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Wide-scale population genomics of Atlantic bluefin tuna (*Thunnus thynnus*) inferred by novel high-throughput technology

Presentata da: Dott.ssa Eleonora Pintus

Coordinatore Dottorato | **Relatore**

Prof. Enrico Dinelli | **Prof. Fausto Tinti**

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INDEX

ABSTRACT..5

CHAPTER 1..9

GBYP PROJECT

1.1 State of world fisheries..9

1.2 Aim of the project..13

1.3 SNP...14

CHAPTER 2..19

TARGET SPECIES: ATLANTIC BLUEFIN TUNA

2.1 Taxonomy and...19

2.2 Geographic distribution, habitat and ecology.......................................20

2.3 Reproduction and spawning...25

2.4 Movement and stock structure..27

CHAPTER 3..32

STATE OF THE ART

3.1 Fishery genetics...32

3.2 Fishery and management of Atlantic bluefin tuna................................36

3.2.1 Fishery...36

3.2.2 Management..39

3.3 Population genetic studies of Atlantic bluefin tuna ……………………..……..44

3.4 Research aims…………………………………………………………………………..47

CHAPTER 4……………………………………………………………………………………….49

HIGH-THROUGHPUT SNP DEVELOPMENT IN ATLANTIC BLUEFIN TUNA USING A COMBINED GDNA AND CDNA SEQUENCING STRATEGY

CHAPTER 5...92

ASSESSING THE ACCURACY AND POWER OF SNPS MARKERS FOR POPULATION GENETICS, INDIVIDUAL ASSIGNMENT AND MIXTURE STOCK ANALYSIS IN *THUNNUS THYNNUS*

CHAPTER 6...141

CONCLUSIONS

REFERENCES..143

AKNOWLEDGEMENTS 161

ABSTRACT

Il mio progetto di dottorato è focalizzato sul tonno rosso, *Thunnus thynnus*, appartenente all'ordine dei Perciformes, e alla famiglia degli Scombridae. Questa specie, distribuita nell'Oceano Atlantico settentrionale e centrale e nel Mar Mediterraneo, presenta due principali aree di riproduzione (il Golfo del Messico per lo stock occidentale e il Mar Mediterraneo per quello orientale) e compie ampie migrazioni transatlantiche tra le zone di alimentazione e quelle di riproduzione, mostrando alta fedeltà alle zone di nascita, dove torna per riprodursi (natal homing). Benché il tonno rosso sia stato pescato in modo continuativo nel Mar Mediterraneo per migliaia di anni, questa specie ha subito un forte incremento dello sfruttamento negli ultimi decenni, a causa del miglioramento delle tecniche di pesca, dello sviluppo del mercato giapponese e della nascita delle tuna farm. Si è infatti passati da una pesca di tipo artigianale ad una di tipo industriale, raggiungendo livelli che secondo alcune recenti valutazioni del WWF non consentirebbero la sostenibilità della risorsa. Questo sta portando a rischio di collasso la pesca e gli stock, tanto che il comitato scientifico ICCAT (Commissione Internazionale per la Gestione del Tonno Atlantico) ha avviato, attraverso un regolamento comunitario, un piano quindicinale per il ripristino dello stock (CE N.643/2007). Il mio progetto di ricerca si inserisce all'interno del progetto ICCAT-GBYP 06/2011 (Atlantic-wide Bluefin Tuna Research Program), sviluppato in collaborazione con diversi partner italiani e stranieri, in cui ci si è avvalsi di metodiche molecolari innovative come le nuove tecnologie genomiche, Next Generation Sequencing (NGS). Sono stati sviluppati e utilizzati marcatori SNPs (Single Nucleotide Polymorphisms) legati o inclusi a geni espressi che, potenzialmente soggetti a processi di selezione, possono permettere di studiare i meccanismi di adattamento delle popolazioni ai cambiamenti delle condizioni ambientali, al prelievo, all'inquinamento ed ad altri disturbi antropici.

Il primo step della ricerca ha visto la costruzione di librerie di cDNA specifiche per dieci individui rappresentativi del polimorfismo interspecifico nel Mediterraneo e nell'Atlantico (4 provenienti dal Golfo del Messico, 3 dal Mediterrraneo Occidentale e 3 da quello Orientale). La scelta dei campioni è stata fatta valutando i requisiti necessari per il sequenziamento 454 (come quantità e qualità dell' RNA totale, ricchezza in mRNA). Queste librerie sono state ottenute mediante retrotrascrizione di mRNA isolato da tessuto

muscolare, e il sequenziamento è stato condotto mediante tecnica di pirosequenziamento implementata dalla tecnologia 454. Queste librerie sono state successivamente purificate e filtrate per eliminare trascritti mitocondriali e ribosomiali, vettori e adapter. Oltre all'utilizzo del trascrittoma, è stata utilizzata anche la risorsa genomica per costruire una sequenza di riferimento (dato che il tonno rosso non è una specie modello e quindi non si hanno informazioni relative al suo genoma in banche dati), partendo da 4 individui provenienti dalle due principali regioni dell'areale del tonno rosso (2 dal Golfo del Messico e 2 dalle Baleari). Il sequenziamento è stato condotto avvalendosi di uno strumento di ultima generazione, l' HiSeq 2000 dell'Illumina. Una volta ottenuto questo genoma di riferimento, tutte le cDNA reads, derivate dal trascrittoma, sono state mappate contro tale genoma, e, utilizzando diversi software bioinformatici e diversi parametri restrittivi, è stato ottenuto un pool di 4000 contigs, usato come riferimento per la successiva fase di SNP detection. Mappando nuovamente le cDNA reads contro questi 4000 contigs selezionati, sono stati identificati 5412 SNPs candidati, in 1350 contigs.

A questo punto è stato necessario validare gli SNPs identificati, per essere sicuri che non fossero dovuti ad errori di sequenziamento, in modo tale da ottenere il pannello definitivo dei 384 SNPs rispondenti ai criteri di selezione *in silico*. Per fare ciò sono stati applicati diversi criteri, 2 dei quali richiesti dalla piattaforma Illumina che verrà utilizzata per la genotipizzazione, che sono la presenza di una regione fiancheggiante lo SNP di almeno 60bp e un Illumina ADT score (Assay Design Tool) > 0,6. In aggiunta a questi parametri, sono stati scelti SNPs che presentano il polimorfismo anche a livello genomico (in modo tale da avere sovrapposizione di informazioni tra cDNA e gDNA) e che, a livello del cDNA, siano presenti in almeno in un individuo con una minima copertura (4 reads presenti in quella data posizione, 2 delle quali portanti l'allele alternativo).

Il pannello di 384 SNP così ottenuto è stato genotipizzato in 960 individui di diversa taglia (larve, age 0, juveniles, medium e large), campionati lungo l'intero range di distribuzione del tonno rosso (Golfo del Messico, Nord-Est Atlantico, Mediterraneo occidentale, centrale e orientale). Il campionamento è stato effettuato principalmente nel corso del 2011, ma sono state aggiunte alle analisi anche diverse repliche temporali, in modo da ottenere un ampio dataset composto da 23 campioni di popolazione. Sei di questi sono stati identificati come campioni di riferimento, in quanto costituiti da larve e age 0, per le quali quindi si è certi dell'origine geografica e della diretta correlazione con le unità

riproduttive. Sono stati utilizzati 40 individui per ogni "strata" di tonno rosso, campione definito dalla combinazione della taglia e dell'area di provenienza, e il DNA genomico di tutti gli individui è stato estratto dal tessuto muscolare, dalla pinna o direttamente dalle larve, e successivamente è stato sottoposto ad un controllo qualitativo e quantitativo. Tutti gli individui sono stati genotipizzati mediante il saggio Illumina Golden Gate Assay e i risultati ottenuti sono stati visualizzati e analizzati mediante un software specifico. Per ottenere una selezione di loci e di individui rappresentativi e idonei alle analisi successive, è stato effettuato un accurato controllo qualitativo, mirato ad eliminare gli SNPs non funzionanti e monomorfici e gli individui non genotipizzati per almeno l'80% dei loci disponibili. Si è così raggiunto un dataset finale costituito da 848 individui e 287 SNPs.

Una volta completata la genotipizzazione, è iniziata l'analisi dei dati ottenuti, finalizzata a valutare la diversità genetica e la struttura di popolazione nel tonno rosso. Sono stati calcolati quindi i principali indici di diversità genetica, come le frequenze alleliche, l'eterozigosità attesa e osservata, la percentuale di loci polimorfici e l'indice di fissazione; sono stati inoltre valutati sia la deviazione dall'equilibrio di Hardy Weinberg che il linkage disequilibrium. Sono stati effettuati successivamente studi sulla struttura di popolazione attraverso il calcolo degli F_{ST}, per valutare la distanza genetica mediante un confronto tra coppie di popolazioni. Le analisi sono state condotte sia utilizzando l'intero pannello di SNPs che un pannello ridotto di loci che presentano indici di differenziamento sopra la soglia dello 0,1%, per riuscire ad avere un maggior potere risolutivo e riuscire a individuare un segnale di differenziazione genetica tra i campioni analizzati. Inoltre la distanza genetica tra i campioni è stata testata attraverso la PCoA, Principal Coordinate Analysis, condotta con il pannello selezionato di SNPs, e sono state anche effettuate analisi filogeografiche per valutare le relazioni tra i campioni esaminati. Tutte queste analisi sono state eseguite sia sulle 23 popolazioni che sulle 6 popolazioni di riferimento. Continuando ad avvalersi dei due pannelli di SNPs e dei due dataset di popolazioni, lo studio è stato approfondito tramite la DAPC (Discriminant Analysis of Principal Components) e utilizzando un approccio Bayesiano, per valutare la presenza di diversi gruppi all'interno dei nostri campioni, non ottenendo però chiare evidenze di struttura genetica. Un debole segnale di differenziazione è stato trovato soltanto nell'analisi condotta utilizzando le 6 popolazioni di riferimento e il pannello ristretto di loci, suggerisce la presenza di 3 cluster genetici corrispondenti alle tre possibili aree di riproduzione del tonno rosso (Golfo del Messico, Mediterraneo occidentale e orientale).

Infine, utilizzando il pannello ristretto di loci, sono stati assegnati tutti gli individui del nostro dataset alle grandi aree riproduttive del Mediterraneo e del Golfo del Messico, non ottenendo però un assegnamento con alti valori di significatività statistica, ma un'indicazione di un maggiore contributo del mar Mediterraneo alle popolazioni adulte. Si è cercato anche di individuare i loci outlier, che, potenzialmente sotto selezione divergente, possono essere in correlazione con le variabili ambientali. Le analisi, condotte con due diversi software, non hanno però prodotto nessun risultato, mettendo in luce l'assenza di loci potenzialmente sotto selezione, dato che si riflette anche nell'assenza di marcata differenziazione genetica.

La mia attività di ricerca ha portato quindi allo sviluppo di risorse genomiche e trascrittomiche per il tonno rosso e alla identificazione e genotipizzazione di un ampio pannello di marcatori SNPs. Attraverso lo studio condotto si è ottenuto un segnale di basso differenziamento nelle popolazioni riproduttrici, associato alla mancanza di struttura genetica tra le popolazioni adulte campionate, portando ad ipotizzare la presenza di una popolazione panmittica nel Mediterraneo e non una strutturazione in meta popolazioni distinte come suggerito dagli studi precedenti.

CHAPTER 1

GBYP PROJECT

1.1 STATE OF WORLD FISHERIES

Sea fishing is a productive old reality characterized by a globally strong complexity which makes it particularly difficult to manage, requiring a multidisciplinary and often multinational approach. The marine biotic resources are classified as potentially renewable, however renewable resources can run out if the rate of exploitation exceeds the rate at which they are regenerated by natural processes. Fishing takes part in natural balance of fish populations, that, in the absence of withdrawal, depends exclusively on the biological properties of the populations and the characteristics of the environment in which they live. Over-exploitation of the fish resource may affect its ability to regenerate and therefore the possibility of using it in the future. Thus, it's necessary to reconcile the expansion of human activities with the need not to alter the natural asset, using the resources in a balanced way without affecting their availability for future generations and maintaining the exploitation at sustainable levels.

Data provided by the Food and Agriculture Organization of the United Nations (FAO), which monitors the state of world fisheries, showed that global capture fisheries supplied the world with about 90.4 million tons of fish in 2011 although there have been some considerable changes in catch trends by country, fishing area and species. World fish food supply has grown dramatically in the last five decades, with an average growth rate of 3.2% per year in the period 1961-2009.

The Northwest Pacific is still by far the most productive fishing area with 20.9 million tons (27% of the global marine catch) in 2010. Catch peaks in the Northwest Atlantic, Northeast Atlantic and Northeast Pacific temperate fishing areas were reached many years ago, and afterwards total production had declined continuously from the early and mid-2000s, but in 2010 this trend was reversed in all three areas. As for mainly tropical areas, total catches grew in the Western and Eastern Indian Ocean and in the Western Central Pacific. In contrast, the 2010 production in the Western Central Atlantic decreased, with a

reduction in United States catches by about 100000 tons, probably mostly attributable to the oil spill in the Gulf of Mexico. Since 1978, the Eastern Central Pacific has shown a series of fluctuations in capture production with a cycle of about 5-9 years. The latest peak was in 2009, and a declining phase has started in 2010. Both the Mediterranean-Black Sea and the Southwest Atlantic have seen declining catches, with decreases of 15 and 30%, respectively, since 2007.

Total global capture production in inland waters has increased dramatically since the mid-2000s with reported and estimated total production at 11.2 million tons in 2010, an increase of 30% since 2004. Inland waters are considered as being overfished in many parts of the world, human pressure and changes in the environmental conditions have seriously degraded important bodies of freshwater. Growth in the global inland water catch is entirely attributable to Asian countries. Asia's share is approaching 70% of global production, with the remarkable increases reported for 2010 production by India, China and Myanmar.

The world's marine fisheries increased markedly from 16.8 million tons in 1950 to a peak of 86.4 million tons in 1996, and then declined before stabilizing at about 80 million tones, ranging between 72.1 and 73.3 million tons in the last seven years (2004-2010).

The relationship between the spawned biomass and the fishing mortality is commonly used to the connection between the stock, recruitment, natural mortality, and growth, and to assess the status of a stock (Figure 1) (Beddington *et al.* 2007).

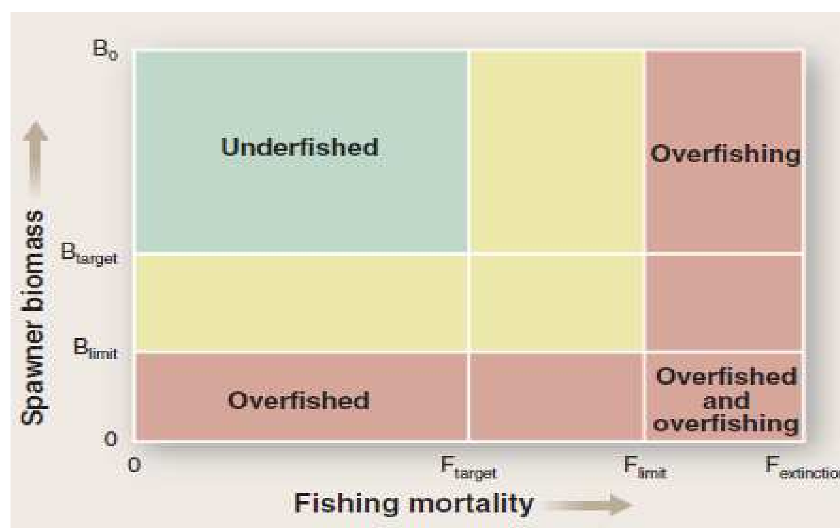


Figure 1. Stock status definitions for stock biomass and fishing mortality. F extinction is the limit of fishing mortality that generates biological extinction (Beddington *et al.* 2007).

The proportion of non-fully exploited stocks has decreased gradually since 1974 when the first FAO assessment was completed. In contrast, the percentage of overexploited stocks has increased, especially in the late 1970s and 1980s, from 10% in 1974 to 26% in 1989. After 1990, the number of overexploited stocks continued to increase, although at a slower rate. Most fish stocks are fully exploited at a level very close to their maximum sustainable yield (MSY), the optimal volume of catches that can be taken each year without threatening the future reproductive capacity; these stocks have no room for further expansion and require effective management to avoid decline. The fraction of these stocks has shown the smallest change over time, with its percentage stable at about 50% from 1974 to 1985, then falling to 43% in 1989 before gradually increasing to 57% in 2009. Among the remaining stocks, 29.9% were overexploited and 12.7% non-fully exploited in 2009 (Figure 2). Overexploited stocks produced lower yields than their biological and ecological potential and required strict management plans to restore their full and sustainable productivity in accordance with the Johannesburg Plan of Implementation that resulted from the World Summit on Sustainable Development (Johannesburg, 2002), which demands all overexploited stocks to be restored to the level that can produce maximum sustainable yield by 2015. The Mediterranean and Black Sea had 33% of assessed stocks fully exploited, 50% overexploited, and the remaining 17% non-fully exploited in 2009 (FAO 2012).

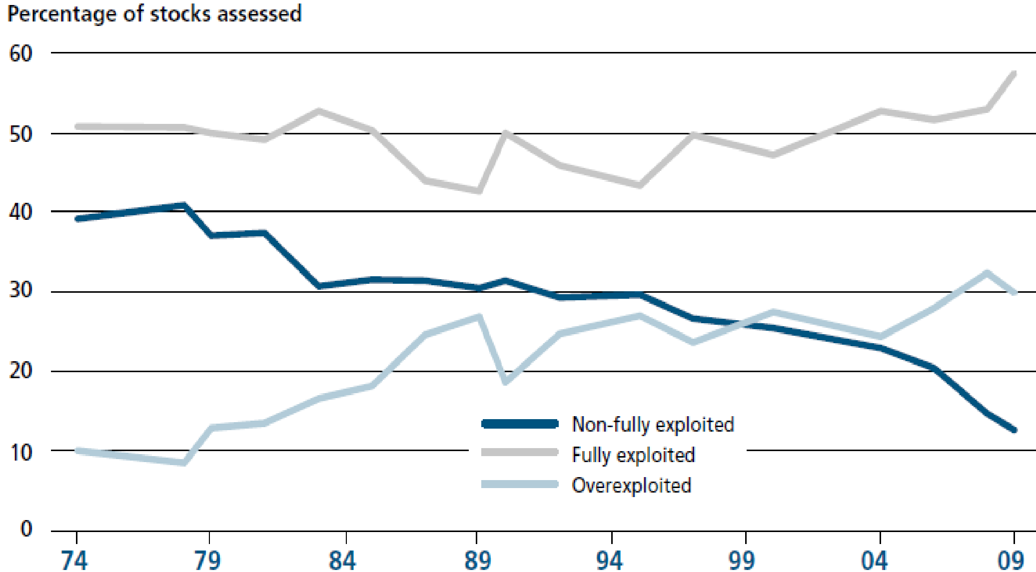


Figure 2. Global trends in the state of world marine fish stocks since 1974 (FAO 2012).

The declining global marine catch over the last few years, the increased percentage of overexploited fish stocks and the decreased proportion of non-fully exploited species around the world convey the strong message that the state of world marine fisheries is worsening and has had a negative impact on fishery production. Overexploitation not only causes negative ecological consequences, but it also reduces fish production, which further leads to negative social and economic consequences. To increase the contribution of marine fisheries to the food security, economies and well-being of the coastal communities, effective management plans must be put in place to rebuild overexploited stocks. Regional fishery bodies (RFBs) are the primary organizational mechanism through which States can work together to ensure the long-term sustainability of shared fishery resources, and they embraces regional fisheries management organizations (RFMOs), which have the competence to establish proper conservation and management measures. The most significant action is the setting of Total Allowable Catches (TAC) for the year and the consequent closure of the fishery when the year's cumulative catch has reached this TAC. Other effective measures adopted as a supplement to TAC are restrictions on fishing gears, fishing seasons, and fishing areas (Beddington *et al.* 2007).

Efforts to ensure long-term sustainable fisheries and promote healthier and more robust ecosystems are weakened by Illegal, Unreported and Unregulated (IUU) fishing and fraudulent activities, as fishing without permission, catching in protected areas, ignoring catch quotas and fishing undersize products. IUU fishing is a serious global problem and one of the main impediments to the achievement of sustainable world fisheries. This business depletes fish stocks, increases fish mortality, destroys marine habitats, penalizes honest fishers and impairs coastal communities, particularly in developing countries. Most RFBs promote and implement measures to fight IUU fishing, that range from more passive activities, such as awareness and dissemination of information, to aggressive programs as surveillance of ports, air and surface. The European Union and the United States of America, as leaders in the global fish trade, in 2011 started a bilateral cooperation in order to fight IUU fishing by keeping illegally caught fish out of the world market. The European Commission (EC) is working hard to prevent any illegal operators from making money out of legal activities, establishing that only marine fisheries products validated as legal by the relevant flag state or exporting state can be imported to or exported from the EU, and fixing

substantial penalties for everyone who fish illegally anywhere in the world, that are proportionate to the economic value of their catch, so that they deprive them of any profit.

An effective reduction in fishing effort, the participation of fishers and state authorities in the science and decision-making process, and a deep knowledge of species biology are important factors affecting successful recovery of depleted fish stocks.

1.2 AIM OF THE PROJECT

The Atlantic-wide research program on bluefin tuna, conventionally ICCAT-GBYP, is an international research project adopted by the Standing Committee on Research and Statistics (SCRS) and Commission of ICCAT in 2008. It's structured as a six years program, divided in several phases, beginning in 2010, and has the purpose to provide fishery independent data to overcame several limits and uncertainties of the current system of the bluefin tuna assessments and management.

Main aims of this project are to enhance knowledge about Atlantic bluefin tuna population structure and the mixing between fish of eastern and western Atlantic origin, and to focus on age and reproductive dynamics. To achieve these objectives, the first goal was aimed at mining historical data sets and at recovering data missing, in order to improve basic data collection through information from traps, observers and vessel management system. Another goal was to set-up an aerial surveys on bluefin tuna spawning aggregation for obtaining indices for the spawning stock biomass and for recruitment. These studies was based on a statistical survey design covering the most relevant areas for spawners in the Mediterranean Sea with a fleet of aircraft and a real time monitoring of the oceanographic conditions. A intense tagging program was also included in the GBYP since the beginning, using conventional, electronic satellite pop-up and internal electronic archival tags, with the aim of updating some essential population parameters necessary for the assessment.

To fulfill purposes of the project, it's also important to enhance understanding of key biological and ecological processes, determining habitat and migration routes, developing methods to estimate sizes of caged fish, implementing a large scale of genetic tagging experiment, carrying out histological analyses to determine bluefin tuna reproductive state and potential, and biological and genetic analyses to investigate population structure. Therefore, the GBYP Phase 2, begun on 22 December 2010, covered a wide range of

activities, based on broad and hard biological samplings that are an essential part of the project, particularly to understand the origin of the various individuals and the potential presence of sub-populations within the ICCAT convention area.

The population structure is of higher hierarchical importance, but several other important uncertainties in biological parameters and processes have been identified for ABFT, as maturity, growth and recruitment success, age composition of the catches (Fromentin and Powers 2005) and they need to be estimated within each new potential management unit (or sub-population). Therefore, GBYP activities included ageing determinations from the portion of the otolith corresponding to the first year of life and the first dorsal fin rays (spines), identification of spawning grounds along the Mediterranean and fecundity through study of gonads, and sophisticated microchemistry analyses on various tissues for defining the origin of each fish.

Population structure and individual assignment to the origin population have the highest priority in marine fish species with high potential for dispersal, as the careful identification and monitoring of population diversity can make possible to develop strategies to maximize and preserve genetic resources for adaption to natural and human-induced environmental alteration. To do this, many efforts of GBYP Phase 2 have focused on genetic sampling and related analyses, through the discovery of novel DNA polymorphisms and the use of new high-throughput technologies.

1.3 SNP

SNPs (Single Nucleotide Polymorphisms) are co-dominant markers and represent polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus (Figure 3). For such a base position with sequence alternatives in genomic DNA to be considered as a SNP, it’s considered that the least frequent allele should have a frequency of 1% or greater.

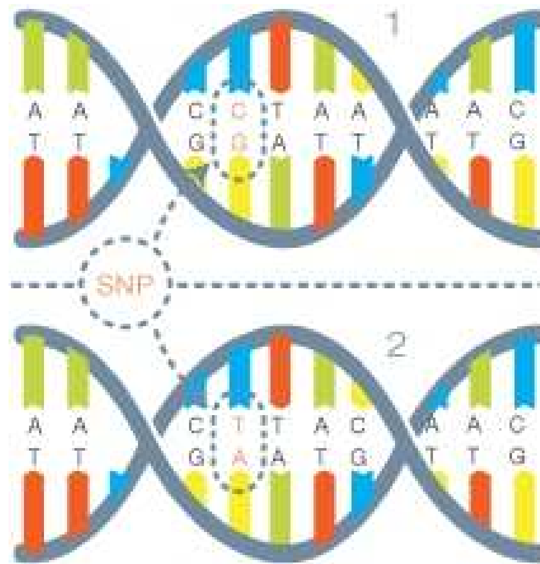


Figure 3. SNP.

Such sequence differences due to base substitutions have been well characterized since the beginning of DNA sequencing in 1977, but the ability to genotype SNPs rapidly in large numbers of samples was not possible until the application of gene chip technology in the late 1990s (Liu *et al.* 2004). Theoretically, these markers could be bi-, tri-, or tetra-allelic polymorphisms, producing as many as four alleles, each containing one of four bases at the SNP site (A, T, C, G), but practically, tri-allelic and tetra-allelic SNPs are rare almost to the point of non-existence, and so SNPs are sometimes simply referred to as bi-allelic markers. One of the reasons for this is the low frequency of single nucleotide substitutions at the origin of SNPs, estimated to be between 1×10^{-9} and 5×10^{-9} per nucleotide and per year at neutral positions in mammals. Another reason is due to a bias in mutations, leading to the prevalence of transition purine-purine (A $\leftrightarrow$ G) or pyrimidine-pyrimidine (C $\leftrightarrow$ T) (Vignal *et al.* 2002, Morin 2004).

SNPs are abundant in genomes and in many species occur every 200-500 bp. The lower heterozygosity values of single locus SNPs as compared to microsatellites imply the use of higher numbers of markers, because microsatellite loci typically have many alleles, whereas two is the norm for SNP loci. The required number of loci is difficult to assess a priori because each study has a different evolutionary context and simulation studies are needed to further elucidate SNP numbers and characteristics for population genetic studies, but 5-10 SNPs per microsatellite locus is considered the threshold to attain similar

discriminatory power. However, there are several advantages in the use of SNPs compared to microsatellites. One technical problem with microsatellites is that it isn't always possible to compare data produced by different laboratories, due to the eventuality of inconsistencies in allele size calling caused by a variety in sequencing machine, fluorescent dye and allele calling software. On the other hand SNPs can be transferred between laboratories easily, because SNP genotypes are based on detection of nucleotide sequence differences rather than PCR product size differences, so that genotype data are universally comparable and portable. Moreover, allele definition for microsatellites is done by assuming that size variation of PCR products is directly correlated with differences in repeat numbers of the simple motif. Although this is generally true, in some instances, size variations can be due to small deletions or insertions in flanking sequences and two PCR products of identical sizes can in reality be different alleles. The allele nomenclature problem is much simpler in the case of SNPs, for which the results can just be coded as a YES/NO problem, where each of the two alleles can be simply considered as being present or absent (Vignal *et al.* 2002).

Thus, the many advantages of SNP markers include abundance in any organism, increased accuracy and ease of automation and transferability of data sets across national and international laboratories. Another asset of using SNPs as population-level markers is the ability to efficiently target coding and non-coding regions of the genome simultaneously and even to predict the functional importance of the SNP depending on the position of the polymorphism (i.e. amino acid changing, silent, regulatory mutation). SNPs can be found in coding and non-coding areas, whereas most of the microsatellites used in population genetics, for example, are typically in non-coding regions of the genome that is expected to be less influenced by selection.

SNP discovery is the process of finding the polymorphic sites in the genome of the species and populations of interest. In humans and in model organisms, most of SNP discovery procedures have been realized "in silico", meaning that genomic information from multiple individuals in the public databases is screened for the identification of putative polymorphisms. As concern non-model organisms, for which genomic resources are lacking or insufficient, another approach needs to be used: SNPs can be found by sequencing and comparing genome-wide regions from multiple individuals. Genomic resources from which SNPs can be derived include Expressed Sequence Tags (EST), sequences of expressed genes, which have been identified from partial sequencing of a messenger RNA (mRNA) pool that

has subsequently been reverse transcribed into cDNA. In the last years, the growing availability of EST resources made possible to detect SNPs through direct alignment of ESTs obtained from multiple individuals representing different geographical regions. By generating SNPs from coding sequences, it's possible to find polymorphisms in functional genes, to identify loci under selection and to study the dynamics of these genes in natural populations. This approach is now becoming easier with the advent of next-generation sequencing methods that provide access to a wealth of sequence information on non-model organisms (Margulies *et al.* 2005; Seeb *et al.* 2011). Transcriptome sequencing provides rich sources of SNPs (Barbazuk *et al.* 2007), facilitating identification and study of the genes involved in adaptive change (Renaut *et al.* 2010; Hemmer-Hansen *et al.* 2011; Williams and Oleksiak 2011).

These new markers can be used in many types of researches. SNPs have in fact been employed for individual identification and paternity; studies of Anderson and Garza (2006) showed that 60-100 SNPs may allow accurate pedigree reconstruction, even in situations involving thousands of potential mothers, fathers, and offspring, while Hauser *et al.* (2011) demonstrated that a panel of 80 SNPs is sufficient to determine parentage in a wild population.

SNPs have also the great power to detect population structure at several levels, as proved in a study of Morin *et al.* (2009) where it was demonstrated that 30 SNPs should be sufficient to detect moderate (F_{ST} = 0.01) levels of differentiation, but 80 or more SNPs may be required to reveal demographic independence (F_{ST} < 0.005) and that increasing the sample size has a strong effect on power rather than the number of SNP loci. Also, including loci suspected to be under selection may increase the power to detect differentiation.

The power of SNPs concerning the assignment of individuals to the population of origin has been widely investigated and, for example, it has been demonstrated that indicated that as few as 22 SNPs for wolves (Seddon *et al.* 2005) and 51 SNPs for chum salmon (Smith and Seeb 2008) provide high probability of correct population assignment, similar to sets of 12 and 15 microsatellites, respectively. Smith *et al.* (2005) showed that 9 polymorphic SNPs are sufficient to assign Chinook salmon to a country of origin with more than 95% accuracy, but their precision decrease when resolving fine-scale relationships. A more recent study on Chinook salmon proved that between 100 and 200 highly informative SNP loci are required to meet management standards (correct assignment > 90%) for

resolving genetic stock identification in finer-scale scenarios (Hess *et al.* 2011). In a study carried out by Glover *et al.* (2010) on wild and domesticated strains of Atlantic salmon, assignment was best (80% correct) when at least 100 SNP loci were used. In the last years, researchers have been using outlier loci (loci more highly differentiated than could be expected under a neutral model), potentially under diversifying selection, to increase the accuracy of assignment tests. This was demonstrated in a recent study on Atlantic salmon, where Freamo *et al.* (2011) obtained 85% of correct assignment with 14 outlier loci against 67% with neutral loci.

Many studies and researches have been carried out to detect SNPs possibly involved in local adaptation in various fish species, as herring (Limborg *et al.* 2012), threespine stickleback (Deagle *et al.* 2012), cod (Hemmer-Hansen *et al.* 2011; Nielsen *et al.* 2009a; Poulsen *et al.* 2011), lake whitefish (Renaut *et al.* 2010; Renaut *et al.* 2011) and several salmonid species (Freamo *et al.* 2011; Gomez-Uchida *et al.* 2011; Limborg *et al.* 2011; Seeb *et al.* 2011). The improvement of genome scan techniques increases the chance to identify candidate loci subject to selection, providing a more direct way of linking genotypes to physiological functions.

CHAPTER 2

TARGET SPECIES: ATLANTIC BLUEFIN TUNA

2.1 TAXONOMY AND DESCRIPTION

The Atlantic Bluefin tuna (*Thunnus thynnus*, Linnaeus 1758) is the largest tuna species, belonging to the Family Scombridae, which includes 15 genera and approximately 48 species of epipelagic fish. Seven species belong to the genus *Thunnus*, included *T. thynnus* (Figure 4).

PHYLUM: CHORDATA
SUBPHYLUM: VERTEBRATA
SUPERCLASS: GNATHOSTOMATA
CLASS: OSTEICHTHYES
SUBCLASS: ACTINOPTERYGII
ORDER: PERCIFORMES
SUBORDER: SCOMBROIDEI
FAMILY: SCOMBRIDAE
TRIBE: THUNNINI
GENUS: *THUNNUS*
SPECIES: *THUNNUS THYNNUS*

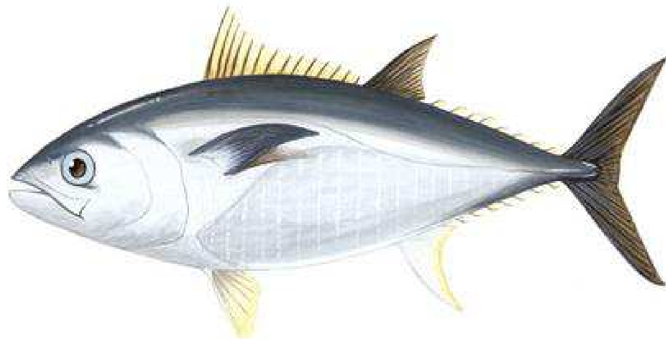


Figure 4. *Thunnus thynnus*.

The Atlantic bluefin Tuna grows to over 300 cm and it can reach a maximum length of 4 m. Its official maximum weight is 726 kg, but weights up to 900 kg have been reported in various fisheries of the West Atlantic and Mediterranean Sea (Mather *et al.* 1995). Its physical characteristics make it an excellent swimmer with speeds up to 90 km/h. It has a fusiform body, deepest near the middle of the first dorsal fin base, with a triangular pyramid-shaped head and a small mouth compared to the development of the skull. Its skin

is very hard, resistant, and covered by small scales that decrease in size going from front to rear of the body. The skin is also lubricated by a mucus which reduces friction with water. Bluefin tuna displays 39 vertebrae and 12 to 14 dorsal spines and 13 to 15 dorsal soft rays. It has two dorsal fins separated by a small space: the front is triangular with spines and the rear is sickle cell and followed by small fins to the tail. The thin caudal peduncle, with a wide and symmetrical tail at the end, is used as rudder and as a means of propulsion. Dorsal, pectoral and small ventral thoracic fins are flattened allowing aerodynamic and fast swimming. The back is dark blue or black, the sides are a silvery gray-blue and belly is white with translucent patches. The first dorsal fin is yellow, the second, which is higher than the first, is red, small fins are yellowish with brown edges and the caudal fin is dark blue. Fish larvae (around 3-4 mm) are typically pelagic with a yolk sac and a relatively undeveloped body form. The yolk sac is desorbed within few days, then the larvae have to feed on their own.

2.2 GEOGRAPHIC DISTRIBUTION, HABITAT AND ECOLOGY

Atlantic bluefin tuna occurs throughout the North Atlantic, including the Gulf of Mexico and the Mediterranean Sea (Walli *et al.* 2009) (Figure 5).

Its distribution extends over an extraordinarily large area, ranging off the Atlantic coasts of Europe and Africa, from the North Cape to the Cape of Good Hope, and off the North American coasts from Newfoundland to a latitude of 40°S (Mather *et al.* 1995).

Among the tuna, ABFT has the widest geographical distribution and is the only large pelagic fish living permanently in temperate Atlantic waters (Bard *et al.* 1998; Fromentin and Fonteneau 2001).

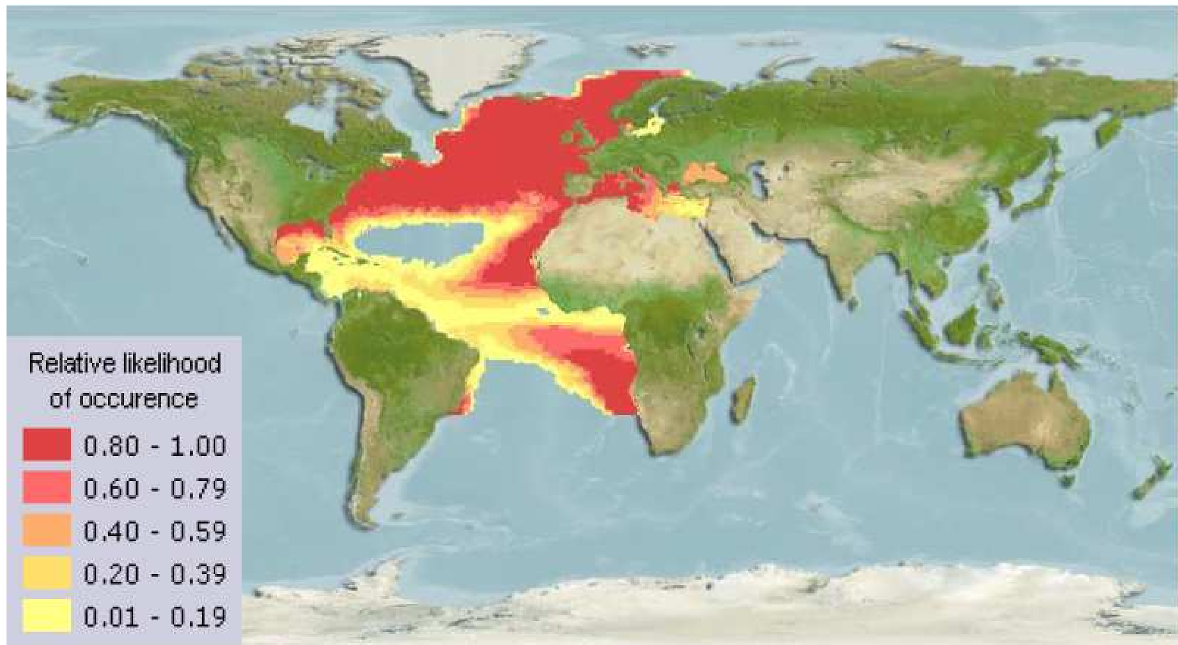


Figure 5. Distribution of *Thunnus thynnus*.

Archival tagging and tracking information confirmed that ABFT can sustain cold (down to 3°C) as well as warm (up to 30°C) temperatures while maintaining stable internal body temperature (Block *et al.* 2001). Data collected by Walli *et al.* (2009) with electronic archival tags on western Atlantic bluefin from ages 7.1 to 14.2 years showed that they spent 87% of occupancy in waters ranging from 10° to 23°C with peak times at 13°-20°C.

T. thynnus is an endothermic fish, so it generates heat as a byproduct of metabolism and maintains its body temperature above that of the surrounding environment. The internal body temperatures for bluefin reporting timeseries data showed a mean of 23.9°C (Walli *et al.* 2009).

The spatial distribution and movement of ABFT are hypothesized to be controlled by preferential ranges and gradients of temperature, similar to Pacific bluefin and other tuna species (Laurs *et al.* 1984; Lehodey *et al.* 1997; Bard 2001; Inagake *et al.* 2001). More works appears to converge toward the opinion that juvenile and adult ABFT frequent and aggregate along ocean fronts (Humston *et al.* 2000; Lutcavage *et al.* 2000; Royer *et al.* 2004). This association is also likely to be related to foraging, ABFT feeding on the abundant vertebrate and invertebrate prey concentrations of these areas. Juvenile and adult ABFT spend the majority of their time in waters less than 200 m but frequently dive to depth of

500-1000 m (Lutcavage *et al.* 2000; Block *et al.* 2001; Stokesbury *et al.* 2004; De Metrio *et al.* 2005). The mean diving depths of bluefin tuna was 34.5 m, with most of their time spent between the surface and 50 meters and an exponential decrease in time spent at greater depths. Maximum depth of 1200 m was recorded by one fish (Walli *et al.* 2009); a similar behaviour has also been reported for southern bluefin tuna, bigeye tuna and swordfish and is generally related to foraging in deep scattering layers or to physiological constraints to cool the body temperature (Carey and Robinson 1981; Holland *et al.* 1992; Musyl *et al.* 2003). During spawning runs, *T. thynnus* shows deep-diving behaviors in the Gulf of Mexico, which likely provide access to cool, oxygen-rich waters as the fish travel to breeding grounds (Stokesbury *et al.* 2004; Teo *et al.* 2007). Once on the spawning area, *T. thynnus* make shallow oscillatory dives at night with frequent visits to the surface. Similar behaviors have been observed for *T. orientalis* (Kitagawa *et al.* 2006) and *T. albacares* (Schaefer 2001) during the breeding phase. *Thunnus thynnus* maintains this behavior for approximately 20 days. Maximum diving depths of *T. thynnus* are significantly less (< 200 m versus > 500 m) during the spawning phase than observed during entry to and exit from spawning grounds in the west.

As larvae and small juveniles, their diet is probably similar to that of *T. orientalis* in the Pacific Ocean, which is comprised primarily of zooplankton with copepods as the main stomach item (Uotani *et al.* 1990). The diet of adults is comprised mainly of fishes, cephalopods (mostly squid) and crustaceans (Sarà and Sarà 2007). These categories may include numerous species, and the particular composition is determined principally by location. In the western Atlantic, the diet is primarily composed of Atlantic herring *Clupea harengus*, Atlantic mackerel *Scomber scombrus*, sand lances *Ammodytes* spp., and silver hake *Merluccius bilinearis* (Nichols 1922; Crane 1936; Dragovich 1970; Mason 1976; Holliday 1978; Eggleston and Bochenek 1990; Chase 2002). In the eastern Atlantic and Mediterranean Sea, ABFT feed on European sprat *Clupea sprattus*, European anchovy *Engraulis encrasicholus* and European pilchard *Sardina pilchardus* (Oren *et al.* 1959; De Jager *et al.* 1963). At tropical latitudes, porcupinefish *Diodon* sp. and flying gurnard *Dactylopterus* sp. are the dominate items observed in the stomachs of *T. thynnus* (Krumholz 1959; Dragovich 1970). No clear relationship has been demonstrated between prey length and the size of ABFT: both small and large ABFT display similar prey-size spectra. Chase (2002) noted that

the largest prey (those > 40 cm) were only consumed by giant ABFT > 230 cm, while Logan *et al.* (2011) observed that prey length was not significantly correlated with ABFT length.

ABFT has a long life span of 40 years. Methods used to estimate age and growth of *T. thynnus* have been based on the examination of calcified structures, length-frequency data or mark-recapture data. Mark-recapture method is limited due to uncertainties in the initial age of a fish at release and the lack of observations and high variability in growth for these sizes. This method used for ageing do not perform well for fish > 200 cm (approximately 10 years old) (Fromentin and Powers 2005). Several different calcified structures have been used to estimate the age and growth of *T. thynnus*: otoliths have the advantage that the central nucleus is not resorbed with age, so they have been used to estimate growth during larval, juveniles and adult phase (Brothers *et al.* 1983; Foreman 1986; Itoh *et al.* 2000; Megalofonou 2006), while the use of spines is limited by the resorption of the medular cavity from age 3 (Compeán-Jimenez and Bard 1983; Mather *et al.* 1995). Growth and mortality of *T. thynnus* during the larval phase has been determined from age data from otolith microstructure analysis (Rooker *et al.* 2007). Scott *et al.* (1993) reported that growth was linear during the larval phase (~2-10 days) at a rate of 0.3-0.4 mm d^{-1}. Similar rates have been reported for congeners from temperate and tropical regions: *T. orientalis* (0.33 mm d^{-1}; Miyashita *et al.* 2001), *T. albacares* (0.47 mm d^{-1}; Lang *et al.* 1994), and *T. maccoyii* (0.28-0.36 mm d^{-1}; Jenkins and Davis 1990; Jenkins *et al.* 1991). Brothers *et al.* (1983) reported a growth rate of 1.4 mm d^{-1} for juveniles in the western Atlantic (267-413 mm FL; ca. 70-200 d). Estimates of growth for juvenile *T. thynnus* (85-555 mm FL) from the Mediterranean Sea are markedly higher, with a mean growth rate of 4.7 mm d^{-1} (Megalofonou 2006). Juvenile growth is rapid for a teleost fish (about 30 cm $year^{-1}$), but somewhat slower than other tuna and billfish species (Fromentin and Fonteneau 2001, Fromentin and Powers 2005). Fish born in June attain a length of about 30-40 cm long and a weight of about 1 kg by October. After one year, fish reach about 4 kg and 60 cm long (Mather *et al.* 1995). Growth in length tends to be lower for adults than juveniles, but growth in weight increases. Therefore, juveniles are relatively slim, whereas adults are thicker and larger, so at 10 years, an ABFT is about 200 cm and 150 kg and at 20 years reaches about 300 cm and 400 kg. West ABFT grow faster after maturity and attain larger sizes than the East and Mediterranean ABFT.

Age structure of adult *T. thynnus* has been studied in both the eastern and western Atlantic, and estimated growth rates are relatively similar between and within regions during

the first five years of life. After age 5, growth trajectories of *T. thynnus* show marked differences between the eastern and western Atlantic, with the length at age being greater in the western Atlantic than the eastern Atlantic. At age 10, mean size in the western Atlantic was 212 cm FL compared to 200 cm FL for the eastern Atlantic (Rooker *et al.* 2007).

Also seasonal growth patterns have been better documented, so both juveniles (Mather and Schuck 1960; Furnestin and Dardignac 1962; Farrugio 1980) and adults ABFT (Tiews 1963; Butler *et al.* 1977) grow rapidly during summer and early autumn (up to 10% per month), while growth is negligible in winter. The existence of a slowdown in growth during the winter has been confirmed for the southern bluefin tuna (Evenson *et al.* 2004) and the pacific bluefin tuna (Bayliff 1993). Seasonal variations in length and growth rates of older *T. thynnus* are less apparent, probably due to the weak relationship between age and length for individuals more than 15 years of age (Hurlbut and Clay 1988).

Sex-specific differences both in length at age and weight at age have been reported, with differential growth in weight being more pronounced between males and females. Past studies shown that males grow more rapidly than females and reach a slightly greater size at a given age, with these differences becoming apparent by approximately age 10 (Rivas 1976; Caddy *et al.* 1976). In the recent study of Santamaria *et al.* (2009), based on sampled over an 8-year period from 1998 to 2005 in several central Mediterranean Sea sites (North Ionian, South Adriatic, South Tyrrhenian seas and Ionian waters around Malta), is shown that after sexual maturity, reached above 135 cm FL, the female weight-at-length is higher than the male's.

Natural mortality rates (M) of ABFT are poorly known. However, the mortality rates is lower and less variable in long-lived fish, such as ABFT, than in short-lived ones; it's higher during juvenile stages than during the adult phase and it also varies with population density, size, sex, predation and environment (Fromentin and Powers 2005). Scott *et al.* (1993) estimated a natural mortality rate of 0.20 d^{-1} for larvae from the western stock, and rates are lower than values reported for more tropical tunas during comparable periods: *T. albacares* (M = 0.33 d^{-1}; Lang *et al.* 1994) and *T. maccoyii* (M = 0.66 d^{-1}; Davis *et al.* 1991). Tagging from Southern bluefin tuna (*Thunnus maccoyii*) tends to confirm that M is higher for juveniles (between 0.49 and 0.24) compared to that of adults (around 0.1). In the absence of direct and consistent estimates of M for Atlantic bluefin tuna, the natural mortality vector of the Southern bluefin tuna is generally used for the East-Atlantic and Mediterranean stock

assessment, whereas a constant M of 0.14 is assumed for the West Atlantic bluefin tuna (ICCAT 1999; ICCAT 2003a).

2.3 REPRODUCTION AND SPAWNING

Bluefin tuna is oviparous and iteroparous like all tuna species (Schaefer 2001).

Ovaries of *T. thynnus* consist of ovigerous lamellae with follicles at different stages of development (Corriero *et al.* 2003). The simultaneous presence of all oocyte developmental stages during the spawning period (Medina *et al.* 2002; Corriero *et al.* 2003) indicates that *T. thynnus* has asynchronous oocyte development and, similar to other temperate and tropical tunas, is a multiple or batch spawner (Wallace and Selman 1981). Spawning frequency or interval for *T. thynnus* has been estimated at 1.2 days (Medina *et al.* 2002). This interval is similar to the observed frequencies of other members of the genus *Thunnus*: yellowfin tuna *T. albacares* (1.27 to 1.99; Schaefer 1998; Itano 2000), bigeye tuna *T. obesus* (1.05; Chu 1999), and southern bluefin tuna *T. maccoyii* (1.62; Farley and Davis 1998). It is generally assumed that bluefin tuna spawns every year, but electronic tagging experiments, as well as experiments in captivity, suggest that individual spawning might occur only once every two or three years (Lutcavage *et al.* 1999).

The testis of *T. thynnus* is comprised of lobules radiating from the longitudinal main sperm duct toward the periphery (Abascal *et al.* 2003). The testicular structure is cystic, each cyst being comprised of a clone of germ cells branched by the cytoplasm of Sertoli cells.

Egg production appears to be age (or size) dependent: a 5 years old female produces an average of 5 million eggs (approximately 1 mm), while a 15-20 years female can carry up to 45 million eggs (Rodríguez-Roda 1967). Estimated relative batch fecundity of *T. thynnus* is greater (> 90 oocytes g^{-1} of body weight) than those estimated for other tunas in the genus *Thunnus*, which are typically less than 70 oocytes g^{-1} of body weight: *T. obesus* 31 oocytes g^{-1} (Nikaido *et al.* 1991), *T. maccoyii* 57 oocytes g^{-1} (Farley and Davis 1998), and *T. albacares* 67 oocytes g^{-1} (Schaefer 1998).

Rodriguez-Roda (1967) estimated that 50% of female *T. thynnus* in the Mediterranean Sea were reproductively active at approximately 103 cm (age 3) and 100% maturity was reached between 115 and 121 cm (age 4 or age 5). Corriero *et al.* (2005) confirmed results of this study, reporting that 50% of *T. thynnus* in the Mediterranean Sea

reached sexual maturity at 104 cm (age 3 or age 4) and 100% at 130 cm (age5). Instead Heinesh *et al.* (2008) studied the growth of the gonads in adults tuna in several areas of the Mediterranean Sea, verifying a mean body length of 200 cm (age 8). In the western Atlantic, histological examination of ovaries from females showed delayed maturation schedules, and individuals were unlikely to reach sexual maturity before age 8 (Baglin 1982). More recent studies indicate that juvenile tuna, tagged in North Carolina and that return in the Mediterranean during the spawning season, didn't pass the Strait of Gibratar before 9-10 years old (Block *et al.* 2005).

The reproductive cycle of *T. thynnus* has been reconstructed on the basis of the histological descriptions of the gonads of fish captured in different periods. In the central and western Mediterranean, *T. thynnus* is reproductively inactive from August to April, when only unyolked oocytes are present in the ovaries, and mainly spermatogonia and meiotic cells have been found in the seminiferous epithelium. Active non-spawning individuals have been observed in May, with yolked oocytes in the ovaries and seminiferous lobules progressively filled with spermatozoa. Hydrated oocytes and post-ovulatory follicles, signs of imminent and recent ovulation, respectively, have been found in actively spawning individuals captured in late June to early July. From late July to September, *T. thynnus* are reproductively inactive, as ovaries show unyolked oocytes and late stages of atresia of yolked oocytes; only residual spermatozoa are present in the testes. The presence of actively spawning fish, with hydrated oocytes and post-ovulatory follicles, was reported in the eastern Mediterranean Sea from mid May to mid June (Karakulak *et al.* 2004b), while spawning occurs in the central and western Mediterranean from mid June to early July (Susca *et al.* 2001; Corriero *et al.* 2003).

There are two regional spawning areas for *T. thynnus*, one in the east and one in the west (Mediterranean Sea and Gulf of Mexico, respectively), as confirmed by electronic tagging studies (Stokesbury *et al.* 2004; Block *et al.* 2005; Teo *et al.* 2007). The timing of spawning in both the east and west is linked to temperature. Sea surface temperatures reported for *T. thynnus* on putative spawning grounds in the Gulf of Mexico and Mediterranean Sea range from approximately 22.6°C-27.5°C and 22.5°C-25.5°C, respectively (Karakulak *et al.* 2004a, 2004b; Garcia *et al.* 2005; Teo *et al.* 2007). Because the waters of the Gulf of Mexico are above the 24°C spawning threshold in early spring (Block *et al.* 2001, 2005; Teo *et al.* 2007), *T. thynnus* begin spawning earlier in the Gulf of Mexico than in the

Mediterranean Sea (April versus May) (Baglin 1982; Nishida *et al.* 1998; Medina *et al.* 2002; Corriero *et al.* 2003; Karakulak *et al.* 2004a).

In the Mediterranean Sea there are three spawning areas: the waters of southern Italy around Sicily, nearby the Sicilian Channel and the Malta Channel (Sella 1929; Sanzo 1932; Piccinetti and Manfrin 1970; Nishida *et al.* 1998), the Balearic Islands, a transitional zone between Mediterranean and eastern Atlantic waters, mostly in the Mallorca Channel and in the south of Menorca (Rodriguez-Roda 1975; Nishida *et al.* 1998; Garcia *et al.* 2005) and areas north of Cyprus along the coast of Turkey (Karakulak *et al.* 2004a, 2004b; Oray and Karakulak 2005).

In the west, the spawning grounds of *T. thynnus* in the Gulf are located along the northern slope waters between the 200 m and 3000 m contours from 85°W and 95°W (Block *et al.* 2005; Teo *et al.* 2007). Apart from the northern Gulf, *T. thynnus* larvae have been reported from the southern Gulf to the Yucatan Channel (Richards and Potthoff 1980; McGowan and Richards 1986) and from the Straits of Florida to the Bahamas (Rivas 1954; Richards 1976; Richards and Potthoff 1980; Brothers *et al.* 1983).

2.4 MOVEMENT AND STOCK STRUCTURE

The interest on the behavior of bluefin tuna and its migration goes back to the past. Bluefin tuna migration in the Mediterranean Sea has been described long ago by the ancient Greek and Latin philosophers, especially Aristotle (IV B.C.) and Pliny the Elder (Ith A.C.). A migratory connection between oceans was first mentioned by Cetti (1777), who suggested that bluefin tuna come into the Mediterranean from the North Atlantic to spawn around Sicily and then go back by the same routes. The first works are attributed to M. Sella (1926, 1927, 1929; cited by Brunenmeister 1980): he suggested that tuna had moved from the east of the Atlantic to the Mediterranean, and that after breeding they had moved from South of Spain to Norway.

New innovative tools promoted a better knowledge of migratory behaviors of this species. Mark-recapture studies with identification tags (“conventional tagging”) have provided valuable information on key aspects of the biology of *T. thynnus*, focusing more on the western North Atlantic than on the eastern Atlantic. From several studies it emerged that juveniles tuna (< 4 years) didn’t move out of the place where they were tagged, while

adults tuna performed long distance movement across the ocean (trans atlantic movement) (Rooker *et al.* 2005). Similar evidence of movement were reported in the eastern Atlantic (Magnuson *et al.* 1994; Fromentin 2001). Conventional tags provide valuable data on a range of life history parameters, but their utility is limited by the lack of information on locations between release and recapture. Alternatively, electronic tags, recording ambient light level, water and body temperature, and pressure at frequent intervals throughout the deployment duration, allowing estimation of position in association with diving behavior and thermal physiology, yielded important insights about bluefin seasonal movements, aggregations and diving behaviors (Teo *et al.* 2004; Block *et al.* 2005; Walli *et al.* 2009). Studies of Block *et al.* (2001, 2005) have highlighted the phenomenon of "spawning site fidelity" (fidelity of individuals to the breeding site), demonstrating that adolescent and mature western Atlantic bluefin tuna (with size > 200 cm) move to the Gulf of Mexico and the eastern Mediterranean Sea during the known breeding season. The observed pattern of migration supports the hypothesis of "homing behavior", according to which bluefin tuna would migrate in specific and well-defined areas, returning to the same spawning area of origin, both in the Mediterranean and in the Gulf of Mexico. In particular, for bluefin tuna would seem more plausible theory the "repeat homing", a process related to spatial learning of young individuals from those adults, rather than the "natal homing", in which the fidelity to the site of birth is due imprinting, during the early stages of life, of specific environment (Fromentin and Powers 2005) (Figura 6). Ravier and Fromentin suggested in their work of 2004 a reproductive strategy, known as "opportunistic homing", halfway between the idea of strict loyalty to origin breeding site and the reproductive opportunism, according to which individuals choose the site of deposition in relation to optimal environmental conditions: during periods when temperatures rise, bluefin tuna may be able to reproduce in areas other than those traditionally described (for example in North Atlantic), where you could create environmental conditions favorable to the course of last stages of gametogenesis, whereas during periods of low temperatures the activity reproduction would be limited to the permanent sites of deposition (Mediterranean and the Gulf of Mexico).

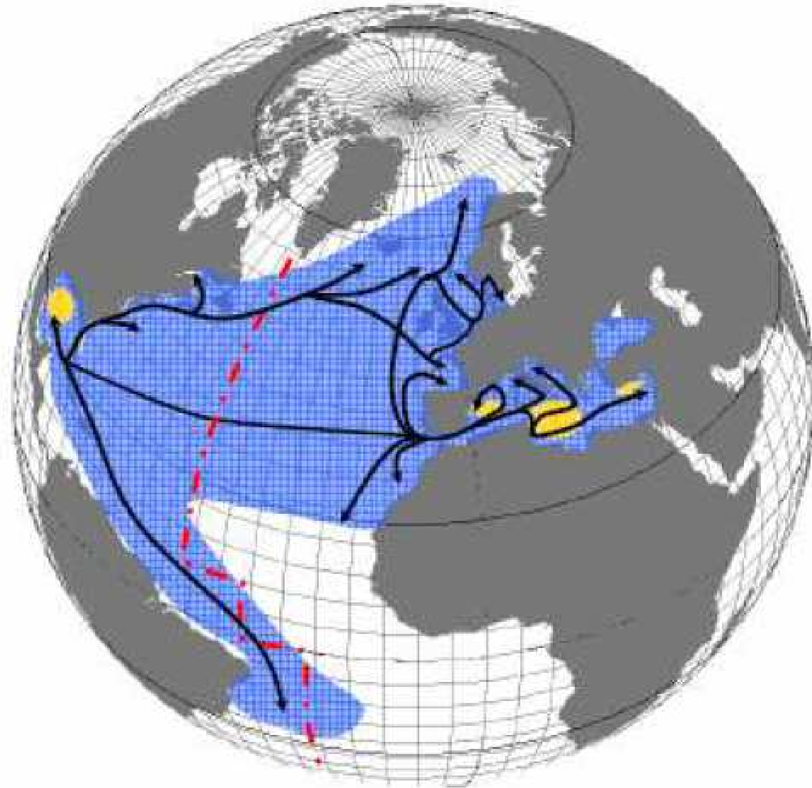


Figure 6. Map of the spatial distribution of Atlantic bluefin tuna (blue shading) and main migration routes (black arrows). The vertical red dotted line depicts the stock delimitation between the two current ICCAT management units and the yellow areas indicate the main spawning grounds (Fromentin and Power 2005).

Tuna are capable of moving from the continental shelf of North America into the eastern Atlantic in 40 days and back again in the same year. These large scale movements between feeding and spawning grounds are comparable to those of Pacific and Southern bluefin tuna. Pacific bluefin migrate from the western Pacific to the North American continental shelf and remain residents for 2 to 5 years before returning to the western Pacific to spawn (Bayliff *et al.* 1991; Gunn 2001). *T. thynnus* moved from 1.6 to 71.6 km/day (average = 16.2 ± 2) with a maximum distance traveled of 5820 km in 304 days. Rapid movements of thousands of kilometers are common in tunas and other highly migratory species. This suggests that the metabolic costs for endothermic fish swimming across ocean basins are low in comparison to the ecological benefits.

Tagging campaigns using electronic tags have also been initiated in the Mediterranean Sea over the last decade, with several studies of De Metrio *et al.* (2002, 2005), that didn't detect evidence of trans-Atlantic migration but suggested that movement

patterns or displacement distance were linked to size, with larger individuals (> 150 kg) being more likely to move out of the Mediterranean. Yamashita and Miyabe (2001) also reported that young *T. thynnus* tagged with archival tags in the Adriatic Sea remained close to the deployment area within the Mediterranean. Movements of *T. thynnus* tagged in the central and western Mediterranean Sea were more pronounced than in the east. Electronic tagging also revealed that the Northwest Atlantic (especially the area being delimited by the Gulf of Maine, Newfoundland and the Gulf Stream) has become a key feeding ground for bluefin tuna of both Western and Eastern origins during the 1990s and early 2000s (Block *et al.* 2001; Block *et al.* 2005; Royer *et al.* 2008). Moreover, Stokesbury *et al.* (2007) reported that giant *T. thynnus* tagged in the eastern Atlantic off Ireland moved from these areas across the 45th W stock boundary over short periods of time, demonstrating connectivity between eastern foraging grounds and western Atlantic fisheries.

A recent work of Walli *et al.* (2009) has shown clear evidence of mixing between eastern and western populations in foraging aggregation zones in the North Atlantic, dependent on the productivity and high abundance of prey species in a given area. This is well supported by results of analysis based on carbon and oxygen stable isotope in otolith ($\delta^{13}C$ and $\delta^{18}O$). Otolith material deposited during the first year of life serves as a natural tag of the individual's place of origin or nursery habitat, it varies regionally and reflects water composition differences in nurseries. Stable $\delta^{18}O$ signatures in otoliths of yearlings from each nursery were distinct, with enriched $\delta^{18}O$ values observed for *T. thynnus* from the cooler, more evaporative Mediterranean basin relative to the western Atlantic. (Rooker *et al.* 2007, 2008; Schloesser *et al.* 2010). Rooker and Secor (2004) demonstrated that the discriminatory power of stable isotopes in otoliths of yearling *T. thynnus* was high, with well over 90% of individuals classified correctly to eastern and western Atlantic nurseries. In a followup study, Rooker *et al.* (2006a) compared otolith core material (corresponding to the first year of life) of large school, medium, and giant *T. thynnus* collected in both the western Atlantic and the Mediterranean Sea. Results from this preliminary assessment indicated that a large fraction (> 50%) of the adolescent *T. thynnus* collected in the western Atlantic fishery originated from nurseries in the Mediterranean Sea. Alternatively, adult *T. thynnus* collected in the Mediterranean Sea were almost entirely of eastern Atlantic origin (> 90%), indicating strong natal homing to spawning/nursery grounds in the Mediterranean Sea. Experiments carried out using eight microsatellite in the eastern North Atlantic Ocean south of Iceland for

ABFT collected during 1999 and 2002 demonstrated genetic divergence between collections of fish caught early and late in the fishing season over the two years. These results confirmed that the northeast Atlantic fishery represents a mixed-stock fishery including animals migrating from different areas and recruited from different spawning grounds (Carlsson *et al.* 2007).

CHAPTER 3

STATE OF THE ART

3.1 FISHERY GENETICS

Fisheries management is currently considered a necessity to ensure the long-term stability of this activity, recovery of fish stocks, sustainability of resources and to avoid the collapse of natural populations. To manage economically important marine species it's necessary to define individual units, as stocks with specific mortality and recruitment levels.

Scientific information represent the focus for a correct management of living marine resources, thus a variety of international organizations have been established to facilitate collection and interpretation of scientific data for marine species in a management context, as International Council for the Exploration of the Sea (ICES), International Whaling Commission (IWC), and International Commission for the Conservation of Atlantic Tunas (ICCAT). It's important to preserve the population diversity, needful for a sustainable utilization of exploited stocks and for adaption to environmental changes. The field of fishery genetics has greatly expanded in recent decades (Sweijd *et al.* 2000; Ward 2000; Hauser and Carvalho 2008), in parallel with rapidly developing technologies in the field of human genetics, changed the understanding of population dynamics and structuring in marine fish. Genetic tools are widely used in many aspects of global biodiversity conservation, including phylogenetic classification, species identification, genetic structure of natural populations and identification of management units for conservation, assessment of genetic diversity within species or population, especially of small ones or at risk, and interactions between environmental contamination and biology and health of organisms.

Whereas classical fisheries approaches are typically focused on factors driving short-term demographic changes in populations (quantitative changes), genetic approaches examine the extent to which changes in the composition of populations (qualitative change) influence both short-term alterations in phenotypic traits and longer-term response to natural and anthropogenic perturbations (Frankham 2005). Better integration of genetic

information and traditional methods of fisheries stock assessment could substantially improve the quality of management advice.

The aim of sustainable fisheries management is to identify the spatial and temporal scale of population structuring, to devise tools to monitor its dynamics and to contribute to overall fisheries production. Even apparently small genetic differences among populations of marine fishes could translate into important adaptive variation distributed among populations (Conover *et al.* 2006). Genetic diversity is required for populations to adapt to environmental changes. Large populations have a significant proportion of genetic diversity, but this is considerably reduced in species and overexploited populations, that may lead to a decline in their capacity to adapt to new circumstances and to the environmental changes (Hauser *et al.* 2002).

The first studies on the structure of fish populations with molecular genetics initiated around 1950 with the study of blood groups, of tuna, salmon and cod (Ligny 1969). Thanks to the development of new techniques, as the DNA polymerase chain reaction, in the last decade of the 20th century different molecular markers are increasingly being used, playing an important role in animal genetics studies. Now large amounts of genetic data from many marine species have been generated, focusing on fish species harvested by humans and overfished, and relevant information for efficient management of fish stocks was provided.
Allozymes are allelic variants of proteins produced by a single gene locus and have long been used due to the ease of use across species (Nevo 1990), but their statistical power is shrink by the limited number of loci and low variability. Mitochondrial DNA (mtDNA) was the first widely used DNA marker and has been employed extensively to investigate stock structure in a variety of fishes including eels (Avise *et al.* 1986), bluefish (Graves *et al.* 1992), red drum (Gold *et al.* 1993), snappers (Chow *et al.* 1993), and sharks (Heist and Gold 1999), providing many insights into the demography of natural populations thanks to its power for genealogical and evolutionary studies. However, due to its non-Mendelian mode of inheritance (it's maternally inherited), it must be considered a single locus and its ability to resolve population structure is relatively restricted (Avise 1994). Most recent genetic studies of natural populations have used microsatellites, multiple copies of tandemly arranged simple sequence repeats. Microsatellites are inherited in a Mendelian fashion as codominant markers, they are very abundant, occurring as often as once every 10 kb in fishes, have an evenly genomic distribution, being in the genome on all chromosomes and all regions of the

chromosome, have small locus size, and showed a high polymorphism, based on size differences due to varying numbers of repeat units contained by alleles at a given locus (Liu and Cordes 2004). Due to its easy use by simple PCR, followed by a denaturing gel electrophoresis for allele size determination, and to the high degree of information provided by its large number of alleles per locus, microsatellites provides high statistical power for population genetics ability to detect population-genetic structure, to test parentage and relatedness, to assess genetic diversity, and to study recent population history. They suffer from two drawbacks: first, they require species-specific marker development, and second, they undergo a high potential for null alleles and are prone to genotyping errors due to their size-based nature (homoplasy) (Jarne and Lagoda 1996; Vignal *et al.* 2002; Oleksiak 2010). Amplified fragment length polymorphisms (AFLP) have been largely used since first described (Vos *et al.* 1995) due to their ease of use in species with no prior sequence information: many AFLP markers can be easily amplified and scored. AFLP analyses, however, require high-quality DNA and provide dominant markers so that heterozygotes cannot be directly measured (Campbell *et al.* 2003; Oleksiak 2010).

A new marker type, named SNP (Single Nucleotide Polymorphism) is now on the scene and has gained high popularity (Vignal *et al.* 2002; Morin 2004). Neutral DNA markers have been extensively used for elucidating demographic population relationships, but the distribution of neutral variation among populations reveals little about the adaptive genetic variation, critical in order to define management units and setting priorities for conservation (Nielsen *et al.* 2009). So now there is an increasing interest in identifying molecular genetic markers under selection that can detect adaptive local events and define different units of population with greater resolution than neutral markers (Nielsen 2001; Beaumont 2005; Schlötterer & Dieringer 2005; Storz 2005; Joost *et al.* 2007). Analysis of variation in or around genes is specifically targeted by expressed sequence tag (EST) sequencing, providing a more focused effort at describing functional genomic variation (Bouck and Vision 2007; Bonin 2008). ESTs are single-pass sequences generated from random sequencing of cDNA clones and represent a partial sequence of the much longer RNA expressed in a cell. Because the mRNAs have been processed and edited in the cell, ESTs encode genes that are actively transcribed without intervening intron sequences and so can be more informative about the ultimate function of the gene. They offer a rapid and valuable first look at genes expressed in specific tissue types, under specific physiological conditions, or during specific

developmental stages (Liu and Cordes 2004). In teleosts fishes, three-dozen species in diverse orders have EST collections that contain more than 10000 sequences: *D. rerio* have the most ESTs, followed by *O. latipes*, then the salmoniformes (*S. salar* and *O. mykiss*) and finally three-spine stickleback. ESTs often are sequenced with the end goal of using them for gene expression analyses, but also are a rich source for discovering microsatellites and SNPs. However, it's necessary be cautious, because one cannot always be certain that a particular SNP in an EST is due to true polymorphism or to sequence error. EST-derived microsatellites have been used for linkage mapping in *P. maxima*, *S. salar*, *O. mykiss* (Rexroad *et al*. 2005; Bouza *et al*. 2008; Moen *et al*. 2008), and, more recently, Kucuktas *et al*. (2009) combined both microsatellites and SNPs derived from ESTs, to construct a genetic linkage map of the *Ictalurus punctatus* (Rafinesque) genome. Other uses for these EST-derived microsatellites and SNPs include population-genomic analyses thanks to the advent of whole genome sequencing projects.

Genomics is a field of science that deals with the structure, function and evolution of genomes. Genomics often simply implies the use of high throughput DNA- or RNA- based methods. It comprises comparative, functional and environmental genomics. Comparative genomics examines whole genomes, their gene content, gene order, structure, evolution and taxonomy. Functional genomics investigates the biochemical and physiological role of gene products and their interactions on a large or small scale. Environmental genomics encompasses studies molecular variation in natural or artificial populations of different taxa and their response to environmental conditions such as temperature or pollutants (Wenne *et al.* 2007). Previously, fish genomics was restricted to fish species like Japanese pufferfish (*Takifugu rubripes*) and zebrafish (*Danio rerio*), both well-known model species, with reference genomes, for comparative and developmental genomics. Although marine fish genomics is still in its infancy, now other species have been sequenced, as medaka, *Oryzias latipes*, spotted green pufferfish, *Tetraodon nigroviridis*, and three-spined stickleback, *Gasterosteus aculeatus*.

A genome-wide coverage would provide a powerful tool to explore the balance between selection and gene flow, and its significance to population connectivity and local adaptation, and to establish selective effects caused by natural and anthropogenic environmental changes (Hauser and Seeb 2008). Concomitant with advances in molecular technology and development of new tools, statistical approaches were also strengthened,

mainly because of higher information content of more variable genetic markers, but also because of the increase in computing power (Beaumont and Rannala 2004; Pearse and Crandall 2004). In the last period there was an increase in sequencing speed and a reduction of sequencing cost achieved by enhancing automation and removing human input. Once limited primarily to model organisms and humans, these techniques are now readily available to fisheries genetics laboratories (Hauser and Seeb 2008).

3.2 FISHERY AND MANAGEMENT OF ATLANTIC BLUEFIN TUNA

3.2.1 Fishery

The oldest method of catching tuna consists of the traditional trap fishery (*tonnara*). They were used in the Mediterranean and along the coasts of the North Atlantic from the 14th century in Sicily, from 16th century in Sardinia and Portugal since the 19th century in Tunisia, Morocco and Spain. The traditional trap fishery were placed along the migration routes of tuna that came in May in the Mediterranean from the North Atlantic for breeding and then resumed in mid-July the way back. Depending on their location along their migration routes, these traps were divided into two categories: the outward and return. The first caught tuna at the beginning or during the period of breeding, the second at the end of such period. Both traps could be of gulf or tip depending on whether they are, within a bay or the end of a promontory. The *tonnara* is formed by a complex system of nets, placed as the barrier to guide and trap the tuna. The trap consisted of two essential structural elements, the *coda* and the *isola*; the *coda*, or the tail, is a long series of nets placed perpendicular to the coast, guiding bluefin toward the trap, and the *isola*, or island, is formed by an elaborate construction of nets that create an elongated rectangular structure. It is made up of many *camera*, or chambers, that divide the large structure into multiple squared pens, where fishes are captured, contained and moved towards final chamber, the *camera della morte* (the chamber of death) (Figure 7) (Longo and Clark 2012). Until the first half of the 20th century, there were hundreds of traps in the Mediterranean, but now they are about ten, due to expansion of exploited areas and evolution of fishing systems.

Figure 7. Scheme of trap fishery (Longo and Clarke 2012).

Around 1960 a new fishing strategy started to spread in Japan, the longline, consisted of a set of hundreds hooks connected at regular intervals to a single support strand called beam. The longliners primarily focused on medium-sized and large fish in temperate waters. These fisheries then expanded in the Mediterranean Sea and the Gulf of Mexico and are nowadays covering most of the bluefin tuna spatial distribution (ICCAT 2007; Fromentin 2009). In the same period purse seine fisheries really took place in Mediterranean Sea, firstly in Yugoslavia (currently Croatia) and Italy, then in France during the 1960s. Following the development of the Japanese sushi-sashimi market during the 1980s, these fisheries considerably increased and new purse seine fisheries appeared, especially in Spain, Tunisia, Turkey and Libya, so that these fisheries are nowadays the most productive ones (Fromentin and Ravier 2005; ICCAT 2007). This tool is formed by a long net, lowered in to the water as a

curtain and closed in a circle around a school of fish. Yet, these fishing methods catch many immature and undersized tunas and fish of other species.

This high increase in purse seine catches is related to the growth of tuna fattening farms, since the purse seine is the best gear type for ensuring the capture and transfer of live tuna (Sumalia and Huang 2012). Bluefin tuna ranching began in earnest in the Mediterranean in the mid-1990s. Up until that time, this practice had been used minimally in the Mediterranean, but the experience of Australian tuna ranches with southern bluefin tuna prompted the expansion of these methods in the Mediterranean. Bluefin ranching saw enormous growth during the 1990s and early 2000s (Miyake *et al.* 2003). Atlantic bluefin tuna farming and fattening in the Mediterranean Sea is a seasonal activity and it involves the capture of fish from the wild and their rearing in sea cages for periods ranging between 3 months to 2 years. According to ICCAT, the Atlantic bluefin tuna rearing operations are classified as "fattening" if rearing is done for a short period (3-7 months) using mature fish (> 30 kg in body weight) to achieve a greater fat percentage in the muscle, which is desirable by the sushi and sashimi markets in Japan, or "farming" if rearing is done for a longer period of time (up to 2 years) and involves juvenile fish (8-30 kg in body weight), reaching a harvest size between 30 and 50 Kg (ICCAT 2008; Mylonas *et al.* 2010). The countries involved in Atlantic bluefin tuna fattening are Cyprus, Greece, Italy, Malta, Spain, Tunisia, and Turkey, while farming takes place only in Croatia. The highest volumes of production in recent years are coming from Malta, Tunisia, Croatia, Italy, and Turkey (Mylonas *et al.* 2010). Yet, this method of production has faced challenges due to its continued reliance on live fish from wild stocks. In addition, the metabolism of bluefin tuna requires high inputs of energy (calories) in order to increase body size and weight. Furthermore, the production and transport of fresh and frozen bluefin tuna to wealthy markets require a big energy expenditures (Longo and Clark 2012).

Development of a proper aquaculture industry for the Atlantic bluefin tuna could be the only way to both satiate the great demand for sushi and conserve the wild stocks of this fish. One of the prerequisites for domestication and the establishment of a sustainable aquaculture industry is the capacity to control reproductive processes of fish in captivity, and to acquire high quality eggs and sperm for grow-out of the marketable product (Mylonas *et al.* 2010). Reproduction in captivity of the bluefin tuna was first accomplished in Japan with the Pacific bluefin tuna (Kumai 1998; Lioka *et al.* 2000; Sawada *et al.* 2005; Masuma *et al.*

2006). Fish are maintained in large cages or enclosures and are allowed to spawn naturally. In June of 2002, artificially bred Pacific bluefin tuna broodstock produced 1 million eggs for the first time. Several studies have been undertaken to develop methods for the control of reproductive maturation in captive-reared Atlantic bluefin tuna (Corriero *et al.* 2007; Mylonas *et al.* 2007; Corriero *et al.* 2009; DeMetrio *et al.* 2010), capturing migrating Atlantic bluefin tuna (5-12 years old) in the Mediterranean Sea. During the natural spawning period (June-July) of two consecutive years, fish were implanted underwater with a controlled-release delivery system loaded with gonadotropin-releasing hormone agonist (GnRHa), demonstrating that it is possible to induce maturation, ovulation/spermiation, and spawning in captivereared Atlantic bluefin tuna using a GnRHa-based therapy. (Mylonas *et al.* 2007, 2010).

3.2.2 Management

Archaeological excavations have shown that fishing on bluefin tuna has occurred in the Mediterranean since the 7th millennium BC (Desse and Desse-Berset 1994). The popularity of Japanese sushi and sashimi worldwide during the 1980s made *T. thynnus* much more economically attractive than before. As the western stock has already been extensively depleted, the eastern Atlantic stock has remained a major source of Atlantic bluefin tuna to supply the global market. Now bluefin tuna is heavily exploited over its whole spatial distribution for a decade, there is thus no more refuge and all the potential sub-populations are currently exploited (Fromentin and Powers 2005; ICCAT 2007). Specific natural characteristics, such as late reproduction, large size at reproduction, long lifespan and the aggregation of the fish that occurs during spawning, make bluefin tuna extremely vulnerable to overexploitation (Safina 2001; Ottolenghi *et al.* 2004). Bluefin tuna are also sensitive to oceanic conditions and disturbances such as those caused by industrial pollution. For an effective management and conservation, it is crucial to know about bluefin tuna population structure and spatial dynamics and their interactions with fishing and environmental conditions (Fromentin 2009).

Observing the BFT historic catch by gear type in the Mediterranean Sea from 1950 to 2010, it has been noted that from the 1950s to the early 1970s, total catches were stable at around 5000 to 8000 t per year, while there was a peak in the mid-1970s (over 15000 t per

year), followed by an unusual drop by the early 1980s. From then on to the mid-1990s, the catches increased steadily from 9000 to 40000 t per year. After that, there was a substantial decrease in catch to 24000 t per year in the most recent decade, which seems to serve as an indication of effective management (Figure 8). So, due to its commercial importance, bluefin tuna is intensely fished and actually overexploited. Since 1970 the biomass of bluefin tuna broodstocks declined by 77% and 14% in the western and eastern populations, respectively (ICCAT 2005). Western Atlantic bluefin spawning stock biomass (adult-aged fish) has declined to about 20-29 per cent of 1970 levels (ICCAT 2010). Eastern Atlantic stocks are between 40% and 57% of spawning stock biomass of the highest known levels in the late 1950s (MacKenzie *et al.* 2009; ICCAT 2010).

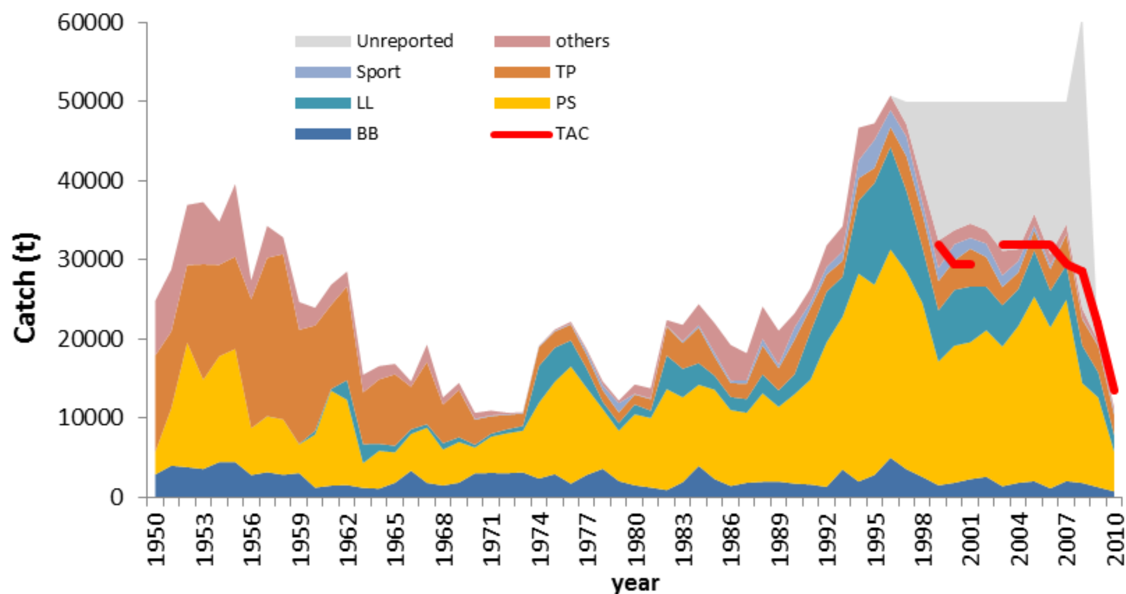


Figure 8. Catch for the East Atlantic and Mediterranean from 1950 to 2010 by gears; LL: longline; BB: bait boat; TP: tuna trap; PS: purse seine; TAC: Total Annual Catch (ICCAT 2012).

To deal with the common-property and shared stock problem of tunas, the International Commission for the Conservation of Atlantic Tunas (ICCAT) was established in 1969 to manage more than 30 tuna and tuna-like species in the Atlantic Ocean and adjacent seas, including the Mediterranean bluefin tuna. The Commission, composed of 48 Contracting Parties (countries/political entities), is a Regional Fisheries Management Organization (RFMO) responsible for combining a wide array of scientific and socio-

economic information into setting the annual total allowable catch (TAC) of Atlantic Tuna species. ICCAT also includes the Standing Commitee on Research and Statistics (SCRS), composed of scientists from various countries, that is responsible for producing models of catch statistics and trends of populations and for providing scientific advice to ICCAT on the TAC and quota allocation among countries' members (Sumalia and Huang 2012). The quota set by ICCAT is then split among member countries who are individually responsible, but not obliged, to manage their fleet in accordance with the TAC. How the shares are divided has undergone changes in two different periods. From 1983 to 1991, ICCAT allocated the TAC among countries mainly according to their historical catches. In addition, the spatial distribution of stock, proximity to coastal states, especially in small and developing countries, have also been taken into consideration. However, CPs (Contracting Parties) without large historical catches argued for changes in the allocation formula in the 1990s and succeeded in getting ICCAT to increase their share in 2001. The allocated quota is transferrable among countries' members, though transfers have to be made under the approval of ICCAT (Grafton *et al.* 2006).

The ICCAT, based on spawning sites of bluefin tuna, recognizes two stocks: those of the west and the east Atlantic (the latter including the Mediterranean Sea), separated by the 45th W meridian (Nemerson *et al.* 2000) although mixing between the two units is known to occur (ICCAT 2002). Both stocks are estimated to be strongly overfished and continue to be overexploited; the 2006 stock assessment points out a substantial risk of fisheries and population collapse (ICCAT 2007). Current advice for bluefin tuna is based upon Virtual Population Analysis (VPA), which assumes that the 2 stocks Eastern and Western considered since 1981 are homogeneous and that there is no sub stock structure within them (Kell *et al.* 2012).Recent evidence indicates, however, that the two populations overlap in the North Atlantic foraging grounds (Block *et al.* 2005).

Illegal, Unreported and Unregulated (IUU) fishing is widely recognized as one of the biggest concerns with BFT management in the Mediterranean Sea and other Atlantic Ocean areas. WWF found huge gaps between national reports on BFT trade and official catch reports to ICCAT, indicating that a large amount of IUU fishing place in the region (WWF 2006). It estimated that the total BFT catches in the Eastern Atlantic Ocean and the Mediterranean Sea recorded through international trade were approximately 45000 t in both 2004 and 2005, which were 40% above the total annual catch (TAC) of 32000 t set by

ICCAT. For 2006-2010, declared catch was 30689 t, 34516 t, 23849 t, 19701 t and 11294 t for the East Atlantic and Mediterranean, of which 23154 t, 26479 t, 16205 t, 13016 t and 6949 t were declared for the Mediterranean for those same years (Figure 9).

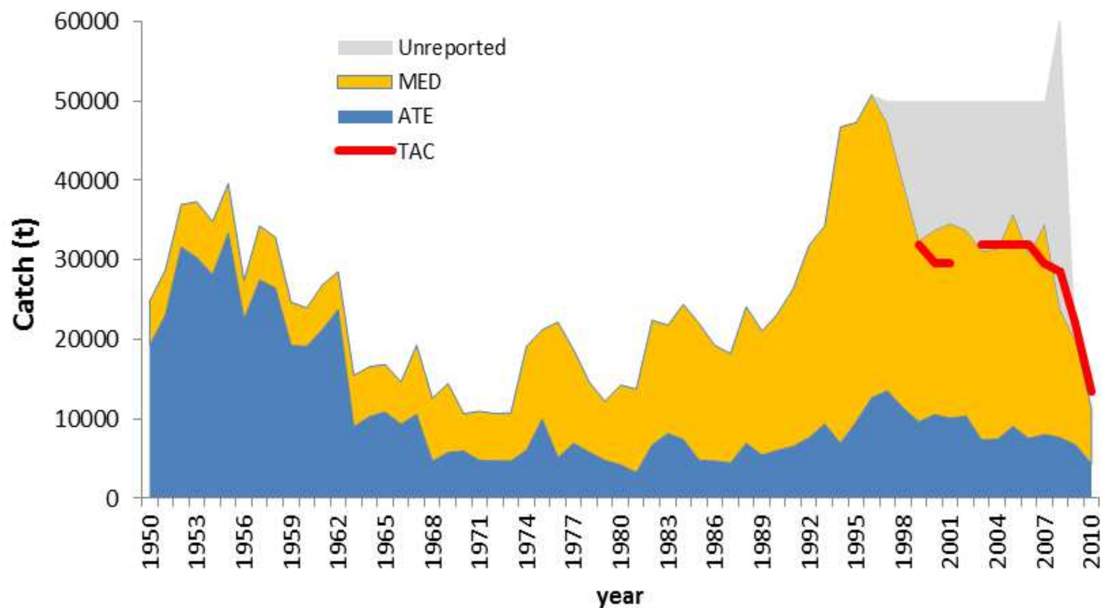


Figure 9. Catches divided by main geographical areas and catches not reported; MED: Mediterranean Sea; ATE: East Atlantic; TAC: Total Annual Catch (ICCAT 2012).

Catches of bluefin tuna from the east Atlantic and Mediterranean were under-reported between the mid-1990s through 2007. During this period, based on the number of vessels operating in the Mediterranean Sea and their catch rates, ICCAT estimated total catches to be close on the order of 50000 t to 61000 in the Mediterranean Sea. Estimates for 2008 and 2009 showed a substantial decrease in the catch, and declared catches in 2010 (11294 t) were significantly below the 2010 TAC of 13500 t (ICCAT 2012).

The pattern of catch at age in the Mediterranean Sea from 1955 to 2010 showed that the catch of age 0 ABFT has decreased since the 1960s and is barely observed today. The catches of other age groups have all increased in weight in 2006 compared to 1950. Increasing BFT catches have led to rapid stock declines over years. According to the stock assessment analyses reported by ICCAT, the spawning stock biomass (SSB), one of the most important indicators of stock abundance and health, is about 57% of the highest estimated SSB levels (1957-1959). Trend in fishing mortality (F) displayed a continuous increase over

the time period for the younger ages (ages 2-5) while for oldest fish (ages 10+) it had been decreasing during the first 2 decades and then rapidly increased during the 1990s.

Bluefin tuna, like other species of fish, are increasingly commodified under the capitalist global market. The lifecycles of bluefin are subsumed under market demands to enhance profit, resulting in unsustainable interactions with bluefin tuna populations. The consequences of this growth imperative include drastic reductions in bluefin populations and the collapse of a sustainable fishing system (Longo and Clark 2012). With the intention of better managing this fishery and protecting the wild stock from over-fishing, during the last decade, the ICCAT adopted a series of measures to control the fishery in the Mediterranean Sea, as an increase in the minimum catch size from 10 to 30 Kg, a reduction in the number of permissible fishing days from 11 to 6 months, the presence of observers at cage facilities, and the prohibition of at-sea transshipment (Commission 2007). Since 2010 ICCAT reduced total allowable catches to 13500 tons (from 32000 tons three years ago), lowered this quota to 12900 tons in 2011, and restricted the purse seine fishing period to only one month. However, it remains to be seen whether the efforts will have the intended consequences of returning the state of the stock to sustainable levels, as, by ICCAT’s own estimates, this gives the stock a 60% chance at recovery by 2022 (ICCAT 2010).

Atlantic US. fisheries for tuna are managed by the National Oceanic and Atmospheric Administration's (NOAA's) National Marine Fisheries Service (NMFS) under the authority of the Atlantic Tunas Convention Act (ATCA) and the Magnuson-Stevens Fisheries Conservation and Management Act (Magnuson-Stevens Act). ATCA authorizes the promulgation of regulations, as may be necessary and appropriate, to implement conservation and management recommendations adopted by the ICCAT. Directed fishing for bluefin on their Gulf of Mexico spawning ground was prohibited by ICCAT in 1982 (NMFS 2006). Western Atlantic catches peaked in 1964 at 18679 t, due to annually fishing of 5000-12000 t mature bluefin off Brazil by Japanese boats from 1962 to 1967, and declining since 2002 until 1523 t in 2005 for a unavailability of fish (Figure 10). In 1998 the commission’s scientific committee determined the annual west-Atlantic catch of 2500 t could not be sustained, 2000 t was likely sustainable, and a quota near zero was necessary to restore the population to 1970s levels within 20 years (Safina and Klinger 2008). In recent years, however, there appears to have been a gradual increase in SSB from the low of 21% in 2003 to an estimated 29% in

2009. The Commission recommended a total allowable catch of 1900 t in 2009, 1800 t in 2010, and 1750 t in 2011 (ICCAT 2012).

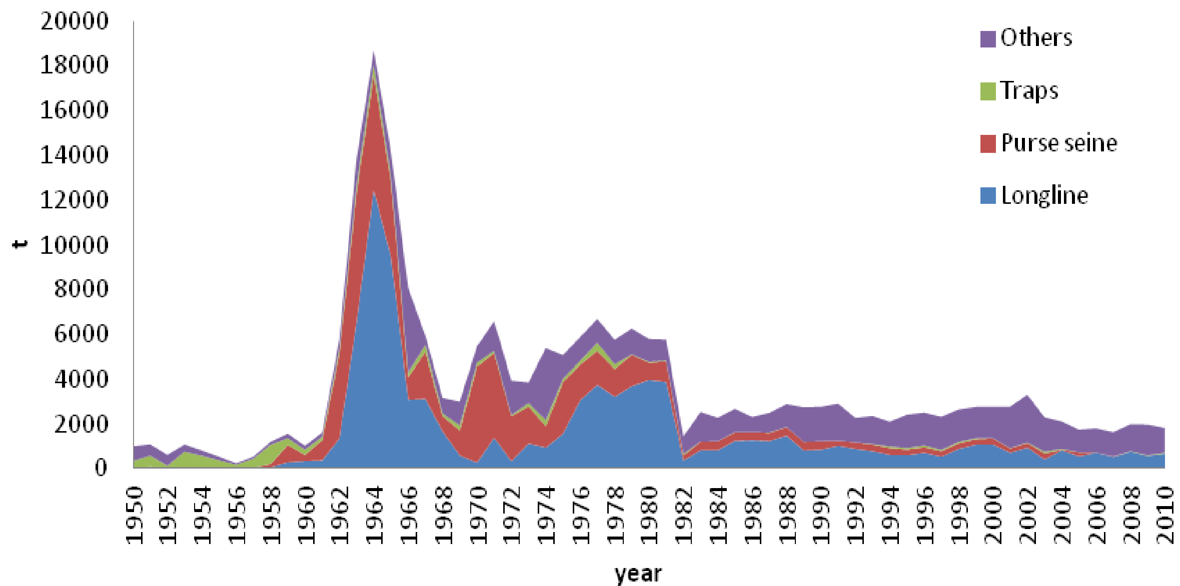


Figure 10. Catches of western bluefin tuna by gear type (ICCAT 2012).

In 2010, the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) discussed the proposal to include the Atlantic bluefin tuna in the Appendix I that includes species that are threatened with extinction, for which, therefore, international trade is prohibited (http://www.cites.org/eng/cop/15/prop/E-15-Prop-19.pdf). The proposal was not adopted by the conference due to solid opposition from nations such as Japan, Korea, Libya, and Turkey.

3.3 POPULATION GENETIC STUDIES OF ATLANTIC BLUEFIN TUNA

Early genetic studies of Atlantic bluefin tuna failed to detect genetic differentiation between eastern and western Atlantic stocks (Edmunds and Sammons 1971, 1973; Thompson and Contin 1980). This is consistent with a species that has a large migratory potential such as the Atlantic bluefin tuna. These preliminary results, indicating a lack of heterogeneity between the two Atlantic stocks, were further supported by studies of nuclear allozymes (Pujolar *et al.* 2003) where spatial or temporal genetic heterogeneity wasn't

observed within the Mediterranean Sea or between the East Atlantic and Mediterranean, indicating the existence of a single genetic grouping on the eastern side of the Atlantic Ocean. No evidence of genetic differentiation between northeast Atlantic and the Mediterranean samples was also found in a study of Alvarado Bremer *et al.* (2005) using the mitochondrial DNA control region (mtDNA CR). This result agrees with the study by Ely *et al.* (2002) that failed to find genetic differences between several temporal Mediterranean samples. Pujolar *et al.* (2003) and Alvarado Bremer *et al.* (2005) analyzed a combination of adult and young individuals using either nuclear allozymes or a short segment of the mtDNA CR respectively, which in both cases might not have the resolution to observe genetic differentiation for such closely related populations. However, one of the major limitations of these studies was that the samples representing the western stock were collected along the United States where mixing of the two stocks may occur and weren't collected in the Gulf of Mexico, the spawning ground for the western stock (Rooker *et al.*2007). A recent study (Viñas *et al.* 2011) conform to the hypothesis of a single panmictic unit of Atlantic bluefin tuna throughout the Mediterranean. This lack of differentiation within the Mediterranean conforms to the general pattern that population structure for large pelagic species, such as the Atlantic bluefin tuna, is only observed on a transoceanic, rather than a local scale (Palumbi 1994; Graves 1998).

Different results were obtained when more representative samples of the stocks were analyzed, including samples from the breeding grounds in the Gulf of Mexico and Mediterranean Sea, so the differentiation between these two populations gained more support (Carlsson *et al.* 2007; Boustany *et al.* 2008). These later findings were in agreement with the natal homing fidelity observed from recent tagging studies (Block *et al.* 2005), microchemical signatures (Rooker *et al.* 2008), and the clear differentiated biology between the eastern and western Atlantic bluefin tunas (Fromentin and Powers 2005). Carlsson *et al.* (2004) observed low levels of genetic differentiation among three regions within the Mediterranean using mtDNA CR sequence data and eight microsatellites. Differentiation was found only between samples in the Ionian Sea and the Tyrrhenian Sea, and it wasn't detected between the Balearic Sea and Ionian Sea. In a second study, Carlsson *et al.* (2007) used the same sample from the Ionian as a reference for the eastern Mediterranean, involving only young of the year (YOY) individuals, and compared them to YOYs from the western Mediterranean and Gulf of Mexico. Significant genetic differentiation at eight

nuclear microsatellite loci (F_{ST} = 0.0059, P = 0.0005) and at the mitochondrial control region (Φ_{ST} = 0.0129, P = 0.0139) was detected among YOY Atlantic bluefin tuna captured on spawning grounds in the Gulf of Mexico versus the western and eastern basins of the Mediterranean Sea. In a more recent study, Boustany *et al.* (2008) combined mtDNA CR sequences and electronic tracking data and found significant population subdivision among the Gulf of Mexico, western Mediterranean and eastern Mediterranean Sea. Finally, in a study by Riccioni *et al.* (2010), the authors analyzed 8 microsatellite loci variation from six contemporary and two historical (80-96 years old) samples of Atlantic bluefin tuna located in the western Mediterranean, detecting significant genetic differentiation. This study also showed that genetic differences between ABFT populations were present long before the development of industrial fisheries and apparently persisted across approximately the past century and several generations. Despite the overexploitation of the Mediterranean population has drastically reduced the census size and changed the population age structure and reproductive demographics (ICCAT 2008; MacKenzie *et al.* 2009), the genetic diversity of the Mediterranean population has been retained over the years and the effective population size (Ne) estimated for Mediterranean populations is about of 500 individuals, a number that is considered to be above the minimum threshold necessary to maintain the genetic diversity and evolutionary potential across generations in natural populations. The same significant differentiation signal (F_{ST} = 0.015) was observed between the two most distant (Alboran Sea and Adriatic Sea) and the two nearest samples (Alboran Sea and Algerian coast). The differentiation pattern between the Alboran Sea and the Adriatic Sea may be related to the fact that the location of the Adriatic Sea belongs to the eastern Mediterranean basin. However, it is very difficult to find a plausible explanation involving life history traits for the genetic differentiation observed between the Alboran Sea and Algerian coast samples. These two locations are part of the same Mediterranean basin and probably share the same breeding ground. In conclusion, although slight evidence of population differentiation within the Mediterranean cannot be denied, in some cases it is very difficult to reconcile the present knowledge of the biology of the species with the results suggesting genetic differentiation (Viñas *et al.* 2011). Moreover, including historical samples from juveniles and adults, the work of Riccioni *et al.* (2010) confirm that the differences that they find in Atlantic bluefin tuna inhabiting the Mediterranean Sea are temporally stable, and not a artifact of high fecundity, so great reproductive success of only a few individuals could

cause genetic variance (F_{ST}'s) to fluctuate widely each generation (commonly called "genetic sweepstakes") (Waples 1998).

Chini *et al.* (2008) started an EST project on *T. thynnus*, developed 10163 sequences, obtained from ovary, testis and liver. They have identified several sequences with known function in other organisms, but not previously described in this species. Among the new genes, 712 were found only in the expression library of the ovary, 613 in that of the testis and 318 in that of the liver, while 324 additional genes were shared by two or more expression libraries; other 127 genes not found in the expression libraries were obtained from the ovary normalized library. Starting from 10163 Expressed Sequence Tags, Ferrara *et al.* (2010) developed 16 EST-linked microsatellite loci for *Thunnus thynnus*, for understanding population structure and investigating the dynamics of local adaptation in Atlantic bluefin tuna.

3.4 RESEARCH AIMS

The changes in fishing rules and the increasing demand of Atlantic bluefin tuna, due to the expansion of Japanese market since the early 80, have driven to need of the adoption of suitable measures for the maintenance of this species.

In the last decades, the exploitation of *Thunnus thynnus* is greatly above the sustainable level, so ICCAT decided to start a plan for the restoration of stocks, in order to get better management of this species and to avoid a collapse of these important commercial resource.

My PhD project was focused on developing new genetic tools for Atlantic bluefin tuna (*Thunnus thynnus*) within the Phase 2 of ICCAT/GBYP project, performed with a consortium of several italian and foreign partners. The research carried out during these three years was aimed at improving the knowledge of population structure of this species, shifting from a neutral variation-based approach to a new concept for population genetic with high-resolution power, based on markers developed in coding regions, so potentially under selection.

I have used novel high-throughput genomic technologies, as Next Generation Sequencing (NGS) and a large number of Single Nucleotide Polymorphisms (SNP) markers, developed from a collection of Expressed Sequence Tags (EST) employing both

transcriptomic and genomic resources. Analysis of population genetic structure was performed between Gulf of Mexico and Mediterranean samples, and within Mediterranean basin, in order to identify different separates genetic units, needing to be manage independently.

Methodologies and results related to these two main research topic are illustrated in chapter 4 and 5, that are manuscripts in preparation to be submitted.

CHAPTER 4

HIGH-THROUGHPUT SNP DEVELOPMENT IN ATLANTIC BLUEFIN TUNA USING A COMBINED GDNA AND CDNA SEQUENCING STRATEGY

High-Throughput SNP development in Atlantic Bluefin Tuna using a combined gDNA and cDNA sequencing strategy

Authors:

Eleonora Pintus[1], Alessia Cariani[1], Fausto Tinti[1], Jeroen van Houdt[2], Gregory Maes[2], Aitor Albaina[3], Pablo Markaide[3], Andone Estonba[3], Rita Cannas[4], Urtzi Laconcha[5], Nicolas Goñi[5], Haritz Arrizabalaga[5].

Affiliations:

[1] Department of Biological, Geological & Environmental Sciences (BiGeA) School of Sciences, University of Bologna, Via Selmi 3, 40126, Bologna, Italy.

[2] Katholieke Universiteit Leuven, Laboratory of Biodiversity and Evolutionary Genomics Fish Genomics Group/BioGenomics division, Ch. Deberiotstraat, 32 B-3000, Leuven, Belgium.

[3] Department of Genetics, Physical Anthropology & Animal Physiology, University of the Basque Country (UPV/EHU), Bº Sarriena s/n 48940, Leioa, Bilbao, Spain.

[4] Department of Life and Enviromental Science, University of Cagliari, Via T. Fiorelli 1, 09126 Cagliari, Italy.

[5] AZTI-Tecnalia / Unidad de Investigación Marina, Herrera kaia portualdea z/g, 20110 Pasaia (Gipuzkoa), Spain.

Introduction

The Atlantic bluefin tuna (*Thunnus thynnus*) is a highly valued, long-lived, and large-bodied marine fish, with geographically restricted spawning sites, as well as relatively short spawning periods of 1 or 2 months (Fromentin and Powers 2005; Rooker *et al.* 2007), all life history traits that make the species susceptible to collapse under continued excessive fishing pressure (De Roos and Persson 2002). *Thunnus thynnus* is an important economic resource that sustains artisanal (with historical and cultural associated value) as well as high capacity fisheries constituting the main resource for a huge number of fishermen and manufacturers (Collette *et al.* 2011). A decline of at least 50% since the 1970s has been estimated and the species is considered overexploited (MacKenzie *et al.* 2009; Juan-Jordá *et al.* 2011) placing *T. thynnus* close to resource collapse. Therefore, improving the management of this resource is a priority and this has to begin by improving the population/stock assessment.

Complex population dynamics, over both spatial and temporal range, and highly migratory behavior, with documented transoceanic and large-scale movements for feeding and reproduction, have been reported from classic tagging experiments and fishery data along with more recently develop methods: otoliths chemistry and molecular marker analyses (Fromentin and Powers 2005; Block *et al.* 2005; Carlsson *et al.* 2007; Rooker *et al.* 2007; Walli *et al.* 2009; Galuardi and Lutcavage 2012). Two main spawning areas were identified, eastern (Mediterranean Sea) and western (Gulf of Mexico) Atlantic, and, currently, *Thunnus thynnus* is managed separately as two stocks, divided by a conventional border in the mid-Atlantic, at 45th W meridian (ICCAT 2002). Further means of assessment of tuna populations are needed (Collette *et al.* 2011) and in this sense an improved understanding of connectivity between individuals from the two main spawning areas is required to properly manage fisheries (Rooker *et al.* 2008). Genetic studies using molecular markers with relatively low resolution (nuclear microsatellites loci and mitochondrial sequences) didn't lead to a definitive conclusion about the *T. thynnus* Mediterranean population structure that still remains undetermined. Recent studies have shown that Mediterranean *T. thynnus* do not represent a single panmictic population thus suggesting genetic structure in the Mediterranean area (Carlsson *et al.* 2004, 2007; Boustany *et al.* 2008; Riccioni *et al.* 2010; Viñas *et al.* 2011). It is therefore necessary to develop and apply more resolving molecular markers to really improve *T. thynnus* management, because

clarifying the population structure as well as achieving the origin traceability of individuals, will allow contrasting fishery frauds and reach the demanded sustainable management of the resource. A molecular tool providing high informative power would contribute to improve the resource management by assessing with certainty the genetic diversity (resilience capacity) of the species, a key factor when determining the species vulnerability to extinction based in its evolutionary potential, as well as by clarifying the stock delimitation. To date the most applied molecular markers when facing genetic diversity and stock delimitation have been microsatellite loci due to the high informative status that can be obtained from a few tens of highly polymorphic loci. However, microsatellites present several drawbacks, mainly of technical nature (Guichoux *et al.* 2011; Ogden 2011) and, nowadays, SNPs (Single Nucleotide Polymorphisms) type markers have become the marker of choice when facing population genetics questions (Helyar *et al.* 2011; Ogden 2011). SNPs are less informative per locus but can overcome microsatellites capacities when enough loci are available, and 5-10 SNPs per microsatellite locus is considered the threshold to attain similar discriminatory power. The avoidance of microsatellite technical associated problems and the cost reduction with recently developed SNP genotyping platforms along with the availability of a large number of SNPs due to the advent of the so-called Next Generation Sequencing (NGS) technologies (Garvin *et al.* 2010), makes designing a panel of SNPs the best selection when trying to produce a highly informative molecular tool.

SNP discovery has experimented a revolution with the advent of NGS technologies, such as Roche´s 454 or Illumina´s HiSeq platforms, allowing exploring genetic variation at a genome-wide scale even in non-model organisms (organisms lacking a reference genome, as most of wild living organisms do) (Garvin *et al.* 2010; Ekblom and Galindo 2011; Nielsen *et al.* 2011; Ogden 2011; Seeb *et al.* 2011a) thanks to the discovery of thousands of SNP markers via NGS, currently possible given a relatively low budget provided. When performing SNPs discovery applying NGS technologies, a common problem is to have enough coverage (number of times each genome position is sequenced) in order to differentiate a real polymorphism from a sequencing error. Different types of errors arise from distinct technologies (insertions and deletions (InDels) more common in 454, substitutions in HiSeq), in order to overcame this drawback and to provide accurate variant calling, a minimum average coverage of 10x to 30-50x per individual, for respectively 454 and HiSeq (due to shorter read lengths in the latter), is generally recommended for a good

transcriptome/genome assembly (Harismendy *et al.* 2009; Kim *et al.* 2011). There are several strategies aiming to obtain validated SNPs in non-model organisms without involving the costs of sequencing the whole genome at a high coverage. One of the most applied is sequencing the transcriptome, that is the very small percentage of the genome but represents the DNA sequences transcribed into RNA molecules. So, SNPs discovered in expressed sequences are located in functionally relevant regions of the genome and probably they are more discriminating between populations than markers found in genomic DNA sequencing. SNPs developed in transcriptome are prone to be under selection (conserved), so they are more informative than neutral ones when aiming the population/origin assignment of individuals (Andre *et al.* 2010; Freamo *et al.* 2011; Gómez-Uchida 2011). It is also possible to annotate the transcripts where SNPs are discovered and thus allowing to associate SNPs to a gene function providing useful information to address adaptation-evolution questions (Stapley *et al.* 2010). This approach was successfully used in the last years in several non model fish species, as lake sturgeon (Hale *et al.* 2009), rainbow trout (Sanchez *et al.* 2009), lake whitefish (Renault *et al.* 2010), catfish (Liu *et al.* 2011), hake (Milano *et al.* 2011), chum salmon (Seeb *et al.* 2011b), turbot (Vera *et al.* 2011), herring (Helyar *et al.* 2012) and common carp (Xu *et al.* 2012), thanks to progress in high-throughput technologies, to improvement of bioinformatic software and to reduction of costs. The main drawback associated to SNPs discovered from transcriptome sequencing is the correct prediction of Intron Exon Boundaries (IEB) proximity that is considered a major cause of genotyping failure (Wang *et al.* 2008). To overcome this issue, in this work we used a combined approach for the discovery and validation of a large set of SNP loci in Atlantic bluefin tuna. We used Roche 454 FLX sequencing to obtain muscle transcriptome sequences, and HiSeq platform, that yield more large output per run and shorter sequences of 100 base pairs, to produce a shallow sequencing (in terms of coverage) of the genome of *Thunnus thynnus*. The availability of this reference allowed us to map the resulting cDNA SNPs on the genome as to avoid the Intron Exon Boundaries trouble.

Thanks to these combined approach, we discovered and validated a large set of SNPs, that could be used to reach the knowledge of genetic structure of Atlantic bluefin tuna and improve its management, with the aim of developing a sustainable fishery of this important commercial species.

Materials and Methods

Atlantic Bluefin Tuna sample

Tuna samples were collected from three geographical regions considered representative of the species putative reproduction areas: Western Mediterranean (Balearic Island), Eastern Mediterranean (Cyprus Island) and Western Atlantic (Gulf of Mexico). Muscle tissue samples were taken from individual belonging to the Young of the Year age class except for the Eastern Mediterranean where adult individuals from fattening cages were available to our study.

Muscle tissues for cDNA sequencing were stored in RNA later at -80°C until RNA extraction, and additional aliquots were preserved in EtOH 96% at -20°C for DNA extraction.

RNA extraction, cDNA library construction and 454 sequencing

Total RNA was extracted with TRIZOL reagent (Invitrogen), and dissolved in Rnase-Free Water from RNeasy Kit (Qiagen). Quantification was performed with the NanoDrop ND-1000 UV/VIS spectrophotometer (Thermo Scientific) and RNA integrity check was assessed on the 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 Nano assay. Suitable samples for concentration and integrity were selected for mRNA isolation, after a DNAse treatment of samples showing a gDNA contamination on the Bioanalizer profile.

Total mRNA was obtained with the mRNA Isolation Kit (Roche) from 10 individuals (3 from Western Mediterranean, 3 from Eastern Mediterranean and 4 from Gulf of Mexico) and used as template for cDNA libraries synthesis according to Clontech's SMARTer cDNA kit. Two series of libraries were produced starting from 100ng of total RNA and 10 ng of isolated mRNA to compare results. Following manufacturer’s protocols, optimization of cDNA amplification condition was performed for each of the ten samples separately, in order to assess the optimal number of cycles ensuring that ds cDNA amplification remains in the exponential phase, as overcycled cDNA might results in concatemerization artifacts due to SMARTer kit reverse transcriptase enzyme activity. Normalization of cDNA libraries was performed with Trimmer Kit (Evrogen) following manufacturer’s guidelines. Libraries were purified with the QiaQuick PCR purification kit (Qiagen) and quantified before being processed according to the Roche protocol for cDNA Rapid Library preparation.

A preliminary test run was performed using only 2 individuals on a ¼ of Roche GS-FLX plate to assess the optimal conditions to be applied on the full run. The test run was performed to i) compare the sequencing outcome of libraries produced from total RNA and mRNA as starting material; ii) evaluate the influence of cycling conditions in the ds cDNA amplification step and iii) compare native to normalized cDNA libraries. To assess the influence of different starting material for cDNA synthesis reads were screened for ribosomal genes by a BLAST search against a local database of Scombridae rDNA sequences. Libraries produced from mRNA showed a considerable lower content of ribosomal transcripts; therefore the synthesis of cDNA from isolated mRNA was preferred. Libraries produced applying different cycling conditions were inspected for the occurrence of PCR oligos in the reads, expecting them only at 5' or 3' ends. In the libraries obtained with higher number of amplification cycles occurrences of SMARTer primers were detected also within the sequence reads, with several repeats, reflecting the production of PCR artifacts due to overcycling. According to these results new libraries were produced with fewer amplification cycles. The normalized samples sequencing didn't produced satisfactory length classes distribution, while the non-normalized ones yielded the expected pattern of reads lengths distributed around 450bp, therefore non-normalized libraries produced from isolated mRNA were used to continue.

The new set of cDNA libraries was produced following the results of the test run. In addition a modified oligo-dT primer (5'-AAGCAGTGGTATCAACGCAGAGTTTTCTTTTTTCTTTTTTV–3') was used for first strand synthesis as described in Meyer *et al.* (2009) and Beldade *et al.* (2006). The poly-T stretch is broken by the inclusion of an internal C to minimize the potential for Roche-454 sequencing problems in this homopolymer stretch. High-throughput sequencing of 10 individual libraries was performed on a full plate run of the Roche GS-FLX DNA Sequencer with Titanium chemistry.

DNA extraction, gDNA library construction and HiSeq2000 sequencing

DNA extraction was performed using the NucleoSpin® 96 Tissue Kit (Machery-Nagel) according to the manufacturer's instructions and DNA quantity and quality were measured using the Nanodrop ND-1000. Four suitable samples (2 from Western Mediterranean and 2 from Gulf of Mexico) were processed following the TruSeq DNA sample preparation protocol

from Illumina. The genomic libraries were sequenced with 2 × 75-bp paired-end module on 3.25 lanes of a HiSeq2000 (Illumina).

Sequence processing (cDNA and gDNA)

Reads produced from the cDNA sequencing were de-multiplexed based on the specific barcoding tags using sff-file tools from the Roche-454 analysis software and binned per individual sample. Reads were trimmed using CLC Genomic Workbench (CLCbio) according to quality value and removing adapter from Roche-454 and SMARTer libraries construction using default settings.

To identify and remove mitochondrial transcripts, trimmed cDNA reads were mapped against complete mitochondrial *Thunnus thynnus* genomes retrieved from NCBI (Accession Numbers GU256522 and AY302574), using gsMapper 2.5 software applying default settings. Local BLAST search was utilized to identify and isolate ribosomal transcripts. The reference database was constituted by all ribosomal sequences available on GenBank for *T. thynnus* and for other Scombridae species when a ribosomal gene was not available for the target species.

Data produced from the gDNA sequencing on the HiSeq2000 were processed with CLC Genomic Workbench to trim reads according to quality value and removing TrueSeq adapters using default settings. No filtering for mitochondrial and ribosomal sequences was performed on this dataset. A two-step approach for de-novo assembly of ABFT genome was performed with CLC (default settings): first reads of each of the sequenced individuals were assembled separately, and then all resulting contigs were de-novo assembled again into a "second order contig" dataset, to be used as reliable genomic reference for ABTF.

SNP discovery procedure

For computational reason it was necessary to select a suitable subset of the genomic reference, to reduce the memory requirements of the transcriptomic data mapping step needed for variants calling. This genomic subset was generated by mapping cDNA reads (filtered for mitochondrial and ribosomal transcripts) to the complete genomic reference using CLC. Genomic contigs were selected applying two criteria: i) a minimum of 10 cDNA reads mapping onto, and ii) a minimum length of 200 bp covered by the mapped reads.

Mapping of cDNA reads to the reduced genomic subset was performed with gsMapper 2.6 (release May 2011), which algorithm can output BAM files. However the BAM file produced is not transferable to any other SNP detection tool, due to the Roche unique way of coding SNP variants. For this reason cDNA reads from each of the ten sequenced individuals were mapped separately and then results pooled to obtain a unique list of not redundant cDNA variants.

Mapping of gDNA reads was performed with CLC to identify genomic SNP for each of the four sequenced individuals, with modified settings of Insertion cost (long reads) = 1, Mismatch cost (long reads) = 3, Similarity = 0.9.

For all candidate cDNA and gDNA SNPs summary statistics at the variant position (i.e. total depth, reference base, alternative allele and frequency/depth of each allele) were obtained for each individual from the cDNA and gDNA mapping output BAM files with custom Perl scripts, as well as ± 60 bp up/down-stream flanking genomic region masking occurring polymorphism with N.

SNP selection procedure

The list of not redundant cDNA variants produced by the cDNA reads mapping was mined to select the best 384 candidate SNPs to be included in the genotyping panel.

Different criteria were assessed and evaluated as:

- the coverage of each candidate SNP in each individual (that carries the variant). The minimum coverage for a SNP to be considered reliable was set to at least 4 reads present at the position and the alternative allele present in at least 2 reads. The higher the number of individuals carrying the variant that accomplished this criterion the more trustworthy as real polymorphism the candidate SNP was considered;

- the presence of the polymorphism in both cDNA and gDNA datasets (i.e. cDNA-gDNA overlap). If a polymorphism is found in two completely independent datasets and approaches the robustness of being a real variant and not a sequencing error or artifact is well supported;

- exclusion of SNP with nucleotidic incongruence between the different individuals analyzed, due to presence of multiple bases (SNP with 3 or 4 allele cannot be scored with the Illumina GoldenGate assay) or indels.

Since the Illumina GoldenGate assay on the VeraCode BeadXpress format was chosen for genotyping, the requirements needed for this assay were properly taken into account in the SNP selection procedure. The main criteria requested for non model organism by the Illumina GoldenGate genotyping assay design are:

1. distance of at least 60 bp between each SNP included in the assay, to avoid interaction and steric hindrance of the genotyping oligos annealing on the same genomic region.
2. conserved flanking region surrounding the SNP position, because suitable stretches are required for oligos design, to avoid mis-annealing on polymorphic sequences.
3. Illumina Assay Design Tool Score (ADT score) > 0.6. This value (assigned between 0 and 1) is obtained submitting the list of variants and 60 bp up/down-stream flanking region to Illumina, which evaluate them with proprietary software. ADT score utilizes factors including template GC content, melting temperature, sequence uniqueness, and self-complementarity to filter the candidates SNP prior to further inspection. The score is indicative of the expected success of the assay when genotyped with the Illumina GoldenGate chemistry.

To accomplish Illumina genotyping assay criteria for non model organism we properly evaluated polymorphism in the genomic region surrounding each candidate cDNA variant to be included in the GoldenGate assay in order to avoid the design of genotyping oligos in a variable portion, which could hamper oligos annealing and resulting in assay fail. When retrieving the flanking regions (± 60 bp) for ADT score evaluation each variant detected by the mapping of both gDNA and cDNA reads was masked with N.

Available SNPs developed for *T. alalunga* and already validated on *T. thynnus* individuals (Albaina et al, in press), accomplishing with the requirements needed for the Illumina GoldenGate assay, were included in the genotyping panel.

SNP genotyping procedure

A total of 384 SNPs were selected to be genotyped in 120 *T. thynnus* individuals, 40 from each of three SNP discovery geographical populations (Eastern Mediterranean, Western Mediterranean and Gulf of Mexico).

To assess the performance of developed markers in a closely related species, 30 individuals of *T. alalunga* (which global distribution was covered by including five individuals from each of the six management units currently accepted) were genotyped for the full 384 SNP panel.

Samples tissue (fin clip, muscle or full larvae) was processed for genomic DNA extraction using the Nucleospin Tissue DNA extraction kit according to manufacturer's conditions (Machery-Nagel GmBH, Düren, Germany) with the semiautomatic 96-well plate equipment Tecan Freedom Evo 150E. PicoGreen (Invitrogen) dsDNA quantification was performed to assess DNA quantity and quality, and DNA was normalized at 50 ng/ul in order to fulfill the requirements of the Illumina assay. Genotyping was performed with the GoldenGate assay on the VeraCode BeadXpress format. Results were visualized and analyzed with the GenomeStudio Data Analysis Software package.

Individual samples with a call rate lower than 0.8 and loci showing poor amplification or clustering were excluded. Accepted SNPs were reviewed and manually re-clustered, to correct errors in allele calling due to inappropriate automatic cluster identification.

Statistical analysis

After removing monomorphic loci applying threshold criteria of Minor Allele Frequency (MAF) and observed heterozygosity (H_O) ≥ 0.01 over the entire dataset. For the successful polymorphic SNP values of observed (H_O) and expected (H_E) heterozygosity were estimated using GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Deviations from Hardy-Weinberg equilibrium (HWE) was assesses using Genepop 4.2 (Rousset 2008). Significance levels for HWE tests were estimated using an MCMC chain of 10000 demorizations, 20 batches and 5000 iterations per batch. Evidence of linkage disequilibrium (LD) was explored using FSTAT 2.9.4 (Goudet 1995) and p-values were adjusted for multiple tests using the algorithm implemented in SGoF+ software (Carvajal-Rodriguez and Uña-Alvarez 2011). Lastly, ascertainment bias, resulting from the non-random exclusion of SNPs with a low Minor Allele Frequency (MAF) from the marker panel, may occur due to the small size (n = 10) of the ascertainment panel. To assess the magnitude of a potential bias, the distribution of MAF in the markers panel was assessed across the data set to check for an elevated non-random exclusion of SNPs with a low MAF. An un-biased SNP panel should exhibit a distribution of MAF categories indicating adequate representation of all MAF categories.

Functional annotation and Gene Ontology

The functional annotation was restricted to the reduced genomic subset and to the contigs, included within the formers, where SNPs were selected for genotyping in order to look for putative biases in the SNP selection procedure. Java web version of Blast2GO suite (Conesa *et al.* 2005; version 2.5.0; http://www.blast2go.com/b2ghome) was selected for functional annotation. Due to the long genomic contigs to be analyzed and the fact that Blast2GO is better suited for cDNA annotation than for gDNA sequences, the ab initio gene predictor Augustus (Stanke *et al.* 2004; version 2.5.5; http://augustus.gobics.de/) was used to predict proteins in both the data sets. The program was set to find complete or partial coding sequences (CDS) on both strands of the contigs, using human (*Homo sapiens*) as nearest species for software training. The predicted amino acid sequence was then annotated usingBlast2GO. Blastp, implemented in Blast2GO, was applied to recover Gene Ontology (GO) terms by searching against the NCBI non-redundant protein database (e-value < 10e-6, low complexity filter ON and Hit Sequence Percentage HSP length cutoff set to 33). For long proteins where Blastp search failed, the NCBI Blast web tool results (applying same parameters) were added to the Blast2GO project. Blast2GO annotation step was carried out with default parameters (e-value 10e-6, annotation cutoff 55 and GO weight 5) except for setting a 30% cutoff for HSP-Hit coverage. The annotated terms obtained with InterProScan, ANNEX and KEGG analysis, as implemented in Blast2GO tool, were added to previously obtained GO terms. Finally, an Enrichment Analysis (Fisher Exact Test corrected for False Discovery Rate, two tailed) was run in order to look for GO terms enrichment bias when comparing the genomic subset and the SNP containing contigs set.

Synonimous/Non-synonimous SNP classification

For the dataset of polymorphic SNPs, the variants putative effect on protein sequence was predicted. Briefly, two contig datasets were created to include both allelic variants for each SNP. Proteins were predicted from these two datasets applying Augustus software with previous parameters and inferred proteins and coding sequences were then compared. Intron/exon boundaries defined in the Augustus gff file were used to detect SNPs located on putative intron/UTR regions. Amino acid changes reported by the two datasets comparison where classified as Minor or Mayor changes, depending whether the corresponding amino acid change corresponded to the same group or not (based on the

polarity of the R group classification). While SNPs located within exons were classified as Non-Synonimous (NS), or Synonimous (S) depending on the SNP resulting in an amino acid change or not, Intronic (I) or Untraslated (UTR) categories applied to the remaining SNPs. The proteins inferred from the different haplotype combinations were taking into account when dealing with contigs containing more than one SNP.

Results

Sequence processing (cDNA and gDNA)

After evaluation of the preliminary test run to assess optimal conditions for cDNA library preparation and sequencing as described in the Methods section, transcriptome sequencing of the selected 10 individuals was carried out using 10 ng of non normalized mRNA and reduced cycling condition (number of cDNA amplification cycles ranging from 17 to a maximum of 20).

From a full 454 sequencing plate run 1182738 cDNA sequences were obtained using GS FLEX Titanium sequencing technology. Reads were assigned to each one of 10 sequenced individuals ranging from 17402 to 264784 reads per individual. After trimming according to quality value and removing adapter from Roche-454 and SMARTer libraries construction, 1021388 reads were retained. Mitochondrial transcripts were removed by mapping trimmed cDNA reads against complete mitochondrial *T. thynnus* genomes, excluding 7.8% of available sequences. Filtering for ribosomal transcripts was performed by local BLAST search and 954599 filtered reads were retrieved for downstream analyses (Table 1).

Sample name	Region	reads	reads after trimming	reads after mitochondrial and ribosomial filtering	reads mapped for variant calling	variant detected
WMED2	Western Mediterranean	78131	67372	63467	46741	1572
WMED4	Western Mediterranean	213680	171303	156492	123222	1483
WMED5	Western Mediterranean	186034	155624	144076	115895	1554
EMED1	Eastern Mediterranean	17402	16093	15097	12429	359
EMED2	Eastern Mediterranean	123670	115483	106886	84972	1749
EMED5	Eastern Mediterranean	23799	6759	5813	3655	162
GOM5	Gulf of Mexico	33567	24118	23022	17444	539
GOM41	Gulf of Mexico	264784	240872	226628	184599	2186
GOM44	Gulf of Mexico	200850	186007	177615	140202	1935
GOM20	Gulf of Mexico	40821	37757	35503	26627	891
	Total	**1182738**	**1021388**	**954599**	**709045**	**12430**

Table 1. Summary of reads data for the transcriptome sequencing: sequences numbers for each of the ten individuals are reported for each processing step as well as the number of variants detected.

From the genome sequencing of 4 individuals on 3.25 lanes of HiSeq2000 more than 833 million reads were produced, of which 826180771 were retained after trimming according to quality value and removal of TrueSeq adapters (Table 2). De-novo assembly was first performed for each of the sequenced individuals separately, which produced approximately 400000 contigs per individual sample. These 1625089 "first order contigs" were then assembled together to produce a "second order assembly" of 508757 contigs (hereafter 500 k contigs reference), used as genomic reference for subsequent analysis.

Sample name	Region	reads	reads after trimming	contigs produced	reads mapped for variant calling	variant detected
WMED1	Western Mediterranean	2,42E+08	239786564	404776	23275538	182149
WMED2	Western Mediterranean	1,88E+08	186439178	394634	18308490	177521
GOM30	Gulf of Mexico	1,8E+08	178215189	404436	20655541	196213
GOM40	Gulf of Mexico	2,24E+08	221739840	421243	20686872	182432
	Total	**8,33E+08**	**826180771**	**1625089**	**82926441**	**738315**

Table 2. Summary of reads data for the genome sequencing: sequences numbers for each of the four individuals are reported for each processing step as well as the number of contig obtained and variants detected.

About 70% of filtered cDNA reads could be mapped against the produced 500 k contigs genomic reference, with 400000 genomic contigs having at least 1 cDNA read mapped onto. To reduce the size of the genomic reference, contigs with at least 10 cDNA reads mapped and length of at least 200 bp were selected; creating a reduced genomic dataset of 4018 contigs (hereafter 4 k contigs reference), that was then used as reference for transcriptomic and genomic reads mapping and variants calling. The average contigs length of the 4 k contigs dataset was 8096 bp, with minimum length of 205 bp and maximum length of 56465 bp.

SNP detection and selection of candidate SNPs for genotyping

Mapping of cDNA reads from each sequenced individuals against the 4 k contigs reference was performed separately and then results pooled to obtain a unique list of 5412 not redundant biallelic SNP variants in 1350 separate genomic contigs. All candidate SNPs positions were also retrieved from the gDNA reads mapping output. Flanking regions of 60 pb were extracted from the contigs sequences, masking occurring polymorphism with N. The list of candidate SNPs was mined to select the best 384 loci to be included in the genotyping panel for validation. The first two criteria applied were those mandatory for the Illumina GoldenGate genotyping assay, i.e. Illumina Assay Design Tool Score (ADT score) > 0.6 and at least 60 bp between SNPs, reducing the number of suitable variants to 1594. Then SNPs were further selected if having i) the same polymorphism detected in both cDNA and gDNA dataset (cDNA and gDNA overlap) and ii) present in at least 1 individual with minimal coverage of 4X (2X for the alternative allele). This combination led to 299 SNPs.

To this subset of 299 SNPs, we added loci selected following less restrictive criteria: iii) 41 variants detected in at least 2 individuals with minimal coverage of 4X (to increase the robustness and therefore the reliability of the locus); iv) 33 SNPs detected in only 1 individual with minimal coverage, but having the highest ADT score.

Moreover, 11 SNPs developed for *T. albacore* and already validated on *T. thynnus* individuals were scored for inclusion in the genotyping panel of 384 loci, after mapping the markers in the 500 k contigs genomic reference and retrieving the corresponding flanking region from the *T. thynnus* genomic data.

The selected 384 SNPs are evenly spread between 277 different contigs, most of them being unique SNP per contig, with a distribution of 212 contigs with 1 SNP, 61 contigs with 2 SNPs, 8 contigs with 3 SNPs, 4 contigs with 4 SNPs and only 2 contigs containing 5 SNPs (Figure 1).

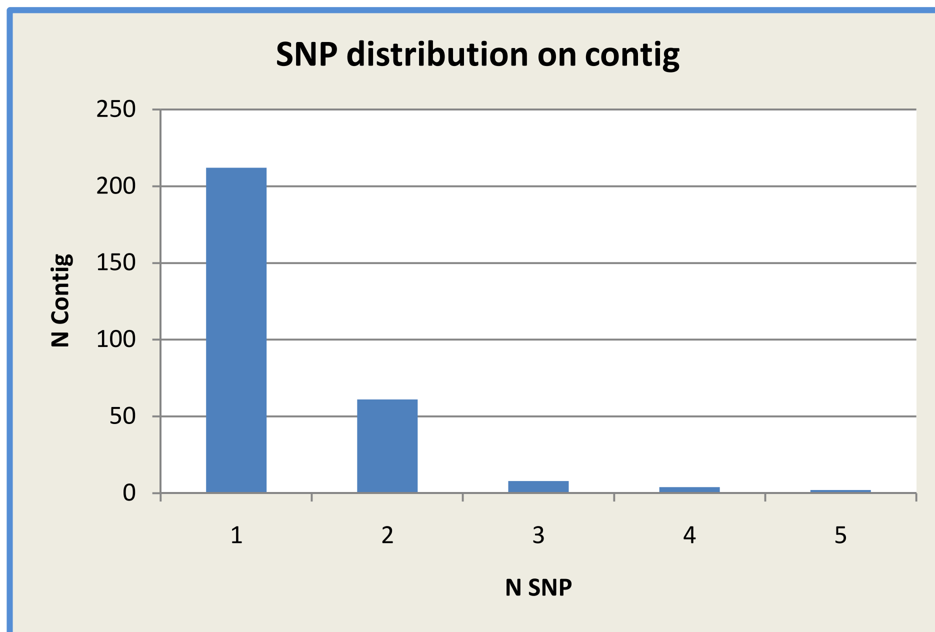


Figure 1. Distribution of SNPs across contigs. On the x-axis, number of SNPs per contig; on the y-axis, number of contigs showing a specific number of SNPs.

SNP validation and cross species amplification

The selected 384 SNPs were validated by genotyping 40 individuals from each of the three geographical populations (Eastern Mediterranean, Western Mediterranean and Gulf of Mexico) targeted for SNP discovery. From the full panel 55 (14%) assays failed because either they did not produce any amplification, or they showed ambiguous clustering of data points. Of the remaining working assays 39 were monomorphic (10%) in the genotyped samples, leading to a dataset of 290 polymorphic SNP and an overall conversion rate of 76%.

Estimates of H_O and H_E revealed very similar level of diversity across the three samples and overall the entire dataset (Table 3 and SI_Table 1); the tests for deviation from HWE for each locus and populations after correction for multiple testing ($\alpha = 0.05$) revealed only one locus retaining significant deviation in each analyzed sample due to a strong excess of heterozygote genotypes. Linkage disequilibrium was assessed for each pair of loci overall the entire dataset and of the 41905 test performed 17 remained significant after correction for multiple tests ($\alpha = 0.05$). Of these linked pairs 14 are constituted by SNPs located on the same contig, suggesting potential evidence of physical linkage that should be further evaluated when using these markers for population genetic applications.

The distribution of SNPs frequencies over the range of MAF categories in the three geographical samples and overall the entire dataset do not suggest an elevated non-random

exclusion of SNPs with low MAF (Figure 2), showing adequate even representation over the entire MAF range.

High percentage of the 290 validated SNPs in *T. thynnus* individuals amplified successfully also in *T. alalunga* samples (272 SNPs) and from this list 107 loci (37%) were polymorphic in the tested individuals (see SI_Table 2).

	H_O	H_E
EMED	0.34332	0.34208
WMED	0.35412	0.34952
GOM	0.34774	0.34535
Overall	**0.34840**	**0.34548**

Table 3. Estimates of mean observed (H_O) and expected (H_E) heterozygosity in the three geographical samples and overall the entire dataset at the 290 polymorphic SNPs ; EMED: Eastern Mediterranean; WMED: Western Mediterranean; GOM: Gulf of Mexico.

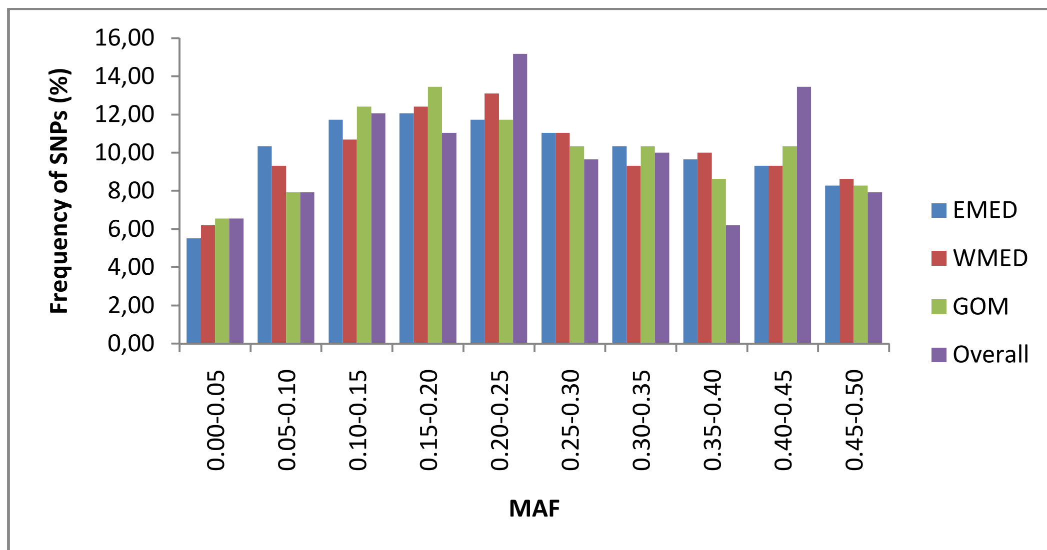


Figure 2. Distribution of Minor Allele Frequency (MAF) of the 290 validated and polymorphic SNPs typed in the three geographical samples and overall the entire dataset; EMED: Eastern Mediterranean; WMED: Western Mediterranean; GOM: Gulf of Mexico.

Protein identification and functional annotation

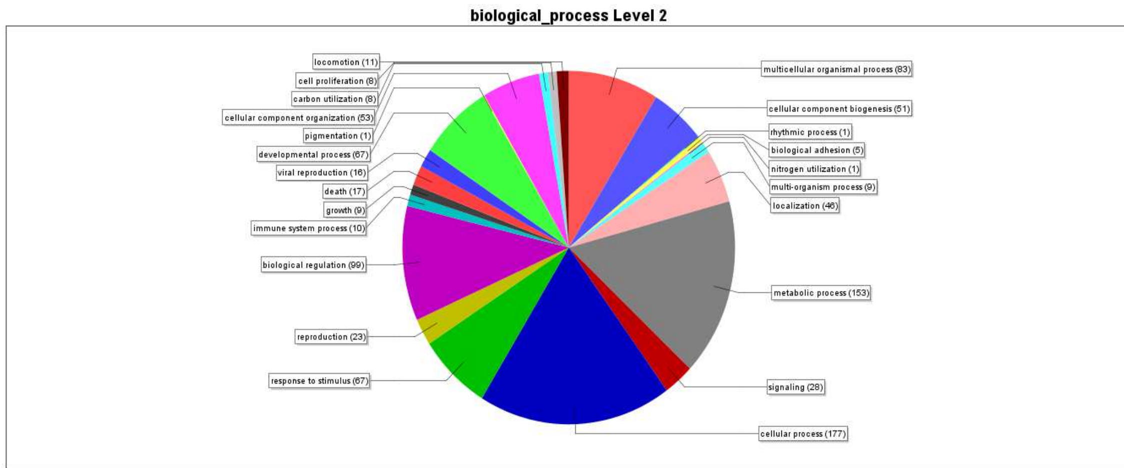
The functional annotation was restricted to the reduced genomic subset (4 k contigs reference) and to the 277 contigs, included within the formers, where SNPs were selected for genotyping in order to look for putative biases in the SNP selection procedure.

Analysis of the 277 contigs dataset

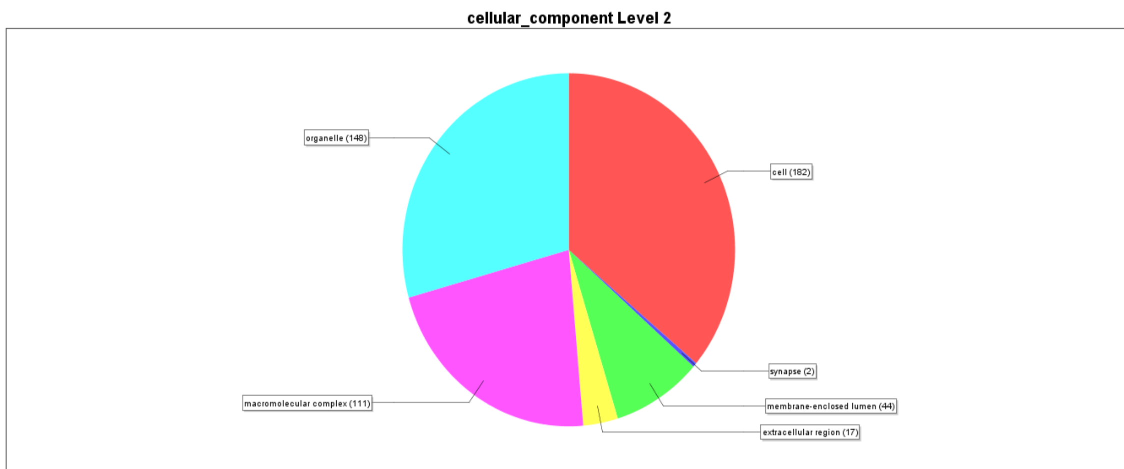
After running an ab initio gene prediction with Augustus software, at least one protein was predicted for 82% of the contigs (229), yielding a total of 279 predicted proteins (mean of 1.21 ± 0.47 proteins per contig), with average length of 270 AA (length range of 25 to 3409 AA).

After running Blast2GO with the predicted proteins as input, a total of 267 proteins (95.6%) showed a significant Blast match against nr protein database, with a majority of the hits presenting e-value scores above 10e-176. Visual inspection of the Blast results showed that the majority of the hits corresponded to teleost species (*Dario rerio*, *Oreochromis niloticus*, *Salmo salar* and *Tetraodon nigroviridis*); apart from this, as expected, hits against well annotated genome species, like *Homo sapiens* and *Mus musculus*, were reported. While *O. niloticus* represented the best match species for 49.8% of the proteins, the first non-teleost species appeared in thirteen place (*H. sapiens*).

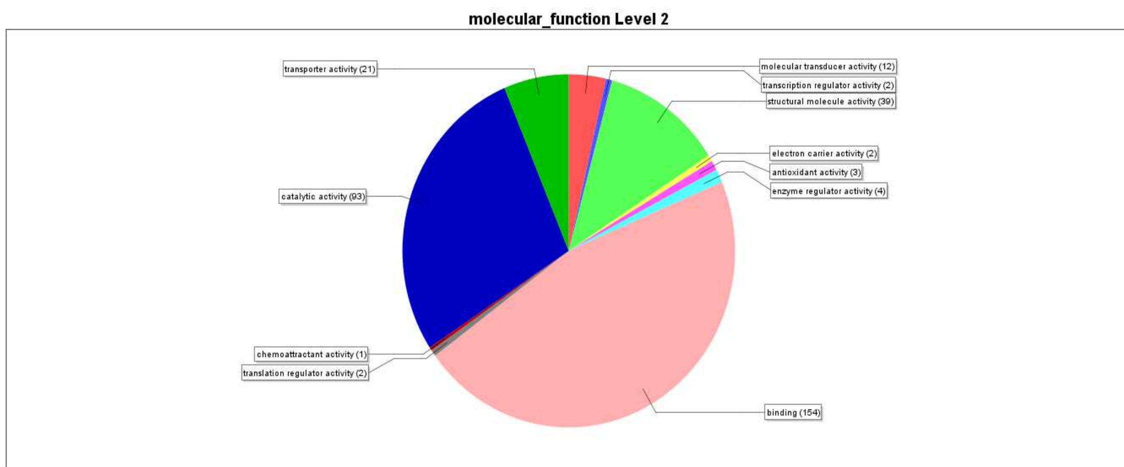
Annotation by similarity was successful for 216 of the 267 proteins presenting a significant Blast match (82%), and yielded a total of 2046 GO terms (9.8 GO terms per protein) annotated, with 1047 of them being unique. An average GO term level of 5.9 (standard deviation 1.7) correspond to the Blast2GO GO term categories (Biological Process (BP), Molecular Function (MF) and Cellular Component (CC)). Among the GO terms corresponding to the BP category, metabolic process, cellular process and biological regulation were the most abundant ones followed by multicellular organismal process and developmental process (Figure 3). Although the presence of GO terms related to Viral reproduction and immune system process could suggest that at least one of the sequenced individuals was under viral infection, manual inspection of the proteins related to those terms showed that all of them were ribosomal proteins with a viral related term associated in the Gene Ontology database. While cell, organelle and macromolecular complex were the most common GO terms corresponding to the CC category, binding and catalytic activity represented the 74.1% of the MF category.



A)



B)



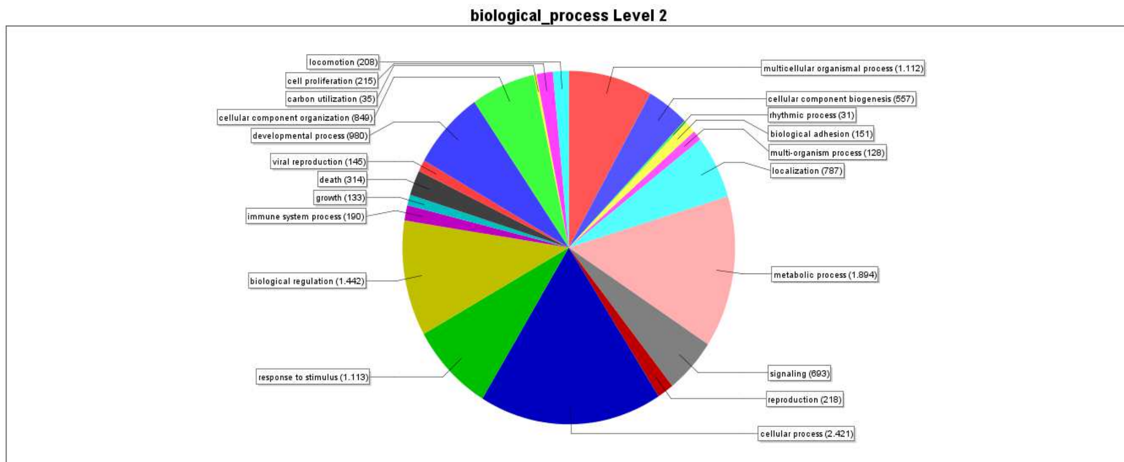
C)

Figure 3. Gene Ontology (GO) assignment (2nd level GO terms) for 277 contig subset. A) Biological Process; B) Cellular Component; C) Molecular Function.

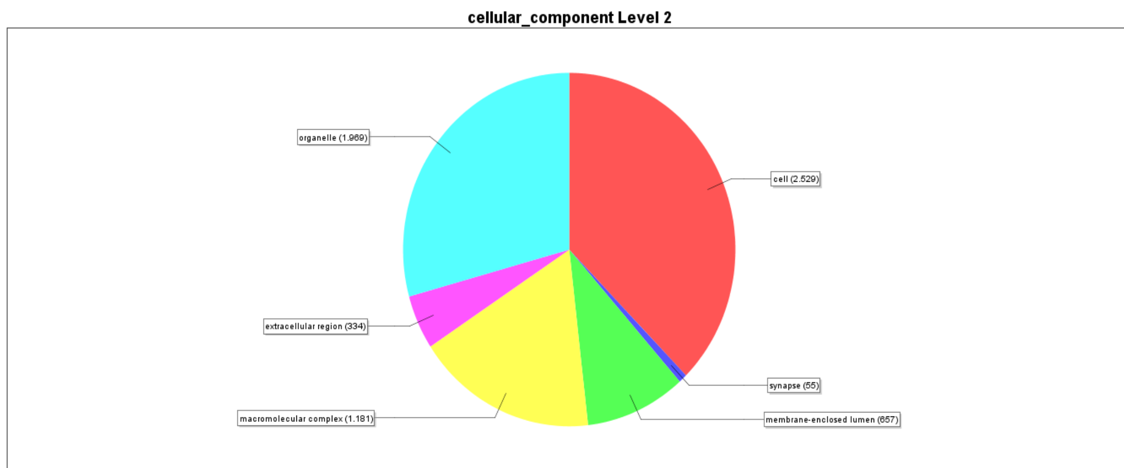
Analysis of the 4k contigs reference dataset

After running Augustus for the reduced genomic dataset of 4018 contigs, used as reference for transcriptomic and genomic reads mapping and variants calling, a total of 4105 proteins from 3141 (78.1%) contigs were in silico predicted (1.30 ± 0.56 proteins per contig). An average length of 377 AA, with a minimum and maximum of, respectively, 11 and 13383 AA, was reported. Blastp positive results were found for 94.2% (3867) of the predicted proteins, with a majority of the hits presenting e-value scores above 10e-176. Species distribution resembled the 277 contigs subset with *D. rerio* presenting the highest number of hits and *O. niloticus* being the species with the best hit for 58.4% of the proteins.

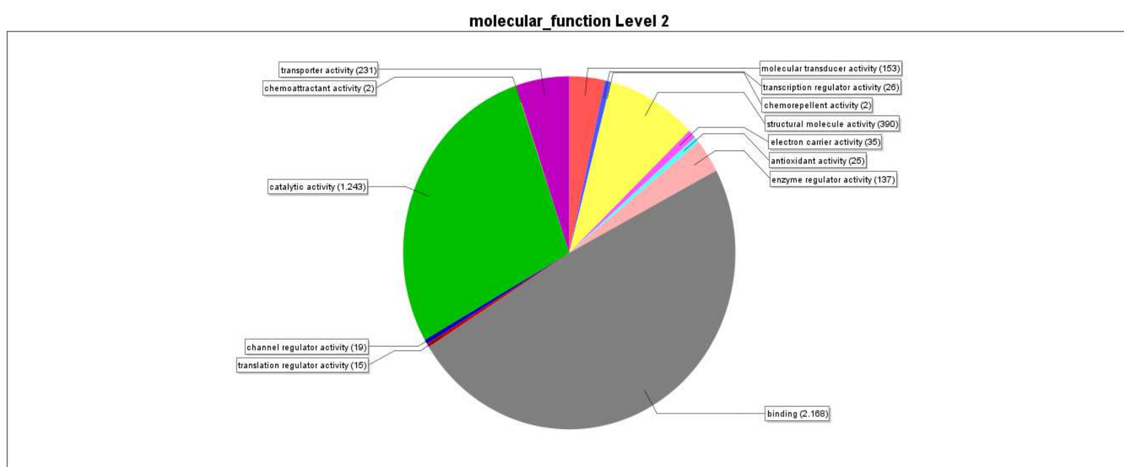
A total of 3052 predicted proteins (78.9%) presented at least one associated GO term, adding up to a total 29927 GO terms (5015 unique) and 7.05 GO terms per contig. An average GO term level of 6 (standard deviation 1.8) is associated to the main Blast2GO GO term categories BP, MF and CC. Figure 4 shows the GO term distribution for the former categories. The Fisher exact test showed no enrichment for any GO term when comparing the 277 and 4018 contigs subsets.



A)



B)



C)

Figure 4: Gene Ontology (GO) assignment (2nd level GO terms) for 4018 contig subset. A) Biological Process; B) Cellular Component; C) Molecular Function.

Synonimous/Non-synonimous SNP classification

From the 290 polymorphic SNPs it was possible to locate 141 loci on coding sequences predicted from the genomic contigs as described above, of which 18 (13%) were identified as putative amino acid replacement substitutions. Considering the class changes of the predicted amino acid substitutions most of them could be classified as mayor changes, which might cause significant functional changes in the encoded protein.

Of the remaining loci, 86 are located in contigs were a protein is predicted, but the variant position is outside the predicted CDS, while 59 SNPs originate in contigs for which no protein prediction could be obtained.

Discussion

Before next generation sequencing technology advent in the field of evolutionary biology, large-scale marker discovery studies have usually concentrated on a restricted number of organisms for which sequenced genomes were available. The currently ongoing technological revolution, that is driving decreasing costs for DNA sequencing and genotyping, allow moving rapidly toward large-scale marker discovery in organisms for which few genomic resources currently exist (Braütigam and Gowik 2010; Seeb *et al.*2011a). With the advent of the new generation of sequencing technologies, genetic⁄genomic resources for nonmodel species have become far more accessible and transcriptome sequencing is becoming one of the most important applications of next-generation sequencing in evolutionary biology (Galindo *et al.* 2010; Metzker 2010).

In this study we provided the *de novo* discovery of 5412 putative SNPs based on 454 transcriptome sequencing of ten individuals covering the species putative reproduction areas, coupled with shallow genome sequencing. Applying a single step approach of validation and genotyping step for a selected panel of 384 assays, we could evaluate 290 loci as polymorphic in the tested samples. The data generated constitute a relevant improvement for genetic analysis in Atlantic bluefin tuna, significantly increasing the omic resources (genomic and transcriptomic) available for this species, as well as novel SNP that could be used to assess genetic structure and improve management, with the aim of developing a sustainable fishery of this important commercial species.

SNPs can be derived by genome or transcriptome resources and, in the latter case, selected from more abundant or rarer expressed transcripts. The clustering and assembly step is critical for SNP mining as it generates the reference for variant detection by mapping reads to the contigs. Not having a validated reference genomes might hamper the correctness of contig assembly and therefore variants calling by mapping, because homologous or paralogous genes sequence potential mis-assemblies cannot be directly sorted out by back-mapping to the species-specific genome. Atlantic bluefin tuna lacks of genome reference and this increases the likelihood of misidentifying polymorphisms between paralogous sequence variants (PSVs) as SNPs. In fact, the occurrence of genome duplication resulted in many assemblies of paralogous sequences that resulted in the identification of a large proportion of false positives (Sanchez *et al.* 2009).

In our work, we use for the first time a combined approach of transcriptome sequencing coupled with shallow genome sequencing to achieve more robust results and overcome the issue of SNPs flanking sequences quality. We use GS FLEX Titanium sequencing technology to obtain more than 1 million of cDNA sequences of ten individuals of Atlantic bluefin tuna from different geographic locations. After adapters trimming and filtering for mitochondrial and ribosomal transcripts, more than 70% of these sequences were available for subsequent analyses. We also generated more than 833 million reads of DNA sequence data by four individuals of *Thunnus thynnus* using Illumina HiSeq2000 and de-novo assembly of these reads was performed to produce a reference genome of more than 500000 contigs.

The first mapping of cDNA reads against this genomic dataset was aimed to reduce this reference, selecting contigs complementary to cDNA sequences, that are most likely coding regions of the genome. We used stringent criteria, as at least 10 cDNA reads mapped and length of at least 200 bp, in order to obtain a final genomic reference of 4000 contig associated to expressed sequences, used for subsequent *in silico* SNP discovery. SNP identification can proceed either from *in vitro* or *in silico* approaches. *In vitro* methods, such as the re-sequencing of targeted amplicons, are costly and time consuming and generally more appropriate when sequence data is limited or when interested in specific polymorphisms or candidate genes. In contrast, *in silico* discovery is the most obvious method for de novo SNP identification, offering a low cost source of abundant SNPs (Lepoittevin *et al.* 2010). In this work, after high-throughput sequence generation, basic SNP discovery was performed mapping cDNA reads against the reference genome dataset, identifying 5412 putative loci. Sequence accuracy is a crucial point to make sure the observed polymorphisms are actually true SNPs and not false positives resulting from sequencing or alignment errors. A robust diagnosis of sequence variation in the vicinity of the target SNPs is also necessary, especially for the GoldenGate assay, which relies on hybridization of allele and locus-specific oligonucleotides on both sides of a given SNP; any sequence ambiguity might compromise their annealing and the subsequent OPA genotyping success. The genomic reference produced in this study allowed to provide reliable 60 bp on either side of the SNP, the minimal requirement of flanking sequence for the Illumina GoldenGate genotyping assay, moreover the masking of occurring polymorphisms in the region allowed to avoid the design of genotyping oligos in variable portion.

For the detection of final panel of 384 SNP we also adopted several restrictive criteria, choosing only variants present in both cDNA and gDNA dataset and with at least 4 reads present at the position and the alternative allele present in at least 2 reads. Minor sequence allele frequency was one of the major factors affecting the validation rates of EST-derived SNPs. In fact, sets of SNP markers developed from a reduced number of individuals are typically affected by bias, which results in MAF spectrum being shifted upwards, with an under-representation of rare SNPs. In small contigs with 2 or 3 sequences, the alternative base is represented only once, and this could be due to sequencing errors; contigs of 4 or more sequences with the minor sequence allele frequency being present at least twice in the contig, provided instead high levels of SNP validation rates (Wang *et al.* 2008). This type of bias is introduced if only the most variable polymorphic sites are selected or if a small panel of individuals is used to discover variation (Brumfield *et al.* 2003). For identification of candidate SNPs, we used an ascertainment panel consisting of DNA from 10 Atlantic bluefin tuna. These samples were taken from a wide range of geographic locations, from Gulf of Mexico to entire Mediterranean Sea, to make the discovering panel as representative of all individuals and populations in those regions as possible and maximize the allelic diversity of the studied species in order to minimize ascertainment bias. It's a common error due to the selection of loci from an unrepresentative sample of individuals which yields loci that are not representative of the spectrum of allele frequencies in a population, as documented in cases where geographically restricted ascertainment samples preferentially identify high heterozygosity SNPs were employed (Morin *et al.* 2004; Smith *et al.* 2005; Rosenblum and Novembre 2007; Helyar *et al.* 2011). In our study, the distribution of minor allele frequencies among the polymorphic SNPs was allocated in a broad window ranging from 0.05 till 0.5, which indicates the successful application of the multiplexing of ten individuals from different geographical regions to avoid ascertainment bias in the selected SNPs. The range of allele frequencies within the SNP panel suggests that the strategy of carefully selecting individuals to maximize the geographical and genetic diversity covered by the SNP development samples has been successful in minimizing ascertainment bias (Vollmer and Rosel 2012).

The selected panel of 384 SNPs was distributed on 277 contigs and 55% of these SNPs originate from different contigs. These selected 384 loci were validated by direct genotyping from genomic DNA of 120 Atlantic bluefin tuna samples, using Illumina GoldenGate assay,

and we obtained a conversion rate of 76% (290 successful polymorphic SNPs over 384). Similar proportions of high-quality genotype calls were reported in Wang *et al.*(2008) and Hubert *et al.* (2010), that achieved a 69.2% and 74.5% conversion rate for the channel catfish and the Atlantic cod respectively. In these studies, SNP validation carried out using Illumina GoldenGate technology, but SNP discovery was based on EST libraries produced with Sanger sequencing, that have higher read quality than 454 sequencing technology, and therefore the results aren't completely comparable. Another works on Atlantic cod and Atlantic herring showed instead a percentage of successful assay conversion of 43% and 38% respectively (Milano *et al.* 2011; Helyar *et al.* 2012), due to lack of reference genome of these species and use of only transcriptome resource, that didn't allow to overcame the Intron Exon Boundaries trouble.

A very high proportion of validated SNPs also amplified in *T. alalunga* even if with a lower polymorphism rate (37%), which is likely to be downwardly biased due to the smaller sample size tested (n= 30). However greater percentage of success was obtained in this study than the reverse amplification test developed by Albaina and colleagues (loci developed in *T. alalunga* cross amplified in *T. thynnus*), which obtained 18% of success (Albaina *et al.* in press). Current results of cross-species amplification support the potential for population genomic studies on *T. alalunga* increasing the number of markers and samples analyzed from each of the six management units currently accepted for the species: North Atlantic, South Atlantic, Indian, North Pacific, South Pacific and Mediterranean (Montes *et al.* 2013). Furthermore, the SNPs validated in Bluefin tuna and Albacore could be tested in other endangered *Thunnus* species to have a "common" genetic tool developed in the genus.

The functional annotation led to an over-representation of ribosomal/translation components as well as cytoskeletal proteins, that is expected when sequencing non-normalized cDNA libraries from skeletal muscle (Milano *et al.* 2011), because protein synthesis is the major cellular process ongoing. This annotation pattern is translated into a larger proportion of SNPs being correlated to these specific functional groups of genes. Additionally, the newly developed transcriptome data resources can be used to develop further tools for gene expression studies such as oligonucleotide microarray or RNA-seq approaches

Resolving the genetic structure of ABFT as well as determining the degree of connectivity (trans oceanic migrations, homing behavior, mix stock aggregates in feeding grounds) between individuals from the main spawning areas are two key questions to understand the ecological and evolutionary dynamics of populations across the entire distribution in order to improve the management of this endangered fishery resource. Population genomics approaches for identifying adaptive population divergences in non-model organisms have become a field of interest as the current high-throughput sequencing technologies allow a genome-wide analysis of genetic variation across populations. Marine fish provide good models for studying adaptive evolution (Nielsen *et al.* 2009) and since genomes have been completely sequenced for only a handful of fish species the establishment of genomic resources like a genome-wide set of genetic markers will provide important contributions for marine genetics and the management of natural and populations.

We demonstrated *de novo* discovery of 5412 putative SNPs based on large-scale transcriptome sequencing of non-normalized muscle samples coupled with shallow genome sequencing, resulting in a set of 290 validated polymorphic and randomly distributed genomic markers. The omic resources and markers developed in this study will foster a broad range of future studies and applications focusing on the ABFT aimed at promoting sustainable fishery management and preventing overexploitation and illegal fishing activities.

References

Albaina A, Iriondo M, Velado I, Laconcha U, Zarraonaindia I, *et al.* SNP discovery in albacore and Atlantic bluefin tuna provides insights into world-wide population structure (accepted in *Animal Genetics*).

Beldade P, Rudd S, Gruber JD, Long AD (2006) A wing expressed sequence tag resource for *Bicyclus anynana* butterflies, an evo-devo model. *BMC Genomics* **7**,130.

Block BA, Teo SL, Walli A, Boustany A, Stokesbury MJ, *et al.* (2005) Electronic tagging and population structure of Atlantic bluefin tuna. *Nature* **434**, 1121-1127.

Boustany AM, Reeb CA, Block BA (2008) Mitochondrial DNA and electronic tracking reveal population structure of Atlantic bluefin tuna (*Thunnus thynnus*). *Marine Biology* **156**,13-24.

Braütigam A, Gowik U (2010) What can next generation sequencing do for you? Next generation sequencing as a valuable tool in plant research. *Plant Biology* **12**, 831-841.

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution* **18**, 249-256.

Carlsson J, McDowell JR, Díaz-Jaimes P, Carlsson JEL, Boles SB, *et al.* (2004) Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean sea. *Molecular Ecology* **13**, 3345-3356.

Carlsson J, McDowell JR, Carlsson L, Graves JE (2007) Genetic identity of YOY bluefin tuna from the eastern and western Atlantic spawning areas. *Heredity* **98**, 23-28.

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

Collette BB, Carpenter KE, Polidoro BA, Juan-Jordá MJ, Boustany A, *et al.* (2011) High Value and Long Life-Double Jeopardy for Tunas and Billfishes. Science 333, 291-292.

Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, *et al.* (2005) "Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research". Bioinformatics 21, 3674-3676.

De Roos AM, Persson L (2002) Size-dependent life-history traits promote catastrophic collapses of top predators. PNAS 99, 12907-12912.

Ekblom R, Galindo J (2011) Application of next generation sequencing in molecular ecology of non-model organism. Heredity 107, 1-15.

Fromentin JM, Powers JE (2005) Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. *Fish and Fisheries* **6**, 281-306.

Galindo J, Grahame JW, Butlin RK (2010) An EST-based genome scan using 454 sequencing in the marine snail Littorina saxatilis. *Journal of Evolutionary Biology* **23**, 2004-2016.

Galuardi B, Lutcavage M (2012) Dispersal Routes and Habitat Utilization of Juvenile Atlantic Bluefin Tuna, *Thunnus thynnus*, Tracked with Mini PSAT and Archival Tags. *PLoS ONE* 7(5): e37829. doi:10.1371/journal.pone.0037829.

Garvin MR, Saitoh K, Gharrett AJ (2010) Application of single nucleotide polymorphisms to non-model species: a technical review. *Molecular Ecology Resources* **10**, 915-934.

Goudet J (1995) FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity* **86**, 485-486.

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, *et al.* (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11**, 591-611.

Hale M, McCormick C, Jackson J, DeWoody JA (2009) Next-generation pyrosequencing of gonad transcriptomes in the polyploid lake sturgeon (*Acipenser fulvescens*): the relative merits of normalization and rarefaction in gene discovery. *BMC Genomics* **10**. 203.

Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, *et al*. (2009) Evaluation of next generation sequencing platforms for population targeted sequencing studies. *Genome Biology* **10**, R32.

Helyar SJ, Hemmer-Hansen J, Bekkevold D, Taylor MI, Ogden R, *et al.* (2011) Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. *Molecular Ecology Resources* **11**, 123-136.

Helyar SJ, Limborg MT, Bekkevold D, Babbucci M, van Houdt J, *et al.* (2012) SNP Discovery Using Next Generation Transcriptomic Sequencing in Atlantic Herring (*Clupea harengus*). *PLoS ONE* **7**(8): e42089. doi:10.1371/journal.pone.0042089.

Hubert S, Higgins B, Borza T, Bowman S (2010) Development of a SNP resource and a genetic linkage map for Atlantic cod (*Gadus morhua*). *BMC Geno*mics **11**, 191.

Juan-Jordá MJ, Mosqueirad I, Cooperf AB, Freirea J, Dulvyc NK (2011) Global population trajectories of tunas and their relatives. *PNAS* **108**, 20650-20655.

Lepoittevin C, Frigerio JM, Garnier-Géré P, Salin F, Cervera MT, *et al.* (2010) *In Vitro* vs *In Silico* Detected SNPs for the Development of a Genotyping Array: What Can We Learn from a Non-Model Species? *PloS ONE* **5**, e11034.

Liu SK, Zhou ZC, Lu JG, Sun FY, Wang SL, *et al.* (2011) Generation of genome-scale gene-associated SNPs in catfish for the construction of a highdensity SNP array. *BMC Genomics* **12**, 13.

MacKenzie BR, Mosegaard H, Rosenberg AA (2009) Impending Collapse of Bluefin Tuna in the Northeast Atlantic and Mediterranean. *Conservation Letters* **2** (1), 25-34.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-380.

Martinsohn JTh, Ogden R, FishPopTrace Consortium (2009) FishPopTrace-Developing SNP-based population genetic assignment methods to investigate illegal fishing. *Forensic Science International: Genetics Supplement Series* **2**, 294-296.

Metzker ML (2010) Sequencing technologies - the next generation. *Nature Reviews Genetics* **11**, 31-46.

Meyer E, Aglyamova GV, Wang S, Buchanan-Carter J, Abrego D, *et al.* (2009) Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. *BMC Genomics* **10**,219.

Milano I, Babbucci M, Panitz F, Ogden R, Nielsen RO, *et al.* (2011) Novel Tools for Conservation Genomics: Comparing Two High-Throughput Approaches for SNP Discovery in the Transcriptome of the European Hake. *PLoS ONE* **6**, e28008.

Montes I, Iriondo M, Manzano C, Arrizabalaga H, Jiménez E, *et al.* (2013) Worldwide genetic structure of albacore *Thunnus alalunga* revealed by microsatellite DNA markers *Marine Ecology Progress Series*, doi: 10.3354/meps09991.

Morin PA, Luikart G, Wayne RK, the Snp wg (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution* **19**, 208-216.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009) Population genomics of marine fishes: identifying adaptive variation in space and time. *Molecular Ecology* **18**, 3128-3150.

Nielsen R, Paul SJ, Albrechtsen A, Song YS (2011) Genotype and SNP calling from next-generation sequencing data. *Nature Review Genetics* **12**, 443-451.

Odgen R (2011) Unlocking the potential of genomic technologies for wildlife forensics. *Molecular Ecology resources* **11** (Suppl.1), 109-116.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **28**, 2537-2539.

Renaut S, Nolte AW, Bernatchez L (2010) Mining transcriptome sequences towards identifying adaptive single nucleotide polymorphisms in lake whitefish species pairs (*Coregonus* spp. Salmonidae). *Molecular ecology* **19**, 115-131.

Riccioni G, Landi M, Ferrara G, Milano I, Cariani A, *et al.* (2010) Spatio-temporal population structuring and genetic diversity retention in depleted Atlantic bluefin tuna of the Mediterranean sea. *PNAS* **107**, 2102-2107.

Rooker JR, Alvarado Bremer JR, Block BA, Dewarc H, de Metrio G, *et al.* (2007) Life history and stock structure of Atlantic Bluefin Tuna (*Thunnus thynnus*). *Reviews in Fisheries Science* **15**, 265-310.

Rooker JR, Secor DH, Metrio G, Schloesser R, Block BA, *et al.* (2008) Natal Homing and Connectivity in Atlantic Bluefin Tuna Populations. *Science* **322**, 742.

Rosenblum EB, Novembre J (2007) Ascertainment Bias in Spatially Structured Populations: A Case Study in the Eastern Fence Lizard. *Journal of Heredity* **98** (4), 331-336.

Sanchez C, Smith T, Wiedmann R, Vallejo R, Salem M, *et al.* (2009) Single nucleotide polymorphism discovery in rainbow trout by deep sequencing of a reduced representation library. *BMC Genomics* **10**, 559.

Seeb JE, Carvalho G, Hauser L, Naish K, Roberts S, *et al.* (2011a) Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular Ecology Resources* **11**(Suppl. 1), 1-8.

Seeb JE, Pascal CE, Grau ED, Seeb LW, Templin WD, *et al.* (2011b) Transcriptome sequencing and high-resolution melt analysis advance single nucleotide polymorphism discovery in duplicated salmonids. *Molecular Ecology Resources* **11**, 335-348.

Seeb LW, Templin WD, Sato S, Abe S, Warheit K, *et al.* (2011) Single nucleotide polymorphisms across a species' range: implications for conservation studies of Pacific salmon. *Molecular Ecology Resources* **11**, 195-217.

Smith CT, Templin WD, Seeb JE, Seeb LW (2005) Single Nucleotide Polymorphisms Provide Rapid and Accurate Estimates of the Proportions of U.S. and Canadian Chinook Salmon Caught in Yukon River Fisheries. *North American Journal of Fisheries Management* **25**, 944-953.

Stanke M, Steinkamp R, Waack S, Morgenstern B (2004) AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Research* **32**, W309-W312.

Stapley J, Reger J, Feulner PGD, Smadja C, Galindo J, *et al.* (2010) Adaptation genomics: the next generation. *Trends in Ecology & Evolution* **25**, 705-712.

Vera M, Alvarez-Dios JA, Milian A, Pardo BG, Bouza C, *et al.* (2011) Validation of single nucleotide polymorphism (SNP) markers from an immune Expressed Sequence Tag (EST) turbot, *Scophthalmus maximus*, database. *Aquaculture* **313**, 31-41.

Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü, *et al.* (2011) Facts and uncertainties about the genetic population structure of Atlantic bluefin tuna (*Thunnus thynnus*) in the Mediterranean. Implications for fishery management. *Reviews in Fish Biology and Fisheries* **21**, 527-541.

Vollmer NL, Rosel PE (2012) Developing genomic resources for the common bottlenose dolphin (*Tursiops truncatus*): isolation and characterization of 153 single nucleotide polymorphisms and 53 genotyping assays. *Molecular Ecology Resources* **12**, 1124-1132.

Xu J, Ji P, Zhao Z, Zhang Y, Feng J, *et al.* (2012) Genome-Wide SNP Discovery from Transcriptome of Four Common Carp Strains. *PLoS ONE* **7**(10), e48140.

Walli A, Teo SLH, Boustany A, Farwell CJ, Williams T, *et al.* (2009) Seasonal movements, aggregations and diving behavior of Atlantic bluefin tuna (*Thunnus thynnus*) revealed with archival tags. *PLoS ONE* **4**, 1-18.

Wang S, Sha Z, Sonstegard TS, Liu H, Xu P, *et al.* (2008) Quality assessment parameters for EST-derived SNPs from catfish. *BMC Genomics* **9**, 450.

SI_Table 1

Estimates of observed (H_O) and expected (H_E) heterozygosity per locus in the three geographical samples and overall the entire dataset; * significant deviation from HWE after correction for multiple testing (α = 0.05). EMED: Eastern Mediterranean; WMED: Western Mediterranean; GOM: Gulf of Mexico.

SNP name	EMED		WMED		GOM		Overall		SNP name	EMED		WMED		GOM		Overall	
	Ho	He	Ho	He	Ho	He	Ho	He		Ho	He	Ho	He	Ho	He	Ho	He
TunaSNP1	0.564	0.490	0.450	0.475	0.553	0.464	0.521	0.473	**TunaSNP194**	0.385	0.399	0.475	0.435	0.289	0.321	0.385	0.387
TunaSNP2	0.231	0.245	0.225	0.202	0.316	0.337	0.256	0.261	**TunaSNP195**	0.359	0.330	0.350	0.380	0.368	0.337	0.359	0.347
TunaSNP6	0.421	0.417	0.475	0.453	0.447	0.405	0.448	0.423	**TunaSNP196**	0.538	0.505	0.600	0.506	0.567	0.481	0.567	0.502
TunaSNP7	0.385	0.505	0.500	0.506	0.553	0.504	0.479	0.502	**TunaSNP197**	0.282	0.281	0.275	0.240	0.237	0.212	0.265	0.243
TunaSNP8	0.026	0.026	0.026	0.026	0.026	0.026	0.026	0.026	**TunaSNP199**	0.359	0.298	0.282	0.281	0.211	0.232	0.284	0.269
TunaSNP9	0.179	0.281	0.375	0.339	0.289	0.321	0.282	0.312	**TunaSNP200**	0.385	0.373	0.462	0.450	0.351	0.483	0.400	0.438
TunaSNP10	0.564	0.490	0.350	0.461	0.447	0.464	0.453	0.469	**TunaSNP201**	0.410	0.503	0.462	0.480	0.553	0.504	0.474	0.502
TunaSNP11	0.308	0.330	0.300	0.292	0.211	0.366	0.274	0.328	**TunaSNP203**	0.436	0.441	0.538	0.399	0.474	0.417	0.483	0.416
TunaSNP13	0.410	0.450	0.425	0.415	0.526	0.472	0.453	0.443	**TunaSNP204**	0.179	0.166	0.231	0.281	0.263	0.232	0.224	0.226
TunaSNP14	0.538	0.485	0.590	0.505	0.579	0.484	0.569	0.490	**TunaSNP205**	0.128	0.122	0.125	0.119	0.083	0.081	0.113	0.107
TunaSNP15	0.658	0.506	0.600	0.505	0.579	0.501	0.612	0.500	**TunaSNP206**	0.436	0.399	0.225	0.240	0.316	0.337	0.325	0.328
TunaSNP16	0.282	0.281	0.375	0.367	0.263	0.269	0.308	0.307	**TunaSNP207**	0.333	0.345	0.350	0.324	0.237	0.251	0.308	0.307
TunaSNP17	0.053	0.052	0.050	0.049	0.000	0.000	0.034	0.034	**TunaSNP208**	0.436	0.345	0.385	0.373	0.368	0.366	0.397	0.359
TunaSNP18	0.462	0.490	0.500	0.501	0.447	0.478	0.470	0.487	**TunaSNP210**	0.487	0.505	0.410	0.506	0.405	0.507	0.435	0.502
TunaSNP19	0.308	0.298	0.325	0.367	0.342	0.287	0.325	0.317	**TunaSNP211**	0.432	0.373	0.333	0.282	0.395	0.380	0.387	0.346
TunaSNP20	0.359	0.360	0.378	0.400	0.417	0.419	0.384	0.390	**TunaSNP213**	0.256	0.226	0.316	0.269	0.342	0.321	0.304	0.271
TunaSNP21	0.289	0.287	0.462	0.432	0.132	0.169	0.296	0.311	**TunaSNP214**	0.154	0.226	0.359	0.298	0.316	0.305	0.276	0.275
TunaSNP22	0.447	0.504	0.675	0.498	0.526	0.484	0.552	0.493	**TunaSNP215**	0.205	0.226	0.350	0.292	0.263	0.269	0.274	0.261
TunaSNP23	0.564	0.503	0.450	0.486	0.526	0.484	0.513	0.489	**TunaSNP217**	0.590	0.501	0.525	0.491	0.526	0.494	0.547	0.492
TunaSNP24	0.103	0.099	0.025	0.025	0.132	0.125	0.085	0.082	**TunaSNP219**	0.308	0.264	0.250	0.258	0.132	0.212	0.231	0.243
TunaSNP25	0.103	0.099	0.051	0.051	0.079	0.125	0.078	0.091	**TunaSNP220**	0.231	0.207	0.333	0.345	0.216	0.195	0.261	0.253
TunaSNP26	0.256	0.264	0.500	0.425	0.474	0.484	0.410	0.407	**TunaSNP221**	0.103	0.099	0.100	0.096	0.079	0.077	0.094	0.090
TunaSNP27	0.718	0.498	0.538	0.485	0.500	0.489	0.586	0.487	**TunaSNP222**	0.154	0.144	0.200	0.182	0.289	0.251	0.214	0.192

TunaSNP30		0.359	0.386		0.275	0.367		0.342	0.321		0.325	0.357			**TunaSNP223**		0.487	0.421		0.500	0.444		0.421	0.438		0.470	0.431	
TunaSNP31		0.385	0.345		0.564	0.498		0.368	0.337		0.440	0.413			**TunaSNP225**		0.436	0.345		0.500	0.475		0.342	0.352		0.427	0.399	
TunaSNP32		0.282	0.399		0.425	0.367		0.368	0.366		0.359	0.374			**TunaSNP226**		0.487	0.485		0.550	0.475		0.474	0.472		0.504	0.473	
TunaSNP33		0.410	0.410		0.308	0.264		0.342	0.287		0.353	0.324			**TunaSNP228**		0.128	0.122		0.175	0.240		0.184	0.212		0.162	0.192	
TunaSNP34		0.385	0.345		0.325	0.367		0.474	0.417		0.393	0.374			**TunaSNP229**	*	0.923	0.503	*	0.950	0.505	*	0.892	0.501	*	0.922	0.499	*
TunaSNP35		0.590	0.495		0.579	0.472		0.342	0.504		0.504	0.496			**TunaSNP230**		0.154	0.144		0.128	0.122		0.278	0.243		0.184	0.168	
TunaSNP36		0.179	0.207		0.231	0.207		0.189	0.257		0.200	0.221			**TunaSNP232**		0.077	0.122		0.075	0.119		0.158	0.147		0.103	0.128	
TunaSNP37		0.359	0.410		0.385	0.441		0.421	0.456		0.388	0.433			**TunaSNP235**		0.282	0.245		0.150	0.222		0.316	0.269		0.248	0.243	
TunaSNP40		0.462	0.450		0.541	0.477		0.447	0.489		0.482	0.470			**TunaSNP238**		0.282	0.315		0.325	0.276		0.378	0.311		0.328	0.298	
TunaSNP41		0.333	0.345		0.500	0.475		0.526	0.438		0.453	0.425			**TunaSNP239**		0.308	0.386		0.250	0.324		0.421	0.393		0.325	0.366	
TunaSNP42		0.026	0.026		0.050	0.049		0.026	0.077	*	0.034	0.050			**TunaSNP240**		0.051	0.051		0.075	0.073		0.079	0.077		0.068	0.066	
TunaSNP44		0.051	0.051		0.075	0.073		0.184	0.169		0.103	0.098			**TunaSNP241**		0.385	0.345		0.375	0.367		0.368	0.393		0.376	0.366	
TunaSNP46		0.385	0.501		0.410	0.490		0.417	0.488		0.404	0.498			**TunaSNP242**		0.231	0.315		0.400	0.380		0.368	0.366		0.333	0.352	
TunaSNP48		0.385	0.421		0.436	0.421		0.622	0.454		0.478	0.429			**TunaSNP243**		0.487	0.495		0.475	0.491		0.500	0.447		0.487	0.477	
TunaSNP49		0.231	0.245		0.359	0.360		0.447	0.405		0.345	0.340			**TunaSNP244**		0.051	0.051		0.100	0.096		0.079	0.077		0.077	0.074	
TunaSNP50		0.359	0.498		0.333	0.485		0.395	0.498		0.362	0.490			**TunaSNP245**		0.205	0.186		0.125	0.119		0.079	0.125		0.137	0.143	
TunaSNP51		0.436	0.441		0.436	0.441		0.526	0.456		0.466	0.442			**TunaSNP246**		0.462	0.466		0.400	0.425		0.500	0.428		0.453	0.437	
TunaSNP52		0.436	0.399		0.425	0.415		0.270	0.344		0.379	0.385			**TunaSNP248**		0.513	0.506		0.590	0.505		0.622	0.507		0.574	0.502	
TunaSNP54		0.132	0.169		0.308	0.264		0.216	0.195		0.219	0.210			**TunaSNP249**		0.256	0.298		0.250	0.222		0.405	0.359		0.302	0.292	
TunaSNP55		0.564	0.480		0.436	0.399		0.500	0.405		0.500	0.430			**TunaSNP250**		0.103	0.099		0.103	0.099		0.053	0.052		0.086	0.083	
TunaSNP59		0.231	0.245		0.179	0.245		0.105	0.101		0.172	0.200			**TunaSNP251**		0.692	0.505		0.550	0.501		0.526	0.494		0.590	0.502	
TunaSNP60		0.410	0.480		0.282	0.373		0.368	0.417		0.353	0.427			**TunaSNP252**		0.026	0.026		0.128	0.122		0.053	0.052		0.069	0.067	
TunaSNP62		0.000	0.000		0.075	0.073		0.053	0.052		0.043	0.042			**TunaSNP253**		0.231	0.245		0.200	0.182		0.447	0.380		0.291	0.273	
TunaSNP63		0.179	0.166		0.175	0.162		0.211	0.191		0.188	0.171			**TunaSNP254**		0.237	0.287		0.333	0.345		0.486	0.373		0.351	0.334	
TunaSNP64		0.462	0.450		0.333	0.485		0.526	0.438		0.440	0.456			**TunaSNP256**		0.103	0.099		0.150	0.141		0.158	0.191		0.137	0.143	
TunaSNP65		0.342	0.352		0.300	0.353		0.395	0.321		0.345	0.340			**TunaSNP257**		0.128	0.122		0.175	0.162		0.211	0.269		0.171	0.185	
TunaSNP67		0.564	0.480		0.550	0.505		0.368	0.494		0.496	0.492			**TunaSNP258**		0.487	0.473		0.385	0.485		0.432	0.489		0.435	0.478	
TunaSNP68		0.487	0.373		0.375	0.309		0.447	0.352		0.436	0.342			**TunaSNP259**		0.179	0.166		0.225	0.202		0.158	0.147		0.188	0.171	
TunaSNP70		0.410	0.466		0.625	0.491		0.421	0.507		0.487	0.490			**TunaSNP262**		0.459	0.470		0.450	0.495		0.658	0.498		0.522	0.485	

TunaSNP71		0.308	0.298		0.325	0.339		0.237	0.321		0.291	0.317			TunaSNP263		0.256	0.298		0.425	0.392		0.474	0.366		0.385	0.352	
TunaSNP72		0.282	0.245		0.150	0.182		0.289	0.251		0.239	0.224			TunaSNP264		0.513	0.506		0.564	0.506		0.447	0.498		0.509	0.501	
TunaSNP73		0.410	0.450		0.400	0.353		0.368	0.393		0.393	0.399			TunaSNP265		0.231	0.315		0.350	0.380		0.263	0.393		0.282	0.361	
TunaSNP74		0.462	0.432		0.425	0.392		0.447	0.447		0.444	0.421			TunaSNP266		0.410	0.386		0.375	0.339		0.395	0.321		0.393	0.347	
TunaSNP76		0.333	0.345		0.256	0.264		0.289	0.287		0.293	0.298			TunaSNP268		0.128	0.122		0.158	0.147		0.053	0.052		0.113	0.107	
TunaSNP77		0.077	0.166		0.300	0.258		0.263	0.232		0.214	0.218			TunaSNP269		0.513	0.432		0.436	0.495		0.553	0.447		0.500	0.459	
TunaSNP78		0.077	0.075		0.150	0.141		0.211	0.191		0.145	0.135			TunaSNP270		0.385	0.441		0.400	0.425		0.421	0.472		0.402	0.443	
TunaSNP79		0.282	0.245		0.250	0.292		0.105	0.101		0.214	0.218			TunaSNP271		0.231	0.207		0.205	0.226		0.316	0.269		0.250	0.233	
TunaSNP80		0.538	0.495		0.436	0.506		0.395	0.478		0.457	0.494			TunaSNP272		0.128	0.122		0.000	0.000		0.250	0.222		0.122	0.115	
TunaSNP81		0.462	0.410		0.400	0.380		0.395	0.428		0.419	0.403			TunaSNP273		0.359	0.298		0.375	0.309		0.237	0.212		0.325	0.273	
TunaSNP82		0.395	0.506		0.361	0.504		0.447	0.506		0.402	0.501			TunaSNP274		0.436	0.441		0.500	0.425		0.579	0.456		0.504	0.437	
TunaSNP83		0.308	0.298		0.350	0.292		0.237	0.287		0.299	0.290			TunaSNP276		0.256	0.298		0.150	0.292		0.421	0.337		0.274	0.307	
TunaSNP84		0.103	0.099		0.100	0.096		0.053	0.052		0.085	0.082			TunaSNP277		0.132	0.212		0.308	0.264		0.132	0.169		0.191	0.215	
TunaSNP86		0.359	0.386		0.325	0.392		0.263	0.366		0.316	0.379			TunaSNP279		0.205	0.186		0.450	0.425		0.389	0.318		0.348	0.321	
TunaSNP87		0.179	0.166		0.200	0.324		0.368	0.305		0.248	0.267			TunaSNP280		0.410	0.450		0.450	0.461		0.500	0.447		0.453	0.449	
TunaSNP89		0.641	0.485		0.436	0.495		0.500	0.498		0.526	0.489			TunaSNP281		0.564	0.466		0.450	0.495		0.541	0.504		0.517	0.487	
TunaSNP90		0.462	0.506		0.650	0.505		0.526	0.505		0.547	0.501			TunaSNP282		0.436	0.421		0.475	0.392		0.421	0.472		0.444	0.428	
TunaSNP91		0.590	0.495		0.450	0.486		0.421	0.484		0.487	0.484			TunaSNP283		0.410	0.410		0.333	0.441		0.263	0.366		0.336	0.405	
TunaSNP94		0.359	0.330		0.385	0.421		0.324	0.400		0.357	0.383			TunaSNP285		0.051	0.051		0.026	0.026		0.152	0.142		0.072	0.070	
TunaSNP95		0.359	0.330		0.150	0.182		0.395	0.380		0.299	0.301			TunaSNP286		0.410	0.386		0.450	0.380		0.342	0.321		0.402	0.361	
TunaSNP96		0.359	0.330		0.225	0.240		0.421	0.417		0.333	0.333			TunaSNP288		0.231	0.315		0.538	0.441		0.342	0.287		0.371	0.354	
TunaSNP97		0.487	0.485		0.459	0.483		0.526	0.505		0.491	0.490			TunaSNP290		0.256	0.264		0.225	0.202		0.237	0.212		0.239	0.224	
TunaSNP98		0.333	0.315		0.275	0.240		0.316	0.305		0.308	0.285			TunaSNP291		0.231	0.245		0.300	0.258		0.105	0.191		0.214	0.231	
TunaSNP99		0.154	0.186		0.125	0.119		0.263	0.232		0.179	0.178			TunaSNP292		0.564	0.450		0.579	0.456		0.378	0.424		0.509	0.440	
TunaSNP100		0.308	0.360		0.325	0.392		0.289	0.287		0.308	0.347			TunaSNP294		0.333	0.506		0.300	0.475		0.361	0.504		0.330	0.496	*
TunaSNP101		0.359	0.360		0.462	0.410		0.459	0.387		0.426	0.383			TunaSNP297		0.282	0.281		0.375	0.367		0.237	0.251		0.299	0.301	
TunaSNP103		0.410	0.330		0.231	0.245		0.105	0.147		0.250	0.245			TunaSNP298		0.526	0.507		0.500	0.495		0.432	0.504		0.487	0.499	
TunaSNP105		0.487	0.495		0.436	0.501		0.421	0.501		0.448	0.501			TunaSNP299		0.622	0.470		0.513	0.432		0.459	0.454		0.531	0.448	
TunaSNP106		0.231	0.207		0.205	0.186		0.162	0.151		0.200	0.181			TunaSNP300		0.513	0.498		0.500	0.486		0.405	0.412		0.474	0.489	

TunaSNP107		0.538	0.441		0.450	0.486		0.316	0.456		0.436	0.460			**TunaSNP301**		0.436	0.399		0.300	0.425		0.526	0.456		0.419	0.425	
TunaSNP109		0.282	0.345		0.400	0.324		0.395	0.380		0.359	0.347			**TunaSNP302**		0.103	0.144		0.300	0.292		0.211	0.269		0.205	0.237	
TunaSNP110		0.026	0.026		0.179	0.207		0.079	0.077		0.095	0.106			**TunaSNP303**		0.385	0.485		0.450	0.461		0.500	0.478		0.444	0.471	
TunaSNP111		0.154	0.264		0.250	0.258		0.263	0.269		0.222	0.261			**TunaSNP305**		0.436	0.495		0.538	0.506		0.526	0.494		0.500	0.497	
TunaSNP114		0.513	0.410		0.333	0.399		0.237	0.287		0.362	0.368			**TunaSNP307**		0.179	0.166		0.150	0.222		0.158	0.191		0.162	0.192	
TunaSNP115		0.487	0.501		0.538	0.501		0.395	0.506		0.474	0.502			**TunaSNP308**		0.462	0.450		0.289	0.352		0.421	0.438		0.391	0.415	
TunaSNP116		0.564	0.498		0.436	0.421		0.553	0.447		0.517	0.459			**TunaSNP309**		0.462	0.450		0.375	0.503		0.526	0.501		0.453	0.487	
TunaSNP117		0.564	0.506		0.513	0.498		0.316	0.484		0.466	0.497			**TunaSNP311**		0.487	0.421		0.475	0.481		0.421	0.366		0.462	0.428	
TunaSNP118		0.333	0.281		0.375	0.392		0.474	0.393		0.393	0.357			**TunaSNP313**		0.462	0.490		0.525	0.491		0.556	0.507		0.513	0.495	
TunaSNP119		0.487	0.485		0.500	0.506		0.447	0.428		0.479	0.483			**TunaSNP314**		0.590	0.485		0.425	0.468		0.500	0.478		0.504	0.473	
TunaSNP122		0.359	0.298		0.350	0.324		0.342	0.287		0.350	0.301			**TunaSNP315**		0.359	0.360		0.263	0.366		0.306	0.441		0.310	0.388	
TunaSNP123		0.333	0.315		0.359	0.360		0.368	0.393		0.353	0.354			**TunaSNP316**		0.385	0.399		0.400	0.380		0.237	0.287		0.342	0.357	
TunaSNP125		0.385	0.345		0.436	0.399		0.474	0.366		0.431	0.368			**TunaSNP318**		0.371	0.506		0.425	0.481		0.237	0.498		0.345	0.492	
TunaSNP126		0.256	0.226		0.275	0.276		0.158	0.147		0.231	0.218			**TunaSNP319**		0.256	0.410		0.410	0.330		0.316	0.417		0.328	0.385	
TunaSNP127		0.538	0.485		0.625	0.506		0.526	0.501		0.564	0.496			**TunaSNP320**		0.256	0.360		0.275	0.276		0.289	0.287		0.274	0.307	
TunaSNP129		0.128	0.122		0.100	0.096		0.158	0.191		0.128	0.135			**TunaSNP321**		0.231	0.207		0.150	0.182		0.316	0.269		0.231	0.218	
TunaSNP131		0.308	0.466		0.462	0.450		0.368	0.417		0.379	0.442			**TunaSNP323**		0.487	0.441		0.225	0.309		0.368	0.366		0.359	0.374	
TunaSNP132		0.282	0.315		0.275	0.276		0.184	0.169		0.248	0.255			**TunaSNP324**		0.154	0.186		0.125	0.119		0.158	0.147		0.145	0.150	
TunaSNP134		0.538	0.459		0.487	0.506		0.541	0.477		0.522	0.485			**TunaSNP325**		0.436	0.473		0.667	0.506		0.500	0.489		0.534	0.493	
TunaSNP137		0.103	0.099		0.128	0.122		0.054	0.053		0.096	0.091			**TunaSNP326**		0.538	0.501		0.550	0.501		0.395	0.504		0.496	0.501	
TunaSNP139		0.462	0.386		0.385	0.399		0.395	0.428		0.414	0.401			**TunaSNP327**		0.487	0.506		0.590	0.506		0.342	0.464		0.474	0.498	
TunaSNP140		0.436	0.501		0.525	0.481		0.395	0.478		0.453	0.484			**TunaSNP328**		0.333	0.315		0.325	0.276		0.395	0.321		0.350	0.301	
TunaSNP141		0.282	0.281		0.300	0.324		0.395	0.380		0.325	0.328			**TunaSNP329**		0.487	0.441		0.308	0.410		0.474	0.456		0.422	0.433	
TunaSNP143		0.487	0.459		0.600	0.461		0.263	0.269		0.453	0.410			**TunaSNP330**		0.385	0.399		0.410	0.360		0.263	0.232		0.353	0.335	
TunaSNP144		0.462	0.466		0.450	0.461		0.579	0.494		0.496	0.471			**TunaSNP332**		0.368	0.337		0.350	0.444		0.421	0.438		0.379	0.409	
TunaSNP145		0.256	0.226		0.275	0.309		0.263	0.232		0.265	0.255			**TunaSNP333**		0.077	0.075		0.075	0.073		0.079	0.077		0.077	0.074	
TunaSNP147		0.077	0.075		0.075	0.073		0.081	0.079		0.078	0.075			**TunaSNP334**		0.026	0.026		0.100	0.096		0.053	0.052		0.060	0.058	
TunaSNP148		0.474	0.494		0.500	0.475		0.553	0.504		0.509	0.489			**TunaSNP335**		0.333	0.441		0.475	0.435		0.395	0.447		0.402	0.437	
TunaSNP149		0.308	0.330		0.333	0.373		0.421	0.393		0.353	0.363			**TunaSNP336**		0.103	0.099		0.075	0.119		0.053	0.052		0.077	0.090	

TunaSNP150		0.579	0.472		0.450	0.444		0.342	0.498		0.457	0.471			**TunaSNP341**		0.410	0.466		0.410	0.432		0.368	0.337		0.397	0.416	
TunaSNP152		0.154	0.144		0.200	0.258		0.105	0.147		0.154	0.185			**TunaSNP342**		0.333	0.315		0.282	0.245		0.211	0.191		0.276	0.251	
TunaSNP154		0.359	0.450		0.564	0.450		0.342	0.428		0.422	0.439			**TunaSNP343**		0.513	0.410		0.333	0.399		0.263	0.305		0.371	0.372	
TunaSNP155		0.179	0.207		0.175	0.162		0.132	0.125		0.162	0.164			**TunaSNP344**		0.154	0.144		0.250	0.222		0.184	0.287		0.197	0.218	
TunaSNP158		0.436	0.473		0.615	0.506		0.500	0.489		0.517	0.493			**TunaSNP345**		0.487	0.506		0.525	0.503		0.500	0.506		0.504	0.502	
TunaSNP159		0.538	0.501		0.487	0.485		0.447	0.447		0.491	0.479			**TunaSNP346**		0.306	0.263		0.256	0.226		0.250	0.222		0.270	0.235	
TunaSNP160		0.342	0.287		0.375	0.339		0.421	0.393		0.379	0.340			**TunaSNP347**		0.410	0.386		0.487	0.399		0.378	0.373		0.426	0.383	
TunaSNP161		0.436	0.441		0.436	0.459		0.378	0.344		0.417	0.418			**TunaSNP348**		0.538	0.485		0.600	0.501		0.526	0.507		0.556	0.497	
TunaSNP162		0.564	0.498		0.550	0.486		0.389	0.493		0.504	0.488			**TunaSNP349**		0.128	0.207		0.250	0.258		0.237	0.212		0.205	0.224	
TunaSNP163		0.538	0.506		0.550	0.495		0.500	0.478		0.530	0.494			**TunaSNP351**		0.154	0.186		0.282	0.281		0.237	0.287		0.224	0.251	
TunaSNP164		0.538	0.441		0.553	0.464		0.405	0.505		0.500	0.474			**TunaSNP352**		0.342	0.405		0.385	0.399		0.270	0.344		0.333	0.381	
TunaSNP165		0.333	0.441		0.400	0.461		0.395	0.428		0.376	0.440			**TunaSNP353**		0.154	0.226		0.175	0.162		0.079	0.125		0.137	0.171	
TunaSNP166		0.462	0.490		0.410	0.498		0.447	0.506		0.440	0.496			**TunaSNP355**		0.205	0.386		0.513	0.466		0.395	0.405		0.371	0.420	
TunaSNP167		0.410	0.498		0.400	0.475		0.500	0.498		0.436	0.487			**TunaSNP356**		0.385	0.345		0.225	0.202		0.237	0.251		0.282	0.267	
TunaSNP168		0.436	0.373		0.436	0.345		0.395	0.321		0.422	0.344			**TunaSNP357**		0.436	0.459		0.400	0.353		0.514	0.387		0.448	0.401	
TunaSNP169		0.282	0.245		0.282	0.373		0.342	0.405		0.302	0.344			**TunaSNP359**		0.385	0.345		0.333	0.281		0.395	0.321		0.371	0.314	
TunaSNP170		0.564	0.506		0.395	0.498		0.514	0.501		0.491	0.498			**TunaSNP360**		0.359	0.506		0.405	0.494		0.579	0.494		0.447	0.496	
TunaSNP172		0.103	0.144		0.175	0.202		0.132	0.169		0.137	0.171			**TunaSNP361**		0.308	0.264		0.450	0.404		0.263	0.269		0.342	0.317	
TunaSNP173		0.436	0.473		0.400	0.425		0.395	0.478		0.410	0.457			**TunaSNP362**		0.077	0.075		0.125	0.162		0.132	0.125		0.111	0.121	
TunaSNP174		0.359	0.386		0.462	0.450		0.553	0.447		0.457	0.427			**TunaSNP363**		0.179	0.245		0.211	0.191		0.158	0.191		0.183	0.208	
TunaSNP175		0.256	0.330		0.275	0.309		0.447	0.405		0.325	0.347			**TunaSNP364**		0.231	0.207		0.200	0.182		0.184	0.212		0.205	0.198	
TunaSNP176		0.615	0.490		0.513	0.503		0.632	0.494		0.586	0.502			**TunaSNP365**		0.436	0.495		0.385	0.495		0.514	0.483		0.443	0.487	
TunaSNP177		0.359	0.480		0.575	0.503		0.500	0.504		0.479	0.499			**TunaSNP366**		0.385	0.485		0.590	0.495		0.667	0.507		0.544	0.495	
TunaSNP179		0.462	0.432		0.475	0.392		0.368	0.366		0.436	0.395			**TunaSNP367**		0.256	0.386		0.375	0.468		0.526	0.472		0.385	0.443	
TunaSNP180		0.462	0.360		0.375	0.367		0.368	0.337		0.402	0.352			**TunaSNP368**		0.154	0.186		0.105	0.101		0.237	0.212		0.165	0.167	
TunaSNP182		0.308	0.432		0.525	0.498		0.421	0.494		0.419	0.477			**TunaSNP370**		0.436	0.399		0.300	0.324		0.421	0.337		0.385	0.352	
TunaSNP183		0.590	0.459		0.474	0.456		0.514	0.454		0.526	0.452			**TunaSNP371**		0.289	0.287		0.308	0.298		0.447	0.405		0.348	0.332	
TunaSNP184		0.359	0.506		0.475	0.506		0.526	0.505		0.453	0.502			**TunaSNP374**		0.462	0.432		0.351	0.454		0.447	0.447		0.421	0.440	
TunaSNP185		0.436	0.505		0.500	0.505		0.526	0.507		0.487	0.502			**TunaSNP377**		0.605	0.464		0.605	0.464		0.500	0.498		0.570	0.474	

TunaSNP186		0.333	0.373		0.359	0.330		0.316	0.337		0.336	0.344			**TunaSNP379**		0.128	0.166		0.175	0.162		0.105	0.101		0.137	0.143	
TunaSNP189		0.051	0.051		0.100	0.096		0.053	0.052		0.068	0.066			**TunaSNP380**		0.205	0.330		0.300	0.353		0.368	0.393		0.291	0.357	
TunaSNP190		0.359	0.450		0.325	0.392		0.429	0.401		0.368	0.413			**TunaSNP381**		0.256	0.226		0.385	0.345		0.211	0.191		0.284	0.257	
TunaSNP191		0.333	0.281		0.300	0.292		0.316	0.366		0.316	0.312			**TunaSNP382**		0.205	0.186		0.154	0.226		0.211	0.191		0.190	0.200	
TunaSNP192		0.385	0.399		0.333	0.345		0.368	0.393		0.362	0.377			**TunaSNP383**		0.385	0.501		0.425	0.491		0.579	0.494		0.462	0.492	
TunaSNP193		0.282	0.245		0.205	0.186		0.132	0.169		0.207	0.200			**TunaSNP384**		0.103	0.099		0.158	0.147		0.158	0.191		0.139	0.145	

SI_Table 2

Results of cross-species testing of the 290 validated and polymorphic SNPs in *T. alalunga* individuals.

SNP name	Locus ID	Cross species validation	
TunaSNP1	tuna04_contig108622_4256	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP2	tuna02_contig129997_405	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP6	tuna_contig74714_929	*Working in T. alalunga*	
TunaSNP7	tuna_contig52293_1028	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP8	tuna_contig56694_2291	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP9	tuna02_contig122979_2582	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP10	tuna_contig149883_1983	*Working in T. alalunga*	
TunaSNP11	tuna_contig56436_2296	*Working in T. alalunga*	
TunaSNP13	tuna02_contig122146_3467	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP14	tuna02_contig128705_3411	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP15	tuna_contig81111_1351		
TunaSNP16	tuna_contig154550_3037	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP17	tuna02_contig92196_1257		
TunaSNP18	tuna02_contig122979_107	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP19	tuna_contig45879_1468	*Working in T. alalunga*	
TunaSNP20	tuna_contig66558_2260	*Working in T. alalunga*	
TunaSNP21	tuna04_contig267259_10949	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP22	tuna_contig23724_3855	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP23	tuna01_contig115709_2224	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP24	tuna_contig54349_593	*Working in T. alalunga*	
TunaSNP25	tuna_contig155323_1999	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP26	tuna_contig57535_921	*Working in T. alalunga*	
TunaSNP27	tuna_contig121382_2923	*Working in T. alalunga*	
TunaSNP30	tuna_contig222172_1461	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP31	tuna_contig115192_1122	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP32	tuna_contig23848_5959	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP194	tuna_contig1807_1464	*Working in T. alalunga*	
TunaSNP195	tuna_contig113966_5180	*Working in T. alalunga*	
TunaSNP196	tuna_contig228262_2189		
TunaSNP197	tuna_contig220997_1104	*Working in T. alalunga*	
TunaSNP199	tuna_contig156245_270	*Working in T. alalunga*	
TunaSNP200	tuna_contig104549_3250	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP201	tuna01_contig101905_4360	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP203	tuna_contig27279_824	*Working in T. alalunga*	
TunaSNP204	tuna_contig154550_3420	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP205	tuna_contig224177_3068	*Working in T. alalunga*	
TunaSNP206	tuna01_contig191719_2339	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP207	tuna_contig114273_1588	*Working in T. alalunga*	
TunaSNP208	tuna04_contig151428_1789	*Working in T. alalunga*	
TunaSNP210	tuna_contig103799_348	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP211	tuna_contig225169_2908	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP213	tuna_contig63575_3883	*Working in T. alalunga*	
TunaSNP214	tuna_contig45275_16096	*Working in T. alalunga*	
TunaSNP215	tuna_contig133101_4816	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP217	tuna01_contig93658_346	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP219	tuna_contig60806_190	*Working in T. alalunga*	
TunaSNP220	tuna_contig167626_2260	*Working in T. alalunga*	
TunaSNP221	tuna_contig47828_2787	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP222	tuna_contig25320_4698	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP223	tuna01_contig151302_4649	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP225	tuna_contig74714_993	*Working in T. alalunga*	
TunaSNP226	tuna_contig21470_954	*Working in T. alalunga*	

TunaSNP33	tuna_contig17065_727	*Working in T. alalunga*	
TunaSNP34	tuna_contig103841_1366	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP35	tuna_contig230664_1022	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP36	tuna_contig215451_8280		
TunaSNP37	tuna_contig105438_909	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP40	tuna_contig159273_2293	*Working in T. alalunga*	
TunaSNP41	tuna_contig227194_1837	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP42	tuna01_contig17323_119	*Working in T. alalunga*	
TunaSNP44	tuna_contig221166_670	*Working in T. alalunga*	
TunaSNP46	tuna_contig60098_899	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP48	tuna_contig74456_78	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP49	tuna_contig137411_3925	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP50	tuna_contig14148_3956	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP51	tuna_contig90889_248	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP52	tuna01_contig93997_9815	*Working in T. alalunga*	
TunaSNP54	tuna_contig158782_2178	*Working in T. alalunga*	
TunaSNP55	tuna_contig112230_3856		
TunaSNP59	tuna_contig65377_1455	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP60	tuna_contig77539_117	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP62	tuna01_contig117316_3273	*Working in T. alalunga*	
TunaSNP63	tuna_contig50048_1747	*Working in T. alalunga*	
TunaSNP64	tuna04_contig242085_534	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP65	tuna_contig219983_1208	*Working in T. alalunga*	
TunaSNP67	tuna_contig59655_1477	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP68	tuna_contig168903_898	*Working in T. alalunga*	
TunaSNP70	tuna_contig216100_3153	*Working in T. alalunga*	
TunaSNP71	tuna_contig35548_2427	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP72	tuna_contig122385_915	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP73	tuna01_contig100627_3442	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP74	tuna01_contig124559_1462	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP228	tuna_contig153727_3677	*Working in T. alalunga*	
TunaSNP229	tuna_contig149068_700		
TunaSNP230	tuna_contig113966_10824	*Working in T. alalunga*	
TunaSNP232	tuna01_contig107453_2161		
TunaSNP235	tuna_contig75178_2809	*Working in T. alalunga*	
TunaSNP238	tuna_contig104143_2111	*Working in T. alalunga*	
TunaSNP239	tuna_contig63177_1281	*Working in T. alalunga*	
TunaSNP240	tuna_contig107346_1369	*Working in T. alalunga*	
TunaSNP241	tuna_contig9675_583	*Working in T. alalunga*	
TunaSNP242	tuna_contig63177_445	*Working in T. alalunga*	
TunaSNP243	tuna_contig106376_2446	*Working in T. alalunga*	
TunaSNP244	tuna_contig136036_439	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP245	tuna_contig157960_1137	*Working in T. alalunga*	
TunaSNP246	tuna_contig215451_7484	*Working in T. alalunga*	
TunaSNP248	tuna_contig29864_3129		
TunaSNP249	tuna_contig55307_234	*Working in T. alalunga*	
TunaSNP250	tuna_contig35902_6157	*Working in T. alalunga*	
TunaSNP251	tuna_contig133101_4921	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP252	tuna01_contig139870_798	*Working in T. alalunga*	
TunaSNP253	tuna_contig45879_1926	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP254	tuna04_contig195158_591	*Working in T. alalunga*	
TunaSNP256	tuna_contig122511_739	*Working in T. alalunga*	
TunaSNP257	tuna_contig133101_1208	*Working in T. alalunga*	
TunaSNP258	tuna_contig221166_2051		
TunaSNP259	tuna_contig108905_2168	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP262	tuna_contig114893_4017	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP263	tuna_contig169727_2465	*Working in T. alalunga*	
TunaSNP264	tuna_contig164772_703		
TunaSNP265	tuna_contig22149_2163	*Working in T. alalunga*	
TunaSNP266	tuna_contig51571_1098	*Working in T. alalunga*	

TunaSNP76	tuna_contig25408_3114	*Working in T. alalunga*	
TunaSNP77	tuna_contig74975_607	*Working in T. alalunga*	
TunaSNP78	tuna_contig166979_4798	*Working in T. alalunga*	
TunaSNP79	tuna01_contig100883_608	*Working in T. alalunga*	
TunaSNP80	tuna_contig34509_3915	*Working in T. alalunga*	
TunaSNP81	tuna_contig133101_7052	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP82	tuna01_contig90497_6655	*Working in T. alalunga*	
TunaSNP83	tuna_contig56694_2694	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP84	tuna_contig166979_4966	*Working in T. alalunga*	
TunaSNP86	tuna_contig27997_2787	*Working in T. alalunga*	
TunaSNP87	tuna01_contig95918_7411	*Working in T. alalunga*	
TunaSNP89	tuna01_contig160715_1552		
TunaSNP90	rpL12-423	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP91	tuna_contig104772_860	*Working in T. alalunga*	
TunaSNP94	tuna_contig30754_3141	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP95	tuna_contig227591_5876	*Working in T. alalunga*	*Polymorphic in T. alalunga*
TunaSNP96	MTF-1-263	*Working in T. alalunga*	*Polymorphic in T. alalunga*
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CHAPTER 5

ASSESSING THE ACCURACY AND POWER OF SNPS MARKERS FOR POPULATION GENETICS, INDIVIDUAL ASSIGNMENT AND MIXTURE STOCK ANALYSIS IN *THUNNUS THYNNUS*

Assessing the accuracy and power of SNPs markers for population genetics, individual assignment and mixture stock analysis in *Thunnus thynnus*

Authors:

Eleonora Pintus[1], Alessia Cariani[1], Fausto Tinti[1], Rita Cannas[2], Gregory Maes[3], Aitor Albaina[4], Andone Estonba[4], Urtzi Laconcha[5], Haritz Arrizabalaga[5].

Affiliations:

[1] Department of Biological, Geological & Environmental Sciences (BiGeA) School of Sciences, University of Bologna, Via Selmi 3, 40126, Bologna, Italy.

[2] Department of Life and Enviromental Science, University of Cagliari, Via T. Fiorelli 1, 09126 Cagliari, Italy.

[3] Katholieke Universiteit Leuven, Laboratory of Biodiversity and Evolutionary Genomics Fish Genomics Group/BioGenomics division, Ch. Deberiotstraat, 32 B-3000, Leuven, Belgium.

[4] Department of Genetics, Physical Anthropology & Animal Physiology, University of the Basque Country (UPV/EHU), Bº Sarriena s/n 48940, Leioa, Bilbao, Spain.

[5] AZTI-Tecnalia / Unidad de Investigación Marina, Herrera kaia portualdea z/g, 20110 Pasaia (Gipuzkoa), Spain.

Introduction

The persistence of many marine fish is threatened by rapid declines; indeed few populations with extensive gene flow and infinite population size recover rapidly but most exhibit little or no change in abundance up to 15 years after a collapse. Reductions in fishing pressure, although clearly necessary for population recovery, are often insufficient. Persistence and recovery are also influenced by life history, habitat alteration, changes to species assemblages, genetic responses to exploitation, and reductions in population growth. In the late twenty years the interest on fishery genetic is enhanced with a large amount of data produced, thanks to rapidly developing technologies in the field of human genetics then applied to others species; so its role on fisheries management and assessment is become prominent, because loss of genetic diversity can lead species to a decline in capacity of adaptation. Progress in the knowledge of ecology of marine species is important not only for improving our basic understanding of natural as well as human-induced evolutionary processes, but also to define management units and setting priorities for conservation. A key aim of sustainable fisheries management is to identify the spatial scale of population structuring, and to find tools to monitor its dynamics. Even apparently small genetic differences among populations of marine fishes at neutral genetic markers could translate into important adaptive variation distributed among populations (Hauser and Carvalho 2008; Nielsen *et al.* 2009).

The Atlantic bluefin tuna (*Thunnus thynnus*) is one of the major components of pelagic ecosystems, being both important predators and forage species that are widely distributed throughout the temperate and tropical epipelagic waters of the world's oceans (Mather *et al.* 1995; Walli *et al.* 2009). Much like the other large tunas, the ABFT shows highly migratory behavior, with well-documented trans-oceanic and large-scale movements for feeding and spawning, high fecundity, large population size and high potential for dispersal during early life stages (Block *et al.* 2005; Fromentin and Power 2005; Rooker *et al.* 2007; Teo *et al.* 2007). Currently, ABTF is managed by the International Commission for the Conservation of Atlantic Tunas (ICCAT) as two stocks, divided at the 45th meridian in the mid-Atlantic (ICCAT 2008), with separated spawning areas in the Gulf of Mexico and the Mediterranean Sea, identified as spawning grounds by the presence of larvae during certain times of the year and adult fish in reproductive condition (National Research Council 1994;

Rooker *et al.* 2007). The two stocks actually mix. All sizes have been documented crossing the hypothetical line of separation among stocks (the 45th W meridian). Data on fisheries and satellite tags provide information on movements that reveal the eastern and western populations of *T. thynnus* share common foraging grounds in different areas of the Atlantic Ocean mainly as adolescent, but sort as adults to the respective breeding grounds (Gulf of Mexico and Mediterranean) a behavior termed natal homing (Rooker *et al.* 2007).

Tuna is a critical worldwide food resource and, although it was caught for thousands of years, only in recent decades, particularly after expansion and growth of Japanese market during 1980s, its use is far above the sustainable level, risking collapse of the fishery and the stock (ICCAT 2010). Both western than eastern Atlantic bluefin spawning stock biomass (SSB) (adult-aged fish) has critically declined in the last fifty years (MacKenzie *et al.* 2009; Juan-Jordá *et al.* 2011). SSB spawning stock biomass peaked over 300000 tons in the late 1950s and early 1970s and then declined to about 150000 tons until the mid-2000s. However, in the most recent period, the SSB showed clear signs of increase, indicating that the stocks are slowly recovering (ICCAT 2012). A main objective of fisheries management is to maintain populations at levels where the spawning stock biomass does not limit the production of new young fish (MacKenzie *et al.* 2009; ICCAT 2010; Reeb 2010). Specific natural characteristics, such as late reproduction, long lifespan and the aggregation of the fish that occurs during spawning, make ABFT extremely vulnerable to overexploitation. Bluefin tuna are also sensitive to oceanic conditions and disturbances such as those caused by industrial pollution (Safina 2001; Ottolenghi *et al.* 2004).

The delineation of population structure in highly migratory pelagic fishes has traditionally been difficult. A highly migratory lifestyle and the lack of clear barriers to gene flow has made the detection of population subdivision within ocean basins difficult (Waples 1998). Currently, the degree of genetic structure among stocks is largely unknown. The differentiation between eastern and western Atlantic stocks is recognized by genetic studies, tagging experiments and microchemical signatures in otoliths of young of the years, in agreement with the spawning site fidelity (Block *et al.* 2005; Carlsson *et al.* 2007; Boustany *et al.* 2008; Rooker *et al.* 2008). However, satellite tags studies (Lutcavage *et al.* 1999; Galuardi *et al.* 2010; Wilson *et al.* 2010) questioned the existence of a unique spawning site for the western population. In fact, adult bluefin tuna have been observed in the central Atlantic and off Bahamas during the spawning season when they were supposed to be in the

Gulf of Mexico. Possible explanations for these 'unexpected' sightings were given: the existence alternative spawning areas and/or the adoption by some mature specimens a skipped spawning strategy (remaining in the foraging ground and skipping spawning in that year) (Lutcavage *et al.* 1999; Goldstein *et al.* 2007; Galuardi *et al.* 2010).

As concerns the Mediterranean Sea, three spawning grounds are traditionally known in the Western basin: around the Balearic Islands, Sicily and Malta (Rooker *et al.* 2007). Recently, the occurrence of a further spawning site in the Levantine Sea has been demonstrated in eastern Mediterranean. Biological data (different spawning times) and tagging studies provided indications for the existence of substructuring within the Mediterranean, with the independence of eastern and western Mediterranean populations (Rooker *et al.* 2007). None of the fish tagged in the Atlantic (Block *et al.* 2005) was ever located east of Malta or as far as the Levantine Sea, hence the spawning migration from the Atlantic did not reach the eastern med basin. Moreover, the fish tagged in east Mediterranean stay confined in that basin (De Metrio *et al.* 2005) leading to hypothesize the existence of a separate, local or resident eastern Mediterranean.

Despite the plethora of studies, the population structure within the Mediterranean Sea is still unclear. Conflicting results were obtained in the genetic studies dealing with the issue of genetic differentiation of stocks (Viñas *et al.* 2011). For instance, Boustany *et al.* (2008) and Carlsson *et al.* (2004, 2007) found significant genetic differentiation between west and east Mediterranean populations, and Riccioni *et al.* (2010) detected structure also within the western basin. On the contrary, Ely *et al.* (2002) and Pujolar *et al.* (2003) did not found significant differentiation, in agreement to the hypothesis of a single panmictic unit of Atlantic bluefin tuna in the Mediterranean basin. Probably these contradictory results could be due to the use of reduced number of molecular markers, differential sampling design and methodological techniques (Viñas *et al.* 2011).

Following many useful suggestions by Viñas et al 2011, in this study we tried to go over these previous inconsistencies, improving the sampling design and testing new powerful markers and analytic approaches.

In particular, a large panel of SNP markers recently derived from Atlantic bluefin tuna transcriptomic and genomic sequences (see chapter 4 of this thesis) was used to genotype to a wide-scale of samples across the entire range of *Thunnnus thynnus*. Single-nucleotide polymorphisms (SNPs) are the more abundant polymorphism in the genome, are

codominant, are usually biallelic, represent a more stable nuclear marker than microsatellites, and are amenable to automation and increasingly cost-effective (Vignal *et al.* 2002; Morin *et al.* 2004; Helyar *et al.* 2011; Odgen 2011). These features have made SNP a marker of choice in modern genomics research and in studies of the ecology and conservation of natural populations because of their capacity to access variability across the genome. Until a short time ago development and genotyping of these markers were not easy for species without reference genome, but now this gap was overcame by next-generation sequencing technologies, that provide access to a wealth of sequence information on non model organisms, thanks to exponentially reduction of DNA-sequencing costs that has led to rapid increase in throughput, allowing sequencing the entire expressed genome of a non-model organism with standard project budgets. (Morin and McCarthy 2007; Hauser and Seeb 2008; Seeb *et al.* 2011; Esteras *et al.* 2012). We use the Illumina Golden Gate assays that genotype 384, 768 or 1536 SNP in parallel and have been the most widely used for high-throughput applications. This genotyping technique has been used extensively in humans (The International HapMap Consortium 2003) and several animal species (McKay *et al.* 2008; Kijas *et al.* 2009; Malhi *et al.* 2011).

In summary, considering that the stock structure of Atlantic bluefin tuna is probably much more complex than originally described, and that more spatially explicit management plans than the simple two stock structure are urgently required, we aimed at an accurate description of its population structure, crucial information required for the sustainable utilization of this important species (Viñas *et al.* 2011; Kell *et al.* 2012). Our study is included in the framework of a wide scientific research program (GBYP "Biological Sampling and Analysis" program) launched in 2010 by ICCAT to obtain new biological and ecological information that can be used to upgrade models for stock assessment, which is the most sensitive process for the conservation of this important resource.

Materials and Methods

Population sampling and SNP genotyping

A broad spatial and multi strata sampling has been realized in 2011 and during the GBYP project phase 2 several temporal replicates, provided by the partners from other projects or private collections, were been added to the analyses. A total of 919 individuals were chosen, collected from the most part of the whole range of Atlantic bluefin tuna. We selected 23 bluefin tuna population samples, 1 from Gulf of Mexico, 4 from Eastern Mediterranean, 4 from Central Mediterranean, 10 from Western Mediterranean and 4 from North East Atlantic, each composed by 40 individuals (except EMED-LS-LA+0 and WMED-BA-0-2010 that have 39 and 35 individuals respectively). Six of these were identified as reference samples of spawning populations (EMED-LS-LA+0, WMED-TY-0, WMED-BA-0-2009, WMED-BA-0-2010, WMED-BA-0 and GOM-LA+0) and 17 represented feeding/breeding aggregates in the regions/areas (Figure 1). Abbreviations cited in the text referred to the code sampling specified in Table 1.

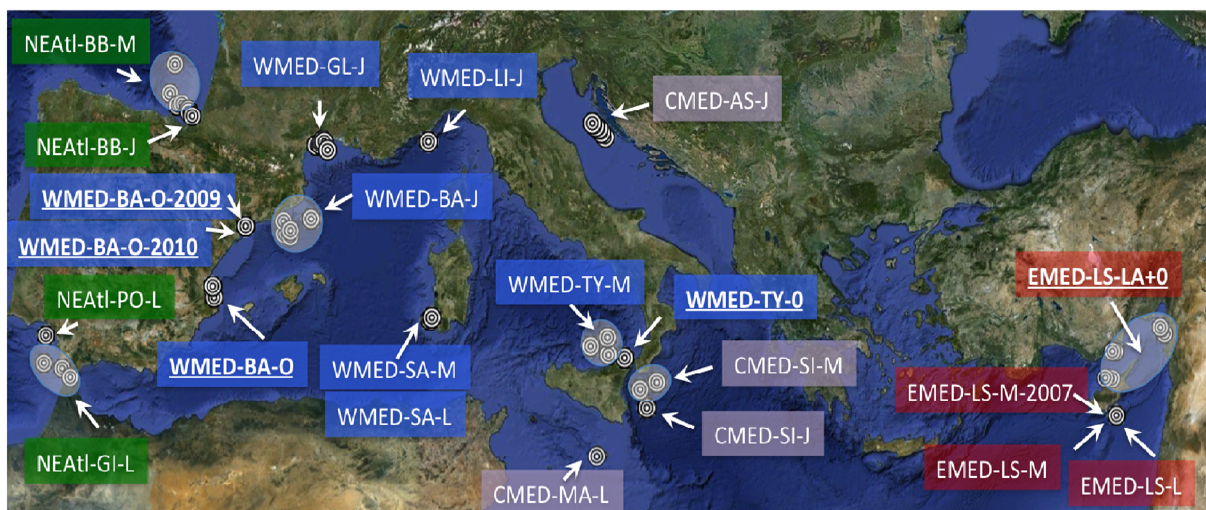


Figure. Geographical representation of sampling locations of Mediterranean Sea, listed in Table 1. Reference samples are in bold and underlined.

REGION	AREA	CODE SAMPLE	Larvae	Age0	Juv[1]	Med[2]	Large[3]	#SNPtyped ind
EMED	LS	EMED-LS-LA+0	10	29				39
EMED	LS	EMED-LS-M				40		
EMED	LS	EMED-LS-L					40	
EMED	LS	EMED-LS-M-2007				40		40
CMED	MA	CMED-MA-L					40	40
CMED	AS	CMED-AS-J			40			40
CMED	SI	CMED-SI-J			40			40
CMED	SI	CMED-SI-M				40		40
WMED	LI	WMED-LI-J			40			40
WMED	SA	WMED-SA-M				40		
WMED	SA	WMED-SA-L					40	
WMED	GL	WMED-GL-J			40			40
WMED	TY	WMED-TY-M				40		40
WMED	TY	WMED-TY-0		40				40
WMED	BA	WMED-BA-0-2009		40				40
WMED	BA	WMED-BA-0-2010		35				40
WMED	BA	WMED-BA-0		40				
WMED	BA	WMED-BA-J			40			
NEAtl	GI	NEAtl-GI-L					40	40
NEAtl	PO	NEAtl-PO-L					40	40
NEAtl	BB	NEAtl-BB-J			40			
NEAtl	BB	NEAtl-BB-M				40		
GOM	GOM	GOM-LA+0	29	16				40
Total General		23	39	205	240	240	200	**919**

Table 1. Sampling information; [1] tunas <25kg, [2] tunas 25-100kg, [3] tunas >100kg.

Samples collected were stored in ethanol 96% at -20°C. The total genomic DNA was extracted from muscle, finclip or full larvae, using the Nucleospin Tissue DNA extraction kit according to the manufacturer's conditions (Machery&Nagel GmBH, Düren, Germany). Quality and quantity of the extracted DNA was checked using PicoGreen (Invitrogen) dsDNA reagents and kit, and DNA was normalized at 50 ng/ul in order to fulfill the requirements of the Illumina assay.

After selection of the panel of 384 high performance SNPs (see chapter 4 of this thesis), all individuals were genotyped using the Illumina GoldenGate assay on the VeraCode

BeadXpress format. Results were visualized and analyzed with the GenomeStudio Data Analysis Software package and then manually re-clustered to obtain highly accurate genotype data.

In order to select a better panel of loci, we excluded SNPs that didn't work, didn't cluster or had a percentage of missing value > 10% and that were monomorphic. We eliminated loci with Minor Allele Frequency (MAF) < 0.01 and observed heterozygosity (H_O) > 0.9, in the 919 bluefin tuna individuals. A quality check was also carried out on individuals and the ones with low-quality results (percentage of missing value over all loci > 20%) were deleted from dataset. Identity analysis, as implemented in Cervus v3.0.5 (Kalinowski *et al.* 2007), was used to find matching genotypes in a genotype file. This analysis is particularly useful in studies where large datasets are used and individuals can be inadvertently resampled. The same software was used to estimate the frequency of null alleles for each locus. The minor allele frequency of loci was calculated with Powermarker v3.25 (Liu and Muse 2005).

Genetic diversity and Hardy-Weinberg Equilibrium

Basic descriptive statistics of genetic diversity in the 23 population samples were calculated over the dataset obtained after the quality check. We calculated allele frequencies, expected (H_E) and observed (H_O) heterozygosity and percentage of polymorphic loci using the package GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Allelic richness was estimated using the method implemented in Fstat 2.9.4 (Goudet 1995). The departure from the Hardy Weinberg Equilibrium (HWE) of each locus in each population was tested using exact probability test implemented in Genepop 4.1.4 (Rousset 2008) with the complete enumeration method, as described by Louis and Dempster (1987), recommended in studies with less than 1000 individuals per sample. In addition, the U tests, both for heterozygote deficiency and heterozygote excess for each locus in each population, were performed. We tested the departure from the Hardy Weinberg Equilibrium (HWE) with the multisample score U test of Rousset and Raymond (1995), which defines a global test across loci and across samples. The Markov chain (MC) algorithm is used (10000 dememorizations, 100 batches and 5000 iterations per batch) to estimate without bias the exact P-value of the U tests (Guo and Thompson 1992). The false discovery rate (FDR) correction, based on Benjamini-Hochberg method (Benjamini and Yekutieli 2001) and implemented in the SGoF+

software (Carvajal-Rodriguez and Uña-Alvarez 2011) was applied to adjust significance levels for multiple simultaneous comparisons.

Linkage disequilibrium (LD) for each pair of SNPs in each population was tested in Genepop 4.1.4 (Rousset 2008). P-values of HWE tests were corrected for multiple tests, using SGoF+ software (Carvajal-Rodriguez and Uña-Alvarez 2011) and the false discovery rate (FDR) correction, based on Benjamini-Hochberg method (Benjamini and Yekutieli 2001), that is expected to provide a large increase in power to identify differentiated populations relative to the Bonferroni method (Narum 2006). F_{IS} values (Weir and Cockerham 1984) were calculated with Fstat 2.9.4 (Goudet 1995) and the significance level was tested with bootstraping over loci.

Outlier loci detection

To identify loci under selection, we availed of two different complementary approaches, implemented in two software, and we performed analyses both for all populations than for six reference samples of the spawning populations.

BayeScan 2.1 (Foll and Gaggiotti 2008) aims at identifying candidate outlier loci using differences between population allele frequencies and a common gene pool. This method based on a scission of locus-population F_{ST} in two component, one shared of all loci and population-specific and other shared of all populations and locus-specific. When the latter is necessary to explain the observed pattern of genetic diversity, we can assume departure from neutrality at a given locus. So, there are two alternative models for each locus, including or not this locus-specific component to model selection. The program calculates a posterior probability for the model including selection, allowing the control of the False Discovery Rate (FDR), defined as the expected proportion of false positives among outlier markers (Foll and Gaggiotti 2008). It has been found that this approach have lower type I (false positive) error rates for divergent selection compared to other outlier detection methods (Narum and Hess 2011).

We performed analyses setting up 20 pilot runs each consisting of 5000 iterations, followed by 5000 iterations with a burn-in of 50000 iterations; the thinning interval, that represent the number of iterations between two samples, was 10, and the prior odds for the neutral model was set to 10, as suggested for the identification of candidate loci with a few hundreds of markers. Posterior Odds (PO), indicating how more likely the model including

selection is compared to the neutral model, were interpreted according to the Jeffreys' scale of evidence for Bayes Factors (Jeffreys 1961).

The other method based on FDIST approach (Beaumont and Nichols 1996) evaluated the relationship between F_{ST} and H_E (expected heterozygosity) in an island model of migration with neutral markers. So, this was possible to identify outlier loci that have higher values of genetic distance than expected from a neutral distribution. We applied this method implemented in Lositan (Looking for Selection In a TANgled dataset), a selection detection workbench constructed around FDIST (Antao *et al.* 2008), with 50000 simulations. The weakness of this process is the incorrect identification of several neutral markers as outlier, and much of this type I error for balancing selection. We used a confidence interval of 0.95 for the expected null differentiation meaning that loci over this interval had to be in the upper 0.025 tail of the distribution to be considered as potentially under directional or divergent selection or in the lower 0.025 tail of the distribution to be considered as potentially under balancing selection (Bourret *et al.* 2012). Lositan also implements a multitest correction based on false discovery rates (FDR) that is fundamental to avoid high overestimation of the percentage of outliers.

Population genetic structure

Genetic distances among samples were also explored by Principal Coordinate Analysis (PCoA) based on the pairwise F_{ST} matrix using GenAlEx 6.5 (Peakall and Smouse 2006, 2012).

Analysis of molecular variance (AMOVA) was used to calculate locus-by-locus F_{ST} and to partition the genetic variance between populations between populations (F_{ST}) within groups within groups (F_{SC}) and among groups (F_{CT}) using Arlequin 3.5 (Excoffier and Lischer 2010).

In particular, individual locus F_{ST} values were calculated for the reference samples (pooling together the ones from the western Mediterranean) and used to rank SNPs in order to select a subpanel of markers with the highest power in discriminating tunas from the three spawning areas. The subpanel of SNPs with $F_{ST} > 0.01$ were compared with the full set of markers for accuracy.

To assess genetic diversity among populations pairwise F_{ST} were calculated with the software Arlequin 3.5 (Excoffier and Lischer 2010) (10000 permutations); a matrix of

pairwise F_{ST} was generated by R-script implemented in the software. The significance level of multiple comparisons was adjusted with the FDR method as described above.

To investigate on the genetic similarity of the 23 population samples, D_A genetic distances (Nei *et al.* 1983) between all pairs of populations were computed with Powermarker v3.25 (Liu and Muse 2005), and a neighbor-joining tree was produced to visualize their relationships using Splitstree4 (Huson 1998; Huson and Bryant 2006). The pattern of phylogeographic structure was visualized with GenGIS v2.02, an application that provides a 3D graphical interface for the merging of information on molecular diversity with the geographic location from which the sequences were collected (Parks *et al.* 2009).

We used also the method successfully adopted by Willing *et al.* (2010) and Kraus *et al.* (2013) to display reticulate relationships among individuals using SNP data: the NEIGHBOUR-NET algorithm. The phylogeographic analysis uses the method Neighbor-net (Bryant and Moulton 2004) implemented in Splitstree4 (Huson 1998; Huson and Bryant 2006). According to Kraus *et al.* (2013) for each individual, the genotype at each SNP was collapsed into a single base character and concatenated to a sequence of nucleotides. Heterozygote genotypes were coded according to IUPAC and missing data denoted 'N'. Uncorrected_P distance was used as metric.

Detection of genetic clusters

Discriminant analysis of principal components (DAPC; Jombart *et al.* 2010) from Adegenet (Jombart 2008) was used to detect the number of genetic clusters and assignment of individuals.

This method transforms data using principle component analysis (PCA) to create uncorrelated variables for input into Discriminant Analysis (DA). DA maximizes between-group variation and minimizes within-group variation for assessment of between-group variation. DAPC is free of assumptions about Hardy-Weinberg equilibrium or linkage disequilibrium and provides graphical representation of divergence among populations. DAPC was implemented using the Adegenet R package (Jombart 2008) version 1.2.8 in R (R Development Core Team 2009). DAPC allowed the search for the most likely number of clusters/groups in the dataset. This can be achieved using k-means, a clustering algorithm which finds a given number (k) of groups maximizing the variation between groups, B(X). To identify the optimal number of clusters, k-means is run sequentially with increasing values of

k, and different clustering solutions are compared using Bayesian Information Criterion (BIC). Ideally, the optimal clustering solution should correspond to the lowest BIC. In practice, the 'best' BIC is often indicated by an elbow in the curve of BIC values as a function of k. Moreover, being based on the Discriminant Analysis, DAPC also provides membership probabilities of each individual for the different groups based on the retained discriminant functions, giving indications of how clear-cut genetic clusters are. Loose clusters will result in fairly flat distributions of membership probabilities of individuals across clusters, pointing to possible admixture.

We used the function 'find.clusters' to determine the most likely number of genetic clusters in the data, using all available principal components (PCs). To calculate the probability of assignment of individuals to each of these clusters using DAPC, we determined the optimal number of PCs. In a preliminary DAPC run we retained only a limited number of PCs (sample size divided by three) used all discriminant functions, in order to avoid unstable assignments of individuals to clusters. The 'optim.a.score' function determined the optimal number of PCs, that were used in the final DAPC.

In addition to DAPC, the cluster analyses were also performed with another approach based on the Bayesian algorithm, implemented in Structure 2.3.4 (Pritchard *et al.* 2000). This software, in contrast to DAPC, assumes Hardy-Weinberg and Linkage Equilibrium and estimates the number of k clusters of individuals. To estimate the more likely number of clusters, a posterior probability is calculated for each inferred k. The algorithm was run assuming the admixture model and correlated allele frequencies among populations, and providing the sampling information as prior with the option LOCPRIOR, in order to improve accuracy in detecting population structure. For each analysis we used 5 iterations per k value, for values of k between 1 and 8, a burnin period length of 10000, and 50000 MCMC repetitions. The optimal k was selected according to the two criteria: the ΔK (Evanno *et al.* 2005), and the ΔF_{ST} (Campana *et al.* 2011) both implemented in the R package CorrSieve (Campana *et al.* 2011). When a K was selected, Clumpp v.1.1 (Jakobsson and Rosenberg, 2007) was used to average the assignment scores over the 5 runs. Results were displayed using Distruct v.1.1 (Rosenberg 2004).

Assignment tests

Assignment tests were used to estimate the origin of each individual fish.

In general Bayesian and maximum likelihood-based methods have proven to be significantly more effective at assignment than previous distance-based methods (Cornuet *et al.* 1999).

The reference sample populations (that comprise larvae and age-0 of known origin) were considered as baseline populations to assign the other 17 samples, made up of juvenile/adults of unknown origin. Due to the lack of differentiation among the four reference samples of Western Mediterranean origin (see Results section), they were put together and only three reporting groups were considered (EMED, WMED and GOM).

The performance of different panels of SNPs (that is the ability to increase the percentage of correct assignment of individuals to the baseline populations) were compared.

In first instance, the 'leave one out' test of the accuracy of assignment tests was performed in Oncor (Anderson *et al.* 2008). This test evaluates how well individuals can be assigned to their population of origin. During the test each individual in each baseline is sequentially removed from the baseline and its origin is estimated using the rest of the baseline. Tunas with incomplete genotypes (because of missing data) were dropped from the analysis but remained in the baseline in order to estimate the origin of other individuals. Oncor records the fraction of assignments for each population that were correct and the population to which individuals were most often incorrectly assigned to. The accuracy of assignment was assessed through self-assignment of larvae and age-0 individuals to their respective reference populations by using the 'leave-one-out' cross-validation test. Since the origin of assigned individuals was known, the probability of assignment to the population of origin was reported for assignment accuracy (Storer *et al.* 2012).

The second step was to perform the genetic assignment of the juvenile/adult tunas to the reference populations using the programs Oncor (Anderson *et al.* 2008) and Geneclass2 (Piry *et al.* 2004).

Classic genetic assignment tests were performed in Geneclass2 (Piry *et al.* 2004) according to the methods of Rannala and Mountain (1997). Each fish was assigned to the reference population with the highest assignment score, and assignment probabilities for each individual were recorded for reference of assignment confidence. To determine

whether any individuals should be excluded from the stock to which they were assigned, we used an exclusion-based counterpart method based on simulations (Manel *et al.* 2005). We used the resampling method of Paetkau *et al.* (2004) in Geneclass2 to simulate 10000 individuals; this procedure allowed to determine whether the probability of assigning a given fish fell into the tail of the distribution ($P < 0.05$), which would indicate that the individual should be excluded from the population to which it was previously assigned by the classic tests. This is important to counteract the possibility of false assignment in the case of a potentially incomplete baselines.

Assignment tests were performed by means of the conditional maximum likelihood approach in Oncor (Millar 1987; Anderson *et al.* 2008). Oncor assigns individuals in a mixture sample to the baseline population that would have the highest probability of producing the given genotype in the mixture. Oncor uses the method of Rannala and Mountain (1997) to estimate this probability. The program uses both genotype frequencies and mixture proportions when estimating the origin of individuals thus is expected to yield more realistic estimates of assignment accuracy than Geneclass2. In our study the ability of SNPs to assign individuals to the most likely population was assessed based on the 'best-estimate' reporting group to which individuals were assigned, that is the reporting group to which a given individual had the highest proportional assignment.

Mixture analysis

Mixture analysis uses baseline genetic data to estimate the composition of a sample, that is the proportion of fish that belong to different 'stocks'. Due to the lack of differentiation among the four reference samples of Western Mediterranean origin, they were pooled together, therefore the mixture analysis was used to assign proportions of the feeding/breeding aggregates to the 3 spawning reporting groups (EMED, WMED and GOM). To examine how accurate mixture analysis Is likely to be, the 100% simulation feature was used. The effect of population size on average accuracy of estimated mixture was investigated for different baseline sample sizes along with the empirical baseline sample sizes. The closer the probability was to 1.0 and the lower the variance, the greater was the reliability of the reference for mixed-stock analysis. In simulations with sample sizes as empirical baselines Oncor uses the method of Anderson *et al.* (2008) to simulate mixture

genotypes and to estimate their probability of occurrence in baseline populations. When a different sample size is employed the method of Kalinowski *et al.* (2007) is used. The method of Anderson *et al.* (2008) is currently preferred for examining the accuracy because the other method has not been tested extensively, and therefore they should be used with some caution. To estimate mixture proportions of the 23 samples, Oncor used conditional maximum likelihood (Millar 1987). Genotype probabilities were calculated using the method of Rannala and Mountain (1997). Ninety-five per cent confidence intervals (CIs) of mixture proportions were determined through 1000 bootstraps.

Results

Genotyping

We genotyped 919 bluefin individuals for 384 SNPs. During a quality check step of the genotypes, we excluded 62 SNPs that didn't work (60) or had a percentage of missing value > 10% (2). Further 35 loci were eliminated because they resulted to be monomorphic (16), with MAF < 0.01 (14) or H_E > 0.9 (5).This yielded a conversion rate of ~ 75%. We also removed 59 individuals because of the low quality of their genotypes (percentage of missing values over all loci > 20%), prevalently from the CMED-MA-L, NEAtl-GI-L and WMED-BA-J samples. The average percentage of missing value for each population ranged from 0.3 in EMED-LS-M-2007 to 8.5 in NEAtl-GI-L (Table 2). Twelve individuals with identical (or highly similar) genotypes, likely due to unintentional re-genotyping, were excluded (Table 2). So, we obtained a final dataset with 848 individuals genotyped at 287 SNPs.

Sample	N	#_call rate <80%_ind	duplicated	N final dataset	Average of % MV_ind
EMED-LS-LA+0	39			39	0,331
EMED-LS-M	40			40	1,585
EMED-LS-L	40			40	1,002
EMED-LS-M-2007	40	1	1	38	0,257
CMED-AS-J	40			40	0,775
CMED-MA-L	40	19		21	7,748
CMED-SI-J	40	4		36	4,394
CMED-SI-M	40	1		39	5,503
WMED-TY-0	40			40	2,570
WMED-TY-M	40	2		38	4,924
WMED-LI-J	40		1	39	0,393
WMED-SA-M	40	2	3	35	4,619
WMED-SA-L	40	1		39	1,385
WMED-GL-J	40	1		39	2,368
WMED-BA-0-2009	40			40	1,228
WMED-BA-0-2010	40	1		39	1,035
WMED-BA-0	40		1	39	1,626
WMED-BA-J	40	12	5	23	6,434
NEAtl-GI-L	40	11		29	8,483
NEAtl-BB-J	40			40	1,002
NEAtl-PO-L	40	1	1	38	1,962
NEAtl-BB-M	40	1		39	0,581
GOM-LA+0	40	2		38	0,651
Total/Average	**919**	**59**	**12**	**848**	**2,646**

Table 2. Quality check of the 919 genotyped specimens. N =total number of individual genotyped per population, #_call_rate < 80%_ind = individuals genotypes complete for less than 80%, duplicated = individuals genotyped twice, N final dataset = individuals included in the final dataset, average of % MV_ind = average of % missing values per population.

Genetic diversity and Hardy-Weinberg Equilibrium

We obtained a very high percentage of polymorphic loci, the proportion of polymorphic loci averaged 99.1% and varied from 97.2% in NEAtl-GI-L to 100% in WMED-SA-L. Estimates of H_O and H_E across the samples ranged from 0.272 to 0.352 (mean 0.332) and 0.326-0.344 (mean 0.335), respectively. Observed heterozygosity within the reference populations revealed similar levels of diversity to the 17 feeding/breeding samples, with WMED-BA-J, CMED-MA-L and NEAtl-GI-L having the lowest H_O values while WMED-BA-0-2009 the highest (Table 3). Global multisample score test of Rousset and Raymond (1995), over all loci and populations revealed, after FDR correction for multiple tests, 14 loci were out of equilibrium (9 loci for heterozygotes deficiency and 4 for heterozygotes excess). However, the "exact HW test" for deviation from equilibrium for each locus in each population revealed none was significant after FDR correction. Global U test showed that, after FDR correction for multiple tests, four samples (CMED-MA-L, NEAtl-GI-L, WMED-BA-J, WMED-SA-M) had significant deviations from HWE. The same populations showed significantly value of $F_{IS} > 0$ (Table 3).

Population sample	%P	H_E	H_O	AR	F_{IS}	P F_{IS}>observed	P HWE deficit
EMED-LS-LA+0	99,30%	0,333	0,335	1,930	0,008	0,232	0,3297
EMED-LS-M	98,95%	0,329	0,342	1,931	-0,027	0,995	0,994
EMED-LS-L	98,26%	0,335	0,335	1,932	0,014	0,082	0,1286
EMED-LS-M-2007	99,65%	0,337	0,342	1,939	-0,001	0,526	0,4168
CMED-AS-J	99,65%	0,335	0,336	1,935	0,01	0,1568	0,1876
CMED-MA-L	97,91%	0,328	0,276	1,932	0,185	**0**	**0**
CMED-SI-J	98,95%	0,340	0,345	1,939	-0,002	0,5716	0,5276
CMED-SI-M	99,30%	0,332	0,338	1,931	-0,006	0,718	0,4956
WMED-TY-0	99,30%	0,335	0,331	1,936	0,024	0,0093	0,0325
WMED-TY-M	99,30%	0,328	0,338	1,930	-0,018	0,9527	0,9648
WMED-LI-J	99,30%	0,338	0,338	1,941	0,013	0,0968	0,1415
WMED-SA-M	98,26%	0,328	0,318	1,927	0,047	**0**	**0,0003**
WMED-SA-L	100,00%	0,339	0,342	1,946	0,003	0,3687	0,2615
WMED-GL-J	99,65%	0,336	0,345	1,940	-0,014	0,9157	0,8975
WMED-BA-0-2009	99,65%	0,344	0,352	1,947	-0,01	0,8385	0,8371
WMED-BA-0-2010	98,95%	0,332	0,333	1,936	0,012	0,1312	0,1208
WMED-BA-0	99,30%	0,337	0,345	1,937	-0,012	0,8783	0,8554
WMED-BA-J	97,91%	0,333	0,293	1,921	0,14	**0**	**0**
NEAtl-GI-L	97,21%	0,326	0,272	1,913	0,184	**0**	**0**
NEAtl-BB-J	98,95%	0,333	0,332	1,935	0,018	0,0382	0,0522
NEAtl-PO-L	99,65%	0,339	0,348	1,941	-0,012	0,8813	0,7494
NEAtl-BB-M	99,65%	0,339	0,342	1,944	0,004	0,363	0,557
GOM-LA+0	99,65%	0,340	0,345	1,944	-0,004	0,6329	0,5186

Table 3. Descriptive statistics of genetic diversity in the 23 population samples at the 287 SNP loci. H_E = expected heterozygosity, H_O = observed heterozygosity, AR = allelic richness, F_{IS} = inbreeding fixation index and relative P- value, P HWE deficit = probability associated to the HWE test for heterozygotes deficiency. F_{IS} values significantly > 0 are in bold underlined.

Twenty-eight pairs of loci showed significant linkage disequilibrium ($p < 0.05$) in all the reference populations. Due to the high number of tests (i.e., 41041 for each population), no correction for multiple tests was performed since this approach would be overly conservative and likely underestimate truly significant relationships (Limborg *et al.* 2012a). None of these loci pairs were isolated from the same contig, suggesting that distinct demographic or selective forces may be associated with the observed linkage disequilibrium rather than merely physical associations (Helyar *et al.* 2012; Zakas *et al.* 2012). In many cases, linked loci appear to provide redundant information, measuring the same allele frequencies across populations (i.e. providing the power to differentiate between the same populations) (Storer *et al.* 2012), and hence potentially leading to upward bias in assignment success.

However, because we wanted testing all available loci for the species and there are only limited pairs of loci which were not in linkage equilibrium and not in all populations, we retained all of them in downstream analyses, and treated them as independent markers.

Outlier loci detection

We searched for outlier loci, that are loci showing higher levels of interspecific genetic differentiation than expected under neutrality, by two complementary and exhaustive methods. First, we used the hierarchical Bayesian method described in Beaumont and Balding (2004), implemented in Bayescan software (Foll and Gaggiotti 2008). Secondly, we used the Beaumont and Nichols (1996) FDIST approach, implemented in Lositan (Antao *et al.* 2008).

Eleven outlier loci under selection were detected by FDIST (Lositan) (Figure 2, Table 4); none was significant after FDR correction. Preliminary analyses did not revealed differences in allele frequencies among the populations studied at any of these loci.

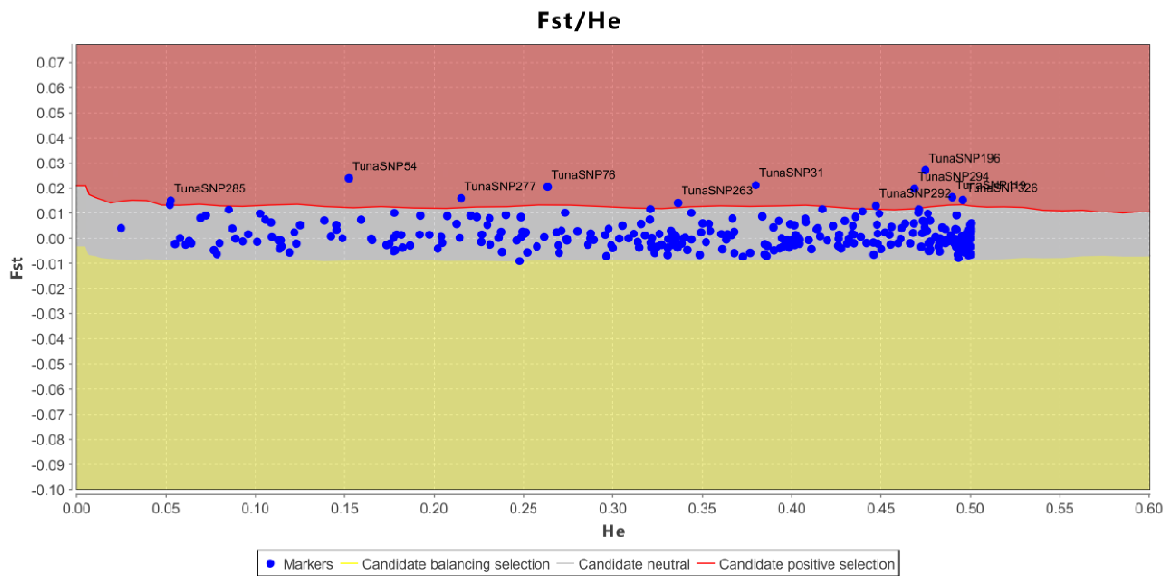


Figure 2. Comparison of F_{ST} and heterozygosity (H_E) in polymorphic loci to identify outliers and potential candidates for selection using Lositan. Loci highlighted in red are candidates for positive selection (loci highlighted in grey are selectively neutral).

Locus	H_E	F_{ST}	P(Simul F_{ST} < sample F_{ST})
TunaSNP31	0,37988	0,021216	0,997291
TunaSNP54	0,15236	0,023948	0,99924
TunaSNP76	0,263474	0,02051	0,996211
TunaSNP119	0,489827	0,016331	0,985804
TunaSNP196	0,474801	0,027128	0,999686
TunaSNP263	0,336313	0,014131	0,979717
TunaSNP277	0,215053	0,015913	0,991643
TunaSNP285	0,0526	0,015026	0,981575
TunaSNP292	0,447185	0,013011	0,981883
TunaSNP294	0,468699	0,019748	0,996564
TunaSNP326	0,495753	0,015245	0,981053

Table 4. Outlier loci detected in Lositan.

Bayescan analysis (Figure 3) pointed out the occurrence of one differentiation locus (TunaSNP196).

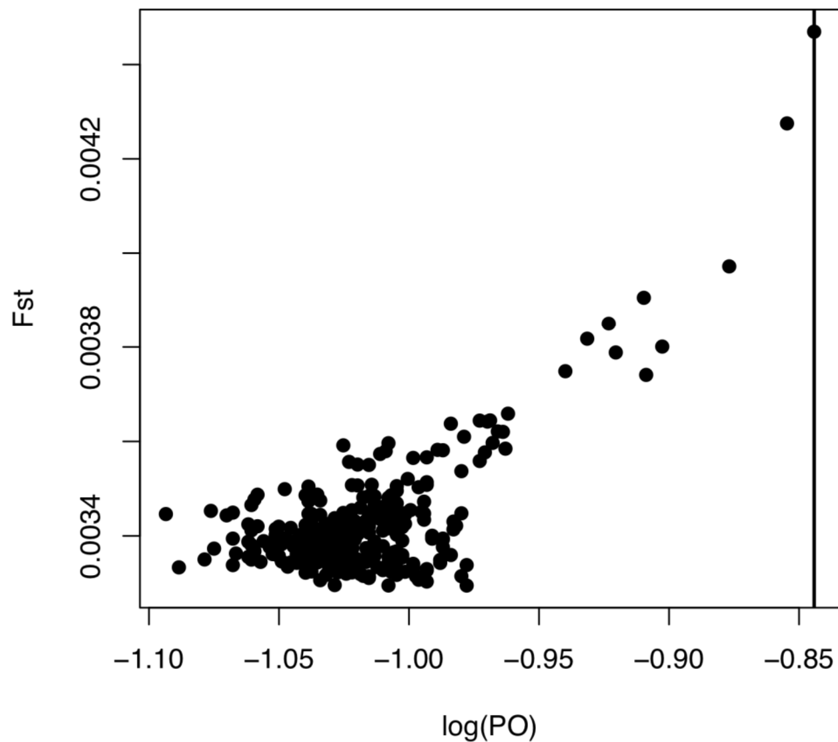


Figure 3. Outlier loci analysis among all 23 populations with the Bayescan approach. Each point corresponds to an SNP locus. F_{ST} is plotted against the log10 of the posterior odds (PO), which provides evidence whether the locus is subject to selection or not. The vertical dashed line shows the decisive threshold value (log10 PO = 0.5) used for identifying outlier loci.

Only one locus (TunaSNP196) was identified by both Lositan and Bayescan as outlier. In general, when a locus is identified contemporary by two approaches that differ in algorithms and assumptions, it is likely to be truly adaptative (Wang *et al.* 2012). However, in our study, TunaSNP196 in Lositan was not significant after FDR and in Bayescan was significant only at a threshold of log10 PO = 0.5 (corresponding to a posterior probability of 0.76). This threshold is considered as being a “substantial” evidence for selection although generally considered as a very weak signal in classical statistics (Fischer *et al.* 2011).

In summary, all the 287 SNPs were used in the following population genetic analyses, none was eliminated because out of HW equilibrium, in linkage disequilibrium or identified as outlier. As concerns the four samples out of HW equilibrium, the analyses were performed both retaining and excluding them with no substantial differences in the results, so in the present paper the main findings for all the 23 population samples are presented.

Population genetic structure

The genetic differentiation measured among all population samples at the 287 loci was not significant, with an overall F_{ST} value of -0.00024 (P-value > 0.05). The pairwise F_{ST} values among samples calculated over the 287 SNP loci were very low, with only one significant pairwise value after FDR correction (F_{ST} = 0.00448) between CMED-SI-J and EMED-LS-M. According to these results, the distribution of locus-by-locus AMOVA F_{ST} estimates showed that about half of the 287 loci (145 SNPs) displayed negative values and only 54 loci were above a value F_{ST} > 0.005.

As for all 23 samples, the distribution of locus-by-locus F_{ST} estimates considering only the six reference samples of the spawning populations (i.e. EMED-LS-LA+0, WMED-TY-0, WMED-BA-0-2009, WMED-BA-0-2010, WMED-BA-0 and GOM-LA+0) showed that more than half of the 287 loci (155 SNPs) displayed negative values, 68 loci were with F_{ST} > 0.005 and 35 loci had a value of F_{ST} > 0.01. When the four reference samples of Western Mediterranean origin were pooled together, 62 loci had F_{ST} > 0.005 and 31 loci F_{ST} > 0.01. All these different panels of loci (54, 68, 35, 62, 31 loci with the highest individual F_{ST} values) were tested for their capacity in differentiating among the three known spawning areas (Eastern Mediterranean, Western Mediterranean, Gulf of Mexico, henceforth indicated as EMED, WMED and GOM). The 31 SNPs allowed to measure the highest overall value of F_{ST} (0.00945, with P-value = 0.04106 ± 0.00572) among the reference samples with almost all significant pairwise F_{ST} values between EMED-LS-LA+0, GOM-LA+0 and the other Western Mediterranean reference samples (Table 5, Figure 4).

	EMED-LS-LA+0	WMED-BA-0	WMED-BA-0-2009	WMED-BA-0-2010	WMED-TY-0	GOM-LA+0
EMED-LS-LA+0		0	0,0001	0,04297	0	0
WMED-BA-0	**0,02006**		0,73211	0,66439	0,96773	0,0002
WMED-BA-0-2009	**0,01636**	-0,00253		0,7126	0,50718	0
WMED-BA-0-2010	0,0072	-0,00162	-0,00249		0,7322	0,00396
WMED-TY-0	**0,02032**	-0,00592	-0,00077	-0,00249		0,0001
GOM-LA+0	**0,01817**	**0,01758**	**0,0199**	**0,0141**	**0,0184**	

Table 5. Matrix of pairwise F_{ST} values (below the diagonal) and P-values (above the diagonal) among reference populations using the 31 SNPs. Significant values after FDR correction are in bold underlined.

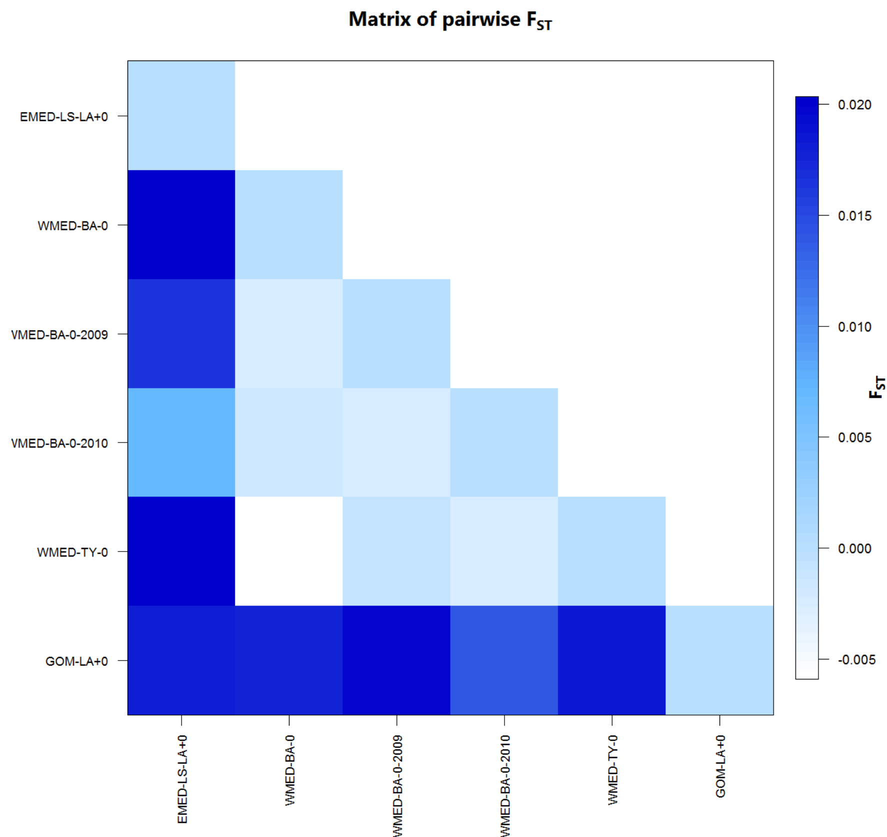


Figure 4. Graphic of the pairwise F_{ST} values between the six reference samples using the restricted panel of 31 loci. The F_{ST} values are coded with a color code showed in the legend on the right side.

The Principal Coordinates Analysis (PCoA) showed EMED-LS-LA+0 and GOM-LA+0 well separated from the other 4 reference Mediterranean populations (Figure 5).

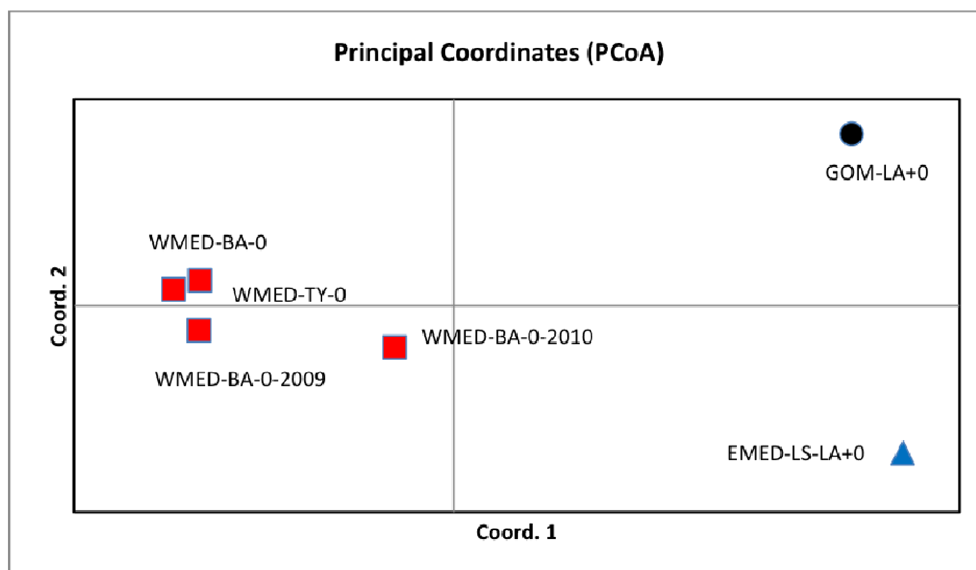


Figure 5. PCoA plot obtained with the restricted panel of 31 loci on the six reference samples.

A weak degree of differentiation was pointed out by the Discriminant Analysis of Principal Components (DAPC), with GOM-LA+0 and EMED-LS-LA+0 only partially separated from the four western Mediterranean samples (Figure 6).

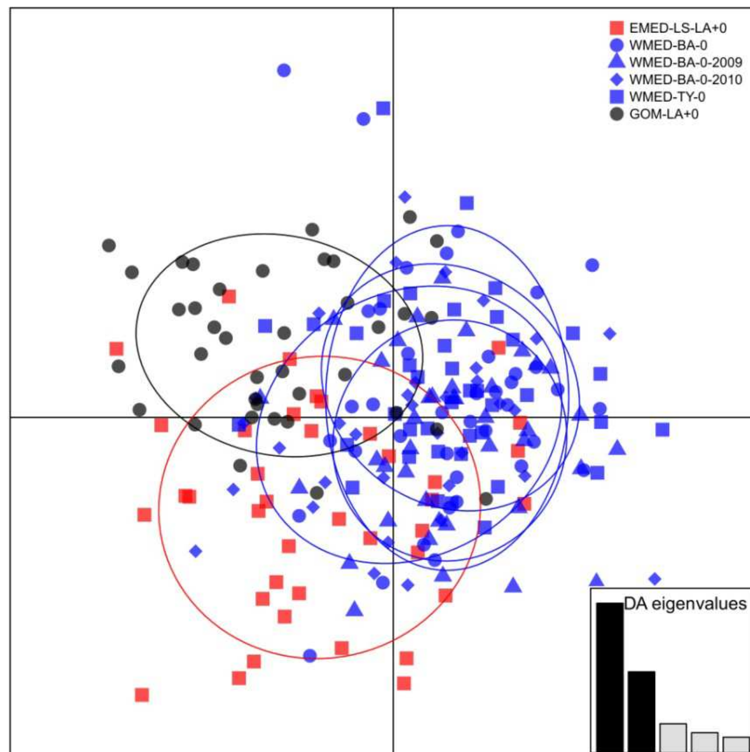


Figure 6. DAPC obtained with the restricted panel of 31 loci on the six reference samples.

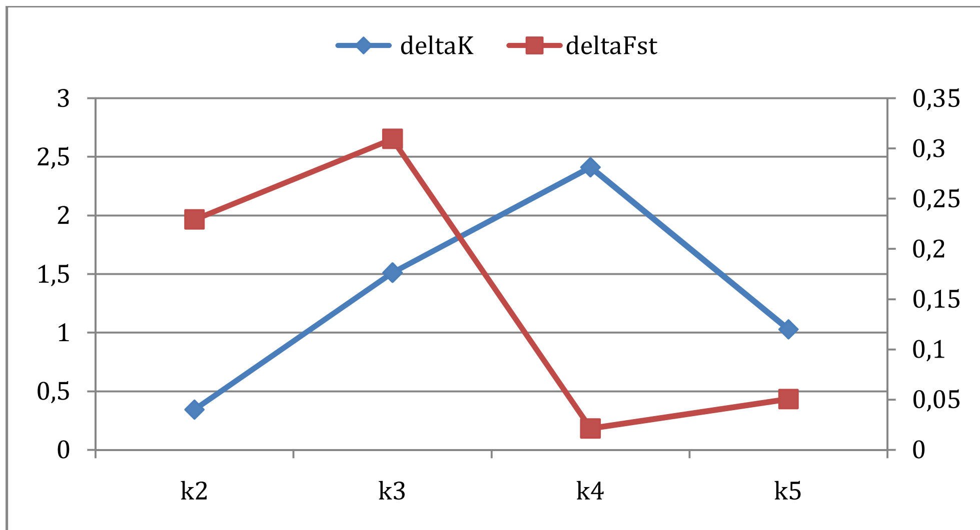
However, when all populations were included in the analyses, the panel of 31 loci failed in identifying significant clustering of samples both with PCoA and DAPC analyses (data not shown). The overall value of F_{ST} was 0.00191 (P-value = 0.01760 ± 0.00439), with a few significant pairwise F_{ST} values (17 out of 270 comparisons).

We tested different genetic structures pooling population in 3 to 5 groups according to the location they were caught or the putative spawning area. The results of the AMOVA analysis are shown in Table 6. Only using the 31 SNPs both the 3-group structure (grouping the samples in three group according to their sampling location: EMED, WMED, GOM) and the 5-group structure (EMED, CMED, WMED, NEAtl, GOM) were statistically significantly differentiated.

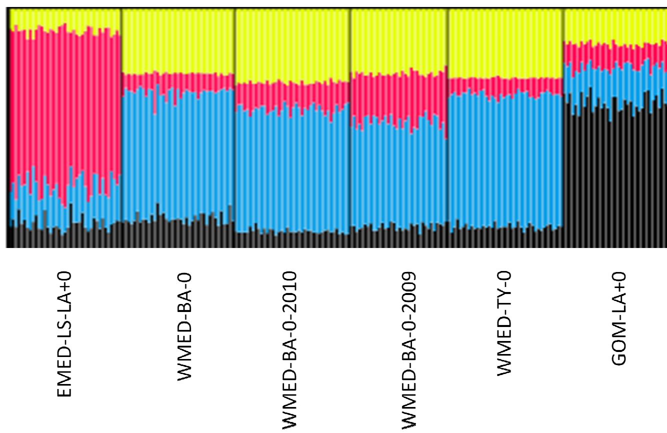
STRUCTURE TESTED	SAMPLES GROUPING	F_{ST}	F_{SC}	F_{CT}
287 loci reference pops				
One group	all reference pops	-0.00043		
3 groups	(EMED) (WMED) (GOM)	-0.00036	-0.00052	0.00016
287 loci all pops				
One group	all pops	-0.00036		
3 groups	(EMED) (CMED, WMED, NEAtl)(GOM)	-0.00046	-0.00030	-0.00016
5 groups	(EMED) (CMED) (WMED) (NEAtl) (GOM)	-0.00036	-0.00037	0.00001
31 loci reference pops				
One group	all reference pops	**0.00938*** **		
3 groups	(EMED) (WMED) (GOM)	**0.01721*** **	-0.00270	0.01986
31 loci all pops				
One group	all pops	**0.00191***		
3 groups	(EMED) (CMED, WMED, NEAtl)(GOM)	**0.00391***	0.00064	**0.00327***
5 groups	(EMED) (CMED) (WMED) (NEAtl) (GOM)	**0.00238***	0.00050	**0.00187***
Significance tests (10100 permutations)				

Table 6. Hierarchical analysis of molecular variance AMOVA. Fixation indices: F_{ST} (variance among populations), F_{SC} (variance among populations within groups) F_{CT} (variance among groups). ***P > 0.001, *P < 0.05. Values statistically significantly are in bold.

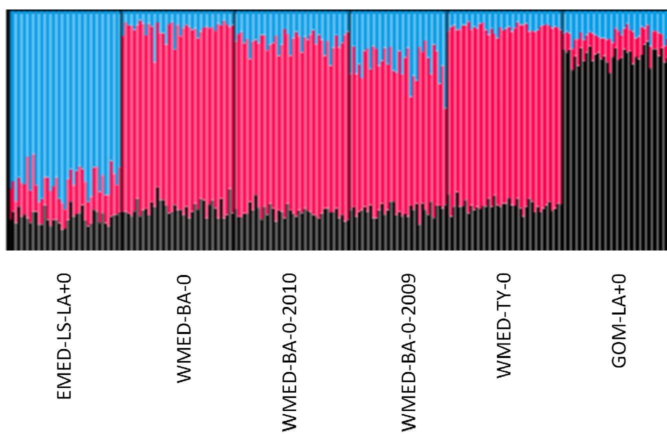
Considering these results, the cluster analysis based on Bayesian algorithm was performed in Structure only with restricted panel of 31 SNPs, the most discriminant among the three main spawning areas. Firstly, we analyzed the reference populations only. The ΔK method identified the optimum number of genetic units as K = 4, while ΔF_{ST} method found K = 3 (Figure 7). Actually, in both cases, three main clusters were identified: the EMED-LS-LA+0 and GOM-LA+0 genetically divergent from a third major cluster including the WMED-BA-0, WMED-BA-0-2009, WMED-BA-0-2010 and WMED-TY-0 samples. The loci we tested for their capacity in discriminating the three areas were able to correctly re-assign the individuals.



A)



B)

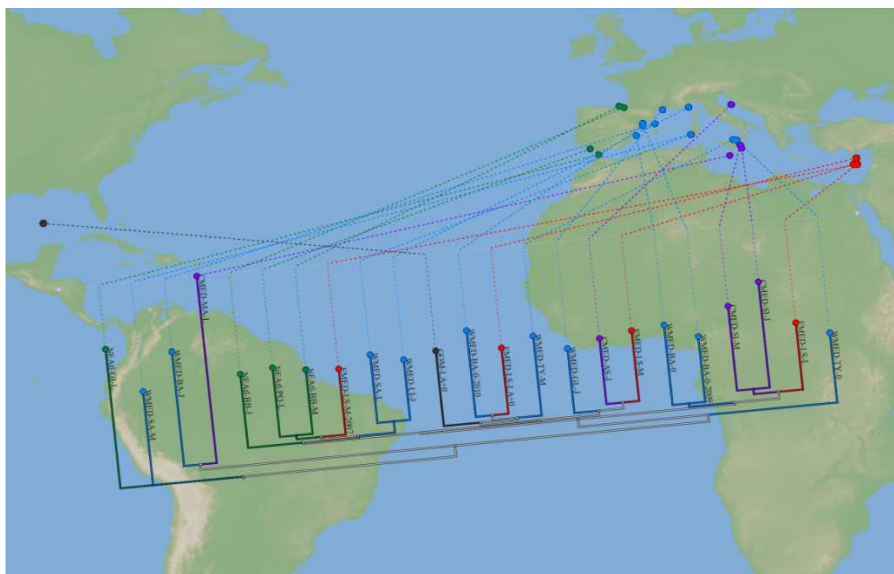


C)

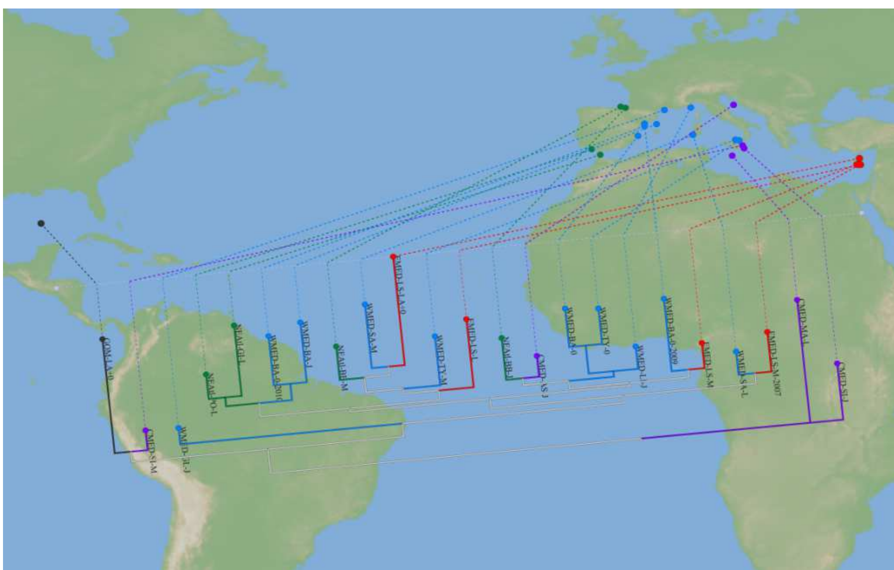
Figure 7. Results of the Bayesian cluster analysis performed with Structure 2.3.4 based on the restricted panel of 31 SNP loci on the 6 reference populations. A) CorrSieve output ΔK versus ΔF_{ST}. ΔK and ΔF_{ST} have different maxima at K = 4 and K=3, respectively. Structure bar plot results obtained with K = 4 (B) and K = 3 (C).

However, when the 31 SNPs were used to identify clusters on the 17 breeding/feeding aggregates the results didn't provide any signal of genetic structuring.

The relative genetic similarity among the 23 samples was investigated also by phylogenetic analyses using the D_A distances (Nei 1983). The relationships among samples are shown schematically in Figure 8, using the whole dataset of 287 SNPs and the restricted panel of 31 loci, respectively. No geographical clustering of populations is evident. Analogously, no groups composed of genetically similar individuals could be detected in the phylogenetic network obtained in Splitstree (data not shown).



A)



B)

Figure 8. The figure shows the neighbour-joining tree on top of the map, based on D_A distances calculated with the full set of SNPs (A) and the 31 loci (B).

Assignment test and mixture analyses

Firstly, we tested for performance in assignment both the full set of SNPs and the different panels of loci.

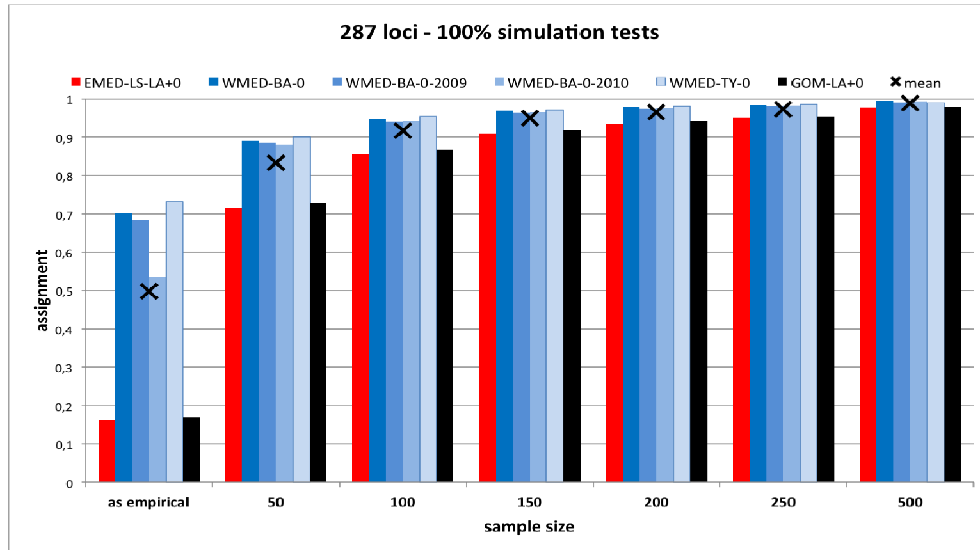
The results of the 'leave-one-out' cross-validation test and 100% fishery simulation tests realized with Oncor are reported in Table 7. In general, the highest percent of correct assignment was recorded for the 31 SNPs panel compared with the full set of loci and other panels of loci (data not shown). However, even for the 31 SNPs the percent scores from the cross-validation test were low (only 46.9% for GOM, 47.2% for EMED, and ranged from 67.9% to 84.6% for WMED). The percentage of correct scores in 100% simulations was greater than in the validation tests (in general ≥ 79%) but often below the 90%, a value which is used by many authors as a threshold for indicating that baseline populations have been adequately delineated for assigning individuals from mixed fisheries (Hess *et al.* 2011 and references therein).

	31 SNP		**287 SNP**	
Reference populations	Leave-one-group out test	100% simulation	Leave-one-group out test	100% simulation
EMED-LA+0	0.472	0.7882	0.250	0.1603
WMED-BA-0	0.846	0.9546	0.700	0.7005
WMED-BA-0-2009	0.813	0.9623	0.462	0.6842
WMED-BA-0-2010	0.679	0.8157	0.786	0.5345
WMED-0-TY	0.846	0.9844	0.375	0.7343
GOM-LA+0	0.469	0.7917	0.083	0.1691

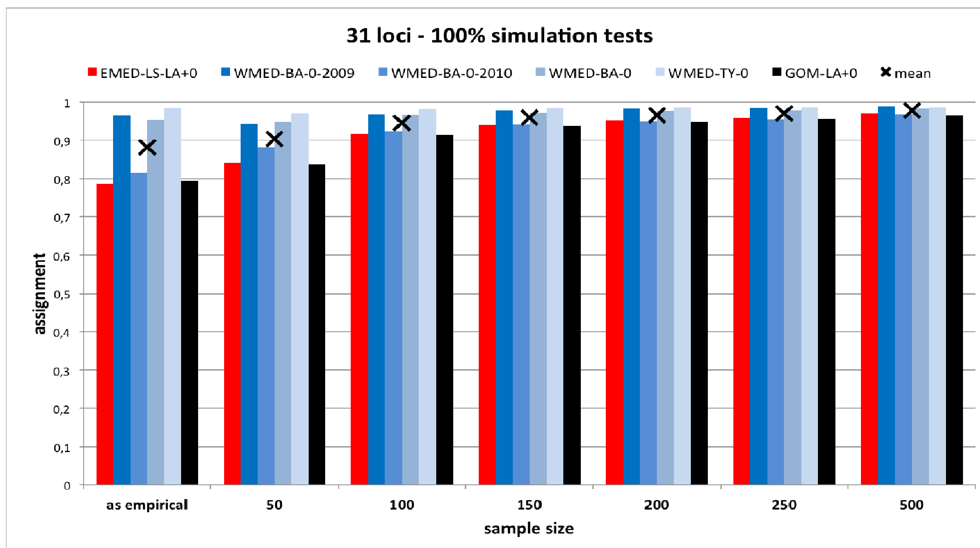
Table 7. Test of the accuracy of genetic stock identification using a 'leave-one-out' cross-validation test and a 100% fishery simulation test in Oncor. Percent correct values refer to the percentage of individuals correctly assigned to each of the 3 reporting groups (spawning areas: EMED, WMED and GOM).

Secondly, we tested if larger population baseline sample sizes would allow to increase the power and reach a good level of accuracy in assignments. We evaluated the effect of average sample sizes on average accuracy using simulations (sampling without replacement) for population samples sizes ranging from 40 (as in empirical samples) to 500. In general, for the same sample size, the full data set of loci provided the less accurate estimates than the 31 loci (Figure 9). Incremental gain in accuracy with larger sample sizes

was measured. The average correct re-assignment in the 100% simulation tests reached the threshold of the 90% (critical level to determine whether the reference population is acceptably identifiable, Seeb *et al.* 2000) with a sample size of 100 fish and 50 fish sampled per populations for the full data set and the 31 SNPs, respectively (Figure 9).



A)



B)

Figure 9. Results of 100% simulations test in Oncor comparing the effect on the accuracy of self-assignment of increasing baseline sample sizes using (A) the full data set of 287 loci and (B) the 31 selected SNPs.

Considering the findings previously described, for the assignment and mixture analyses of all populations only the ‘best performing’ panel of 31 SNPs panel was tested.

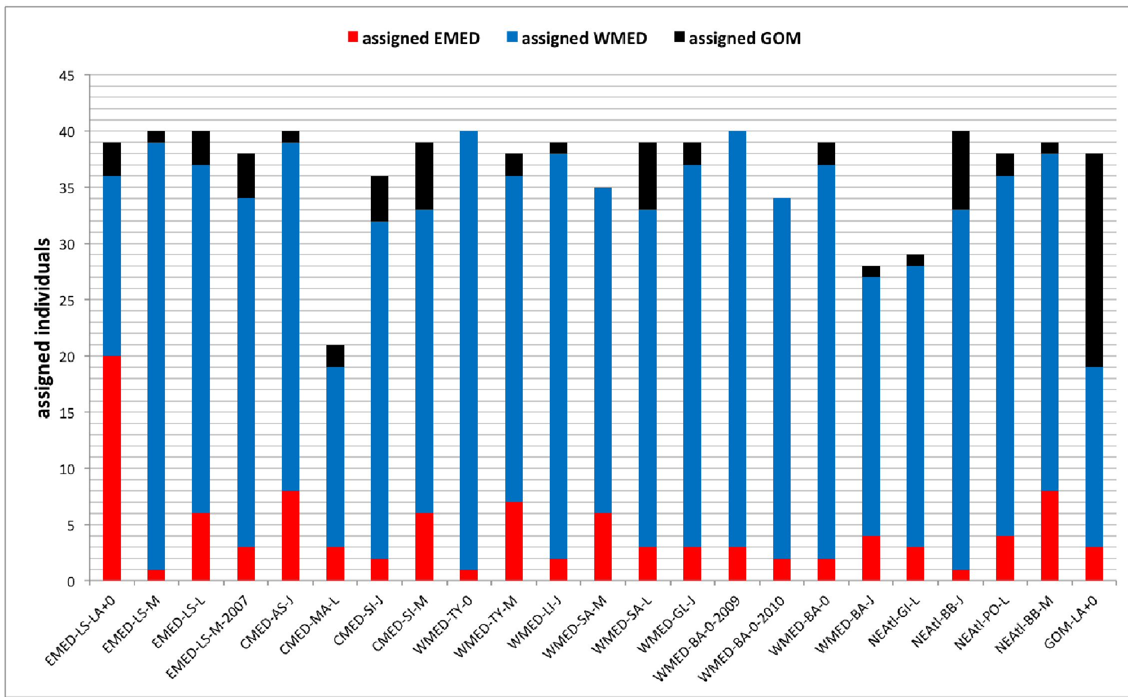
The assignment test performed in Geneclass2 with the 31 SNPs indicated a prevalent contribution of the WMED reference samples to the feeding/breeding aggregates, except, as expected, in the reference populations from the Levantine Sea and Gulf of Mexico (Table 8).

Exclusion analysis found that for 4 tunas, the probability of their assigning to the reference population was less than 0.05, indicating that they are to be excluded from the population to which they were assigned. However, the fishes that exceeded the assignment probability threshold of 90% was only 84 (9.9% of the total individuals in the dataset) confirming the low performance (accuracy) of the select SNPs.

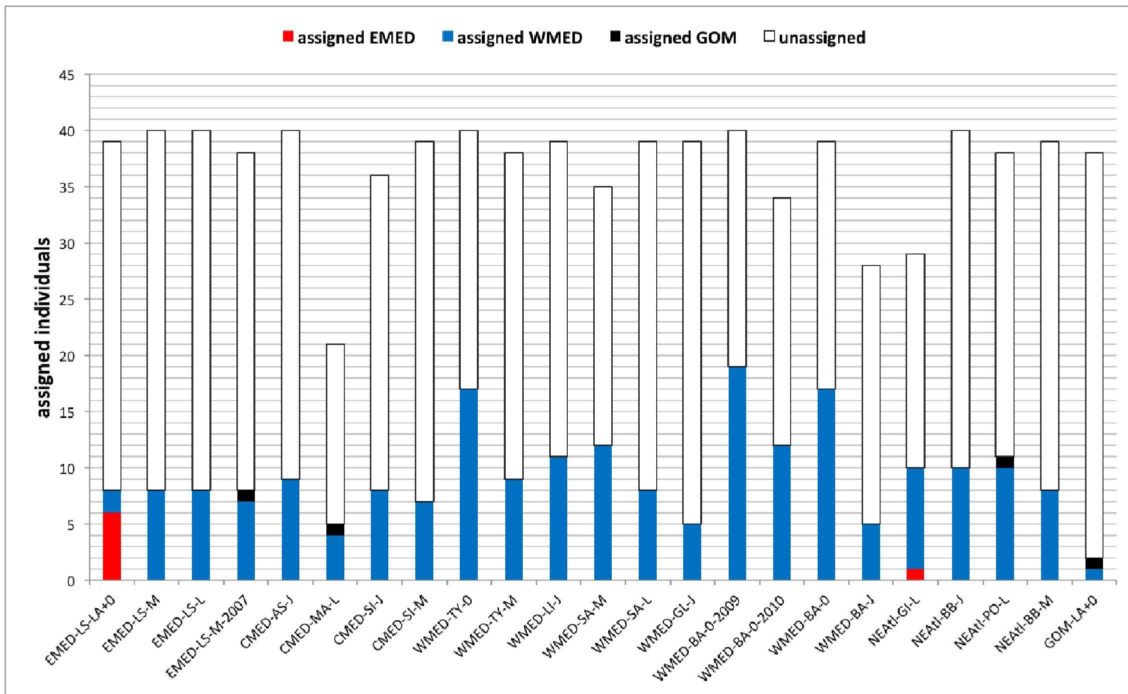
Similarly, the assignment analyses performed in Oncor assigned the majority of tunas from the breeding/feeding samples to the WMED area. Still, the individuals with > 90% probability of assignment to their 'best-estimate' reporting group were only 191 (21% of the total individuals in the dataset) (Figure 10).

Sample	N	EMED	WMED	GOM	EMED%	WMED%	GOM%
EMED-LS-LA+0	39	34	3	2	87,18	7,69	5,13
EMED-LS-M	40	6	30	4	15,00	75,00	10,00
EMED-LS-L	40	10	25	5	25,00	62,50	12,50
EMED-LS-M-2007	38	4	26	8*	10,53	68,42	21,05
CMED-AS-J	40	8	25	7	20,00	62,50	17,50
CMED-MA-L	21		13	8	0,00	61,90	38,10
CMED-SI-J	36	3	28	5	8,33	77,78	13,89
CMED-SI-M	39	6	23	10	15,38	58,97	25,64
WMED-TY-0	40	5	35		12,50	87,50	0,00
WMED-TY-M	38	7	27	4	18,42	71,05	10,53
WMED-LI-J	39	6	26	7	15,38	66,67	17,95
WMED-SA-M	35	7	26	2	20,00	74,29	5,71
WMED-SA-L	39	7	24	8*	17,95	61,54	20,51
WMED-GL-J	39	4*	26	9	10,26	66,67	23,08
WMED-BA-0-2009	40	5	31	4	12,50	77,50	10,00
WMED-BA-0-2010	34	5	26	3	14,71	76,47	8,82
WMED-BA-0	39	2	35	2	5,13	89,74	5,13
WMED-BA-J	28	7	18	3	25,00	64,29	10,71
NEAtl-GI-L	29	3	21	5	10,34	72,41	17,24
NEAtl-BB-J	40	5	29	6	12,50	72,50	15,00
NEAtl-PO-L	38	9*	21	8	23,68	55,26	21,05
NEAtl-BB-M	39	8	25	6	20,51	64,10	15,38
GOM-LA+0	38	1	3	34	2,63	7,89	89,47
Total	**848**	**152**	**546**	**150**	**17,92**	**64,39**	**17,69**

Table 8. Geneclass assignment test. Individual assignment analysis of the 23 population samples towards the geographical reference populations. In this analysis, the four reference samples from the WMED were pooled. * indicates that 1 individual has to be excluded (see text for details).



A)



B)

Figure 10. Percentage of tunas assigned by the assignment test performed by Oncor to the three spawning areas EMED, WMED, GOM. A) individuals assigned to their 'best-estimate' reporting group. B) individuals assigned with < 90% of probability of correct assignment are reported as unassigned. See text for further details.

Finally, a mixture analysis on our samples was performed in Oncor; Table 9 illustrates the main results. On the overall, the vast majority of juveniles/adults were assigned to the

WMED reference population (~ 69%), ~ 19% of individuals were assigned to EMED and ~ 12% to GOM. The very broad confidence intervals for these assignments (often including 0% and hence to be regarded as not significant according to Habicht *et al*. 2010) confirm that the loci have a very low resolutive power also for this application.

Population sample	EMED % (95% CI)	WMED % (95% CI)	GOM % (95% CI)
EMED-LS-M	0.0045 (0.111, 0.829)	0.9457 (0, 0.556)	0.0497 (0.001, 0.652)
EMED-LS-L	0.2943 (0.196, 0.831)	0.595 (0, 0.432)	0.1108 (0.023, 0.665)
EMED-LS-M-2007	0.0736 (0.085, 0.68)	0.6168 (0, 0.421)	0.3097 (0.203, 0.787)
CMED-AS-J	0.235 (0.183, 0.761)	0.7018 (0, 0.503)	0.0633 (0.069, 0.673)
CMED-MA-L	0.1669 (0.004, 0.794)	0.6479 (0, 0.596)	0.1852 (0.042, 0.866)
CMED-SI-J	0.1135 (0.091, 0.82)	0.6977 (0, 0.503)	0.1888 (0.088, 0.712)
CMED-SI-M	0.2131 (0.082, 0.661)	0.4811 (0, 0.381)	0.3057 (0.221, 0.874)
WMED-TY-M	0.3613 (0.217, 0.846)	0.637 (0, 0.503)	0.0017 (0, 0.653)
WMED-LI-J	0.0107 (0.062, 0.695)	0.8927 (0, 0.617)	0.0966 (0.039, 0.68)
WMED-SA-M	0.3168 (0.329, 0.988)	0.6832 (0.001, 0.634)	0 (0, 0.264)
WMED-SA-L	0.2827 (0.153, 0.718)	0.4613 (0, 0.384)	0.256 (0.126, 0.774)
WMED-GL-J	0.1267 (0.058, 0.769)	0.7395 (0, 0.474)	0.1338 (0.119, 0.798)
WMED-BA-J	0.3217 (0.219, 0.905)	0.6777 (0, 0.579)	0.0006 (0, 0.639)
NEAtl-GI-L	0.1301 (0.001, 0.663)	0.8386 (0.005, 0.75)	0.0313 (0, 0.72)
NEAtl-BB-J	0.0004 (0, 0.586)	0.719 (0, 0.524)	0.2807 (0.214, 0.882)
NEAtl-PO-L	0.0863 (0.043, 0.728)	0.7936 (0, 0.574)	0.1201 (0.031, 0.75)
NEAtl-BB-M	0.438 (0.342, 0.951)	0.5616 (0.001, 0.474)	0.0003 (0, 0.421)
EMED-LS-LA+0	0.998 (0.771, 1)	0.0019 (0, 0.066)	0.0001 (0, 0.218)
WMED-TY-0	0 (0, 0.494)	0.9998 (0.286, 0.975)	0.0002 (0, 0.496)
WMED-BA-0-2009	0.0004 (0.001, 0.571)	0.9996 (0.291, 0.965)	0 (0, 0.433)
WMED-BA-0-2010	0.099 (0.019, 0.734)	0.901 (0.082, 0.812)	0 (0, 0.52)
WMED-BA-0	0.0276 (0, 0.47)	0.9724 (0.232, 0.898)	0.0001 (0.001, 0.537)
GOM-LA+0	0 (0, 0.24)	0.0111 (0, 0.041)	0.9889 (0.752, 1)

Table 9. Percentage and 95% confidence intervals of juvenile/adult samples assigned by mixture analysis performed in Oncor to the three reporting areas (EMED, WMED, GOM). Reference population samples are in red.

Discussion

The Atlantic bluefin tuna (*Thunnus thynnus*) is one of the most depleted species of tunas; even though it has been continually exploited for thousands of years, only in these last decades the exploitation rate was reported to be far beyond the sustainable level, with a quite high risk of fisheries and stock collapse (Safina 2008).

The species used to be distributed widely throughout the north Atlantic Ocean, Mediterranean Sea and Black Sea, but its biogeographic range has contracted since the 1950s (MacKenzie and Mariani 2012). After large catches in the 1960s, the species collapsed from the coasts off Norway and Brazil (Fromentin and Powers 2005; MacKenzie and Myers 2007; ICCAT 2012) and, in the late 1980s, it disappeared also from the Black Sea (MacKenzie and Mariani 2012). According to these authors, this population should had peculiar phenotypic (genetic) adaptations in order to reproduce successfully in the specific hydrographic (estuarine) conditions of the Black Sea. The disappearance of past fisheries suggest that important changes in the spatial dynamics of bluefin tuna may have resulted from fishing but also from interactions between biological factors and environmental variations (ICCAT 2012). In general, the loss of this locally-adapted reproducing populations is alarming because it represents a dangerous decline in population richness and an increase in species vulnerability to human-driven perturbations (exploitation and environmental change) (MacKenzie and Mariani 2012).

The identification of substructuring and local populations is of paramount importance for the proper management of *T. thynnus*, but it is a very complex task. The need to elucidate appropriate management units for ABFT and the actual existence of separate stocks led to several genetic studies. The genetic structure of Atlantic bluefin tuna was early studied using molecular markers with low discriminating power, as allozymes (Pujolar *et al.* 2003) and mithocondrial sequences (Ely *et al.* 2002; Alvarado Bremer *et al.* 2005), unsuitable to detect population differentiation. More recently, markers more used to investigate population structure of this species were microsatellites (Carlsson *et al.* 2004, 2007), that present a high polymorphism degree but have the technical drawback because it isn’t always possible to compare data produced by different laboratories, due to the eventuality of inconsistencies in allele size calling caused by variety in sequencing machine, fluorescent dye and allele calling software (Vignal *et al.* 2002; Guichoux *et al.* 2011). In recent years, SNPs

have demonstrated more suitable markers in studies of population genetics and they are already applied to research in various fish species (Poulsen *et al.* 2011; Deagle *et al.* 2012; Hess *et al.* 2012; Limborg *et al.* 2012b). A single multiallelic microsatellites has more statistical power than one bi-allelic SNP, so it's necessary to use a large numbers of these markers to obtain a comparable power to detect divergences between populations. The aim of this work was to examine a wide set of 384 SNP markers newly developed for Atlantic bluefin tuna (see chapter 4 of this thesis) to choose a panel of high informative loci that provide enough statistical power to detect fine-scale population differences and that could be used to a better management of stocks.

Previous studies on population dynamics of Atlantic bluefin tuna were often conducted on a restricted number of individuals, and this may have been another limiting factor in the identification of a population structure (see Viñas *et al.* 2011 and references therein). To overcome this limits, in our study an intensive sampling effort was performed and we analyzed a total of 919 individuals (23 population samples), collected from Gulf of Mexico to entire Mediterranean basin, including individuals of all size/age classes, from larvae to adults, in order to have a more robust and complete dataset of the reproductive populations and ecological aggregates of the Atlantic bluefin tuna. To avoid the confounding effect of mixed populations (typically occurring at foraging grounds) adult spawners at advanced maturation stage were collected during the spawning season in 2011. Larvae and age-0 from the EMED, WMED and GOM were analyzed, because they are unlikely to have undertaken long distance movements and thus they are representative of their respective spawning populations. Juveniles, medium-sized as well as large adult specimens were also collected from various locations.

As regards the molecular markers, we used a set of species-specific high performance genetic loci developed by novel Next Generation Sequencing (NGS) technologies, that offered the opportunity to obtain several hundreds/thousands of Single Nucleotide Polymorphisms (SNPs) in expressed gene sequences in non-model species-with cheaper and more reliable high throughput genotyping technologies (Garvin *et al.* 2010; Ekblom and Galindo 2011; Nielsen *et al.* 2011). Using a combined approach of transcriptomic and genomic resources, we can obtained a conversion rate (number of working and polymorphic SNPs) of 70%, more higher than that achieved in previous studies for non validated SNPs

developed from EST-sequencing of non model organism, where the result was approximately 30% (Milano *et al.* 2011).

In our study the analysis of 848 individual with 287 SNP led to detect an extremely low and not significant level of genetic differentiation among all bluefin tuna population samples. The adult samples resulted genetically undifferentiated between them and from the reference samples of the spawning populations, using both entire set of loci than the restricted panel of SNP. The pairwise F_{st} values observed were very low, and ranged between -0.5% to 0.5%. These results are consistent with the values found in previous studied on *Thunnus thynnus*, with mitochondrial sequences (0.2% < F_{st} > 3%) and microsatellites, both neutral (0.5% < F_{st} > 2%) and EST-linked (-0.5% < F_{st} > 0.3%) (Carlsson *et al.* 2004, 2007; Boustany *et al.* 2008; Ferrara *et al.* 2010; Riccioni *et al.* 2010). The lack of genetic differentiation is expected in *T. thynnus*, a highly vagile species with large populations size (Ely *et al.* 2002; Palumbi 2003; Viñas *et al.* 2011,). Atlantic bluefin tuna, similar to other large pelagic fishes, have in fact the potential to migrate over extensive distances (Mather *et al*. 1995; Block *et al*. 2001) and sampling in a location may be composed of individuals originating from more than one spawning area, preventing the detection of genetic population structure. The occurrence of even very low levels of mixing among supposed isolated regions, that can be under noticed due to the limits in resolution power of tagging studies and other techniques, are enough to genetically homogenize the populations (Viñas *et al.* 2011).

Our results pointed out also the lack of significant genetic differences among temporal replicate samples of age-0 and larvae from the western Mediterranean. This is indicative of an absence of family effect (Allendorf-Phelps effect), validating the assumption that the samples were not the progeny of a few breeding adults rather than entire population (Allendorf and Phelps 1981; Waples 1998).

Loci influenced by selection could have provide a more precise indication of genetic structure than other loci. Such loci could have been particularly helpful for assessing relative differences in levels of gene flow, especially in high gene flow species and highly migratory behavior, as showed in several papers (Renaut *et al.* 2010; Freamo *et al.* 2011; Hess *et al.* 2011; Guichoux *et al.* 2012; Renaut *et al.* 2012). In fact, if overall gene flow is high, differences in levels of diversity or in allele frequencies among populations might be slight and error-prone (Waples 1998; Neigel 2002). In contrast, as selection can reduce effective

gene flow and increase divergence, the signature of asymmetric gene flow should be strong at loci under divergent selection (Guichoux *et al.* 2012). However, in our study, the different used approaches demonstrated an insufficient sensitivity to identify loci that were under weak selection, that can cause small differences in allele frequencies of candidate loci among populations, resulting in lower values of F_{ST} (Narum and Hess 2011). Our scenario could have been worse by presence of loci under balancing selection, that drive to homogenize differences between population more than neutral loci (Helyar *et al.* 2011; Narum and Hess 2011).

According to Kalinowski *et al.* (2011), when the goal of a genetic study is to summarize genetic differences among populations, traditional methods can be very effective for displaying population structure, even when populations have not had a hierarchical history of population fragmentation. Phylogenetic trees can contain much more information about population structure than results from more popular approaches such as those implemented in Structure (Kalinowski *et al.* 2011). In our study, we used the method Neighbor-net, successfully adopted by Willing *et al.* (2010) and Kraus *et al.* (2013) to display reticulate relationships among individuals and populations using SNP data. If populations are differentiated from each other, the Neighbor-net algorithm would display reticulate relationships more densely within less differentiated groups and less densely in more differentiated groups (Kraus *et al.* 2013). The networks obtained from our data were 'bush-like' (sensu Kraus *et al.* 2013) and confirmed once more the lacking of any population genetic structure.

Finally, because individual assignment tests based on genetic data have proven to be effective stock identification tools in many studies (Habicht *et al.* 2010; Beacham *et al.* 2011; Hess *et al.* 2011; Templin *et al.* 2011), this approach was also tested.

At present, two main classes of genetic markers are commonly used in genetic stock identification (GSI) applications: microsatellites and SNPs. High resolution of stock composition estimation is a function of accurate estimation of allele frequencies of the genetic markers used (''sampling error''), and of the degree of genetic differentiation among populations in the suite of genetic markers applied in estimation of stock composition (''genetic error''). Both factors influence the accuracy of estimates of stock composition when applied to mixed-stock fishery samples compositions (Beacham *et al.* 2011). Previous studies have shown that for populations with very low F_{ST} (< 0.1), assignment programs can

be unreliable (Latch *et al.* 2006). A value of F_{ST} = 0.05 is recommended by the same authors for 97% accuracy of assignment even if most GSI (Genetic Stock Identification) applications demand a lower level of accuracy (e.g. 90% correct assignment) to be useful in management decisions (Beacham *et al.* 2011).

In our study the low level of differentiation observed for the markers used (see results) resulted in insufficient power to apply such methods adequately (Vasemägi and Primmer 2005). Our panel of SNP loci lacks this accuracy; in fact, even if we consider the most resolutive panel (31 SNPs), the overall F_{ST} for all samples included in the baseline was 0.0094 and 0.0019 for the overall dataset. Furthermore, the sample sizes of our baseline collections (especially EMED and GOM) were relatively small (< 50 individuals), which may have decreased the accuracy of estimates of allele frequencies.

In general, if genetic differentiation among populations is limited, larger baseline population sample sizes may be required and more markers incorporated in the panel for stock identification to provide the maximum differentiation possible (Beacham *et al.* 2011).

The slight differences measured with the restricted panel of 31 SNPs among the reference populations between western and eastern spawning samples, partially confirm the genetic results obtained by Carlsson *et al.* (2004, 2007) and Boustany *et al.* (2008), and, combined with the finding of mature bluefin tuna in this latter area (Karakulak *et al*. 2004a) and the presence of a resident tuna component in the eastern Mediterranean throughout the year (Di Natale *et al.* 2005; Oray and Karakulak 1997), suggests the presence of a genetically independent stock of bluefin tuna in the eastern basin of the Mediterranean Sea. Differences in spawning times among Mediterranean regions could also support the existence of distinct populations within this sea, in fact spawning occur in June-July in the western Mediterranean (Susca *et al.* 2001; Corriero *et al.* 2003), and on May-June in the eastern basin (Karakulak *et al.* 2004b). This hypothesis could be confirmed by tagging data, as none of the individuals tagged in the western Atlantic and western Mediterranean were ever spotted in the Levantine, Aegean or Adriatic Seas, and the most eastern locations were found in the central Mediterranean (Block *et al.* 2005; De Metrio *et al.* 2005).

Currently, Atlantic bluefin tuna was managed by ICCAT as two different stocks, divided in the Atlantic ocean at 45th W meridian. This separation was supported by identification of two spawning area, one in the Gulf of Mexico e one in the Mediterranean sea (Mather *et al.* 1995; ICCAT 2002; Rooker *et al.* 2008). The Mediterranean basin is treated

as a single unit, but our preliminary results could be suggested a possible division in two different areas that may require a separate approach to avoid the impact of a type II error that could lead to the possible loss of the regional subpopulations. Future studies of bluefin tuna population genetics are essentials to go deeper in the structure of this important commercial species for a better understanding of strategies that will be adopted for its conservation. It should be necessary to incorporate more samples from eastern Mediterranean, that is the area less investigate of entire range of Atlantic bluefin tuna. If the existence of one or more genetically independent bluefin tuna stocks in the eastern basin of the Mediterranean Sea will be confirmed, this should be taken into consideration when making decisions concerning the management and conservation of the species.

References

Allendorf FW, Phelps SR (1981) Use of allelic frequencies to describe population-structure. *Canadian Journal of Fisheries and Aquatic Sciences* **38**,1507-1514.

Alvarado-Bremer JR, Viñas J, Mejuto J, Ely B, Pla C (2005) Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. *Molecular Phylogenetics and Evolution* **36**, 169-187.

Anderson EC, Waples RS, Kalinowski ST (2008). An improved method for estimating the accuracy of genetic stock identification. *Canadian Journal of Fisheries and Aquatic Sciences* **65**, 1475-1486.

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics* **9**, 323.

Beacham TD, McIntosh B, Wallace CG (2011) A comparison of polymorphism of genetic markers and population sample sizes required for mixed-stock analysis of sockeye salmon (*Oncorhynchus nerka*) in British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences* **68**(3), 550-562.

Beaumont MA, Nichols RA (1996) Evaluating Loci for Use in the Genetic Analysis of Population Structure. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **263**, 1619-1626.

Beaumont MA, Balding DJ (2004) Identifying adaptive genetic divergence among populations from genome scans. *Molecular Ecology* **13**, 969-980.

Benjamini Y, Yekutieli D (2001) The Control of the False Discovery Rate in Multiple Testing under Dependency. *The Annals of Statistics* **29**, 1165-1188.

Block BA, Dewar H, Blackwell SB, Williams TD, Prince ED, *et al.* (2001) Migratory movements, depth preferences, and thermal biology of Atlantic bluefin tuna. *Science* **293**, 1310-1314.

Block BA, Teo SL, Walli A, Boustany A, Stokesbury MJ, *et al.* (2005) Electronic tagging and population structure of Atlantic bluefin tuna. *Nature* **434**, 1121-1127.

Bourret V, Kent MP, Primmer CR, Vasemägi A, Karlsson S, *et al.* (2012) SNP-array reveals genome-wide patterns of geographical and potential adaptive divergence across the natural range of Atlantic salmon (*Salmo salar*). *Molecular ecology* DOI: 10.1111/mec.12003.

Boustany AM, Reeb CA, Block BA (2008) Mitochondrial DNA and electronic tracking reveal population structure of Atlantic bluefin tuna (*Thunnus thynnus*). *Marine Biology* **156**, 13-24.

Bryant D, Moulton V (2004) Neighbor-Net: An Agglomerative Method for the Construction of Phylogenetic Networks. *Molecular Biology and Evolution* **21**, 255-265.

Campana MG, Hunt HV, Jones H, White J (2011) *CorrSieve*: software for summarizing and evaluating Structure output. *Molecular Ecology Resources* **11**, 349-352.

Carlsson J, McDowell JR, Díaz-Jaimes P, Carlsson JEL, Boles SB, *et al.* (2004) Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean sea. *Molecular Ecology* **13**, 3345-3356.

Carlsson J, McDowell JR, Carlsson L, Graves JE (2007) Genetic identity of YOY bluefin tuna from the eastern and western Atlantic spawning areas. *Heredity* **98**, 23-28.

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

Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M (1999) New Methods Employing Multilocus Genotypes to Select or Exclude Populations as Origins of Individuals. *Genetics* **153**, 1989-2000.

Corriero A, Desantis S, Deflorio M, Acone F, Bridges CR, *et al.* (2003) Histological investigation on the ovarian cycle of the bluefin tuna in the western and central Mediterranean. *Journal of Fish Biology* **63**, 108-119.

Deagle BE, Jones FC, Chan YF, Absher DM, Kingsley DM, *et al.* (2012) Population genomics of parallel phenotypic evolution in stickleback across stream-lake ecological transitions. *Proceedings of the Royal Society B:Biological Sciences* **279,** 1277-1286.

De Metrio G, Arnold GP, De la Serna JM, Block BA, Megalofonou P, *et al*. (2005) Movements of bluefin tuna (*Thunnus thynnus* L.) tagged in the Mediterranean Sea with pop-up satellite tags. *ICCAT Col. Vol. Sci. Pap.* **58,** 1337-1340.

Di Natale A, Mangano A, Asaro B, Bacone B, Celona A, *et al.* (2005) Bluefin tuna (*Thunnus thynnus* L.) catch composition in the Tyrrhenian sea and in the straits of Sicily in 2002 and 2003. *ICCAT Coll Vol Sci Pap* **58**, 1296-1336.

Ekblom R, Galindo J (2011) Application of next generation sequencing in molecular ecology of non-model organism. *Heredity* **107**, 1-15.

Ely B, Stoner DS, Alvarado-Bremer JR, Dean JM, Addis P, *et al.* (2002) Analyses of Nuclear *ldhA* Gene and mtDNA Control Region Sequences of Atlantic Northern Bluefin Tuna Populations. *Marine Biotechnology* **4**, 583-588.

Esteras C, Gómez P, Monforte AJ, Blanca J, Vicente-Dólera N, *et al.* (2012) High-throughput SNP genotyping in Cucurbita pepo for map construction and quantitative trait loci mapping. *BMC Genomics* **13**, 80.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564-567.

Ferrara G, Zane L, Van Houdt J, Milano I, Cariani A, *et al.* (2010) Isolation, characterization and multiplex genotyping of 16 EST-SSR loci for the Atlantic bluefin tuna, *Thunnus thynnus*. *Molecular Ecology Resources* **10**, 576-579.

Fischer MC, Foll M, Excoffier L, Heckel G (2011) Enhanced AFLP genome scans detect local adaptation in high-altitude populations of a small rodent (*Microtus arvalis*). *Molecular Ecology* **20**, 1450-1462.

Foll M, Gaggiotti O (2008) A Genome-Scan Method to Identify Selected Loci Appropriate for Both Dominant and Codominant Markers: A Bayesian Perspective. *Genetics* **180**, 977-993.

Freamo H, O'Reilly P, Berg PR, Lien S, Boulding EG (2011) Outlier SNPs show more genetic structure between two Bay of Fundy metapopulations of Atlantic salmon than do neutral SNPs. *Molecular Ecology Resources* **11**, 254-267.

Fromentin JM, Powers JE (2005) Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. *Fish and Fisheries* **6**, 281-306.

Galuardi B, Royer F, Golet W, Logan J, Neilson J, *et al*. (2010) Complex migration routes of Atlantic bluefin tuna (*Thunnus thynnus*) question current population structure paradigm. *Canadian Journal of Fisheries and Aquatic Sciences* **67**(6), 966-976.

Garvin MR, Saitoh K, Gharrett AJ (2010) Application of single nucleotide polymorphisms to non-model species: a technical review. *Molecular Ecology Resources* **10**, 915-934.

Goldstein J, Heppell S, Cooper A, Brault S, Lutcavage M (2007) Reproductive status and body condition of Atlantic bluefin tuna in the Gulf of Maine, 2000-2002. *Marine Biology* **151**, 2063-2075.

Goudet J (1995) FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity* **86**, 485-486.

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, *et al.* (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* **11**, 591-611.

Guichoux E, Garnier-Géré P, Lagache L, Lang T, Boury C, *et al.* (2012) Outlier loci highlight the direction of introgression in oaks. *Molecular Ecology* **22**, 450-462.

Guo SW, Thompson EA (1992) Performing the Exact Test of Hardy-Weinberg Proportion for Multiple Alleles. *Biometrics* **48**, 361-372.

Habicht C, Seeb LW, Myers KW, Farley EV, Seeb JE (2010) Summer-Fall Distribution of Stocks of Immature Sockeye Salmon in the Bering Sea as Revealed by Single-Nucleotide Polymorphisms. *Transactions of the American Fisheries Society* **139**, 1171-1191.

Hauser L, Carvalho GR (2008) Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. *Fish and Fisheries* **9**, 333-362.

Hauser L, Seeb JE (2008) Advances in molecular technology and their impact on fisheries genetics *Fish and Fisheries* **9**, 473-486.

Helyar SJ, Hemmer-Hansen J, Bekkevold D, Taylor MI, Ogden R, *et al.* (2011) Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. *Molecular Ecology Resources* **11**, 123-136.

Helyar SJ, Limborg MT, Bekkevold D, Babbucci M, van Houdt J, *et al.* (2012) SNP Discovery Using Next Generation Transcriptomic Sequencing in Atlantic Herring (*Clupea harengus*). *PLoS ONE* **7**(8): e42089. doi:10.1371/journal.pone.0042089.

Hess JE, Matala AP, Narum SR (2011) Comparison of SNPs and microsatellites for fine-scale application of genetic stock identification of Chinook salmon in the Columbia River Basin. *Molecular Ecology Resources* **11** (Suppl. 1), 137-149.

Hess JE, Campbell NR, Close DA, Docker MF, Narum SR (2012) Population genomics of Pacific lamprey: adaptive variation in a highly dispersive species. *Molecular Ecology* doi: 10.1111/mec.12150.

Huson DH (1998) SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14** (1),68-73.

Huson DH, Bryant D (2006) Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology and Evo*lution **23**(2), 254-267.

ICCAT (2002) ICCAT workshop on bluefin tuna mixing. *Collective Volume of Scientific Papers ICCAT* **54,** 261-352.

ICCAT (2008) Report of the 2008 Atlantic Bluefin Stock Assessment Session (SCRS/2008/019). (International Commission for the Conservation of Atlantic Tunas, Madrid).

ICCAT (2010) Report of the Standing Committee on Research and Statistics (SCRS). Madrid: International Commission for the Conservation of Atlantic Tuna.

ICCAT (2012) Report for Biennial Period, 2010-11, Part II (2011), Vol. 2. Madrid, Spain: SCRS, 2012.

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23** (14), 1801-1806.

Jeffreys H (1961) *Theory of probability* Clarendon Press, Oxford.

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**, 1403-1405.

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* **11**, 94.

Juan-Jordá MJ, Mosqueirad I, Cooperf AB, Freirea J, Dulvyc NK (2011) Global population trajectories of tunas and their relatives. *PNAS* **108**, 20650-20655.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* **16**, 1099-1006.

Kalinowski ST, Novak BJ, Drinan DP, DeM Jennings R, Vu NV (2011) Diagnostic single nucleotide polymorphisms for identifying westslope cutthroat trout (*Oncorhynchus clarki lewisi*), Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) and rainbow trout (*Oncorhynchus mykiss*). *Molecular Ecology Resources* **11**, 389-393.

Karakulak S, Oray I, Corriero A, Aprea A, Spedicato D, *et al.* (2004a) First information the reproductive biology of the bluefin tuna (*Thunnus thynnus*) in the Eastern Mediterranean. *ICCAT Col. Vol. Sci. Pap.* **56**, 1158-1162.

Karakulak S, Oray I, Corriero A, Deflorio M, Santamaria N, *et al.* (2004b) Evidence of a spawning area for the bluefin tuna (*Thunnus thynnus* L.) in the Eastern Mediterranean. *Journal of Applied Ichthyology* **20**, 318-320.

Kell LT, Fromentin JM, Bonhommeau S (2012) An evaluation of the implications of population structure on the current bluefin tuna advice framework. *ICCAT Col. Vol. Sci. Pap* **68**(1), 370-378.

Kijas JW, Townley D, Dalrymple BP, Heaton MP, Maddox JF, *et al.* (2009) A genome wide survey of SNP variation reveals the genetic structure of sheep breeds. *PLoS ONE* **4**(3), e4668.

Kraus RHS, Van Hooft P, Megens HJ, Tsvey A, Fokin SY, *et al.* (2013) Global lack of flyway structure in a cosmopolitan bird revealed by a genome wide survey of single nucleotide polymorphisms. *Molecular Ecology* **22**, 41-55.

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes Jr. OE (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conservation Genetics* **7**, 295-302.

Limborg MT, Blankenship SM, Young SF, Utter FM, Seeb LW, *et al.* (2012a) Signatures of natural selection among lineages and habitats in *Oncorhynchus mykiss*. *Ecology and Evolution* **2**, 1-18.

Limborg MT, Helyar SJ, De Bruyn M, Taylor MI, Nielsen EE, *et al.* (2012b) Environmental selection on transcriptome-derived SNPs in a high gene flow marine fish, the Atlantic herring (*Clupea harengus*). *Molecular Ecology* **21**, 3686-3703.

Liu K, Muse SV (2005) PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* **21**, 2128-2129.

Louis EJ, Dempster ER (1987) An Exact Test for Hardy-Weinberg and Multiple Alleles. *Biometrics* **43**, 805-811.

Lutcavage ME, Brill WR, Skomal GB, Chase BC, Howey PW (1999) Results of pop-up satellite tagging of spawning size class fish in the Gulf of Maine: do North Atlantic bluefin tuna spawn in the mid-Atlantic? *Canadian Journal of Fisheries and Aquatic Sciences* **56**(2), 173-177.

MacKenzie BR, Myers RA (2007) The development of the northern European fishery for north Atlantic bluefin tuna *Thunnus thynnus* during 1900–1950. *Fisheries Research* **87**, 229-239.

MacKenzie BR, Mosegaard H, Rosenberg AA (2009) Impending Collapse of Bluefin Tuna in the Northeast Atlantic and Mediterranean. *Conservation Letters* **2** (1), 25-34.

MacKenzie BR, Mariani P (2012) Spawning of Bluefin Tuna in the Black Sea: Historical Evidence, Environmental Constraints and Population Plasticity. *PLoS ONE* **7**(7), e39998.

Malhi RS, Trask JS, Shattuck M, Johnson J, Chakraborty D, *et al.* (2011) Genotyping single nucleotide polymorphisms (SNPs) across species in Old World Monkeys. *American Journal of Primatology* **73**, 1031-1040.

Manel S, Gaggiotti OE, Waples RS (2005) Assignment methods: matching biological questions with appropriate techniques. *Trends in Ecology & Evolution* **20**, 136-142.

Mather FJ, Mason JM, Jones AC (1995) Historical document: life history and fisheries of Atlantic bluefin tuna. U.S. Dep. Comm., NOAA Technical Memorandum, NMFS-SEFSC 370.

McKay SD, Schnabel RD, Murdoch BM, Matukumalli LK, Aerts J, *et al.* (2008) An assessment of population structure in eight breeds of cattle using a whole genome SNP panel. *BMC Genetics* **9**, 37.

Milano I, Babbucci M, Panitz F, Ogden R, Nielsen RO, *et al.* (2011) Novel Tools for Conservation Genomics: Comparing Two High-Throughput Approaches for SNP Discovery in the Transcriptome of the European Hake. *PLoS ONE* **6**, e28008.

Millar RB (1987) Maximum Likelihood Estimation of Mixed Stock Fishery Composition. *Canadian Journal of Fisheries and Aquatic Sciences* **44**, 583-590.

Morin PA, Luikart G, Wayne RK, the Snp wg (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution* **19**, 208-216.

Morin PA, McCarthy M (2007) Highly accurate SNP genotyping from historical and low-quality samples. *Molecular Ecology Notes* **7**, 937-946.

Narum SR (2006) Beyond Bonferroni: Less conservative analyses for conservation genetics. *Conservation Genetics* **7**, 783-787.

Narum SR, Hess JE (2011) Comparison of FST outlier tests for SNP loci under selection. *Molecular Ecology Resources* **11**, 184-194.

National Research Council (1994) An assessment of Atlantic bluefin tuna. *National Academies Press*, Washington, 148 pp.

Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution* **19**, 153-170.

Neigel JE (2002) Is F_{ST} obsolete? *Conservation Genetics* **3**, 167-173.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009) Population genomics of marine fishes: identifying adaptive variation in space and time. *Molecular Ecology* **18**, 3128-3150.

Nielsen R, Paul SJ, Albrechtsen A, Song YS (2011) Genotype and SNP calling from next-generation sequencing data. *Nature Review Genetics* **12**, 443-451.

Odgen R (2011) Unlocking the potential of genomic technologies for wildlife forensics. *Molecular Ecology resources* **11** (Suppl.1), 109-116.

Oray IK, Karakulak FS (2005) Further evidence of spawning of bluefin tuna (*Thunnus thynnus* L., 1758) and the tuna species (*Auxis rochei* Ris., 1810, *Euthynnus alletteratus* Raf., 1810) in the Eastern Mediterranean Sea: preliminary results of TUNALEV larval survey in 2004. *Journal of Applied Ichthyology* **21**, 236-240.

Ottolenghi F, Silvestri C, Giordano P, Lovatelli A, New MB (2004) Capture-Based Aquaculture. Rome: Food and Agriculture Organization.

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology* **13**, 55-65.

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. *Ecological Applications* **13**(1), S146-S158.

Parks DH, Porter M, Churcher S, Wang S, Blouin C, *et al.* (2009) GenGIS: A geospatial information system for genomic data. *Genome Research* **19**, 1896-1904.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **28**, 2537-2539.

Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, *et al.* (2004) GeneClass2: A Software for Genetic Assignment and First-Generation Migrant Detection. *Journal of Heredity* **95**, 536-539.

Poulsen N, Hemmer-Hansen J, Loeschcke V, Carvalho G, Nielsen E (2011) Microgeographical population structure and adaptation in Atlantic cod *Gadus morhua*: spatio-temporal insights from geneassociated DNA markers. *Marine Ecology Progress Series* **436**, 231-243.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of Population Structure Using Multilocus Genotype Data. *Genetics* **155**, 945-959.

Pujolar JM, Roldán MI, Pla C (2003) Genetic analysis of tuna populations, *Thunnus thynnus thynnus* and *T. alalunga*. *Marine Biology* **143**, 613-621.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *PNAS* **94**, 9197-9201.

Reeb CA (2010) Genetic discontinuity of big fish in a small sea. *PNAS* **107**, 2377-2378.

Renaut S, Nolte AW, Bernatchez L (2010) Mining transcriptome sequences towards identifying adaptive single nucleotide polymorphisms in lake whitefish species pairs (*Coregonus* spp. Salmonidae). *Molecular Ecology* **19** (Suppl. 1), 115-131.

Renaut S, Maillet N, Normandeau E, Sauvage C, Derome N, *et al.* (2012) Genome-wide patterns of divergence during speciation: the lake whitefish case study. *Philosophical Transactions of the Royal Society B* **367**, 354-363.

Riccioni G, Landi M, Ferrara G, Milano I, Cariani A, *et al.* (2010) Spatio-temporal population structuring and genetic diversity retention in depleted Atlantic bluefin tuna of the Mediterranean sea. *PNAS* **107**, 2102-2107.

Rooker JR, Alvarado Bremer JR, Block BA, Dewarc H, de Metrio G, *et al.* (2007) Life history and stock structure of Atlantic Bluefin Tuna (*Thunnus thynnus*). *Reviews in Fisheries Science* **15**, 265-310.

Rooker JR, Secor DH, Metrio G, Schloesser R, Block BA, *et al.* (2008) Natal Homing and Connectivity in Atlantic Bluefin Tuna Populations. *Science* **322**, 742.

Rosenberg NA (2004) Distruct: a program for the graphical display of population structure. *Molecular Ecology Notes* **4**, 137-138.

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. *Genetics* **140**, 1413-1419.

Rousset F (2008) Genepop’007: a complete reimplementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources* **8**, 103-106.

Safina C (2001) 'Tuna Conservation'. In *Tuna. Physiology, Ecology, and Evolution*, ed. Block BA and Stevens ED, 414-57. New York: Academic Press.

Safina C, Klinger DH (2008) Collapse of Bluefin Tuna in the Western Atlantic. *Conservation Biology* **22**, 243-246.

Seeb LW, Habicht C, Templin WD, Tarbox KE, Davis RZ, *et al.* (2000) Genetic diversity of sockeye salmon of Cook Inlet, Alaska, and its application to management of populations affected by the Exxon Valdez oil spill. *Transactions of the American Fisheries Society* **129**,1223-1249.

Seeb LW, Templin WD, Sato S, Abe S, Warheit K, *et al.* (2011) Single nucleotide polymorphisms across a species' range: implications for conservation studies of Pacific salmon. *Molecular Ecology Resources* **11**, 195-217.

Storer CG, Pascal CE, Roberts SB, Templin WD, Seeb LW, *et al.* (2012) Rank and Order: Evaluating the Performance of SNPs for Individual Assignment in a Non-Model Organism. *PLoS ONE* **7**: e49018.

Susca V, Corriero A, Bridges CR, De Metrio G (2001) Study of the sexual maturity of female bluefin tuna: Purification and partial characterization of vitellogenin and its use in an enzyme-linked immunosorbent assay. *Journal of Fish* Biology **58**, 815-831.

Templin WD, Seeb JE, Jasper JR, Barclay AW, Seeb LW (2011) Genetic differentiation of Alaska Chinook salmon: the missing link for migratory studies. *Molecular Ecology Resources* **11** (Suppl 1), 226-246.

Teo SLH, Boustany A, Dewar H, Stokesbury M, Weng KC, *et al.* (2007) Annual migrations, diving behavior and thermal biology of Atlantic bluefin tuna, *Thunnus thynnus*, to breeding grounds in the Gulf of Mexico. *Marine Biology* **151**, 1-18.

The International HapMap Consortium (2003) The International HapMap Project. *Nature* **426**, 789-796.

Vasemägi A, Primmer CR (2005) Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies. *Molecular Ecology* **14**, 3623-3642.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* **34**, 275-305.

Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü, *et al.* (2011) Facts and uncertainties about the genetic population structure of Atlantic bluefin tuna (*Thunnus thynnus*) in the Mediterranean. Implications for fishery management. *Reviews in Fish Biology and Fisheries* **21**, 527-541.

Walli A, Teo SLH, Boustany A, Farwell CJ, Williams T, *et al.* (2009) Seasonal movements, aggregations and diving behavior of Atlantic bluefin tuna (*Thunnus thynnus*) revealed with archival tags. *PLoS ONE* **4**, 1-18.

Wang T, Chen G, Zan Q, Wang C, Su YJ (2012) AFLP Genome Scan to Detect Genetic Structure and Candidate Loci under Selection for Local Adaptation of the Invasive Weed *Mikania micrantha*. *PLoS ONE* **7**, e41310.

Waples RS (1998) Separating the wheat from the chaff: Patterns of genetic differentiation in high gene flow species. *Journal of Heredity* **89**, 438-450.

Weir BS, Cockerham CC (1984) Estimating F-Statistics for the Analysis of Population Structure. *Evolution* **38**, 1358-1370.

Willing EM, Bentzen P, Van Oosterhout C, Hoffmann M, Cable J, *et al.* (2010) Genome-wide single nucleotide polymorphisms reveal population history and adaptive divergence in wild guppies. *Molecular Ecology* **19**, 968-984.

Wilson SG, Lawson GL, Stokesbury MJW, Spares A, Boustany AM, *et al*. (2011) Movements of Atlantic bluefin tuna from the gulf of St. Lawrence to their spawning grounds. *Collect. Vol. Sci. Pap. ICCAT* **66**(3), 1247-1256.

Zakas C, Schult N, Mchugh D, Jones KL, Wares JP (2012) Transcriptome Analysis and SNP Development Can Resolve Population Differentiation of *Streblospio benedicti*, a Developmentally Dimorphic Marine Annelid. *PLoS ONE* **7**, e31613.

CHAPTER 6

CONCLUSIONS

My PhD project, carried out during these three years, allowed to gain knowledge about genetic structure of Atlantic bluefin tuna (*Thunnus thynnus*). Indeed, a wide and thorough sampling has been realized for this work on this valuable commercial species. This sampling had several strengths: the large number of individuals collected, close to thousand samples of tuna, much higher than that reported in previous studies carried out until now, the wide coverage of the range of *T. thynnus*, since the sampling is extended from Gulf of Mexico to the most part of Mediterranean Sea, and the presence of both feeding/breeding aggregate and reference population samples.

Moreover, the work presented in my PhD thesis has showed the great potential of high-throughput sequencing technologies to facilitate the access to genomic resources of non-model species as Atlantic bluefin tuna. New genomic technologies were applied combining two different approach, both transcriptomic and genomic; so, we can develop and validate a large panel of 384 Single Nucleotide Polymorphisms (SNPs), nowadays the most widely used markers in population genetics and conservation studies, thanks to their high statistical power and to possibility to overcame restrictions related to the previous marker.

The absence of significant differences between adult samples and the weak signal of structure between reference populations emerged by our studies suggest the presence of a panmictic population of adults bluefin tuna and genetically independent reproductive populations in the Mediterranean Sea. Also, we didn't detect outlier loci and this occurrence could be contribute to lack of genetic differentiation founded. In fact, loci under divergent selection, with F_{ST} values higher than loci under neutrality, can provide more information about population structure and local adaptation, and can be applied in study of traceability, especially in high migratory fish with a low gene flow and highly migratory behavior, as *T. thynnus*.

Novel genetic strategies and bioinformatic tools are in continuous development, allowing an ever greater decrease of costs for DNA sequencing and genotyping and a growth

of efficiency and accuracy of the results. Currently, studies on Atlantic bluefin tuna are in progress, within the project ICCAT-GBYP Phase 3, that have the aim to go more in depth in the knowledge about Atlantic bluefin tuna population structure and mixing. To do this, the project aimed to extend the sampling design, including new spawning and feeding/breeding population samples from new areas of Mediterranean Sea and new temporal replicates for a better assessment of interannual variation, and increasing the size of samples analyzed, in order to have a statistically more robust representation of genetic variation.

To achieve the best results, new sequencing technologies were employed, as the Reduced Representation Sequencing and Genotyping (RRSG), that permit to develop thousand SNPs and to select a large panel of outlier and high-divergent loci, useful to the identification of evolutionary units and to the correct assignment of all individuals to the geographic basin of origin.

Future results of these works on *Thunnus thynnus* could be led to an effective improvement to fisheries control and traceability of this species, because the resolution of population structure is essential to the identification and preservation of local populations and adaptive diversity. These kind of approach can be also applied to other species heavily damaged by intensive exploitation, demonstrating the applicability of these new molecular and genetic technologies to real-world problems, and providing a considerable contribution to management, persistence and stability of fish species.

REFERENCES

Abascal FJ, Megina C, Medina A (2003) Testicular development in migrant and spawning bluefin tuna (*Thunnus thynnus* (L.) from the eastern Atlantic and Mediterranean. *Fishery Bulletin* **102**, 407-417.

Alvarado-Bremer JR, Viñas J, Mejuto J, Ely B, Pla C (2005) Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. *Molecular Phylogenetics and Evolution* **36**, 169-187.

Anderson EC, Garza JC (2006) The power of Single-Nucleotide Polymorphisms for large-scale parentage inference. *Genetics* **172**, 2567-2582.

Avise JC, Helfman GS, Saunders NC, Hales LS (1986) Mitochondrial DNA differentiation in North Atlantic eels: population genetic consequences of an unusual life history pattern. *PNAS* **83**, 4350-4354.

Avise J (1994) Molecular Markers, Natural History, and Evolution. New York, NY: Chapman and Hall.

Baglin REJ (1982) Reproductive biology of western Atlantic bluefin tuna. *Fishery Bulletin* **80**, 121-134.

Barbazuk WB, Emrich SJ, Chen HD, Li L, Schnable PS (2007) SNP discovery via 454 transcriptome sequencing. *The Plant Journal* **51**, 910-918.

Bard FX (2001) Extension of geographical and vertical habitat of albacore (*Thunnus alalunga*) in the north atlantic. Possible consequences on true rate of exploitation of this stock. *SCRS/00/119, ICCAT Col Vol Sci. Pap* **52** (4), 1447-1456.

Bayliff WH, Ishizuka Y, Deriso RB (1991) Growth, movement, and attrition of northern bluefin tuna (*Thunnus thynnus*) in the Pacific Ocean as determined by tagging. *Inter-Amer. Trop. Tuna Comm Bull.* **20**, 1-94.

Bayliff WH (1993) Growth and age composition of northern bluefin tuna, *Thunnus thynnus*, caught in the eastern Pacific Ocean, as estimated from length-frequency data, with comments on trans-Pacific migration. *Inter-American Tropical Tuna Commission. Special Report* **20**, 523-540.

Beaumont MA (2005) Adaptation and speciation: what can F_{ST} tell us? *Trends in Ecology & Evolution* **20**, 435-440.

Beddington JR, Agnew DJ, Clark CW (2007) Current problems in the management of marine fisheries. *Science* **316**, 1713-1716.

Block BA, Dewar H, Blackwell SB, Williams TD, Prince ED, *et al.* (2001) Migratory movements, depth preferences, and thermal biology of Atlantic bluefin tuna. *Science* **293**, 1310-1314.

Block BA, Teo SL, Walli A, Boustany A, Stokesbury MJ, *et al.* (2005) Electronic tagging and population structure of Atlantic bluefin tuna. *Nature* **434**, 1121-1127.

Bonin A (2008) Population genomics: a new generation of genome scans to bridge the gap with functional genomics. *Molecular Ecology* **17**, 3583-3584.

Bouck A, Vision T (2007) The molecular ecologist's guide to expressed sequence tags. *Molecular Ecology* **16**, 907-924.

Boustany AM, Reeb CA, Block BA (2008) Mitochondrial DNA and electronic tracking reveal population structure of Atlantic bluefin tuna (*Thunnus thynnus*). *Marine Biology* **156**, 13-24.

Bouza C, Hermida M, Millan A, Vilas R, Vera M, *et al.* (2008) Characterization of EST-derived microsatellites for gene mapping and evolutionary genomics in turbot. *Animal Genetics* **39**, 666-670.

Brothers EB, Prince ED, Lee DW (1983) Age and growth of young-of-the-year bluefin tuna, *Thunnus thynnus*, from otolith microstructure. **In**: *Proceedings of the International Workshop on Age Determination of Oceanic Pelagic Fishes: Tunas, Billfishes, and Sharks*, 49-59 (Prince EE and Pulos LM, Eds.). U.S. Dep. Comm., NOAA Tech. Rep. NMFS 8.

Bruneinmeister S (1980) A summary and discussion of technical information pertaining to the geographical discreteness of Atlantic bluefin tuna resources. *SCRS/79/95 ICCAT Col Vol Sci Pap* **IX**(2), 506-527.

Butler MJA, Caddy JF, Dickson CA, Hunt JJ, Burnett CD (1977) Apparent age and growth, based on otolith analysis of giant bluefin tuna (*Thunnus thynnus thynnus)* in the 1975-1976 Canadian catch. *ICCAT Col. Vol. Sci. Pap.* **5**, 318-330.

Caddy JF, Dickson CA, Butler JA (1976) Age and growth of giant bluefin tuna (*Thunnus thynnus thynnus*) taken in Canadian waters in 1975. *Journal of the Fisheries Research Board of Canada* MS Rep. No. 1395.

Campbell D, Duchesne P, Bernatchez L (2003) AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology* **12**, 1979-1991.

Carey FG, Robinson BH (1981) Daily patterns in the activities of swordfish, *Xiphias gladius*, observed by acoustic telemetry. *Fishery Bulletin* **79**, 277-292.

Carlsson J, McDowell JR, Díaz-Jaimes P, Carlsson JEL, Boles SB, *et al.* (2004) Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean sea. *Molecular Ecology* **13**, 3345-3356.

Carlsson J, McDowell JR, Carlsson L, Graves JE (2007) Genetic identity of YOY bluefin tuna from the eastern and western Atlantic spawning areas. *Heredity* **98**, 23-28.

Chase BC (2002) Differences in the diet of Atlantic bluefin tuna (*Thunnus thynnus*) at five seasonal feeding grounds on the New England continental shelf. *Fishery Bulletin* **100**, 168-180.

Chini V, Cattaneo AG, Rossi F, Bernardini G, Terova G, *et al.* (2008) Genes expressed in Blue Fin Tuna (*Thunnus thynnus*) liver and gonads. *Gene* **410**, 207-213.

Chow S, Clarke ME, Walsh PJ (1993) PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): A simple method for species and stock identification. *Fishery Bulletin* **91**, 619-627.

Chu SL (1999) Reproductive biology of bigeye tuna *Thunnus obesus* in the western Pacific Ocean. MS thesis, Institute of Oceanography, National Taiwan University.

Commission E (2007) Establishing a multi-annual recovery plan for bluefin tuna in the Eastern Atlantic and Mediterranean (a proposal to the Commision for a Council Regulation). COM (2007) 169 final.

Compeán-Jiménez G, Bard FX (1983) Growth increments on dorsal spines of eastern Atlantic bluefin tuna (*Thunnus thynnus* (L.)) and their possible relation to migration patterns. **In**: *Proceedings of the International Workshop on Age Determination of Oceanic Pelagic Fishes: Tunas, Billfishes, and Sharks*, pp. 77-86 (E. E. Prince and L. M. Pulos, Eds.). U.S. Dep. Comm., NOAA Tech.Rep. NMFS 8.

Conover DO, Clarke LM, Munch SB, Wagner GN (2006) Spatial and temporal scales of adaptive divergence in marine fishes and the implications for conservation. *Journal of Fish Biology* **69**, 21-47.

Corriero A, Desantis S, Deflorio M, Acone F, Bridges CR, *et al.* (2003) Histological investigation on the ovarian cycle of the bluefin tuna in the western and central Mediterranean. *Journal of Fish Biology* **63**, 108-119.

Corriero A, Karakulak S, Santamaria N, Deflorio M, Spedicato D, *et al.* (2005) Size and age at sexual maturity of female bluefin tuna (*Thunnus thynnus* L. 1758) from the Mediterranean Sea. *Journal of Applied Ichthyology* **21**, 483-486.

Corriero A, Medina A, Mylonas CC, Abascal FJ, Deflorio M, *et al*. (2007) Histological study of the effects of treatment with gonadotropin-releasing hormone agonist (GnRHa) on the reproductive maturation of captive-reared Atlantic bluefin tuna (*Thunnus thynnus* L.). *Aquaculture* **272**, 675-686.

Corriero A, Medina A, Mylonas CC, Bridges CR, Santamaria N, *et al.* (2009) Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) treated with gonadotroping releasing hormone agonist (GnRHa). *Animal Reproduction* Science **116**, 346-357.

Crane J (1936) Notes on the biology and ecology of giant tuna *Thunnus thynnus*, L., observed at Portland, Maine. *Zoologica* **21**, 207-212.

Davis TLO, Lyne V, Jenkins GP (1991) Advection, dispersion, and mortality of a patch of southern bluefin tuna larvae *Thunnus maccoyii* in the East Indian Ocean. *Marine Ecology Progress Series* **73**, 33-45.

Deagle BE, Jones FC, Chan YF*,* Absher DM, Kingsley DM, *et al.* (2012) Population genomics of parallel phenotypic evolution in stickleback across stream-lake ecological transitions. *Proceedings of the Royal Society B:Biological Sciences* **279**, 1277-1286.

De Jager B, Van D, de V. Nepgen CS, Van Wyk RJ (1963) A preliminary report on tuna in west coast waters of South Africa. Part II. *S. Afr. Shipp. News Fish. Ind. Rev.* **18**, 61-69.

De Metrio G, Arnold GP, Block BA, de la Serna JM, Deflorio M, *et al.* (2002) Behaviour of post-spawning Atlantic bluefin tuna tagged with pop-up satellite tags in the Mediterranean and eastern Atlantic. *ICCAT Col. Vol. Sci. Pap.* **54**, 415-424.

De Metrio G, Arnold GP, De la Serna JM, Block BA, Megalofonou P, *et al.* (2005) Movements of bluefin tuna (*Thunnus thynnus* L.) tagged in the Mediterranean Sea with pop-up satellite tags. *ICCAT Col. Vol. Sci. Pap.* **58**, 1337-1340.

De Metrio G, Bridges CR, Mylonas CC, Caggiano M, Deflorio M, *et al.* (2010) Spawning induction and large-scale collection of fertilized eggs in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) and the first larval rearing efforts. *Journal of Applied Ichthyology* **26**, 596-599.

Desse J, Desse-Berset N (1994) Stratégies de pêche au 8ème millénaire : les poissons de Cap Andreas Kastros (Chypre), Pages 335-360 in Le Brun, A., ed. Fouilles récentes à Khirokitia. Paris, Editions Recherche sur Civilisations.

Dragovich A (1970) The food of bluefin tuna (*Thunnus thynnus*) in the western North Atlantic Ocean. *Transactions of the American Fisheries Society* **99**, 723-731.

Edmunds PH, Sammons JI (1971) Genic polymorphism of tetrazolium oxidase in bluefin tuna, *Thunnus thynnus*, from western north Atlantic. *Journal of the Fisheries Research Board of Canada* **28**, 1053-1055.

Edmunds PH, Sammons JI (1973) Similarity of genic polymorphism of tetrazolium oxidase in bluefin tuna (*Thunnus thynnus*) from Atlantic coast of France and western north Atlantic. *Journal of the Fisheries Research Board of Canada* **30**, 1031-1032.

Eggleston DB, Bochenek EA (1990) Stomach contents and parasite infestation of school bluefin tuna, *Thunnus thynnus*, collected from the middle Atlantic bight, Virginia. *Fishery Bulletin* **88**, 389-395.

Ely B, Stoner DS, Alvarado-Bremer JR, Dean JM, Addis P, *et al.* (2002) Analyses of Nuclear *ldhA* Gene and mtDNA Control Region Sequences of Atlantic Northern Bluefin Tuna Populations. *Marine Biotechnology* **4**, 583-588.

Eveson JP, Laslett GM, Polacheck T (2004) An integrated model for growth incorporating tag-recapture, length-frequency, and direct aging data. *Canadian Journal of Fisheries and Aquatic Sciences* **61**, 292-306.

FAO (2012) The state of world fisheries and aquaculture 2012.

Farley JH, Davis TLO (1998) Reproductive dynamics of southern bluefin tuna, *Thunnus maccoyii*. *Fishery Bulletin* **96**, 223-236.

Farrugio H (1980) Age et croissance du thon rouge (*Thunnus thynnus*) dans la pecherie Francaise de surface en Mediterranee. *Cybium* **9**, 45-59.

Ferrara G, Zane L, Van Houdt J, Milano I, Cariani A, *et al.* (2010) Isolation, characterization and multiplex genotyping of 16 EST-SSR loci for the Atlantic bluefin tuna, *Thunnus thynnus*. *Molecular Ecology Resources* **10**, 576-579.

Foreman TJ (1986) An assessment of age determination techniques of northern bluefin tuna *Thunnus thynnus* L. from the eastern Pacific Ocean. MS Thesis, San Diego State University, San Diego, CA.

Frankham R (2005) Stress and adaptation in conservation genetics. *Journal of Evolutionary Biology* **18**, 750-755.

Freamo H, O'Reilly P, Berg PR, Lien S, Boulding EG (2011) Outlier SNPs show more genetic structure between two Bay of Fundy metapopulations of Atlantic salmon than do neutral SNPs. *Molecular Ecology Resources* **11**, 254-267.

Fromentin JM (2001) Descriptive analysis of the ICCAT bluefin tuna tagging database. *ICCAT Col. Vol. Sci. Pap.* **54**, 353-362.

Fromentin JM, Fonteneau A (2001) Fishing effects and life history traits: a case study comparing tropical versus temperate tunas. *Fisheries Research* **53**, 133-150.

Fromentin JM, Powers JE (2005) Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. *Fish and Fisheries* **6**, 281-306.

Fromentin JM, Ravier C (2005) The East Atlantic and Mediterranean Bluefin Tuna Stock: Looking for Sustainability in a Context of Large Uncertainties and Strong Political Pressures. *Bulletin of Marine Science* **76**(2), 353-361.

Fromentin JM (2009) Lessons from the past: investigating historical data from bluefin tuna fisheries. *Fish and Fisheries* **10**, 197-216.

Furnestin J, Dardignac J (1962) Le thon rouge de Maroc Atlantic (*Thunnus thynnus*). *Revue des Travaux de l'Institut des Peches Maritimes* **26**, 381-397.

Garcia A, Alemany F, De la Serna JM, Oray I, Karakulak S, *et al.* (2005) Preliminary results of the 2004 bluefin tuna larval surveys off different Mediterranean sites (Balearic Archipelago, Levantine Sea, and the Sicilian Channel). *ICCAT Col. Vol. Sci. Pap.* **58**, 1420-1428.

Glover K, Hansen M, Lien S, Als TD, Høyheim B, *et al.* (2010) A comparison of SNP and STR loci for delineating population structure and performing individual genetic assignment. *BMC Genetics* **11**, 2.

Gold JR, Richardson LR, Furman C, King TL (1993) Mitochondrial DNA differentiation and population structure in red drum (*Sciaenops ocellatus*) from the Gulf of Mexico and Atlantic Ocean. *Marine Biology* **116**, 175-185.

Gomez-Uchida D, Seeb JE, Smith MJ, Habicht C, Quinn TP, *et al.* (2011) Single nucleotide polymorphisms unravel hierarchical divergence and signatures of selection among Alaskan sockeye salmon (*Oncorhynchus nerka*) populations. *BMC Evolutionary Biology* **11**, 48.

Grafton RQ, Hannesson R, Shallard B, Sykes D, Terry J (2006) The economics of allocation in Tuna Regional Fisheries Management Organizations (RFMOs). Economics and Environment Network Working Paper EEN0612. Australian National University.

Graves JE, McDowell JR, Jones ML (1992). A genetic analysis of weakfish *Cynoscion regalis* stock structure along the mid-Atlantic coast. *Fishery Bulletin* **90**, 469-475.

Graves JE (1998) Molecular insights into the population structures of cosmopolitan marine fishes. *Journal of Heredity* **89**, 427-437.

Gunn J, Block BA (2001) Advances in acoustic, archival and satellite telemetry. In: *Tuna: Physiology, Ecology and Evolution*, 167-224 (Block BA, Stevens ED, Eds.). New York: Academic Press.

Hauser L, Adcock GJ, Smith PJ, Ramírez JHB, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *PNAS* **99**, 11742-11747.

Hauser L, Carvalho GR (2008) Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. *Fish and Fisheries* **9**, 333-362.

Hauser L, Seeb JE (2008) Advances in molecular technology and their impact on fisheries genetics *Fish and Fisheries* **9**, 473-486.

Hauser L, Baird M, Hilborn RAY, Seeb LW, Seeb JE (2011) An empirical comparison of SNPs and microsatellites for parentage and kinship assignment in a wild sockeye salmon (Oncorhynchus nerka) population. *Molecular Ecology Resources* **11**, 150-161.

Heinisch G, Corriero A, Medina A, Abascal FJ, de la Serna JM, *et al.* (2008) Spatial-temporal pattern of bluefin tuna (*Thunnus thynnus* L. 1758) gonad maturation across the Mediterranean Sea. *Marine Biology* **154**, 623-630.

Heist EJ, Gold JR (1999) Microsatellite DNA variation in sandbar sharks (*Carcharhinus plumbeus*) from the Gulf of Mexico and mid-Atlantic bight. Copeia 1, 182-186, published by: American Society of Ichthyologists and Herpetologists (ASIH).

Hemmer-Hansen J, Nielsen EE, Meldrup D, Mittelholzer C (2011) Identification of single nucleotide polymorphisms in candidate genes for growth and reproduction in a nonmodel organism; the Atlantic cod, *Gadus morhua*. *Molecular Ecology Resources* **11** (Suppl. 1), 1-10.

Hess JE, Matala AP, Narum SR (2011) Comparison of SNPs and microsatellites for fine-scale application of genetic stock identification of Chinook salmon in the Columbia River Basin. *Molecular Ecology Resources* **11** (Suppl. 1), 137-149.

Holland KN, Brill RW, Chang RKC, Sibert JR, Fournier DA (1992) Physiological and behavioural thermoregulation in bigeye tuna (Thunnus obesus). *Nature* **358**, 410-412.

Humston R, Olson DB, Ault JS (2004) Behavioral Assumptions in Models of Fish Movement and Their Influence on Population Dynamics. Transactions of the American Fisheries Society 133.

Hurlbut T, Clay D (1988) A review of age and growth of Canadian giant bluefin as estimated from otoliths. *ICCAT Col. Vol. Sci. Pap.* **28**, 192-195.

ICCAT (1999) 1998 SCRS detailed report on bluefin tuna. *Collective Volume of Scientific Papers ICCAT* **49**, 1-191.

ICCAT (2002) ICCAT workshop on bluefin tuna mixing. *Collective Volume of Scientific Papers ICCAT* **54,** 261-352.

ICCAT (2003) Basic Texts, 3rd revision. International Commission for the Conservation of Atlantic Tuna (ICCAT), Madrid, 113 pp.

ICCAT (2005) Report for biennial period, 2004–05, Part I (2004), Vol. 2. Madrid, Spain: SCRS, 2005.

ICCAT (2007) Report of the 2006 Atlantic Bluefin Tuna Stock Assessment Session. *Collective Volume of Scientific Papers ICCAT* **60,** 652-880.

ICCAT (2008) Report of the 2008 Atlantic Bluefin Stock Assessment Session (SCRS/2008/019). (International Commission for the Conservation of Atlantic Tunas, Madrid).

ICCAT (2010) Report of the Standing Committee on Research and Statistics (SCRS). Madrid: International Commission for the Conservation of Atlantic Tuna.

ICCAT (2012) Report for Biennial Period, 2010-11, Part II (2011), Vol. 2. Madrid, Spain: SCRS, 2012.

Inagake D, Segawa K, Okazaki M, Mitta A, Itoh T (2001) Migration of Young Bluefin Tuna, Thunnus orientalis Temminck et Schlegel, through Archival Tagging Experiments and its relation with oceanographic condition in the western north Pacific. *Bulletin of the National Research Institute of Far Seas Fisheries* **38**, 53-81.

Itano DG (2000) The reproductive biology of yellowfin tuna (*Thunnus albacares*) in Hawaiian waters and the western tropical Pacific Ocean: Project summary. SOEST 00-01, JIMAR Contribution 00-328.

Itoh T, Shiina Y, Tsuji S, Endo F, Tezuka N (2000) Otolith daily increment formation in laboratory reared larval and juvenile bluefin tuna *Thunnus thynnus*. *Fishery Science* **66**, 834-839.

Jarne P, Lagoda PJL (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* **11**, 424-429.

Jenkins GP, Davis TLO (1990) Age, growth rate, and growth trajectory determined from otolith microstructure of southern bluefin tuna *Thunnus maccoyii* larvae. *Marine Ecology Progress Series* **63**, 93-104.

Jenkins GP, Young JW, Davis TLO (1991) Density dependence of larval growth of a marine fish, the southern bluefin tuna, *Thunnus maccoyii*. *Canadian Journal of Fisheries and Aquatic Sciences* **48**, 1358-1363.

Joost S, Bonin A, Bruford W, Després L, Conord C, *et al.* (2007) A spatial analysis method (SAM) to detect candidate loci for selection: towards a landscape genomics approach to adaptation. *Molecular Ecology* **16**, 3955-3969.

Karakulak S, Oray I, Corriero A, Aprea A, Spedicato D, *et al.* (2004a) First information the reproductive biology of the bluefin tuna (*Thunnus thynnus*) in the Eastern Mediterranean. *ICCAT Col. Vol. Sci. Pap.* **56**, 1158-1162.

Karakulak S, Oray I, Corriero A, Deflorio M, Santamaria N, *et al.* (2004b) Evidence of a spawning area for the bluefin tuna (*Thunnus thynnus* L.) in the Eastern Mediterranean. *Journal of Applied Ichthyology* **20**, 318-320.

Kell LT, Fromentin JM, Bonhommeau S (2012) An evaluation of the implications of population structure on the current bluefin tuna advice framework. *ICCAT Col. Vol. Sci. Pap* **68**(1), 370-378.

Kitagawa T, Kimura S, Nakata H, Yamada H (2006) Thermal adaptation of Pacific bluefin tuna *Thunnus orientalis* to temperate waters. *Fisheries Science* **72**, 149-156.

Krumholz LA (1959) Stomach contents and organ weights of some bluefin tuna, *Thunnus thynnus* (Linneaus), near Bimini, Bahamas. *Zoologica* **44**, 127-131.

Kucuktas H, Wang S, Li P, Li C, Xu P, *et al.* (2009) Construction of Genetic Linkage Maps and Comparative Genome Analysis of Catfish Using Gene-Associated Markers. *Genetics* **181**, 1649-1660.

Kumai H (1998) Studies on bluefin tuna artificial hatching, rearing and reproduction. *Nipp. SuisanGakk.* **64**, 601-605 (in Japanese, with English abstract).

Lang KL, Grimes CB, Shaw RF (1994) Variations in the age and growth of yellowfin tuna larvae, *Thunnus albacares*, collected about the Mississippi River plume. *Environmental Biology of Fishes* **39**, 259-270.

Laurs RM, Fiedler PC, Montgomery DR (1984) Albacore tuna catch distributions relative to environmental features observed from satellites, Deep Sea Research Part A. *Oceanographic Research Papers* **31**, 1085-1099.

Lehodey P, Bertignac M, Hampton J, Lewis A, Picaut J (1997) El Niño Southern Oscillation and tuna in the western Pacific. *Nature* **389**, 715-718.

Ligny W (1969) Serological and biochemical studies in fish populations. *Marine Biology Annual Review* **7**, 411-513.

Limborg MT, Blankenship SM, Young SF, Utter FM, Seeb LW, *et al.* (2011) Signatures of natural selection among lineages and habitats in *Oncorhynchus mykiss*. *Ecology and Evolution* **2**, 1-18.

Limborg MT, Helyar SJ, De Bruyn M, Taylor MI, Nielsen EE, *et al.* (2012) Environmental selection on transcriptome-derived SNPs in a high gene flow marine fish, the Atlantic herring (*Clupea harengus*) *Molecular Ecology* **21**, 3686-3703.

Lioka C, Kani K, Nhhala H (2000) Present status and prospects of technical development of tuna sea-farming, pp. 275–285. In: *Cahiers Options Méditerranéennes, vol. 47: Mediterranean Marine Aquaculture Finfish Species Diversification* (Basurco, B., Ed.). Zaragoza, Spain, CIHEAM, Instituto Agronomico de Zaragoza.

Liu ZJ, Cordes JF (2004) DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* **238**, 1-37.

Logan JM, Rodríguez-Marín E, Goñi N, Barreiro S, Arrizabalaga H, *et al.* (2011) Diet of young Atlantic bluefin tuna (*Thunnus thynnus*) in eastern and western Atlantic foraging grounds. *Marine Biology* **158**, 73-85.

Longo SB, Clark B (2012) The Commodification of Bluefin Tuna: The Historical Transformation of the Mediterranean Fishery. *Journal of Agrarian Change* **12**, 204-226.

Lutcavage ME, Brill RW, Skomal GB, Chase BC, Goldstein JL, *et al.* (1999) Tracking adult North Atlantic bluefin tuna (*Thunnus thynnus*) in the northwestern Atlantic using ultrasonic telemetry. *Marine Biology* **137**, 347-358.

MacKenzie BR, Mosegaard H, Rosenberg AA (2009) Impending Collapse of Bluefin Tuna in the Northeast Atlantic and Mediterranean. *Conservation Letters* **2** (1), 25-34.

Magnuson JJ, Block BA, Deriso RB, Gold JR, Grant WS, *et al.* (1994) An assessment of Atlantic bluefin tuna, National Academy Press.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-380.

Masuma S, Tezuka N, Koiso M, Jinbo T, Takebe T, *et al.* (2006) Effects of water temperature on bluefin tuna spawning biology in captivity. *Bull. Fish. Res. Agen.*, Supplement **4**, 157-172 (in Japanese, with English abstract).

Mather FJ, Shuck HA (1960) Growth of bluefin tuna of the western North Atlantic. *Fishery Bulletin* **179**, 39-52.

Mather FJ, Mason JM, Jones AC (1995) Historical document: life history and fisheries of Atlantic bluefin tuna. U.S. Dep. Comm., NOAA Technical Memorandum, NMFS-SEFSC 370.

McGowan MF, Richards WJ (1986) Distribution and abundance of bluefin tuna (*Thunnus thynnus*) larvae in the Gulf of Mexico in 1982 and 1983 with estimates of the biomass and population size of the spawning stock for 1977, 1978, and 1981-1983. *ICCAT Col. Vol. Sci. Pap.* **24**, 182-195.

Medina A, Abascal FJ, Megina C, Garcia A (2002) Stereological assessment of the reproductive status of female Atlantic northern bluefin tuna during migration to Mediterranean spawning grounds through the Strait of Gibraltar. *Journal of Fish Biology* **60**, 203-217.

Megalofonou P (2006) Comparison of otolith growth and morphology with somatic growth and age in young-of-the-year bluefin tuna. *Journal of Fish Biology* **68**, 1867-1878.

Miyake PM, de la Serna JM, Di Natale A, Farrugia A, Katavic I, *et al.* (2003) *General Review of Bluefin Tuna Farming in the Mediterranean Area.* Madrid: ICCAT.

Miyashita S, Sawada Y, Okada T, Murata O, Kumai H (2001) Morphological development and growth of laboratory-reared larval and juvenile *Thunnus thynnus* (Pisces: Scombridae). *Fishery Bulletin* **99**, 601-616.

Moen T, Hayes B, Baranski M, Berg PR, Kjoglum S, *et al.* (2008) A linkage map of the Atlantic salmon (*Salmo salar*) based on EST-derived SNP markers. *BMC Genomics* **9**, 223.

Morin PA, Luikart G, Wayne RK, the Snp wg (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution* **19**, 208-216.

Morin PA, Martien KK, Taylor BL (2009) Assessing statistical power of SNPs for population structure and conservation studies. *Molecular Ecology Resources* **9**, 66-73.

Musyl MK, Brill RW, Bogg CH, Curran DS, Kazama TK, *et al.* (2003) Vertical movements of bigeye tuna (*Thunnus obesus*) associated with islands, buoys, and seamounts near the main Hawaiian Islands from archival tagging data. *Fisheries Oceanography* **12**, 152-169.

Mylonas C, Bridges CR, Gordin H, Belmonte Ríos A, García A, *et al.* (2007) Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*). *Reviews in Fisheries Science* **15**, 183-210.

Mylonas C, De La Gándara F, Corriero C, Belmonte Ríos A (2010) Atlantic Bluefin Tuna (*Thunnus Thynnus*) Farming and Fattening in the Mediterranean Sea. *Reviews in Fisheries Science* **18**(3), 266-280.

Nemerson D, Berkely S, Safina C (2000) Spawning site fidelity in Atlantic bluefin tuna,*Thunnus thynnus*: the use of size-frequency analysis to test for the presence of migrant east Atlantic bluefin tuna on Gulf of Mexico spawning grounds. *Fishery Bulletin* **98**, 118-126.

Nevo E (1990) Molecular evolutionary genetics of isozymes: pattern, theory, and application. *Progress in Clinical and Biological Research* **344**, 701-742.

Nichols JT (1922) Color of the tuna. *Copeia* **1922**, 74-75.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009) Population genomics of marine fishes: identifying adaptive variation in space and time. *Molecular Ecology* **18**, 3128-3150.

Nielsen R (2001) Statistical tests of selective neutrality in the age of genomics. *Journal of Heredity* **86**, 641-647.

Nikaido H, Miyabe N, Ueyanagi S (1991) Spawning time and frequency of bigeye tune, *Thunnus obesus*. *Bulletin of the National Research Institute of Far Seas Fisheries* **28**, 47-73.

Nishida T, Tsuji S, Segawa K (1998) Spatial data analyses of Atlantic bluefin tuna larval surveys in the 1994 ICCAT BYP. *ICCAT Col. Vol. Sci. Pap*. **48**, 107-110.

Oleksiak MF (2010) Genomic approaches with natural fish populations. *Journal of Fish Biology* **76**, 1067-1093.

Oray IK, Karakulak FS (2005) Further evidence of spawning of bluefin tuna (*Thunnus thynnus* L., 1758) and the tuna species (*Auxis rochei* Ris., 1810, *Euthynnus alletteratus* Raf., 1810) in the Eastern Mediterranean Sea: preliminary results of TUNALEV larval survey in 2004. *Journal of Applied Ichthyology* **21**, 236-240.

Oren OH, Ben-Tuvia A, Gottleib E (1959) Experimental tuna fishing cruise in the eastern Mediterranean. *Proc. Gen. Fish. Conc. Mediterranean* **5**, 269-279.

Ottolenghi F, Silvestri C, Giordano P, Lovatelli A, New MB (2004) *Capture-Based Aquaculture*. Rome: Food and Agriculture Organization.

Palumbi SR (1994) Genetic-divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology, Evolution, and Systematics* **25**, 547-572.

Pearse DE, Crandall KA (2004) Beyond F_{ST}: Analysis of population genetic data for conservation. *Conservation Genetics* **5**, 585-602.

Piccinetti C, Manfrin GP (1970) Osservazioni sulla biologia dei primi stadi giovanili del tonno (*Thunnus thynnus* L.). *Boll. Pesca Piscic. Idrobiol.* **25**, 223-247.

Poulsen N, Hemmer-Hansen J, Loeschcke V, Carvalho G, Nielsen E (2011) Microgeographical population structure and adaptation in Atlantic cod *Gadus morhua*: spatio-temporal insights from geneassociated DNA markers. *Marine Ecology Progress Series* **436**, 231-243.

Pujolar JM, Roldán MI, Pla C (2003) Genetic analysis of tuna populations, *Thunnus thynnus thynnus* and *T. alalunga*. *Marine Biology* **143**, 613-621.

Ravier C, Fromentin JM (2001) Long-term fluctuations in the eastern Atlantic and Mediterranean bluefin tuna population. ICES *Journal of Marine Science* **58**, 1299-1317.

Renaut S, Nolte AW, Bernatchez L (2010) Mining transcriptome sequences towards identifying adaptive single nucleotide polymorphisms in lake whitefish species pairs (Coregonus spp. Salmonidae). *Molecular Ecology* **19**, 115-131.

Renaut S, Nolte AW, Rogers SM, Derome N, Bernatchez L (2011) SNP signatures of selection on standing genetic variation and their association with adaptive phenotypes along gradients of ecological speciation in lake whitefish species pairs (Coregonus spp.). *Molecular Ecology* **20**, 545-559.

Rexroad CE, Rodriguez MF, Coulibaly I, Gharbi K, Danzmann RG, *et al.* (2005) Comparative mapping of expressed sequence tags containing microsatellites in rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics* **6**, 54.

Riccioni G, Landi M, Ferrara G, Milano I, Cariani A, *et al.* (2010) Spatio-temporal population structuring and genetic diversity retention in depleted Atlantic bluefin tuna of the Mediterranean sea. *PNAS* **107**, 2102-2107.

Richards WJ (1976) Spawning of bluefin tuna (*Thunnus thynnus*) in the Atlantic Ocean and adjacent seas. *ICCAT Col. Vol. Sci. Pap.* **5**, 267-278.

Richards WJ, Potthoff T (1980) Distribution and abundance of bluefin tuna larvae in the Gulf of Mexico in 1977 and 1978. *ICCAT Col. Vol. Sci. Pap.* **9**, 433-441.

Rivas LR (1954) A preliminary report on the spawning of the western North Atlantic bluefin tuna (*Thunnus thynnus*) in the Straits of Florida. *Bull. Mar. Sci. Gulf Carib*. **4**, 302-321.

Rivas LR (1976) Variation in sex ratio, size differences between sexes, and change in size and age composition in western North Atlantic giant bluefin tuna (*Thunnus thynnus*). *ICCAT Col. Vol. Sci.Pap.* **5**, 297-301.

Rodríguez-Roda J (1967) Fecundidad del atún, *Thunnus thynnus* (L.), de la costa sudatlántica de España. *Investigacion Pesquera (Spain)* **31**, 33-52.

Rodríguez-Roda J (1969) Resultados de nuestras marcaciones de atúnes en el Golfo de Cadiz durante lo años 1960 a 1967. *Publ. Téc. Junta Est. Pesca, Dir. Gen. Pesca Mar.* **8**, 153-158.

Rooker JR, Secor DH (2004) Stock structure and mixing of Atlantic bluefin tuna: evidence from stable δ^{13}C and δ^{18}O isotopes in otoliths. *ICCAT Col. Vol. Sci. Pap.* **56**, 1115-1120.

Rooker JR, Secor DH, De Metrio G, Rodriquez-Marin E (2006) Evaluation of population structure and mixing rates of Atlantic bluefin tuna from chemical signatures in otoliths. *ICCAT Col. Vol.Sci. Pap.* **59**, 813-818.

Rooker JR, Alvarado Bremer JR, Block BA, Dewarc H, de Metrio G, *et al.* (2007) Life history and stock structure of Atlantic Bluefin Tuna (*Thunnus thynnus*). *Reviews in Fisheries Science* **15**, 265-310.

Rooker JR, Secor DH, Metrio G, Schloesser R, Block BA, *et al.* (2008) Natal Homing and Connectivity in Atlantic Bluefin Tuna Populations. *Science* **322**, 742.

Royer F, Fromentin JM, Gaspar P (2004) The association between bluefin tuna schools and oceanic features in the Western Mediterranean Sea. *Marine Ecology Progress Series* **269**, 249-263.

Safina C (2001) 'Tuna Conservation'. In *Tuna. Physiology, Ecology, and Evolution*, ed. Block BA and Stevens ED, 414-57. New York: Academic Press.

Safina C, Klinger DH (2008) Collapse of Bluefin Tuna in the Western Atlantic. *Conservation Biology* **22**, 243-246.

Santamaria N, Bello G, Corriero A, Deflorio M, Vassallo-Agius R, *et al.* (2009) Age and growth of Atlantic bluefin tuna, *Thunnus thynnus* (Osteichthyes: Thunnidae), in the Mediterranean Sea. *Journal of Applied Ichthyology* **25**, 38-45.

Sanzo L (1932) Uova e primi stadi larvali di tonno (*Orcynus thynnus* Ltkn.). *Mem. R. Com. Talass. Ital.* **189**, 1-16.

Sarà G, Sarà R (2007) Feeding habits and trophic levels of bluefin tuna (*Thunnus thynnus*) of different size classes in the Mediterranean Sea. *Journal of Applied Ichthyology* **23**, 122-127.

Sawada Y, Okada T, Miyashita S, Murata O, Kumai H (2005) Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temmich et Schlegel) life cycle. *Aquaculture Research* **36**, 413-421.

Schaefer KM (1998) Reproductive biology of yellowfin tuna (*Thunnus albacares*) in the eastern Pacific Ocean. *IATTC Bull.* **21**, 201-272.

Schaefer KM (2001) Reproductive biology of tunas. In: Barbara Block and E. Stevens, Editor(s), *Fish Physiology* **19**, 225-270.

Schloesser RW, Neilson JD, Secor DH, Rooker JR (2010) Natal origin of Atlantic bluefin tuna (Thunnus thynnus) from Canadian waters based on otolith $\delta^{13}C$ and $\delta^{18}O$. *Canadian Journal of Fisheries and Aquatic Sciences* **67**, 563-569.

Schlötterer C, Dieringer D (2005) A novel test statistics for the identification of local selective sweeps based on microsatellite gene diversity. In: Selective Sweep (ed. Nurminski D), 55-64. Eurekah.com and Klüwer Academic/Plenum Publishers, Georgetown, TX, USA.

Scott GP, Turner SC, Grimes CB, Richards WJ, Brothers EB (1993) Indices of larval bluefin tuna, *Thunnus thynnus*, in the Gulf of Mexico; Modeling variability in growth, mortality, and gear selectivity. *Bulletin of Marine Science* **53**, 912-929.

Seddon JM, Parker HG, Ostrander EA, Ellegren H (2005) SNPs in ecological and conservation studies: a test in the Scandinavian wolf population. *Molecular Ecology* **14**, 503-511.

Seeb JE, Carvalho G, Hauser L, Naish K, Roberts S, *et al.* (2011) Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular Ecology Resources* **11**, 1-8.

Seeb LW, Templin WD, Sato S, Abe S, Warheit K, *et al.* (2011) Single nucleotide polymorphisms across a species' range: implications for conservation studies of Pacific salmon. *Molecular Ecology Resources* **11**, 195-217.

Sella M (1929) Biologia e pesca del tonno (*Thunnus thynnus* L.). *Atti Conv. Biol. Mar. Appl. Pesca*, Messina, Giugno 1928, 1-32.

Smith CT, Templin WD, Seeb JE, Seeb LW (2005) Single Nucleotide Polymorphisms Provide Rapid and Accurate Estimates of the Proportions of U.S. and Canadian Chinook Salmon Caught in Yukon River Fisheries. *North American Journal of Fisheries Management* **25**, 944-953.

Smith CT, Seeb LW (2008) Number of Alleles as a Predictor of the Relative Assignment Accuracy of Short Tandem Repeat (STR) and Single-Nucleotide-Polymorphism (SNP) Baselines for Chum Salmon. *Transactions of the American Fisheries Society* **137**, 751-762.

Stokesbury MJW, Teo SLH, Seitz A, O'Dor RK, Block BA (2004) Movement of Atlantic bluefin tuna (*Thunnus thynnus*) as determined by satellite tagging experiments initiated off New England. *Canadian Journal of Fisheries and Aquatic Sciences.* **61**, 1976-1987.

Stokesbury M, Cosgrove R, Boustany A, Browne D, Teo S, *et al.* (2007) Results of satellite tagging of Atlantic bluefin tuna, *Thunnus thynnus* , off the coast of Ireland. *Developments in Hydrobiology* **195**, 91-97.

Storz JF (2005) Using genome scans of DNA polymorphism to infer adaptive population divergence. *Molecular Ecology* **14**, 671-688.

Sumalia UR, Huang L (2012) Managing Bluefin Tuna in the Mediterranean Sea. *Marine Policy* **36**, 502-511.

Susca V, Corriero A, Bridges CR, De Metrio G (2001) Study of the sexual maturity of female bluefin tuna: Purification and partial characterization of vitellogenin and its use in an enzyme-linked immunosorbent assay. *Journal of Fish* Biology **58**, 815-831.

Sweijd NA, Bowie RCK, Evans BS, Lopata AL (2000) Molecular genetics and the management and conservation of marine organisms. *Hydrobiologia* **420**, 153-164.

Teo SLH, Blackwell SB, Boustany A, Walli A, Weng K, *et al.* (2004) Validation of geolocation estimates based on light level and sea surface temperature from electronic tags. *Marine Ecology Progress Series* **283**, 81-98.

Teo SLH, Boustany A, Dewar H, Stokesbury M, Weng KC, *et al.* (2007) Annual migrations, diving behavior and thermal biology of Atlantic bluefin tuna, *Thunnus thynnus*, to breeding grounds in the Gulf of Mexico. *Marine Biology* **151**, 1-18.

Thompson HC, Contin RF (1980) Electrophoretic study of Atlantic bluefin tuna (*Thunnus thynnus*) from the eastern and western north Atlantic ocean. *Bulletin of Marine Science* **30**, 727-731.

Tiews K (1963) Synopsis of biological data on bluefin tuna, *Thunnus thynnus* (Linnaeus) 1758 (Atlantic and Mediterranean). *FAO Fish. Rep.* **6**, 422-481.

Uotani I, Saito T, Hiranuma K, Nishikawa Y (1990) Feeding habit of bluefin tuna *Thunnus thynnus* larvae in the western North Pacific Ocean. *Bulletin of the Japanese Society of Science and Fisheries* **56**, 713-717.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* **34**, 275-305.

Viñas J, Gordoa A, Fernández-Cebrián R, Pla C, Vahdet Ü, *et al.* (2011) Facts and uncertainties about the genetic population structure of Atlantic bluefin tuna (*Thunnus thynnus*) in the Mediterranean. Implications for fishery management. *Reviews in Fish Biology and Fisheries* **21**, 527-541.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.

Wallace RA, Selman K (1981) Cellular and dynamic aspects of oocyte growth in teleosts. *American Zoologist* **21**, 325-343.

Walli A, Teo SLH, Boustany A, Farwell CJ, Williams T, *et al.* (2009) Seasonal movements, aggregations and diving behavior of Atlantic bluefin tuna (*Thunnus thynnus*) revealed with archival tags. *PloS ONE* **4**, 1-18.

Waples RS (1998) Separating the wheat from the chaff: Patterns of genetic differentiation in high gene flow species. *Journal of Heredity* **89**, 438-450.

Ward RD (2000) Genetics in fisheries management. *Hydrobiologia* **420**, 191-201.

Wenne R, Boudry P, Hemmer-Hansen J, Lubieniecki KP, Was A, *et al.* (2007) What role for genomics in fisheries management and aquaculture? *Aquatic Living Resources* **20**, 241-255.

Williams LM, Oleksiak MF (2011) Ecologically and evolutionarily important SNPs identified in natural populations. *Molecular Biology and Evolution* **28**(6), 1817-1826.

WWF (2006) The Plunder of Bluefin Tuna in the Mediterranean and East Atlantic in 2004 and 2005. WWF Mediterranean Programme Office.

Yamashita H, Miyabe N (2001) Report of bluefin tuna archival tagging conducted by Japan in 1999 in the Adriatic Sea. *ICCAT Col. Vol. Sci. Pap.* **52**, 809-823.

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