGGGCTTCTAC	6-FAM	66
Xg-402	$(TCC)_5+(CTT)_2$	F: GCGATTCAGGAGATTCCTAAC R: ATTAACTCGTCATTCAACGGC	6-FAM	66
Xg-56	$(CA)_{16}$	F: ATGGGAAACATCTGGTCAC R: ACTTTCTTATTCTGTTTCTGTCC	NED	53
Xg-66	$(CA)_{11}$	F: TTTTCACCTTGTCAGTGTGCG R: ACAGACGTATACAACCACCTG	PET	53
Xg-144	$(GGA)_7$	F: TTCCACTCATACCTCTGTCATC R: ACCACATCCATTATAGCATGTTG	PET	53
Xg-166	$(CAA)_7$	F: GTGAGTCATGTGTCAGTGTGG R: CCTCTGCCTGAAATACTTCAG	VIC	53

Tab 2.1: List of Microsatellite used with corresponding repeat motif, primer, fluorescent dye and annealing temperature.

The PCR reaction protocol consist in a touch down program, which increases the PCR reaction specificity and minimized the formation of incomplete amplicons, which can cause potential scoring errors.

PCR reaction protocol:

Initial denaturation 92°C for 5 min.

10 cycles:

Denaturation 92°C for 20 s

Annealing (See Table 2.1 for Ta) for 30 s: with each cycle the temperature decreased by 0.5°C

Extension 72°C for 45 s

25 cycles:

Denaturation 90°C for 30 s

Annealing (Ta-5°C) for 50 s

Extension 72°C for 55 s

Final extension 72°C for 5 min

The resulting amplifications were checked with a 2% agarose gel electrophoretic run.

Genotyping was performed by BMR genomics, through capillary electrophoresis using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystem®).

Microsatellite Scoring and Binning

Genotyping allows visualizing each fragment as a peak when it migrates through a detector sending a laser beam. Thanks to the fluorescent primer we can distinguish different loci and its alleles.

The microsatellite scoring aims to determine the size of these alleles, it was performed using the software Peak Scanner 2 (Applied Biosystems) where peaks are visualized in a graph with the electrophoresis migration time in the abscissa and the laser intensity signal in the ordinate. Microsatellites were sized with a GS 500LIZ_3130 size standard.

The scoring process has provided allele sizes with two decimals, caused by not only the fragment length but also the GC content and imperfect linear regression between size standard and PCR product run length (Amos et al., 2007). However, to process data, software that analyzes microsatellite

sequences require length values expressed in integer numbers, and it is made possible with a method called “binning” that round up allele sizes values.

Binning was performed with software FLEXIBIN v2 (Amos et al., 2007) that estimates bin number as relative repeat numbers.

Finally, to check for potentially genotyping errors, like null alleles, large allele dropout and scoring of stutter peak, for each locus was used the software MICRO-CHECKER (ver. 2.02.0003) (Van Oosterhout et al., 2004).

Genetic Diversity Analysis

Genetic diversity has been evaluated through different software:

- FSTAT 2.9.3 (Goudet, 2001) was used to check the number of alleles at each locus (NA), observed (Ho) and expected (H_E) heterozygotes and allelic richness (AR). This software was also used to evaluate global F_{ST} of Weir & Cockerham, 1984.
- ARLEQUIN 3.5.1.3 (Excoffier & Lischer, 2010), because of its implemented exact test from Guo & Thompson (1992), was used in order to statistically evaluate the test of Hardy-Weinberg equilibrium and linkage disequilibrium. Also, the significance of the test was evaluated, using the Markov chain method (1000 batches and 10000 interaction per batch).

Population differentiation was evaluated by calculating pairwise F_{ST} values both between samples and cluster, detected by clusters analysis.

- GENEPOP (Raymond, 1995) used to calculate F_{IS} values (Weir & Cockerham, 1984) in order to verify if the departure from Hardy-Weinberg equilibrium was due to a deficit or surplus of heterozygotes. Significance levels were adjusted using the Bonferroni correction (Rice, 1989).
- BOTTLENECK (Cornuet & Luikart, 1996) was used to evaluate the presence of recent nuclear genetic bottleneck events in Mediterranean swordfish. Bottleneck events reduce allelic diversity faster than heterozygosity, causing populations to display an excess of heterozygotes at a greater number of microsatellite loci that predicted by chance until mutation-drift model (Cornuet & Luikart, 1996). Heterozygotes excess was tested using Wilcoxon signed rank test, and it was performed with the infinite allele model (IAM), stepwise-mutation model (SMM), and two-phase microsatellite evolution model (TPM) with 95% IAM and 5% SMM. With the same software, it was calculated the loss of low-frequency alleles in populations following recent bottleneck, that can also a mode-shift from the expected L-shaped distribution frequency of microsatellite alleles (Luikart et al., 1998).

Bayesian Analysis with STRUCTURE

The software STRUCTURE 2.3.4 (Pritchard et al., 2000) uses iterative computation process to infer the most likely number of population (K) represented in the total sample. The software is a model-based clustering method using multilocus genotype data to infer population structure and assign individuals to populations. The method can be applied to various type of markers, but it assumes that the marker loci are unlinked and at linkage equilibrium with one another within populations. It also assumes Hardy-Weinberg equilibrium within populations (Pritchard et al., 2000). Each individual is assumed to originate in one of K populations, each with a characteristic set of allele frequencies at each locus. (Pritchard et al., 2000). In a matrix each individual is assigned a membership coefficient (q) for each cluster: this q value denotes the admixture proportions for each individual, and represents the probability that an individual or a fraction of its genome has membership in a cluster, which can be used to infer ancestry of individuals (Jakobsson & Rosenberg, 2007).

In this study, the model was set to consider individuals under mixed ancestry (admixture model) and allele frequencies were assumed to be correlated between samples (correlated model). The analysis was based on 10 serial runs

for each number of clusters (k) between 1 and 8. All analyses were run for 5×10^5 generations after a burn-in of 10×10^4 generations.

Subsequently, using the software STRUCTURE HARVESTER (Earl & VonHoldt, 2012) it was possible to quickly summarize output data from STRUCTURE. The most likely number of clusters was estimated using Evanno method (Evanno et al., 2005), a statistic Δk based on the rate of change in log probability of the data among successive K values tested.

Discriminant Analysis of Principal Components

Genetic structure of analyzed samples was also evaluated through the Discriminant Analysis of Principal Components (DAPC).

PCA has been suggested as an alternative to Bayesian clustering algorithms to identify genetic structures in very large datasets with negligible computational time in order to provide assignment of individuals to groups, a visual assessment of between-population differentiation, and contribution of individual alleles to population structuring (Jombart et al., 2010). When group priors are lacking, DAPC uses sequential K-means and model selection to infer genetic clusters. K-means relies on the same model as DA to partition genetic variation into a between-group and a within-group component and attempts to find groups that minimize the latter (Jombart et al., 2010).

DAPC, contrary to STRUCTURE, does not require linkage disequilibrium and Hardy-Weinberg equilibrium moreover, DAPC is more sensitive in detecting genetic differences when F_{ST} value was lower than 0,05.

The package *adegenet* (Jombart 2008) implemented in the R, (RStudio-Team 2015) was used to execute DAPC analysis. This analysis was also performed both with and without prior information on individual populations. The optimal number of clusters (k) was evaluated with the function *find.cluster* and selected based on the lowest Bayesian Information Criterion (BIC). The interpopulation level of gene flow among the clusters identified by the DAPC was quantified by estimating pair-wise and global *F*ST values as a weighted average over 18 loci in ARLEQUIN v.3.5, where statistical significance was assessed following 10,000 permutations.

II. Mitochondrial-DNA analysis (D-loop)

A PCR-RFLP-SSCP analysis was performed to screen mitochondrial genetic variability. 360 bp of the first domain of the mitochondrial control region was amplified using primers L15998 (5'-TAC CCC AAA CTC CCA AAG CTA-3') (Alvarado Bremer 1994) and SWO 5' CCC TGT GAA ATA TGC TGG TTG 3' (designed in this study).

The 25 µl reaction mix was prepared with 5 µl of Master Mix 5X (BioLine), 0,3 µl of BIOTAQ DNA-Polymerase 5u/µl (BioLine), 2 µl of DNA 40 ng/µl and 4 µl of Primer Mix 5 µM.

PCR run was performed with the following parameters:

Initial denaturation 94°C for 5 min

35 cycles:

Denaturation 94°C for 45 s

Annealing 50°C for 30 s

Extension 72°C for 60 s

Extension 72°C for 5 min.

Finally, PCR product was checked through a 2% agarose gel.

RFLP Analysis

Double digestion was performed in order to distinguish specimens between Clade I and Clade II. Two endonucleases were used: *Pac1* a six-cutter

(AT/TAAT) and *Vspl* an eight-cutter (TTTAAT/TAA). Digestion mix reaction was carried out in a volume of 20 µl: 2 µl of Buffer G 10X (ThermoScientific), 4 µl of amplified DNA 40 ng/µl and 0,3 µl of each endonuclease (*Pac1* and *Vspl*) 10 u/µl.

The digestion process was performed in a thermocycler at the temperature of 37 ºC, overnight.

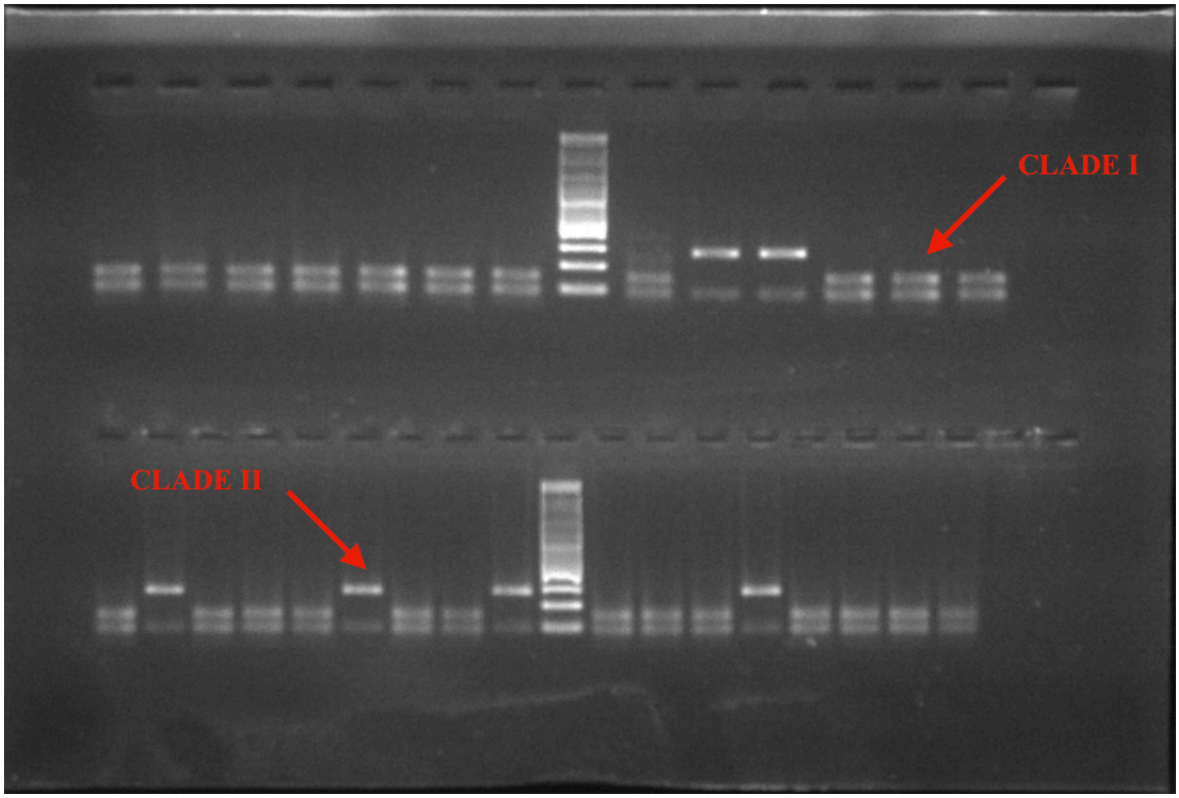


Fig. 2.1: PCR-RFLP Analysis: restriction profiles produced by the digestion of D-loop with the two enzymes, *Pac1* and *Vspl.*

Single Strand Conformation Polymorphism (SSCP) Analysis

Haplotypes identification was carried out thanks to SSCP (single strand conformation polymorphism) analysis, an electrophoretic run through non-denaturing polyacrylamide gel, already tested in several studies (Rehbein 1999, Céspedes 1999). In this study, the polyacrylamide gel electrophoresis run was carried out with the BIO-RAD Sequi-Gen® GT apparatus.

The SSCP analysis separates DNA fragment basing on the secondary structure, instead of the fragment length: in non-denaturing condition single-stranded DNA has a folded structure (secondary conformation) that is determined by nucleotide sequence (primary conformation) (Hayashi, 1992).

5 µl of each amplicon was added to 4 µl loading buffer (98% formamide, 10 mM, EDTA (0,5 M, pH 8), 0.05% bromphenol blue, 0.05% xylene cyanol), heated to 95 °C for 5 min and immediately chilled on ice. Vertical electrophoresis run was performed in a non-denaturing polyacrylamide gel (8% acrylamide/polyacrylamide [49:1], 10% glycerol) at 5 W for 18 hours at room temperature, with 1 × TBE as the running buffer.

After the electrophoretic run, the gel was colored through SILVER STAINING protocol, as follows:

1. Fixation, in 10% absolute ethanol and 0,5% acetic acid solution, for 5 minutes;
2. Impregnation, through 1 Liter solution with 1,5 g $AgNO_3$ (silver nitrate) and 1,5 ml HCOH (formaldehyde), for 7 minutes;
3. Development, in 1 Liter solution with 1 ml HCOH and 15 g NaOH (sodium hydroxide);
4. Second fixation, with the same solution used in step 1, for 2-3 minutes.

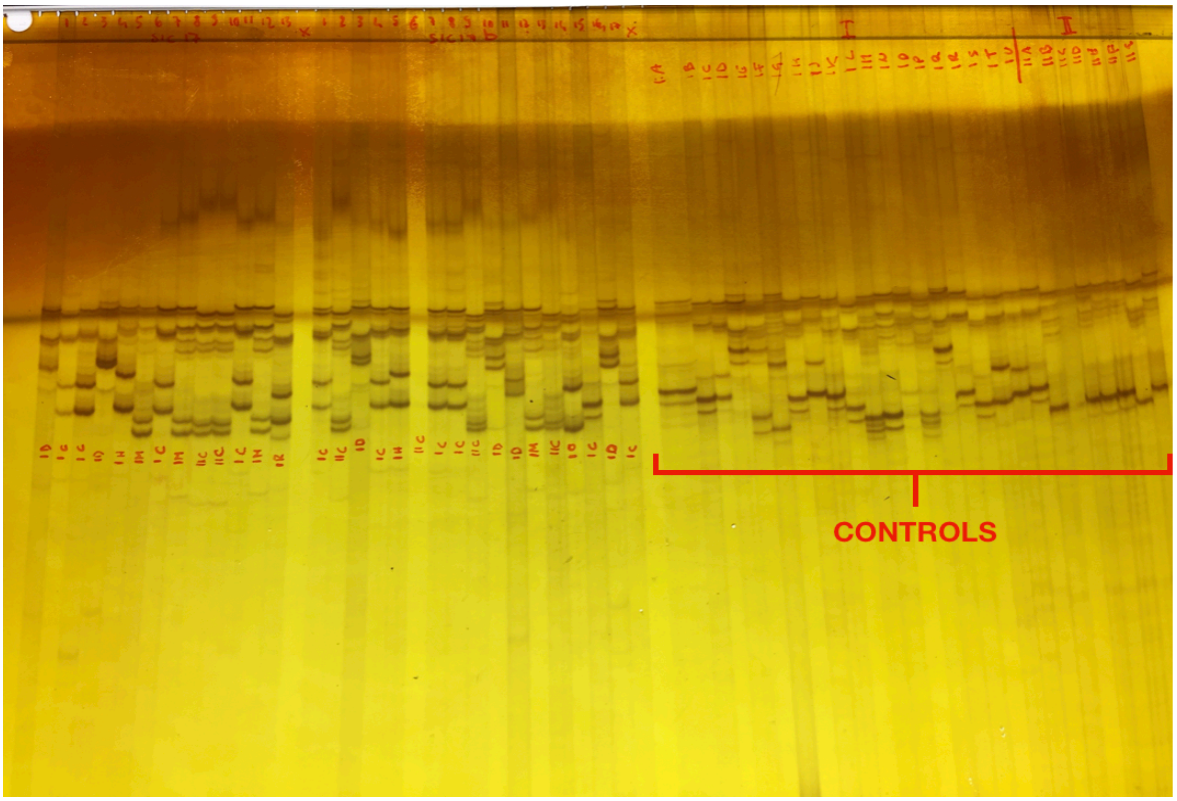


Fig. 2.2: SSCP analysis result: every column is a morph belonging to a sample: same morph, same secondary structure of the DNA fragment

After the silver staining different SSCP profiles (morphs) different morphs were recognized, which correspond to different secondary structures then different mtDNA haplotypes (Figure 2.2).

Sanger sequencing of *D-loop* was performed on a subsample for each SSCP pattern on an Applied Biosystems ABI 3730XL DNA sequencer.

To exclude that the observed haplotypic diversity for Clade II, lower compared to those reported in the literature, could be imputed underestimation of SSCP analysis, all specimens belonging to CLADE II was sequenced. Sequences obtained were compared with sequences on GenBank to identify haplotype already observed or never seen before.

Statistical Analysis

Sequences were aligned using ClustalW (Thompson et al. 1997) and optimized by eye in BIOEDIT (Hall, 1999). “TATA” repetitions were considered as transversion. Two phylogenetic trees, with and without Canadian samples, were build using software MEGA7. The evolutionary history was inferred using the Neighbor-joining method (Saitou & Nei, 1987). The Bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of morphs analyzed (Felsenstein, 1985). As substitution model was used Maximum Composite Likelihood Method. Gaps or missing

were considered setting pairwise deletions option. The cut-off value for the consensus tree was set to 70%. Relationships between haplotypes were estimated using Median-joining (MJ) networks built using NETWORK v4.510 (Bandelt et al., 1999), including haplotypes observed in the Mediterranean Sea by Viñas (2010).

Intra and inter-population variability were estimated incorporating all lineages (pooled Clades I and II), and for each clade separately.

Genetic diversity within the locality and DAPC genetic clusters were estimated by computing haplotype diversity (h) and nucleotide diversity (π) in ARLEQUIN v. 3.5.2. Haplotype diversity represents the probability that two randomly sampled alleles are different, while nucleotide diversity is defined as the average number of nucleotide differences per site in pairwise comparisons among DNA sequences (Nei, 1987).

An analysis of molecular variance, AMOVA (Excofier *et. al.*, 1992), was used to test several hypotheses of population structure swordfish incorporating all lineages (pooled Clades I and II), and for each clade separately. Genetic differentiation was calculated 1) between Mediterranean localities; 2) considering three groups: East Mediterranean, West Mediterranean and Canadian; 3) considering two groups: all the Mediterranean samples and all the Canadian samples; and 4) between genetic cluster detected by DAPC analysis.

Significance levels were determined by 10000 permutations. Finally, pairwise F_{ST} was calculated both among localities and DAPC clusters using the default setting.

3.RESULTS

3.1. Microsatellite Loci

Twenty microsatellite loci were amplified for 323 swordfish samples, with a PCR failure per locus ranged between 0 and of 5.9% with an average of 1.15%. Significant deviations from Hardy-Weinberg equilibrium were detected in 18 out of 140 single locus exact tests at loci Xgl94, Xgl74, Xgl14, Xg66 and Xg166. All deviations were towards heterozygote deficit. MICROCHECKER identified these loci as potentially exhibiting null alleles with the estimated frequencies of null alleles > 0.3 for Xgl74 and Xg66. These loci deviated in mostly population including Canadian sample thus, these loci were removed from further analysis. Much rarer null alleles at Xgl94, Xgl14 and Xg166 were detected via MICROCHECKER at low frequency. Genotype data were retained since population differentiation parameters are not biased by the presence of null allele. In fact, the estimation of FST both using and without using the ENA correction method gave equal results; FST = 0.018 with the respective 95% CI [0.011–0.027]. No consistent evidence for linkage disequilibrium was detected between pairs of loci within populations.

All the remaining 18 loci were polymorphic, with the number of alleles per locus ranging from two, at locus Xg-402, to 21, at locus C8. Both Xg-402 (two alleles) and Xg-394 (three alleles) were monomorphic in three samples GRE SPA and CAN. Mediterranean samples exhibited a significantly lower number of alleles per locus, allelic richness, and expected heterozygosity, compared to Atlantic one. No evidence of geographical pattern was observed for the distribution of genetic diversity among Mediterranean samples. The inbreeding coefficient, F_{IS}, ranged from 0.03 to 0.12 and was highly significant in all samples but two (GRE, TIR).

In the test for a recent bottleneck event, significant heterozygote excess was detected in all samples only considering the IAM mutational model may correspond to a bottleneck signal. However, it is controversial to what extent the IAM model faithfully describes the process of microsatellite mutation (Spong & Hellborg, 2002). On the contrary, all tests performed with SMM and TPM did not produce significant results. Moreover, there was no evidence for a significant mode-shift in microsatellite allele frequencies in all samples, suggesting the population is at mutation-drift equilibrium for these microsatellite loci.

Genetics Structure

The global F_{ST} over all loci detected a great signal of genetic differentiation between the whole Mediterranean and Atlantic samples (F_{ST} = 0.091; 95 % C.I. 0.056-0.133). F_{ST} value drop considering Mediterranean samples separately (F_{ST} = 0.018; 95 % C.I. 0.011-0.027). Pairwise F_{ST} across all samples ranged from –0.005 to 0.097. The lower and no statistically significant values were detected between Mediterranean samples, whereas the highest and significant outcomes were observed in comparison among Atlantic sample.

Two discrete genetic clusters have been revealed by Bayesian clustering analysis implemented in the program STRUCTURE supported by the ΔK score. All Mediterranean swordfish were grouped together and separated from Atlantic ones with a high individual assignment score to the specific cluster (Fig. 3.1).

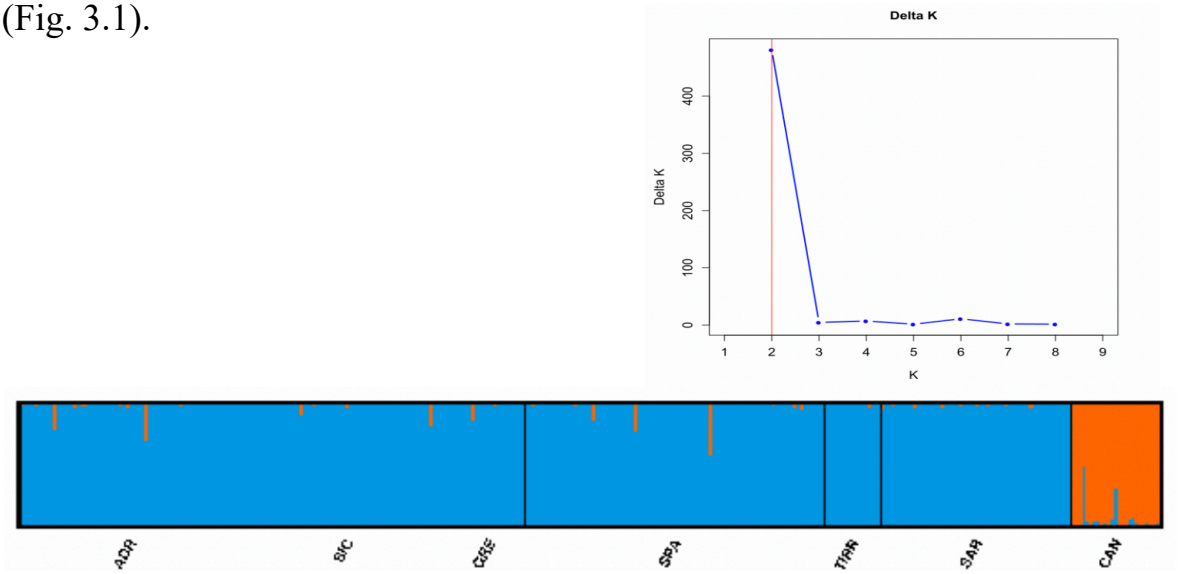


Fig 3.1: Structure bar plot showing the assignment probabilities (K = 2) of each genotyped individual of swordfish from the different sampling locations in the Mediterranean Sea and NW-Atlantic Ocean. Above is reported Delta K graph.

Also, in the DAPC analysis, assuming the sampling locality as *a priori* groups, the Atlantic sample was distinct from the Mediterranean locality, closely related to each other. On the contrary, *a posteriori* assignment of DAPC analysis deviated from the preview results, identifying four genetic clusters. Specifically, the first principal component emphasized the genetic difference among Canadian swordfish (Cluster 2) and all Mediterranean samples, while the second and third principal component split the Mediterranean swordfish into three genetic clusters (1, 3 and 4) (Fig.3.2). Each cluster was composed of individuals from every Mediterranean sampled area. The cluster 1 represented 38.9 % of the Mediterranean dataset, cluster 3 and 4 represented 24.2 % and 36.9 % respectively. Multi-locus pairwise F_{ST} among clusters, detected with DAPC analysis, showed F_{ST} values ranged from 0.044 to 0.130 and all tests showed a high statistical significance (Tab. 3.1).

	Cluster 1	**Cluster 3**	**Cluster 4**
Cluster 1			
Cluster 3	0.06168***		
Cluster 4	0.04424***	0.05896***	
Cluster 2	0.09943***	0.10223***	0.13044***

Tab. 3.1: Pairwise F_{ST} estimated for 18 microsatellite loci between genetic clusters identify by DAPC analyses, Values with "***" are statistically significant (p-value<0,001).

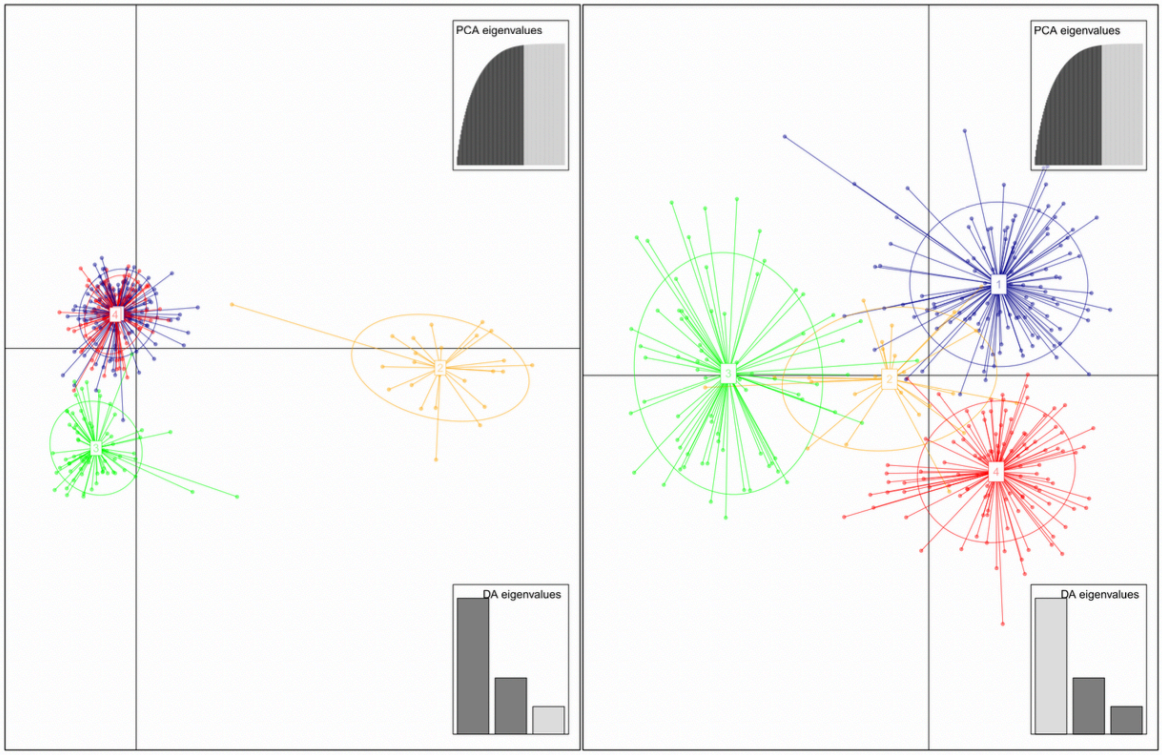


Fig. 3.2: Discriminant analysis of principal components (DAPC) scatterplot of the best number of genetic cluster identified by *find cluster* function in *adegenet* package. The proportion of principal component eigenvalues retained as predictors for linear discriminant analysis (DA; bottom right corner) is highlighted in dark grey.

3.2.Mitochondrial DNA Results

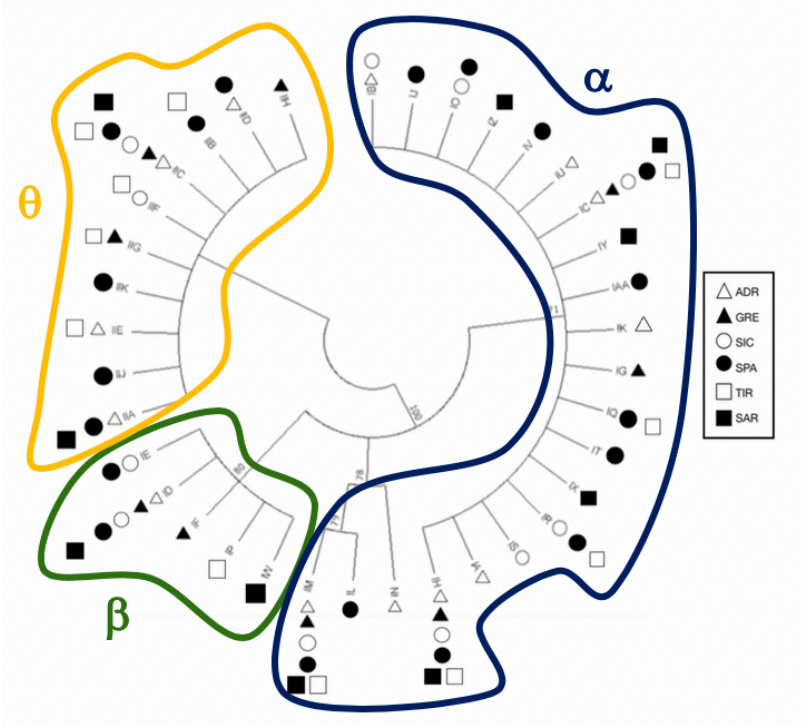


Fig 3.3: Neighbor-Joining tree containing the Mediterranean haplotypes. Locality where haplotypes were observed were represented by different symbols. Haplotypes have been also subdivided in the three subgroups observed in Alvarado-Bremer (1996): blue, α subgroup; green, β subgroup; yellow, θ subgroup.

SSCP analysis of ca 300 bp portion of mtDNA D-loop from 311 swordfish revealed 59 different morphs. For each morph, a subsample has been sequenced for a total of 197 swordfish. Sequence comparison of 298 bp of the mtDNA CR-I revealed 61 polymorphic sites, 35 of which were parsimony informative. These polymorphisms defined 59 distinct haplotypes (see Fig. 3.6), 36 for Mediterranean swordfish and 23 for Atlantic ones. Twenty-four of 59 haplotypes were already observed in previous studies (see Table 3.7), while 35

were never observed before. No haplotype was shared between Mediterranean and Atlantic samples.

According to Alvarado-Bremer (1996) the mtDNA haplotypes of swordfish clustered into two highly divergent clades, Clade I and Clade II. In particular, 48 of the haplotypes observed (81,4%) belong to Clade I, and 11 (18,6%) belong to Clade II. The haplotype frequencies for the pooled Mediterranean locations were 66,4% for Clade I and 33,6 % for Clade II, while the haplotypic frequencies observed in Canadian was 96% for Clade I and 4% for Clade II. Moreover, Clade I may be divided into two subgroups “Alpha” and “Beta”, the first characterized by the presence of RsaI restriction site (Alvarado-Bremer, 1996). Alpha lineage represented the 45,1% of Mediterranean swordfish and grouped 21 haplotypes, while the Beta represented the 21,3 % including 5 haplotypes (ID, IE, IF, IP, IW). The remaining 33,4% of Mediterranean swordfish showed haplotypes belonging to Clade II (10 haplotypes). In the Canadian sample, 96% of swordfish showed haplotypes belonging to Alpha lineage (including 22 haplotypes) and the 4% belong to the Clade II (only one specimen). No haplotype belongs to Beta lineage was found in Canadian swordfish.

Comparison Among Locality

Analysis of haplotypic frequencies within localities showed that the 48,4% of swordfish in the Adriatic Sea was represented by Alpha lineage, the 16,1% by Beta and 35,5% by Clade II; the Sicilian sample (Alpha 50,9%, Beta 27,1% and Clade II 22%); the Greece sample (Alpha 50%, Beta 31,2% and Clade II 18,8%); the Spanish sample (Alpha 45,2%, Beta 17,8% and Clade II 37%); the Sardinian sample (Alpha 32,6%, Beta 28,6% and Clade II 38,8%); the Tyrrhenian sample (Alpha 43,7%, Beta 6,3% and Clade II 50%). Significant differences in Clade I (Alpha and Beta lineage) and Clade II frequencies between pooled Mediterranean samples and Canadian were detected (χ^2 test, p = 0.000). On the contrary, no significant differences have been found among Mediterranean locations (χ^2 test, p = 0.191).

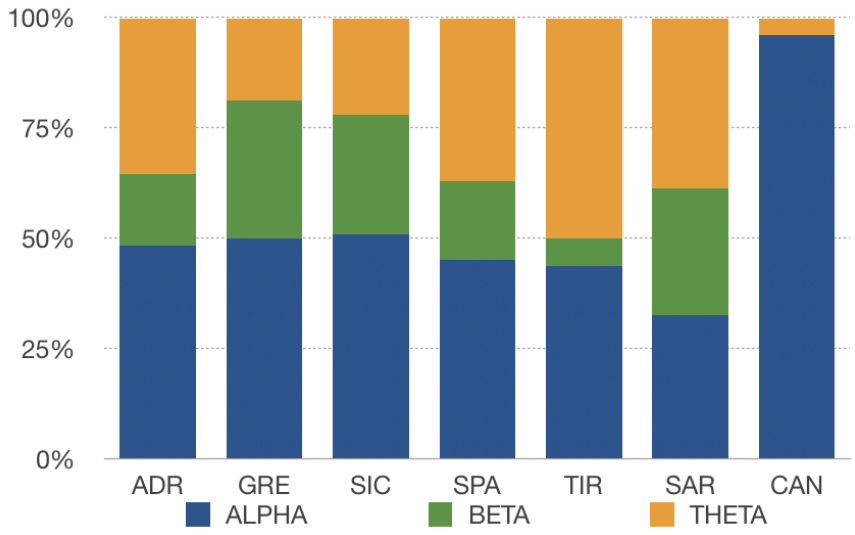


Fig. 3.4: Percentage of the two Clade in sampled localities. Clade I ALPHA (blue), BETA (green), and Clade II (yellow).

The average haplotypic diversity of Mediterranean Sea (*h*= 0,858 s.d. 0,05) was lower than those estimated for Canadian Sample (*h*= 0,993). Mediterranean haplotype diversity ranged from *h*= 0,950 in Tyrrhenian sample to *h*= 0,788 in Sardinian sample. In contrast, the levels of nucleotide diversity were similar throughout the Mediterranean and also similar to the Canadian sample (Table 3.2). When data for the two clades were analyzed separately, a high level of variability has been observed between locality for Clade II in comparison to Clade I. The haplotypic diversity for Clade I ranged from *h*= 0,893 (Tyrrhenian sample) to *h*= 0,740 (Sardinian sample) with a mean value of 0,811 (s.d. 0.05). Haplotypic diversity for Clade II, instead, showed high variability between location ranged from *h*= 0,893 in Tyrrhenian sample *h*= 0,154 in Sicilian sample with a mean value of 0,464 (s.d. 0,28). In table 3.2 are shown all values in detail.

Comparison Among Genetic Clusters Detected with Microsatellites

A comparison among genetic clusters detected by DAPC showed that the values of nucleotide diversity were uniform both pooling the two Clades together and analyzing them separately. The highest values of haplotypic diversity for both Clades has been registered in Cluster 2, corresponding to the

Canadian sample. Within the Mediterranean the largest values of haplotypic diversity for both Clades were observed in Cluster 1 (Clade I h = 0,826; Clade II h = 0,544), followed by the Cluster 4 (Clade I h = 0,799; Clade II h = 0,424) and the Cluster 3 (Clade I h = 0,766; Clade II h = 0,271). In table 3.3 are shown all values in detail.

Locality	N	H	S	h (s.d.)	π (s.d.)
ADR	62	13	36	0,853 (0,022)	0,041 (0,021)
Clade I	40	9	19	0,803 (0,036)	0,017 (0,009)
Clade II	22	4	4	0,463 (0,120)	0,002 (0,002)
GRE	16	8	34	0,883 (0,052)	0,037 (0,020)
Clade I	13	6	17	0,833 (0,071)	0,021 (0,012)
Clade II	3	2	1	0,667 (0,314)	0,003 (0,003)
SIC	59	11	31	0,819 (0,025)	0,036 (0,019)
Clade I	46	9	16	0,764 (0,039)	0,018 (0,009)
Clade II	13	2	1	0,154 (0,126)	0,0006 (0,0009)
SPA	84	21	41	0,855 (0,023)	0,042 (0,021)
Clade I	53	14	22	0,835 (0,031)	0,018 (0,009)
Clade II	31	7	7	0,404 (0,111)	0,002 (0,002)
TIR	16	11	30	0,950 (0,036)	0,043 (0,023)
Clade I	8	6	13	0,893 (0,111)	0,013 (0,008)
Clade II	8	5	5	0,893 (0,086)	0,007 (0,005)
SAR	49	11	33	0,788 (0,03)	0,042 (0,021)
Clade I	30	8	17	0,740 (0,056)	0,018 (0,010)
Clade II	19	3	3	0,205 (0,119)	0,0003 (0,0007)
CAN	25	23	61	0,993 (0,013)	0,032 (0,017)
Clade I	24	22	51	0,993 (0,014)	0,029 (0,015)
Clade II	1	1	-	-	-

Tab. 3.2: Table reported the number of the sample (N), number of haplotypes observed (H), polymorphic site (S), haplotype (h) and nucleotide (π) grouping individual according to sampling locality.

DAPC Cluster	*N*	*H*	*S*	*h* (s.d.)	*π* (s.d.)
Cluster 1	109	27	40	0,876 (0,017)	0,039 (0,020)
Clade I	78	18	21	0,826 (0,028)	0,018 (0,009)
Clade II	31	9	10	0,544 (0,107)	0,003 (0,002)
Cluster 2	25	23	61	0,993 (0,013)	0,032 (0,017)
Clade I	24	22	51	0,993 (0,014)	0,029 (0,015)
Clade II	1	1	-	-	-
Cluster 3	70	15	36	0,822 (0,025)	0,039 (0,020)
Clade I	49	11	20	0,766 (0,043)	0,016 (0,009)
Clade II	21	4	4	0,271 (0,124)	0,001 (0,001)
Cluster 4	102	19	42	0,830 (0,021)	0,042 (0,021)
Clade I	61	12	22	0,0779 (0,032)	0,018 (0,009)
Clade II	41	7	7	0,424 (0,094)	0,002 (0.002)

Tab. 3.3: Table reported the number of the sample (N), the number of haplotypes observed (H), polymorphic site (S), haplotype (h) and nucleotide (π) grouping individual according to DAPC result.

The MJ network (Fig. 3.5) clearly identified the two swordfish mtDNA clades. Clade I included four centroids (morphs IC, ID, IH, IM) represented respectively by 61, 55, 14 and 25 fish, while Clade II is characterized by a single star-like formation featuring one major centroid (morph IIC) with a total of 73 individuals. These five haplotypes have been observed with high frequencies in all Mediterranean localities. The other Mediterranean haplotypes were represented in very few numbers of the individual often representing singletons.

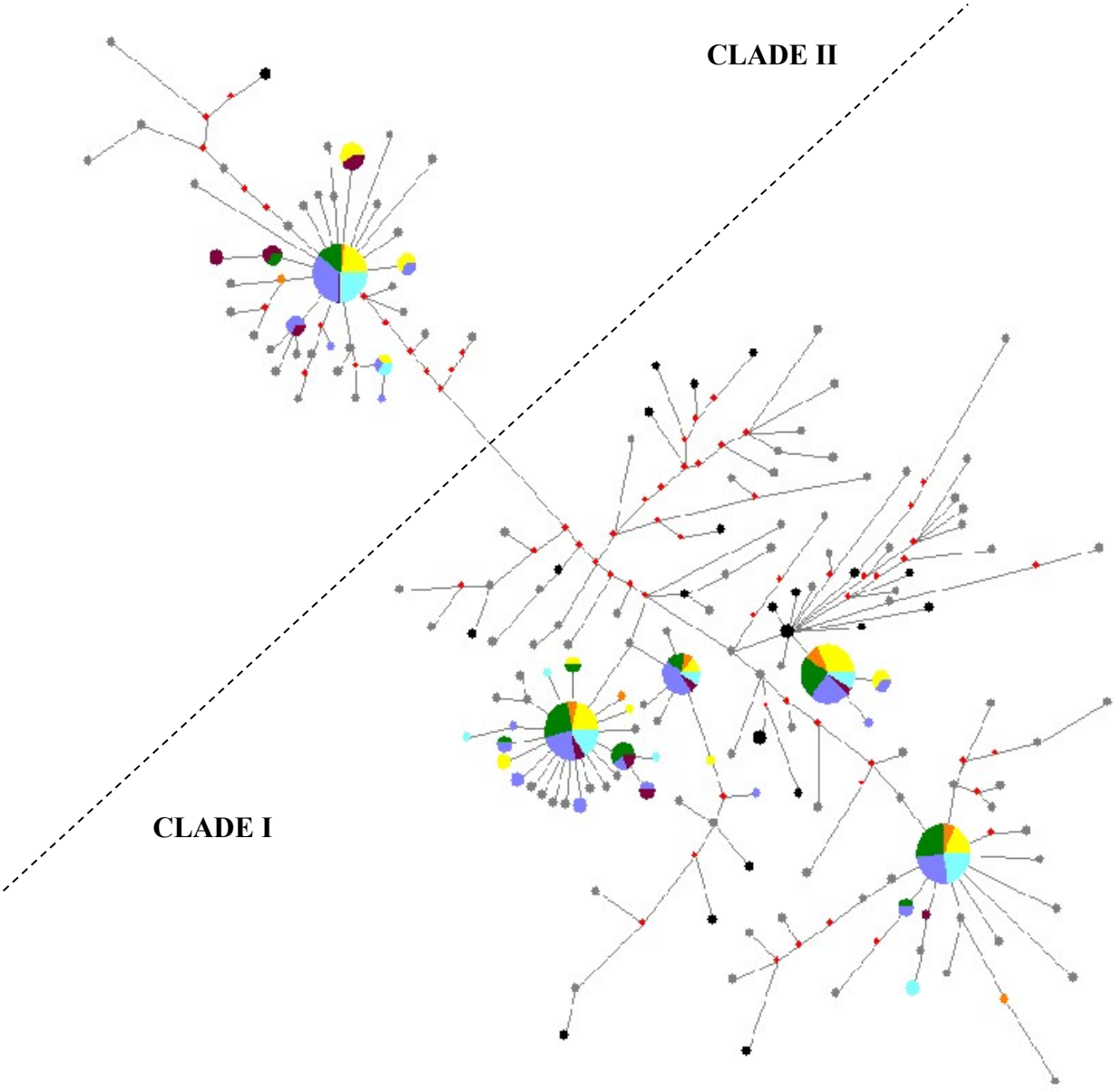


Fig. 3.5: Median-Joining network of haplotypes detected in this study. Colours identify different localities: ADR (yellow), GRE (orange), SIC (light-blue), SPA (blue), TIR (Bordeaux), SAR (green) and CAN (black). Grey dots represented haplotype observed in Mediterranean swordfish by Viñas (2010) but not observed in this study.

Population Structure

Results from AMOVA analysis revealed statistically significant genetic structure between Atlantic and Mediterranean swordfish. Despite the overall genetic variation within populations (> 90 %) was much larger than the variation among populations (8,87%), F_{ST} resulted to be significant (FST = 0.09, p = 0.002). The AMOVA testing within Mediterranean population structure, instead, failed to identify genetic differentiation among locality using complete sequences set and only Clade I. On the other hand, analyses considering Clade II revealed a significant proportion of variation among Mediterranean localities (F_{ST} = 0.087, p = 0.0009).

Pairwise F_{ST} values were all not significant between Mediterranean localities showing a very low F_{ST} value (Table 3.5), while the comparison with Canadian sample were all significant, with F_{ST} value ranged from 0,27 to 0,11.

Same results were obtained from AMOVA and pairwise F_{ST} estimation among genetic cluster identified by DAPC analysis. Significant and high value were observed only in comparison among Cluster 2 (Canadian swordfish) and the other ones (Table 3.6).

Source of variation	Percentage of variation	Fixation index
Populations set according to sampling localities		
Whole dataset, one gene pools (only Mediterranean swordfish)		
Among group	0,22	ΦST=0.002
Within populations	99,78	
Clade I, one gene pools (only Mediterranean swordfish)		
Among group	-0,2	ΦST=-0.02
Within populations	100,2	
Clade II, one gene pools (only Mediterranean swordfish)		
Among group	8,73	ΦST=0.087***
Within populations	91,27	
Whole dataset, two gene pools (Atlantic vs. the Mediterranean)		
Among group	8,87	ΦSC=0.002
Among population within group	0,17	ΦCT=0.09
Within populations	90,96	ΦST=0.089***
Populations set according to DAPC result, two gene pools (Atlantic vs. the Mediterranean)		
Among group	8,73	ΦSC=0.001
Among population within group	0,1	ΦCT=0.087
Within populations	91,17	ΦST=0.088***

Tab. 3.4: AMOVA analysis results of Mediterranean swordfish populations, among Mediterranean and Atlantic populations and among genetic clusters detected by DAPC analyses. Values with "***" are statistically significant (p-value<0,05).

	ADR	GRE	SIC	SPA	TIR	SAR
ADR						
GRE	-0.02614					
SIC	-0.00091	-0.01249				
SPA	0.00420	0.00521	0.00020			
TIR	0.03385	0.00985	0.01478	0.05305		
SAR	0.00394	-0.00547	-0.00542	-0.00361	0.02111	
CAN	0.09935***	0.05972***	0.08087***	0.11493***	0.02771***	0.08064***

Tab. 3.5: Pairwise FST analysis results among sampling locality. Values with "***" are statistically significant (p-value<0,05).

	Cluster 1	Cluster 3	Cluster 4
Cluster 1			
Cluster 3	0.00263		
Cluster 4	0.00100	0.00012	
Cluster 2	0.06992***	0.09799***	0.09542***

Tab. 3.6: *1 Pairwise FST analysis results among genetic clusters detect by DAPC analyses. Values with "***" are statistically significant (p-value<0,05).*

MORPH	LENGTH (bp)	ID (GenBank)	AUTHOR
IC	293	AY650768.1	Alvarado-Bremer, 2005
ID	292	AY650763.1	Alvarado-Bremer, 2005
IF	296	AY650855	Alvarado-Bremer, 2005
IH	293	AY650778	Alvarado-Bremer, 2005
IJ	293	EU827759	Vinas, 2010
IM	293	AY650781.1	Alvarado-Bremer, 2005
IQ	293	AY650821.1	Alvarado-Bremer, 2005
IR	293	AY650836.1	Alvarado-Bremer, 2005
IS	293	AY650860.1	Alvarado-Bremer, 2005
IW	292	AY650861	Alvarado-Bremer, 2005
IZ	293	AY650858	Alvarado-Bremer, 2005
IIA	290	AY650809	Alvarado-Bremer, 2005
IIB	290	AY650805	Alvarado-Bremer, 2005
IIC	290	AY650762	Alvarado-Bremer, 2005
IID	290	AY650814	Alvarado-Bremer, 2005
IIE	290	EU827771.1	Vinas, 2010
IIF	290	EU827787.1	Vinas, 2010
IIG	290	AY650829.1	Alvarado-Bremer, 2005
IIH	290	AY650761.1	Alvarado-Bremer, 2005
IcanE	293	AY650780.1	Alvarado-Bremer, 2005
IcanG	291	AY961269.1	Alvarado-Bremer, 2005
IcanJ	284	AY961143.1	Alvarado-Bremer, 2005
IcanK	293	AY650754.1	Alvarado-Bremer, 2005
IcanV	293	AY961357.1	Alvarado-Bremer, 2005

Tab. 3.7: list of the morphs already observed in previous studies.

4.DISCUSSION

This study aimed to evaluate genetic structure of the swordfish, *Xiphias gladius*, within the Mediterranean Sea comparing information obtained by nuclear and mitochondrial molecular markers. Several works that have described the global genetic structure of swordfish included Mediterranean samples merely as reference samples. These studies were conducted considering single geographic areas (Bremer et al. 1995, 1996, Rosel & Block 1996, Smith et al. 2015) or grouping the samples together (Bremer et al. 2005, Kotoulas et al. 2007). Few studies were focused on the intra-Mediterranean swordfish population structure reaching contrasting results. Kotoulas et al. (2002) shown, basing on mtDNA analysis, how the whole Mediterranean Sea can be viewed as one genetically homogeneous population but less variable compared with the Atlantic population. The same tendencies were observed by Pujolar et al. (2002) using allozyme analysis, that obtained, for Mediterranean population, low genetic variability results, and attributing them to the small effective population size. In contrast, Viñas et al. (2010) analyzing mtDNA, found a genetic differentiation between eastern and western populations. This differentiation was detected when phylogenetic analysis is conducted separately for Clade I and Clade II. Our analysis of mtDNA haplotypes revealed

no genetic differentiation within the Mediterranean Sea, while microsatellite revealed signals of a moderate genetic structure with statistically significant differences between three genetic clusters within the basin. So, while mtDNA analysis show no evidence of geographical genetic pattern and a high level of mixture between Mediterranean samples, microsatellite, instead, reject the hypothesis of a unique and homogeneous Mediterranean swordfish stock.

Mitochondrial Control Region Analysis

Among the 311 samples analyzed, the observed overall haplotypic diversity (*h*) for Mediterranean samples (0,842) is quietly comparable with which ones observed in previous studies like Viñas (2010) where *h*=0,948, or Alvarado-Bremer (2005) (*h*=0,973), and greater than that observed in Kotoulas (1995) (*h*=0,721). For Canadian samples instead the haplotypic diversity is 0,993, perfectly comparable with other previous studies like Alvarado-Bremer (1997, 1995, 2005), where *h* is around 0,990. The number of haplotypes observed in this study (36 in total; 26 Clade I and 10 Clade II) was extremely lower compared with previous works (Alvarado Bremer et al. 2005; Viñas et al., 2010) where the analysis of the same mitochondrial portion (from 275 and 251 Mediterranean swordfish respectively) shown 74 (48 Clade I, 26 Clade II) and 93 (60 Clade I, 33 Clade II) haplotypes. Moreover, in our study the five main

haplotypes showed frequencies way much higher than the ones presented in the above mentioned works. The unbalanced frequencies between haplotypes resulted in low level of haplotypic diversity, especially for Clade II. The comparison between two temporal samples highlighted a loss of mtDNA genetic diversity during only two decades. No evidences of bottleneck event were detected by the microsatellites, however, given the different mode of inheritance of nuclear and mitochondrial loci, we would expect these effects to be stronger for mtDNA than nuclear DNA loci.

Overexploitation can drive the decay of genetic diversity of marine species (Allendorf et al. 2008, Pinsky & Palumbi, 2014). Reduction of genetic diversity may lead to a long term impact on evolutionary potential and adaptive ability of the species, particularly if abundance remains low and diversity continues to decay (Pinsky & Palumbi, 2014).

The degree to which diversity was reduced depends on many factors, including initial effective population size, the length of time and the degree to which effective population size is reduced, and the amount of gene flow (Frankham *et al.* 2002; Allendorf *et al.* 2008; Flight, 2010). Basing on the current knowledge about Mediterranean swordfish we cannot exclude that reduction in haplotypes diversity observed in this work may be ascribed to overexploitation. Mediterranean swordfish stock status assessment (ICCAT, 2018), in fact,

indicate that the stock is overfished throughout the whole period from 1985 and, currently, is subjected to overfishing. Recruitment showed a declining trend in the last decade, while stock biomass remains stable at low levels that are about 1/3 of that in the mid-1980s. Despite in the last six years (2012-2017), following the implementation of the three-month fishery closure and the establishment of the list of authorized vessels, overall fishing effort has been decreased and catches are around 10,000 t, the SSB less than 15% of B_{MSY} and F is almost twice the estimated F_{MSY}. Furthermore, Mediterranean swordfish population is characterized by low effective population size (Koutulas et al, 2007), and no gene flow occurs between Atlantic and Mediterranean stocks (Alvarado Bremer et al. 2005).

Population Structure

Swordfish is a highly migratory species. In the Atlantic Ocean, it is able to cover annually long distances by horizontal movements as reported by pop-up satellite archival tags analysis (Neilson et al. 2014, Abascal et al. 2015). The same ability was also observed within the Mediterranean Sea (Canese et al. 2008). Accordingly, results of both microsatellite and mitochondrial makers did not reveal a spatial genetic segregation among the six Mediterranean localities. This result was in line with previous studies based on allozymes data

(Pujolar et al. 2002), RFLPs of the entire mtDNA (Kotoulas et al. 2007), analyses of a single-copy nuclear calmodulin gene and PCR–RFLP data of the mtDNA CR (Chow & Takeyama 2000). However, sample homogeneity does not necessarily equate to population homogeneity (Ward 2000) and population differentiation may be obscured by population mixture in wintering or feeding areas, especially for highly migratory species (Van Wagner & Baker 1990, Bowen et al. 1992, Wenink et al. 1993, Bremer et al. 2005a).

Population Structure Inferred by Nuclear Markers

STRUCTURE analyses clearly detected genetic differentiation among NW-Atlantic and Mediterranean swordfish populations but failed to find signals of genetic structure within Mediterranean. On the contrary DAPC analysis indicates the presence of three Mediterranean genetic clusters distinct from a fourth group formed by Atlantic swordfish. High level of mixing of these three clusters was observed in all Mediterranean sampling locality. This observation is coherent with a significant excess of homozygotes genotypes observed. Deficit in heterozygotes has often been reported for both marine invertebrates (Zouros & Foltz 1984) and fish (Waldman & McKinnon 1993, Lundy et al. 1999, Maggio et al. 2009) and big pelagic fish such as swordfish (Kotoulas et al. 2007, Muths et al. 2009). In case of an explicit exclusion of the technical

issues (genotyping errors or not appropriate sample size), heterozygotes deficit may be originated when the sample analyzed is composed by a mix of distinct subpopulations (Wahlund effect), as would be expected by highly migratory and structured species. The genetic structure revealed by DAPC was also confirmed by a highly significant multi-locus pairwise FST among the above clusters. The inconsistency between STRUCTURE and DAPC results was due to that the latter is more suitable to unravel the underlying structuring in more complex population genetic models and generally performs better than Bayesian clustering methods when $F_{ST}<0.05$ (Jombart et al. 2010; Latch et al. 2006).

Philopatric behaviour has been identified as the driving force behind structuring of very high migratory pelagic fish *Istiompax indica* (Williams et al. 2016), *Gadus morhua* (Bonanomi et al. 2016) and *Thunnus thynnus* (Rooker et al. 2008, Aranda et al. 2013). As for swordfish, spawning site fidelity is supported by both maximum levels of genetic differentiation obtained comparing separated breeding areas in the Atlantic Ocean (Bremer et al. 2005, 2007). Evidence of strong seasonal site fidelity was also reported into the Mediterranean Sea, thanks to recapturing of tagged individuals, that generally occurred in the same area of tagging also after several years (Garibaldi & Lanteri 2017). Within the Mediterranean Sea, three main spawning areas were

described. The first is located in the western Mediterranean, from the Strait of Gibraltar up to the Balearic Islands; the second one extends from the Strait of Messina to the Gulf of Taranto in the Ionian Sea (Cavallaro et al. 1991, Arocha 2007), and the last one in the Levantine Sea close to Rhodes and Cyprus islands (Tserpes et al. 2001, 2008). Geographical localization of these three discrete spawning areas may explain the three genetic clusters observed in this study, suggesting a more complex scenario compared to population structuring between western and eastern basins as proposed by Viñas (2010). Rejection of a model of panmixia in a relatively small sea basin is not new for large pelagic species (Carlsson et al 2004; Riccioni et al. 2010; Davies et al, 2011).

Population Structure Inferred by Mitochondrial Markers

AMOVA analysis among localities have identify a genetic structure between Atlantic and Mediterranean samples but failed to identify it within Mediterranean basin samples, even when dataset was analyzed both among populations and clusters (detected through DAPC analysis) point of view. Result was in line with the absence of a genetic geographic pattern as observed from microsatellite results. Several other studies have also found greater population differentiation using nuclear than mitochondrial markers (Kim *et al*.

1998; Wilmer *et al*. 1999; Piertney *et al*. 2000; Wirth & Bernatchez 2001). The ability of microsatellite markers to resolve population structure more accurately than mtDNA has been attributed to the rapid mutation rate of microsatellites (DeWoody & Avise 2000; Selkoe & Toonen 2006) and the power of multilocus approaches (Waples 1998). In relation of different modes of evolution, microsatellite markers narrate the phylogenetic history of tens of thousands years while mitochondrial markers shed light on the deeper phylogenetic history (Emerson, 2005). Isolation of Mediterranean swordfish from the Atlantic swordfish has been estimated to occur during the peaks of at least the last two glaciations (Alvarado Bremer et al. 2005). Thus, genetic structure among Atlantic and Mediterranean populations was detected by both molecular markers. In contrast, only microsatellite reach to reveal signals of a more recent genetic differentiation within the Mediterranean Sea.

Conclusion

The results obtained from this thesis work has shown two main outcomes. The comparison between two different molecular markers (microsatellites and D-loop) confirmed the high degree of differentiation between the Atlantic and Mediterranean swordfish stocks, but conflicting results were obtained in the identification of a population structure within the Mediterranean Sea. On one

hand, the microsatellites (nuclear markers) have shown signals of a possible, though weak, structuring in three distinct genetic clusters, and a high level of mixing between these populations in the whole basin. On the contrary, genetic homogeneity was evidenced by mitochondrial DNA analysis. A possible explanation of this discrepancy can be attributed to the different evolution rate of the two markers. The second important outcome was the identification of potential decay of genetic diversity during the last 3 decades, thanks to comparison among temporal samples (data collected from literature). In agreement with the latest stock assessment, we cannot exclude that overexploitation may play a role in this loss of genetic diversity, coupled to the low Mediterranean swordfish population size and no migration from Atlantic population. Given the economically and ecologically importance of this species, other studies should be carried out to improve the knowledge about Mediterranean swordfish and ensure a more sustainable fisheries.

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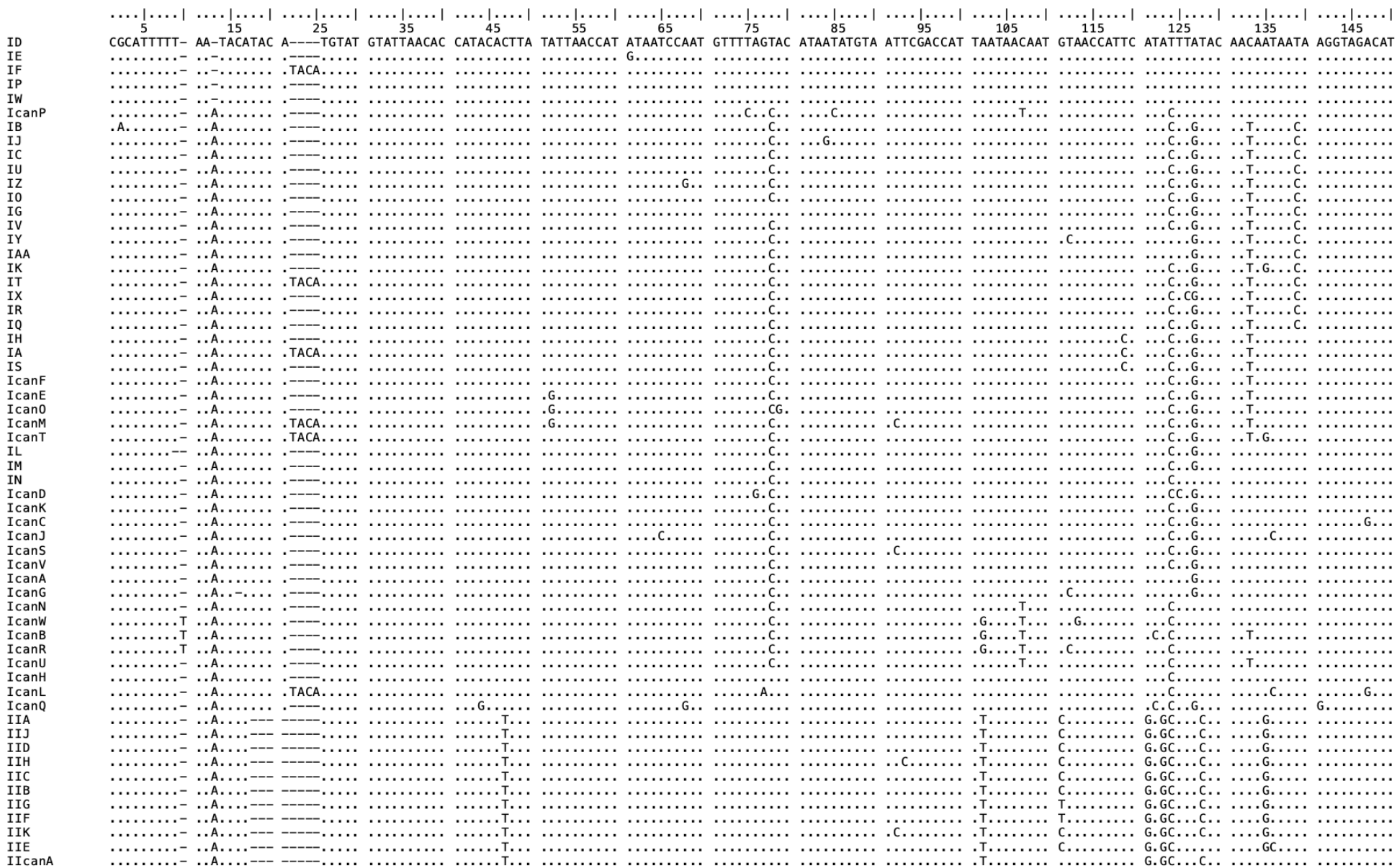

Fig. 3.6: sequences alignment of all haplotypes (continues in next page).