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Are there genetic breaks between Atlantic and Pacific Yellowfin tuna (*Thunnus albacares*) populations? A preliminary study based on microsatellites gene variation

Tesi di laurea in Struttura e connettività delle popolazioni marine

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Abstract

Yellowfin tuna (*Thunnus albacares*, YFT, Bonnaterre 1788) is one of the most important market tuna species in the world. The high mortality of juveniles is in part caused by their bycatch. Indeed, if unregulated, it could permanently destabilize stocks health. For this reason investigating and better knowing the stock boundaries represent a crucial concern.

Aim of this thesis was to preliminary investigate the YFT population structure within and between Atlantic and Pacific Oceans through the analysis of genetic variation at eight microsatellite loci and assess the occurrence of barriers to the gene flow between Oceans.
For this propouse we collected 4 geographical samples coming from Atlantic and Pacific Ocean and selected a panel of 8 microsatellites loci developped by Antoni *et al.*, (2014). Samples 71-2-Y and 77-2-Y, came from rispectively west central pacific ocean (WCPO) and east central pacific ocean (ECPO), instead samples 41-1-Y and 34-2-Y derive from west central atlantic ocean (WCAO) and east central atlantic ocean (ECAO). Total 160 specimens were analyzed (40 per sample) and were carried out several genetic information as allele frequencies, allele number, allelic richness, HWE (using He and Ho) and pairwise Fst genetic distance.

Results obtained, may support the panmictic theory of this species, only one of pairwise Fst obtained is statistically significant (Fst= 0.00927; pV= 0.00218) between 41-1-Y and 71-2-Y samples. Results suggest low genetic differentiation and consequent high level of gene flow between Atlantic and Pacific populations.

Furthermore, we performed an analysis of molecular taxonomy through the use of ATCO (the flaking region between ATPse6 and cytochrome oxidase subunit III genes mt DNA, to discriminate within the gener *Thunnus* two of the related species (Yellofin and bigeye tuna) according with their difficult recognition at certain size (<40 cm).

ATCO analysis in this thesis, has provided strong discriminate evidence between the target species proving to be one of the most reliable genetic tools capable to indagate within the genus *Thunnus.* Thus, our study has provided useful information for possible use of this protocol for conservation plans and management of this fish stocks.

1. Introduction

1.1 Yellowfin Tuna biology and ecology

YELLOWFIN TUNA
Order - Perciformes
Family - Scombridae
Genus - *Thunnus*
Species - *albacares*

Fig. 1 Yellowfin tuna

Yellowfin tuna (*Thunnus albacares*, YFT, Bonnaterre 1788) (Fig. 1) is a pelagic fish widely distributed in tropical and subtropical waters worldwide (Fig. 2). It is one of the most important market tuna species in the world and it is fished in the Indian, Pacific, and Atlantic Oceans, where it has been commercially harvested since the early 1950s (Collette and Nauen, 1983; Miyake *et al.*, 2010).

YFT are torpedo-shaped fish with about 20 broken vertical lines in the belly and a typical dark blue colour on the back and upper sides. They have very long anal and dorsal fins that are bright yellow, from which their common name is derived (Collette and Nauen, 1983). YFT are relatively fast growing, and they can grow up to 200 cm FL and 175 kg with a life span of about 8 years (ISSF 2013).

Tagging experiments indicate that YFT adults are able to undertake wide migrations, according to the fact that they are fairly fast swimming and highly migratory fishes (Schaefer, 2007). Although their potential for trans-oceanic's migrations, the majority of tagged individuals are recovered within several hundred kilometres from their release positions (Schaefer, 2008). YFT usually spend most of their life in the warmest first 30-40 meters of the water column (Block, 1997). This behaviour seems to be strictly related to the reproductive biology of this species, as reviewed by Schaefer (2001) spawning events,

as for other tuna species, can occur in relation to the sea surface temperature over 24ºC. YFT is a batch-spawner characterized by an asynchronous ovary organization (Schaefer, 2001) and an indeterminate fecundity (Zudaire *et al.,* 2013). YFT can spawn with a frequency of approximately 1.52 days (McPherson, 1991; Schaefer, 2001), throughout the year (Itano, 2001; Stéquert *et al.,* 2001). Moreover, sea surface temperature deviations from 24°C could seriously decrease their potential spawning activity (Itano, 2001).

Worldwide studies on the fork length at which 50% of females reach maturation (L_{50}) provide different estimates among oceans and areas. For example, in the Indian Ocean L_{50} was estimated around 100 cm FL (Zhu et al., 2008), McPherson (1991) estimated it as 108 cm FL in the western Pacific Ocean, while Schaefer (1998) estimated this parameter as 92 cm FL in the eastern Pacific Ocean, and Itano (2001) reported a L_{50} of 104 cm FL for the equatorial west Pacific. In all these studies, the L_{50} was defined with macroscopic method by setting maturity limit in advanced vitellogenic ovaries (Itano 2001). Instead, Zudaire *et al.*, (2013) in Indian Ocean, applied a maturity threshold in ovaries in Cortical Alveoli (CA) stage, retrieving a L_{50} value of 75 cm FL. CA stage represents the earliest sign of oocyte maturation (Murua and Motos, 2000), and females in this developmental stage usually go through vitellogenesis and spawn in the upcoming season (Wright, 2007).

During the reproduction, YFTs continue feeding, and its spawning activity has been described to be dependent on the prey availability (Itano, 2001). Thus, they need energy from feeding to carry out ovarian development (Zudaire *et al.*, 2013), and for this reason the species could be described as a capital-income breeder (Alonso-Fernández and Saborido-Rey, 2012). According to this strategy, fishes require energy from feeding, because the energy stored before reproduction is not enough to offset a successful reproduction (Henderson and Morgan, 2002).

YFT diet includes variable prey composition. Poitier *et al.,* (2007), studying the stomach contents of YFT caught by long-line fishery in Indian Ocean, indicated fishes as their main food source, while for YFT caught by Atlantic purse seine fisheries the favourite nutritional resources seem to be mainly small pelagic fishes (Menard and Marchal, 2003). In addition, there are significant differences in prey species composition for the specimens captured around fish aggregating devices (FAD) and free-swimming schools (FSC). Stomach-contents analyses have indicated *Vinciguerria nimbaria* (Photichthyidae, philum Chordata) as the main food source in FAD-associated small YFT, whereas small little

Scombridae, mixed with *Cubicepspauci radiatus* (Nomeidae, philum Chordata) are the main preys in YFT FSC-associated school (Dagorn *et al.*, 2007).

Besides these data, the percentage of empty stomachs found in YFT FAD-associated (85%) is higher than those caught on unassociated schools (25%), underlying the possibility that these fish do not feed under drifting FADs (Menard *et al.*, 2000).

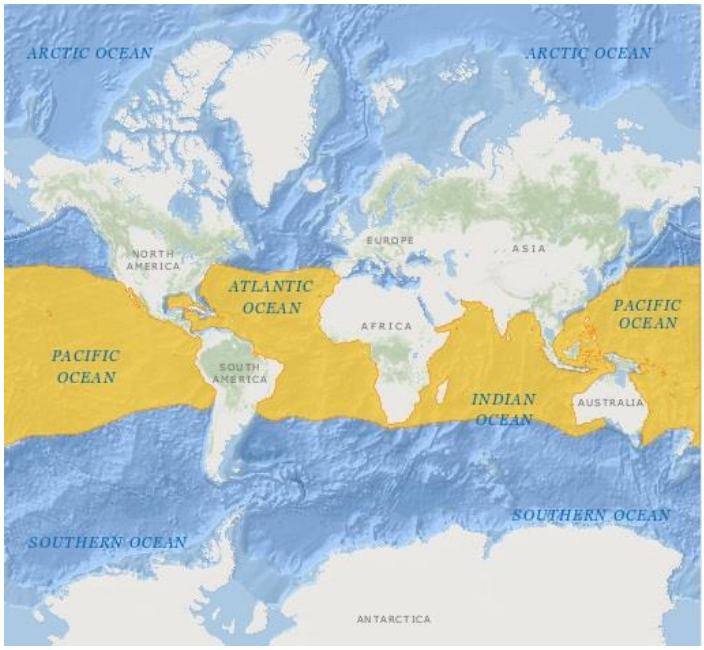

Fig. 2_Yellowfin geographical distribution.

1.2 Tropical tuna fisheries

Tuna fisheries operate on an industrial scale in the Indian, Atlantic and Pacific Oceans (Davies *et al.*, 2012), representing more than 4.1 million tons of the global fisheries catches and are dominated by three fishing gears, i.e. purse-seine, longline, and pole and line, accounting for about 60%, 15% and 11% of the world tuna catches respectively (FAO, 2012).

The European tuna purse-seine fishery targets yellowfin (*Thunnus albacares*), skipjack (*Katsuwonus pelamis*), and bigeye (*Thunnus obesus*) tuna since the early 1980s (Amandè *et al.*, 2012).

European purse-seine fishery total catches of the principal commercially tuna species have achieved a maximum of about 400 000 t in 2003 and have fluctuated around 250 000 t in

recent years (Pianet *et al.*, 2010). This fishing method implies that the fish are pursued and encircled by vessels of a broad range of sizes and capacities (Fig. 3). The net employed has a length that may reach more than 2200 m and its depths are usually from 150 m to 350 m; the mesh size varies from 7.5 cm to 25 cm but the vast majority is employing a 10.8 cm stretched mesh (FAO, 2012).

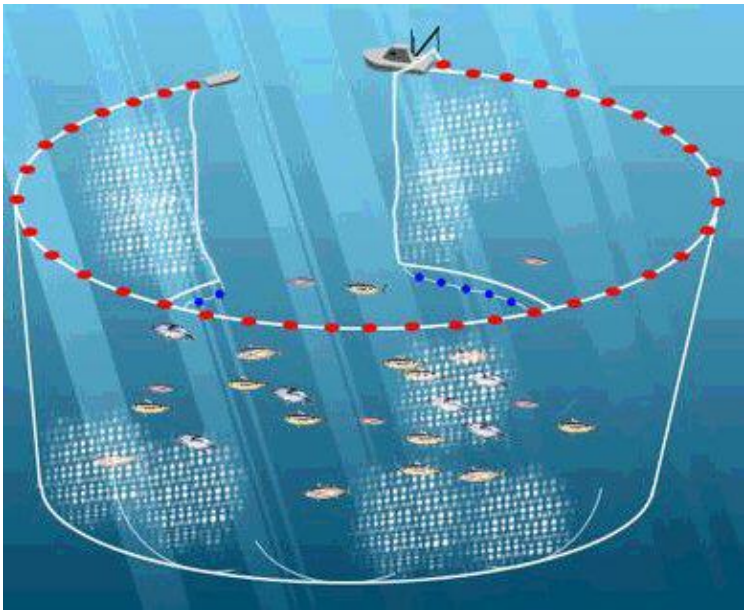

Fig. 3_Purse seiner fishing activity

Tropical tuna purse seine fishery is characterized by two fishing modes with sets made on: 1) tuna schools associated with floating objects (FADs - Fishing aggregating device) and 2) on free schools (FSC - Free swimming schools) (Amandè *et al.*, 2010).

FSC can be detected from signs on the surface of the water, in fact schools usually move close to the surface while chasing. Frequently, the presence of birds close to the surface (blackspot), is a further hint for the presence of free tuna schools (Allen, 2010).

Fig. 4_Free schools catch during landing procedures**.**

Instead, FAD sets are generally less mobile than FSC sets, making their catch easier. FAD could be natural objects or man-made (raft or plastic), with a long net below and can be either anchored (aFADs) or drifted (dFADs) depending on the fishing area (Dagorn, 2011). FADs are easier detectable than FSC, decreasing sighting effort and time required to locate fish schools. (Dempster and Taquet, 2004). The main difference between the two fishing methods is their target; in fact FSC's target is represented by large YFT (Fig. 4) and bigeye tuna (e.g. up to 100 cm FL), instead FAD sets focus on catching adult skipjack tuna (Dagorn *et al.*, 2013).

1.2.1 FAD-based fishery

Tropical tuna purse seine fisheries have globally increased their use of drifting fish aggregating devices (dFADs) since the early 1990s, in order to improve catch levels (Dempster and Taquet, 2004; Dagorn et al., 2013). In fact sets around FADs have shown higher achievement rate (90%), compared with the FSC (50%) (Fonteneau et al., 2000). In addition, in the last years, FAD-based fishery has recorded an impressive technological development, (e.g. echo-sounder, GPS units attached to their buoys) that allow to the fishermen to know their movements and to track their routes, but also to know the fish

composition and abundance below them (Lopez *et al.*, 2014). In this context, in the last years almost half of all principal market tunas were caught by sets on dFADs, of which an estimated 50,000–100,000 are deployed each year (Baske *et al.*, 2012).

The loss of potential yield and the reduction of spawning stock biomass (SSB) can be some of the ecological and biological problems linked to the constant and unchecked increase in the use of FADs (Fig. 5) (Fonteneau and Ariz, 2011).

Dagorn *et al.*, (2012) showed that the total harvested target species associated with dFADs, are composed for 75% by adults SKY. While the by-catch of juvenile BET and YFT (40-65 cm FL) (Fonteneau *et al.*, 2000, Bromhead *et al.*, 2003) inadvertently caught with this fishing gear, counts respectively for 16% and 9% of the global tuna catches. By-catch can be defined as accidental catches, which are not the target of the specific fishing method employed, representing a widespread environmental problem (Hall, 2000). However, accurate data on it are of essential importance for the stock assessment of these species, but currently data are still poor or even unreported, due to the fact that most of juvenile tuna by-caught are discarded or sold at local markets (Romagny *et al.*, 2000).

YFT can grow to sizes that are much larger than those typically caught with FAD sets. The potential yield of YFT population would be much higher if the harvest of small fish will be reduced, improving their status and mitigating the current overfishing (ISSF, 2012).

Fig. 5_Underwater drifting FADs nets.

In addition, juveniles overfishing, as well as adults overfishing, might reduce the spawning stock biomass (SSB) or bring stocks under the Maximum Sustainable Yield (MSY).

For instance, Pacific and Atlantic Ocean stocks that are experiencing high fishing mortality seem to display similar value below the SSB_{MSY} (Dagorn *et al.*, 2013).

FAD-based fishery could also affect tropical tuna ecology and behaviour, driving fishes in areas probably not advantageous for their feeding; constituting what is described as "Ecological Trap", leading fishes in poor-quality habitats (Marsac *et al.,* 2000). In this scenario, population productivity could be reduced as a consequence of maladaptive habitat choice (Schlaepfer *et al.* 2002). On the contrary, natural floating rubble, usually tend to be accumulated in confluent areas, with high nutrients and forage rate (Bromhead *et al.*, 2003). Thus, in general these animals would follow the movement of the natural log to feed, but due to the increasing presence of dFADs in the sea, they are forced into different areas (Marsac *et al.,* 2000).

Finally floating objects can also impact coastal ecosystems when hit by waves and crashed up on coral reefs, causing physical damages on corals (Dagorn *et al.* 2013).

1.3 Discrimination of Juvenile Yellowfin

Among the different species caught accidentally around FADs, small and immature tropical tunas represent the largest group in terms of number of individuals (Amandè et al., 2010). For instance, in Atlantic Ocean the number of tuna juveniles caught as by-catch, between 2003 and 2007, was estimated in 751.000 individuals (Amandè et al., 2010), and most of them were thrown back to the sea (dead or alive), or were traded in local markets (Romagny et al., 2000).

It appears clear how further researches in this field are necessary, to improve scientific knowledge on the growth and natural mortality of juvenile tunas, in order to understand the real impact of the FAD-based fishery on the ecology and biology of these species (Bromhead et al., 2003).

Although adult BET and YFT present different external characteristics which allow easy discrimination (e.g., eyes diameter, body coloration, marks) these differences are less evident when the specimens are below a certain size (<40 cm FL) and even more when specimens are frozen (Fig. 6). Furthermore, species misidentification at their post-larval and early-stage juveniles could be caused by geographical variation in their morphological features (Chow and Inoue, 1993).

Fig. 6_juveniles yellowfin (left) and bigeye (right)

Thus, very high-quality taxonomic skills are required to discriminate between them, in fact it has been underlined that misidentification by fishery-data collectors can be as high as 30% (Chow and Inoue, 1993). An important internal characteristic that allows to discriminate between the two species is the liver morphology (Itano, 2004): asymmetric for YFT without striations vs. symmetric for BET with surface striated (Fig. 7). However during sampling activities is not always possible to work with the whole fish, vanishing any possibility to identify these species using this internal character.

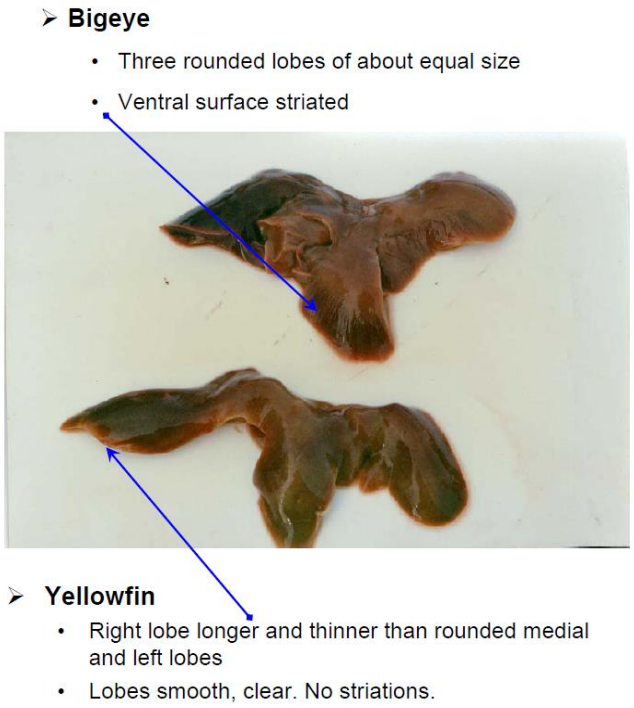

Fig. 7_liver morphology

The specific identification of these tuna is fundamental to improve data about their catches and distribution and provide more accurate information on the real mortality of juveniles

that affects negatively their yield per recruit. Among the different methodologies developed to overcome misidentification issues, DNA-based identification methods represent a very accurate and precise tool because DNA can be extracted by almost all types of samples (fresh, frozen, canned, dried tissues) (Teletchea et al., 2009). Many different DNA markers have been used for tuna and tuna-like species identification, but the analysis of the mtDNA regions, both using restriction endonucleases or through the nucleotide sequence analysis, is the preferred method to identify individuals (Billington and Hebert, 1991).

However, a specific gene marker has to be selected appropriately and detailed knowledge of the DNA sequences from target species is required before setting-up the methodology and validate the final assignation, otherwise species misidentification can occur, especially when within-species and between-species genetic distance are of similar magnitude (Chow et al., 2006).

Different gene regions in mitochondrial DNA or in nuclear DNA have been proven more useful than other to discriminate among these species. For instance the restriction fragment length polymorphism (RFLP) analysis using the cytochrome b (cytb) and 12s RNA (12Sr) gene fragments failed in the attempt to discriminate between tuna (Chow and Inoue, 1993). Instead a possible rapid molecular instrument to the discrimination between different individual is the multiplex-based PCR.

In fact, few multiplex-based analysis were tested to discriminate between different species (i.e, *Thunnus thynnus* from *Sarda sarda*, Lockley and Bardsley, 2000); Michelini et al., (2007) tested a triplex-polymerase chain reaction of the ctyb sequences to discriminate between three tuna species, with successful result.

However, using restriction endonucleases or nucleotide sequence analysis, the flaking region between ATPse6 and cytochrome oxidase subunit III genes (ATCO) was verified as one of the most performing marker in discriminating the eight tuna species (Chow and Inoue 1993).

Hebert et al., (2003) suggested the use of 648 base pair (bp) portion of the mitochondrial DNA gene cytochrome oxidase subunit I (cox1) as universal marker to differentiate a vast spectrum of animal species (DNA barcoding). Therefore, COI has been commonly used for discriminating among fish species, including tuna and tuna-like species, even if many issues have been pointed out in using a single genetic marker for all fish species (Ward et

al., 2005). Moreover, Alvarado-Bremer et al., (1997) have underlined as the mtDNA Control Region (CR) is not capable to detect differences between related species of the genus *Thunnus.*

Thus, Chow et al., (2006) detected no evidence of genetic differences between BET and YFT, through the use of the nuclear genetic marker ITS1 (nuclear fragment rDNA first internal transcribed spacer).

Instead, Vinas and Tudela, (2009) employing a combined approach between this marker together with the mitochondrial genetic marker CR have shown promising results in discriminating the 8 species of Thunnus in general, and YFT and BET in particular.

1.4 YFT population structure and current management

The International Union for Conservation of Nature (IUCN) classify YFT as *Near Treatened* at the global scale, underlining the importance of taking urgent management measures for the conservation of this species to avoid any possibility of populations collapse. Moreover, the Convention on the Law of the Sea (2012) has classified YFT as highly migratory species in its Annex I, underlying how the nations should cooperate in its conservation.

The monitoring and management of YFT populations, as for all tuna and tuna-like species, are under the jurisdiction of four independent Regional Fisheries Management Organizations (RFMOs) (Fig. 8). The West and Central Pacific Fisheries Commission (WCPFC), the Inter-American Tropical Tuna Commission (IATTC), the International Commission for the Conservation of Atlantic Tuna (ICCAT) and the Indian Ocean Tuna Commission (IOTC).

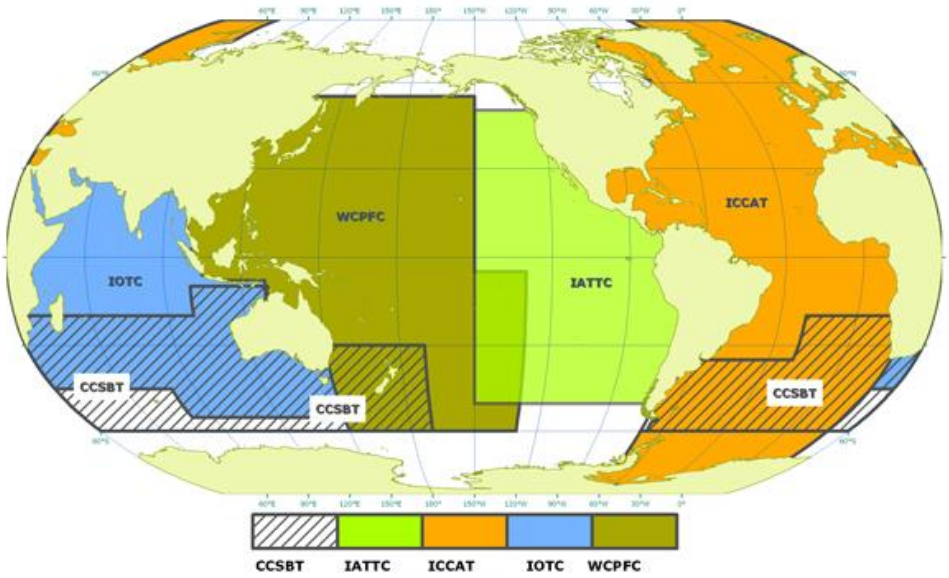

Fig. 8_Tuna worldwide RFMOs

The life-history traits of this species, such as their high mobility and dispersal capacity, extraordinary fecundity and large population sizes, have reduced the possibilities to discriminate clearly its stocks boundaries, leading to consider panmitic populations within each ocean. In fact YFT populations are actually monitored and managed independently by each of the four RFMOs, and management strategies and models often lie on stock assessments that are systematically distorted by insufficient fishery and population biology data.

Understanding the spatial structure of YFT populations is a central key to the assessment and management of these economically and socially important species. The first attempt to delineate YFT stock structure date back to Suzuki (1962), using TG2 blood group antigen in samples of Pacific and the Indian Oceans, which showed no differences between samples.

Many other genetic studies employing both mtDNA and nDNA markers have been conducted to investigate yellowfin population structure. Ward *et al.,* (1994), failed in identifying possible local structuring within the Pacific Ocean using five allozymes. Instead Appleyard *et al.,* (2001), analysed samples from the central / western Pacific Ocean and the eastern Pacific Ocean, employing 5 microsatellite loci (SSRs - simple sequence repeats). Their study showed significant heterogeneity at one of five microsatellite loci

analyzed, although not enough differentiation to prove the presence of multiple stocks within the Pacific Ocean.

Similar results have been showed by Diaz-Jaimes and Uribe-Alcocer, (2006), through the analysis of seven different SSR loci. They did not detect any signs of differentiation within the Pacific Ocean confirming the absence of YFT local partitioning.

The first worldwide genetic study on YFT population structure was made by Ely *et al.*, (2005), using the mtDNA control region CR. Their results present very low differentiation, suggesting the presence of a single large panmictic population at the global scale. However, recent genetic studies in Indian Ocean have suggested that YFT could have a more fragmented population structure than what assumed in its assessment and management process so date. Dammannagoda *et al.*, (2008), suggested the possibility that genetically discrete yellowfin tuna local sub-populations may be present in the north western Indian Ocean, detecting significant genetic differentiation among sites for mitochondrial DNA ($\Phi st = 0.1285$, $P < 0.001$) and at two microsatellite loci ($Fst = 0.0164$, $P < 0.001$ and $Fst = 0.0064$, $P < 0.001$). This issue has been also corroborated by Swaraj *et al.*, (2013) with the proposed presence of at least three genetic stocks of YFT in Indian waters.

These discordant information underline the need to dig deeper into the YFT stock structure, according to the fact that if the stock assessment and management rely on invalid assumptions, there will be a failure of the conservation and the optimal economic use of this resource. Thus, if populations are distinct, some of them could locally collapse and management measures might focus on the wrong populations.

2. Aim of the study

This thesis was developed within the framework of an ongoing collaboration between the University of Bologna and the French Institute of Research for the Development (IRD). The general aim of the shared research is to assess the global population structure and the maternal effects of yellowfin tuna (*Thunnus albacares*, YFT).

According to the fundamental role of tropical tunas for the marine pelagic ecosystem, and their consistent importance as a commodity for the global economy, in this thesis two main science-based goals were identified:

- ✓ To discriminate between juveniles of yellowfin and bigeye, which can be easily misidentified during the sampling process, through the analysis of the nucleotide sequence variation of ATCO fragment (mtDNA).

- ✓ To preliminary investigate the YFT population structure within and between Atlantic and Pacific Oceans through the analysis of genetic variation at eight microsatellite loci and assess the occurrence of barriers to the gene flow between Oceans.

The importance of this preliminary survey is underlined by the fact that nowadays the YFT population structure is still poorly understood and it has been considered to consist of a single panmictic spawning population for the purposes of stock assessment and management. In fact, wrong assumptions on tuna population structure might conduct to the over-exploitation of some populations with consequent serious food security and economic problems.

3. Materials and Methods

3.1 Sampling design

The sampling design was planned to solve the population structures of YFT between Atlantic and Pacific populations and evaluate if there would be factors that could determine a genetic barrier to gene flow among them. For this purpose we analyzed multiple samples from both Oceans in order to quantify the infra-and intra-Oceanic genetic differentiation.

The Food and Agriculture Organization (FAO) divided the Oceans in different fishing areas called "FAO Major Fishing Areas" (Fig. 9). We have collected one sample (40 individuals) from each Atlantic and Pacific fishing area (Tab. 1), 77-2-Y (ECPO – East/Central Pacific Ocean), 71-2-Y (WCPO – West/Central Pacific Ocean) and 41-1-Y (WCAO – West/Central Atlantic Ocean), 34-2-Y (ECAO – East/Central Atlantic Ocean).

Most of the YFT specimens were obtained within a collaborative framework with international research groups. Samples from 77-2-Y and from 41-1-Y were muscle tissue samples, while the 71-2-Y and 34-2-Y were constituted by fin clip tissues. Sampling in area 77-2-Y was carried out by Sofia Ortega (National Polytechnic Institute - Mexico), 71-2-Y area was sampled by Jeff Muir (Hawaii Institute of Marine Biology of the University of Hawaii) and 41-1-Y samples were obtained by Freddy Arocha (Universidad de Oriente – Venezuela) (Tab. 1).

Instead, I carried out personally the sampling in the 34 FAO Fishing Sub-Area, during a research period in Abidjan, the Ivory Coast capital (Guinea Gulf - ECAO) in February/March 2014, hosted by the CRO (Centre de Recherches Océanologiques), inside Abidjan harbour, where we cooperated daily with their staff during sampling sessions. Sampling was made on board of two Spanish puirse seiner anchored in the Abidjan port, where the fishing coordinates of each tank of the vessel were identified. Thanks to this recorded info, we selected the ones that showed the maximal geographical distance from each other and we proceeded with the selection of individuals and morphometric measurements.

Species identification of YFT and BET was made thanks to the help of CRO scientific staff, although the difficulty due to frozen specimens, they were able to detect and separate

both species using morphometric characteristics (e.g., eyes diameter, body coloration, marks). It was impossible to use the liver as morphometric identifier character, because the specimens sampled on board were still frozen and then impossible to dissect.

From each specimen obtained, we selected and sectioned a piece of the pectoral fin, with sterilized instrument to avoid contamination. The collected tissues were transported inside the CRO dry-lab, where all samples were stored in 95% ethanol tubes.

In order to avoid DNA degradation, all samples obtained were immediately checked and stored in -20C° upon receipt.

The size of specimens sampled in this thesis was within a range of 35-55 cm. Some studies on tuna behavior noticed that smallest organisms tend to remain close to the areas where they were born, thus increasing the likelihood of detecting potential local biological populations (Kimely and Holloway 1999).

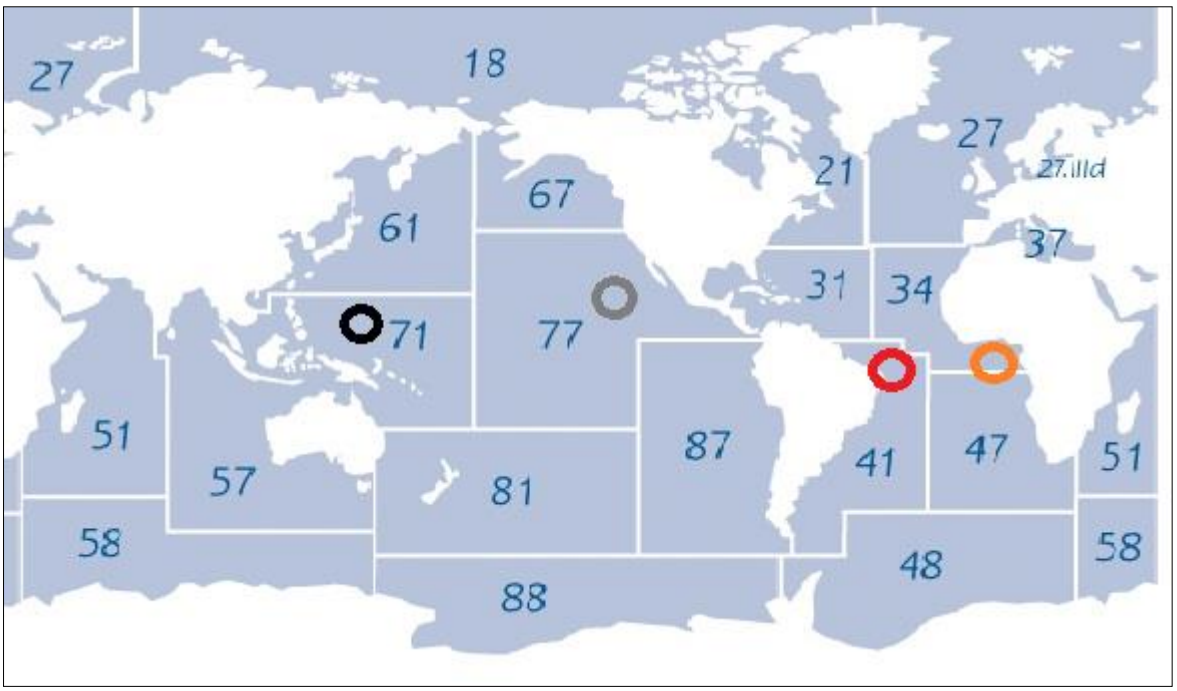

Fig. 9_F.A.O Fishing Areas: Colored circles in the map represents different sampling areas; black (71-2-Y), grey (77-2-Y), red (41-1-Y) and orange (34-2-Y)

3.2 DNA extractions

A total of 160 YFT individuals were collected and analyzed in this study. Total genomic DNA (gDNA) was extracted from 20 mg of tissue using the Invisorb Spin Tissue Kit, or a filter-based protocol (Promega Kit).

With the Insorb Spin Tissue Kit, tissues were lysed at 52C° and continuosly shaken and left in thermostated bath overnight. Few tests were made during lysis phase, testing different time exposure in order to detect the most profitable lysation time range (6-8 hours). Lysis was performed adding 400 µL of Lysis Buffer G and 40 µL of Proteinase K. The lysate was transferred into a new test tubes (excluding pellet phase) adding 40 µl of RNAse, followed by 200 µl of Binding Buffers T. Each lysate was then transferred to an Insorb Spin Filter and gDNA was absorbed onto the membrane as the lysate was drown trough by centrifugal force as contaminants passed through. Remaining contaminants and enzyme inhibitors were efficiently removed after two washing steps using 500 µL of Wash Buffer, followed by 1 minute of centrifuge for each one, while the gDNA remained bound to the membrane. The final gDNA extraction phase, forecast DNA eluted from the Spin Filter, provided the use of 80 µl of Elution Buffer or water, to increase extracted gDNA quantity.

This final step was repeted twice.

The Promega Extraction Kit provided an incubation initial step at 55 °C for (16/18 hours) in wich 275 µL of Digestion Solution Master Mix were added to the tissues. Following incubation phase, 275 µL of the Wizard SV Lysis Buffer was added in each test tube and strongly mixed, pipetting several time. Vacuum Mainfold was set adding the Binding plate in the Vacuum Mainfold Base. Thus the lysates were transferred into the Binding plate wells and applied vacuum until all the lysates passed through the binding plate.

Contaminants and products that were not of DNA origin, were removed during three washing steps, adding each time 1 µL of Wizard SV Wash Solution. When all the wells were emptied, in order to keep the membrane matrix dry, it was necessary to submit vacuum for 6 more minutes.

After turning of the vaccum, the 96-Well Plate was located in the Manifold Bed, and the vacuum Mainfold Collar was placed on top. For the final step it was added 250 µL of

Nuclear-Free Water to each well of the Binding plate and incubated for 2 minutes at room temperature.

Finally, DNA extractions were checked on 1% agarose gel and stored at -20C°.

Tab_1_Samples collection and characteristics: F.A.O= FAO fishing areas; G.Area= Geographic area; F.Gear= Fishing gear

FAO	Species	G.Area	Latitude	Longitude	F.Gear	Year	Tissue	Sampler
71-2-Y	Yellowfin	WCPO	3°24'00.0"S	*166°21'36.0" E*	Purse seiner	2013	fin clips	Jeff Muir
77-2-Y	Yellowfin	EPO	25°15'00.0" N	*114°09'00.0" W*	Purse seiner	2013	White muscle	Sofia Ortega
41-1-Y	Yellowfin	WCAO	11°14'24.0" N	*65°00'00.0"W*	Tagging cruise	2014	White muscle	Freddy Arocha
34-2-Y	Yellowfin	CEA	11°07'48.0" S	*11°36'36.0"E*	Purse seiner	2014	Fin clip	Carlo Pecoraro

3.3 Species identification (ATCO barcoding)

Yellowfin and bigeye juveniles can easily be misidentificated especially after being frozen in purse seiner wells, were they lost most of the discriminatory features. Thus, only skilled operators can differentiate both species through morphometric characteristics and phenotipic markers.

In order to avoid misidentification between the two species made by other operators, mtDNA genetic identification was performed. Presumed samples of yellowfin tuna from 71-2-Y area (66 specimens) were choosen for genetics analysis, and compared with bigeye tuna sample of 34-2-B area (5 specimens) collected by trained researchers.

Among the potential and different mitochondrial molecular markers used for species identification and after an accurate analysis of the existing literature we chose the ATCO fragment. This particular mtDNA marker is the only one able to identify the differences between related *Thunnus* species, due to its high polymorphism (Chow and Inoue, 1993). Additional sequences of target mtDNA were retrieved from Genbank (www.ncbi.nlm.nih.gov/genbank) and compared with the obtained ones.

The primers used in this study were designed from the consensus sequences between human (Anderson et al., 1981), Xenopus (Roe et al., 1985) and salmon (Thomas and Beckenbach, 1989), targeting flanking region between ATPase6 and cytochrome oxidase subunit III genes, called ATCO, the nucleotide sequences were; (L8562) 5'-CTTCGACCAATTTATGAGCCC-3' and (H9432) 5'-GCCATATCGTAGCCCTTTTTG-3' (Chow and Inoue, 1993).

Gene amplification was carried out by a 50 µL reaction mixture; 1x GoTaq Flexi Buffer (Promega) 1.5 mM MgCl2 , 0.4mM dNTPs , 1uM each primers, 2 units of *Taq* Dna Polymerase (Promega), 3-4 µL of DNA template. The reaction mixture was pre-heated at 94°C for 2 minutes followed by 35 cycles of amplification (93°C for 1 min, 52-57,8°C for 1 min and 72°C for 45 sec) after all a last step of 72°C for 8 min (Fig. 10).

Success of amplification was tested by electrophoresis on 2% agarose gel.

Sequencing was performed by a commercial sequence service provider (Macrogen Europe, Amsterdam, Netherlands) with an ABI3730XL and the same primers used for the PCR amplification.

ATCO sequence obtained were assessed and analysed by MEGA 6.0 (Tamura *et al.,* 2013), the sequences were aligned and compared to build a neighbour-joining tree, using Tamura-Nei distance with 1000 bootstrap replicates.

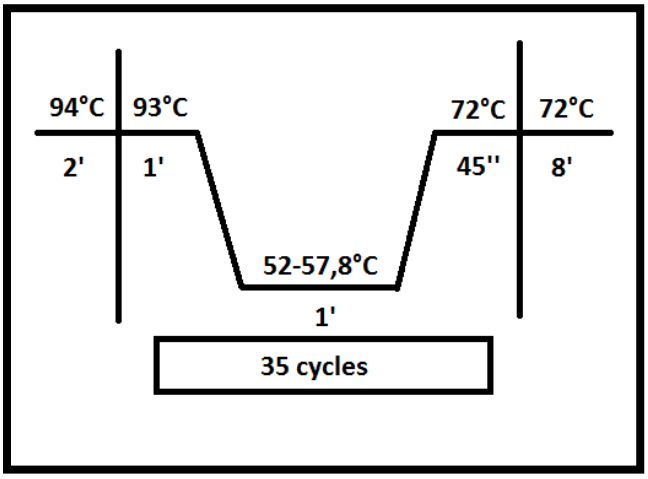

Fig. 10_PCR cycling condition for ATCO amplification.

3.4 Microsatellites loci and genotyping

To investigate genetic population structure and infer possible gene flow between Atlantic and Pacific samples, we selected a panel of eight microsatellite loci, recently isolated for YFT (Antoni *et al*., 2014). Among the published markers eight microsatellites were chosen having the same annealing temperature (62 C°) in order to perform multiplexed amplification.

The QIAGEN MULTIPLEX kit was used for this purpose, this kit is specifically designed for microsatellite analysis, being able to minimize stuttering and prevent large allele drop-out errors. Two multiplex reactions (Multiplex 1 and Multiplex 2) with four loci each were optimized assessing PCR amplification conditions with preliminary tests using few individuals.

PCR reactions were performed in a T-Gradient thermocycler (Biometra), for each reaction (10 μL total volume) 2 μL of DNA were amplified with the following concentrations:

- Master mix Qiagen 1X (5 μL),
- Qiagen Water (2 μL),
- Primers Mix (1 μL): Primers Forward and Reverse 0.20 μM each for every locus. Forward primers labelled as described in (Tab. 2).

The Master mix contains pre-optimized concentrations of HotStarTaq DNA Polymerase, $MgCl_2$, dNTPs and Polymerase buffer.

Use of a master-mix format reduces time and handling for reaction setup and increases reproducibility by eliminating many possible sources of pipetting errors. Moreover no polymerase activity occurs at room temperatures, but the enzyme is activated by a 15-minute at 95°C incubation step, this prevents the formation of improper products and primer-dimers.

The cycling conditions applyed were those for standard multiplex PCR recommended by Qiagen. The temperature profile consisted of an initial heat activation step at 95 °C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 90 seconds and extension 72°C for 90 seconds. The final extension was set at 72 °C for 10 minutes.

Yield of multiplex amplification was assessed by electrophoresis on 2.5% agarose gel.

Tab. 2_Microsatellite panel: Locus: name of the locus; Repeat: motif repeat; Primer sequence: sequence composition; AT°:Anniling temeprature; Label: sample labeling dyes

Locus	Repeat	Primer sequence	AT°	Label
Multiplex 1				
YT4	(CA)	F: CGACTCCTGGCCTTGGTTA	62	PET
		R: GGAGAACAGGCCCTGAATTT		
YT84	(GATA)	F: TTACTTACTGCCGCCTACTGG	62	NED
		R: AAGTTGGAACCAGAGAACCATT		
YT87	(CATA)	F: CGAGATACGCGAAGGACAA	62	6FAM
		R: CAGTGGCATTCTTGGCAATAG		
YT111	(CA)	F: CGTCCAGTAGTGACGAGGAT	62	VIC
		R: GACTGTATCTGCTGAAACCAAA		
Multiplex 2				
YT121	(CA)	F: GAAGGCTCTTCAGCTGGTTG	62	PET
		R: AGTTGTGGCACGATTGTCTG		
YT29	(CA)	F: TGCGTCTTTGAATGGCTAA	62	NED
		R: GGTTGTCAGTCAGGACAAAGG		
YT92	(GA)	F: CCTCAGCCAAGGTGAGAAGA	62	6FAM
		R: CGCTCGCTACTACTCACTCCA		
YT12	(CA)	F: GAGATGTGGCTTCCTCCAAA	62	VIC
		R: GGCGATTTATGGCATCAGTT		

Specimens which amplication were unsuccesful were re-amplified with single locus reaction following conditions described in Antoni *et al*., (2014).

PCR reactions were conducted in a 10 µL total volume, with 2.2 pmol of the forward and reverse primers, 8.4 nmol of MgCl2, 1.1 nmol of dNTPs, 0.28 U of GoTaq Flexi DNA Polymerase (Promega), 2 µL of gDNA and 1 X of buffer. The amplification cycle consisted in an initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 sec, 62°C for 30 sec, 72 °C for 45 sec, and a final extension at 72 °C for 10 min.

The amplicons sizing was performed by a commercial provider (Macrogen Inc, Seoul, Korea), using the GS-500LIZ size standard. The electropherogram files obtained were imported into the software PEAK SCANNER 1.0 (Applied Biosystems), then the microsatellite alleles were sized and scored to individual genotypes (Fig 11).

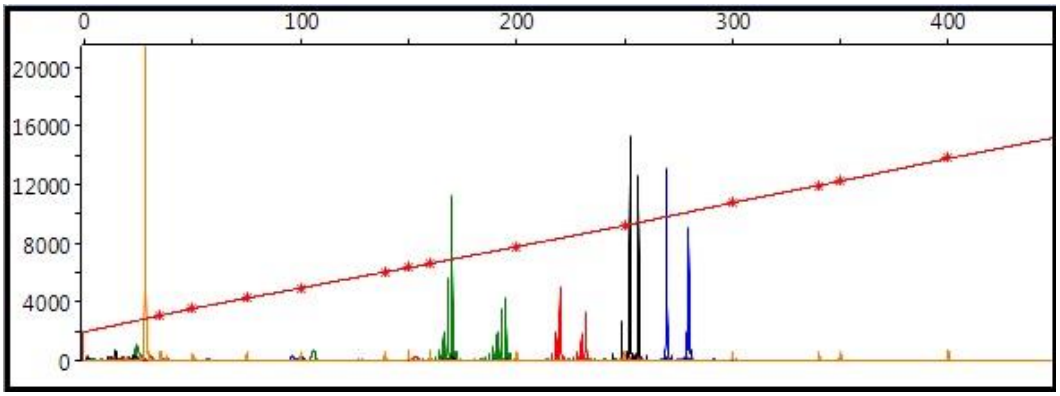

Fig. 11_Example of Multiplex 1 profile

3.6 Binning

Binning is the process of assigning a integer number to a decimal allelic value obtained during the allele calling phase. This part of the data processing is crucial to avoid sistematic errors, mainly related to the fact that fragments lengths raw data are continuous and provided with two decimal that need to be transformed into integers representing very distinct allelic classes.

Errors can be due to the fact that the alleles sizing is influenced by migration fragment speed, which is strongly related to the GC content of the fragment (Wenz *et al.*, 1998). Length fragment estimation may also vary from experiment to experiment due to the stochastic variation of the environmental temperature (Rosenblum *et al.*, 1997). Induced errors during the binning phase can have repercussions on the estimate i of allele frequencies and falsify results or parameters such as expected and observed heterozygosity (Amos *et al.*, 2006).

For this study we used TANDEM (Matschiner and Salzburger, 2009), a specific software to optimize the binning phase and to obtain binned value as reliable as possible from the row dataset. TANDEM operates through an algorithm that fills a gap of the microsatellite workflow by rounding allele sizes to valid integers, depending on the microsatellite repeat units. The module repeat can be either established on the basis of the data observed by the program itself or set manually. During this analysis we performed manual setting for TANDEM selecting for each locus the right module repeat according to the loci motifs.

3.7 Microsatellite dataset analysis

We used MICROCHECKER ver. 2.2.3 (van Oosterhout *et al.,* 2004) to identify scoring errors as null alleles, stutter and large allele drop-out.

The program permit the identification of genotyping errors due to nonamplified alleles (null alleles), short allele dominance (large allele dropout) and the scoring of stutter peaks. MICRO-CHECKER estimates the frequency of null alleles and can adjust the allele and genotype frequencies of the amplified alleles. Furthermore, it can discriminate between inbreeding and wahlund effects, and Hardy–Weinberg deviations caused by null alleles.

Microsatellite polymorphism estimates were calculated using the GENETIX 4.05.2 Software (Belkhir *et al.,* 1996) as the mean number of alleles per locus, allele frequencies, allelic range and observed (Ho) and unbiased expected heterozygosity (HE).

The probabilities of Hardy-Weinberg equilibrium for each locus for each population were estimated using the exact probability test, carried out by the program GENPOP on line version 3.4 (Raymond and Rousset, 1995).

We used Jackknifing over loci, carried out by the software GENETIX, to analyze single-locus effects of the 8 microsatelites loci using the Weir & Cockerham's F-statistics estimators.

Mean and single-population estimation of allelic richness per locus were obtained with the software FSTAT 2.9.3.2 (Goudet, 1995).

The use of ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010) software was necessary for the calculation of pairwise Fst values, with 10,000 permutation and 0.01 significance level as settings, and for the analyisis of the molecular variance (AMOVA) among arbitrary group, (among groups; among populations within groups and within populations).

PCoA (Principal Coordinates Analysis) based on matrix of pairwise Fst values was generated using the program GENALEX v.6.41 (Peakall and Smouse 2006). This program allows through plots to detect the percentage of variability associated to a spatial differentiation between geographical samples considered.

STRUCTURE 2.3.4 software (Pritchard *et al.,* 2000) is one of the most interesting and powerful tool able to describe genetic relationships among populations. This program uses Bayesian clustering algorithm to infer genetic divergence among samples and allows to extrapolate the number of genetic clusters in the dataset without making any *a priori* assumptions on the species characteristics.

The determination of the most likely number of homogenous population (K) and estimation of the posterior probability represent two fundamenal steps for the correct setting of STRUCTURE algorithm. K value is based on the best adaptation of the genotypic data under the conditions of slightest divergence from HW equilibrium and minimal degree of linkage disequilibrium. The posterior probability depends on the probability of each individual's genotype to belong to each defined cluster.

Barplots obtained presents on the y-axis the percentage value of membership to a given cluster for each individual.

Markov Chain Monte Carlo (MCMC) simulated approach generate posterior probability. The parameters used in this study were: Length of burning period= 100.000, Number of iterations= 300.000, Number of K from 1 to 10 each assessed with 5 iterations.

Admixture model was chosen as ancestry model with Correlated Allele frequency as allele frequencies model beacose this configuartion is considered the best in cases of discrete population structure (Falush et al. 2003).

The Admixture model is one of the most recommended in genetic studies as it is very flexible and able to consider the complexity of real populations. This model supposed that specimens originated from the admixture of K ancestral parental populations and have inherited part of their genetic makeup. Indeed, the most important parameters for this model are the ancestry coefficients, calculated for each individual in the sample.

The correlated allele frequency model, supposed that different populations are likely to be similar probably due to migration or shared ancestry. In this way, this model may provides high power to detect distinct populations that are particularly closely related.

Moreover, LOCPRIOR function was set as a prior population information model, using the sampling location as prior information, provide more accurate inference of population structure.

Hubisz et al. (2009) has invented a new model that leads STRUCTURE to use the location information if the data suggest that the locations are informative. In fact, this new model, is particularly useful when the genetic signal is too weak as not to permit a proper individuals clustering. Finally, STRUCTURE results were analyzed with STRUCTURE HARVESTER (web version 0.6.94 July 2014). This software permit to calculate the likelihood function of the data for each K (LnP(K)), its standard deviation over the replicates (Stdev LnP(K)), the first (Ln'(K)) and second (Ln"(K)) rate of change of

LnP(K) with respect to K. Mean of absolute (Ln”(K)) value divided by the Standard deviation LnP(K), permit the estimation of the (ΔK) fundamental to apply Evanno's theory.

There are two types of errors that can occur during analysis: the type I error is the probability to reject the null hypothesis even if true, better know as α (called the significance level of the test), and the error type II to accept the null hypothesis that should be rejected, indicated by β.

When performing multiple tests simultaneously increases the likelihood of making errors of type 1. In order to avoid this error, one strategy is to correct the alpha level when performing multiple tests by adjusting the p-value. Making the alpha level more strict will create less type I errors, but could increase the possibility to underestimate real effects and increase type II errors. The correction model most used is the sequential Bonferroni correction (Rice, 1989), this method permit with scraps sequentially decreasing to correct the P value according to the number of test. This technique is very conservative and is strongly criticized in several ecology studies (Moran, 2003).

5. Results

5.1 ATCO barcoding

We obtained 71 sequences of the mitochondrial DNA fragment ATCO, of which five from to specimens morphologically indentifies as BET and 66 as YFT. We added two homologous YFT sequences retrieved from GenBank (KM055398.1 and AF115278.1), one of BET (AF115274.1) and one of *Katsuwonus pelamis* (GU256527.1), being the last one used as outgroup.

The final dataset consisted of 75 sequences of 759 bp where 109 variable sites (V) and 15 informative sites (Pi) were detected.

The YFT ATCO sequences obtained in this study were totally concordant with those downloaded from GenBank with no variable sites and the same situation was observed for BET sequences. All individuals analyzed were correctly identified and the Neighbour-Joining tree obtained, using Tamura-Nei distance method (Fig. 12), very clearly discriminate the two species YFT and BET with very high bootstrap values, (95% and 99% for YFT and BET clades respectively), supporting the use of ATCO as molecular marker for these tuna species identification.

For this reason it can be asserted that there is a complete congruence between the molecular and morphological identification of the specimens analyzed.

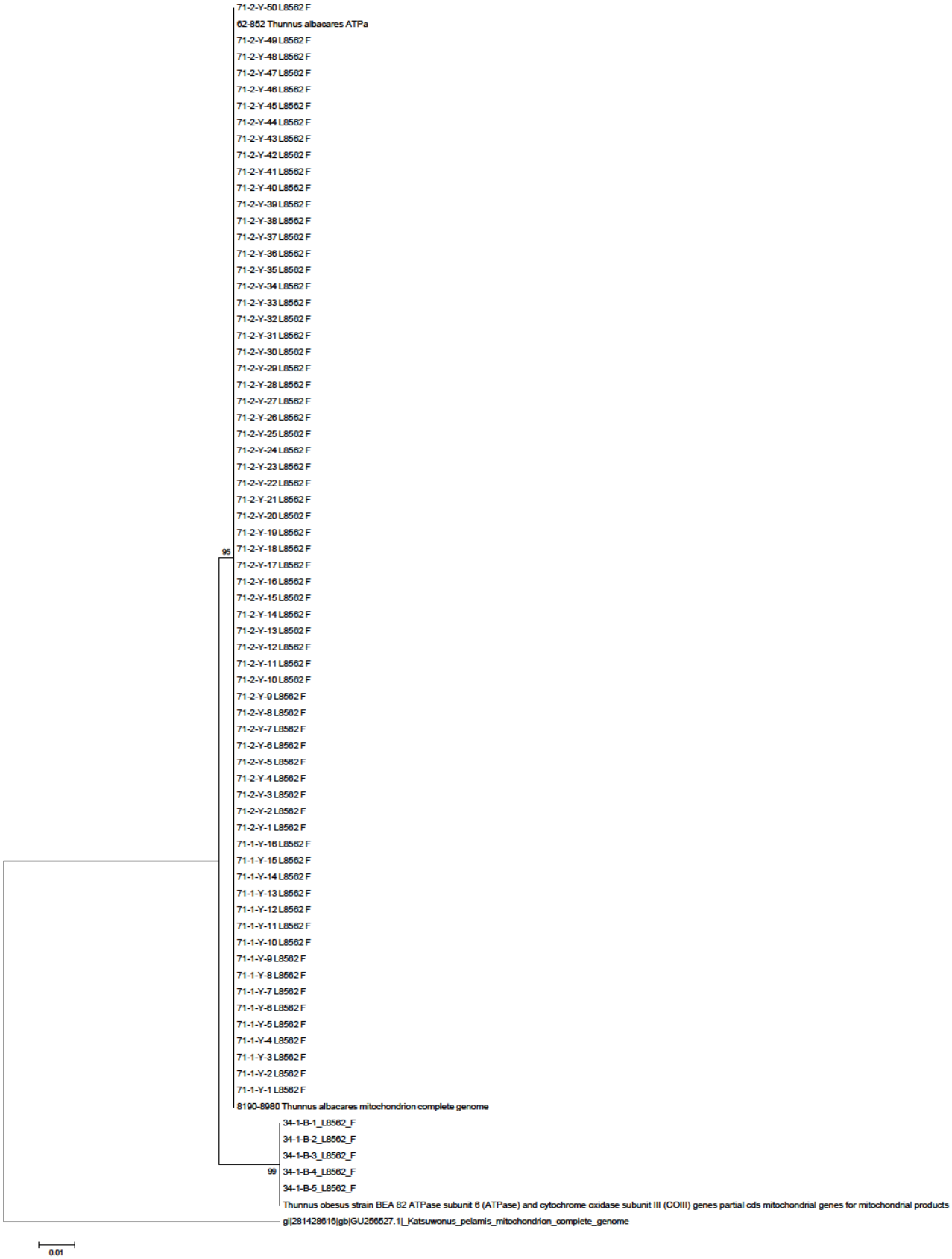

Fig. 12_Neighbour-Joining tree, using Tamura-Nei distance method, based on ATCO sequences of Yellowfin and Bigeye tuna. Skipjack Tuna is considered as outgroup.

5.2 Genetic differences between Pacific and Atlantic specimens.

PCR amplification failures were observed only at the loci YT84 (multiplex 1) and YT29 (multiplex 2) and for only a few specimens of the samples 71-2-Y and 41-1-Y (for a total of 15 individuals re-amplified in single locus).

MICROCHECKER results showed possible presence of null alleles at some loci, but no stuttering problems or large allele dropout were detected. Possible presence of null alleles, revealed by an excess of homozygotes was observed in samples 71-2-Y (at loci YT12, YT29, YT84), 77-2-Y (at loci YT12, YT84), 34-2-Y (at loci YT84, YT12) and 41-1-Y (at loci YT12, YT29, YT84) as summarized in Tab. 3.

Tab. 3_MICROCHECKER Scoring errors test: NA= Null allele presence, ST= Stuttering LD= Large Allele Dropout issue.

Scoring errors	71-2-Y	77-2-Y	34-2-Y	41-1-Y
YT4				
YT84	NA	NA	NA	NA
YT87				
YT111				
YT12	NA	NA	NA	NA
YT29	NA			NA
YT92				
YT121				

High polymorphism was detected in all loci in agreement with literature data. Locus YT92 showed the lowest number of alleles while YT111 the highest one, respectively 8 and 26 (Fig. 13 a). Slight differences were observed in number of alleles and in allele size range at loci YT12 and YT87 between data obtained in this study and previous ones (Antoni *et al.,* 2014). However, the samples used by Antoni *et al. (*2014) were collected from the Ghana coast. The comparison of the number of alleles of the samples from the same geographical area (Fig 13 b) revealed that there are similarities in all the loci assayed except for the locus YT12 that has a lower number of alleles.

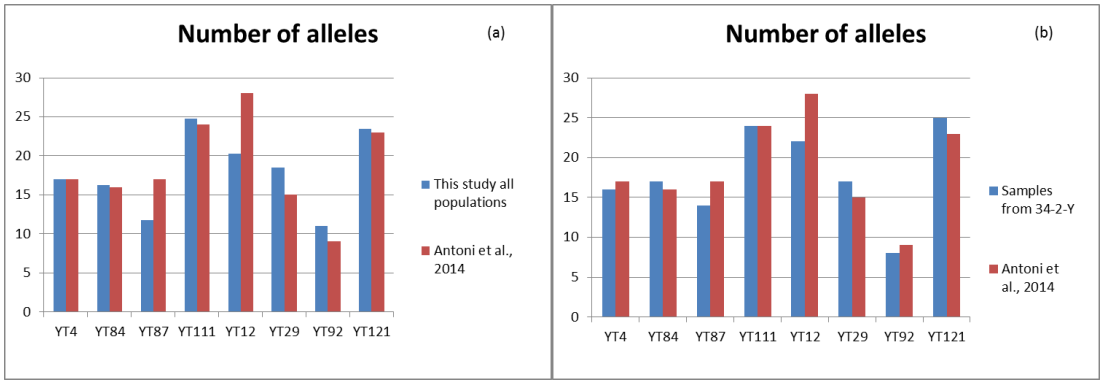

Fig. 13_Total number of alleles obtained in this study compared with Antoni *et al.*, (2014) data (a); comparison between samples of area 34-2-Y and Antoni's data (b).

Allelic richness (Ar) provides a measure of the number of alleles standardized to the smaller sample size, in order to reliably compare different samples with different sample size. Our dataset was balanced with the samples with the lower number of specimens genotyped (36), and therefore Ar values obtained shown similar values to the number of alleles per locus (Na, Tab. 4). All four geographic samples analysed present very similar values of polymorphism for each locus, mean values ranging from 17.625 (71-2-Y and 77-2-Y) to 18.375 (41-1-Y).

Total allelic frequencies are reported in Appendix 1.

Tab. 4_Number of alleles (An) on the left, compared with Allelic richness (Ar) on the right.

Na	71-2-Y	77-2-Y	34-2-Y	41-1-Y	Ar	71-2-Y	77-2-Y	34-2-Y	41-1-Y
YT4	16	18	16	18	**YT4**	15.663	17.580	15.681	17.570
YT84	17	16	17	15	**YT84**	16.300	15.590	16.373	14.886
YT87	10	11	14	12	**YT87**	9.963	10.690	13.663	11.773
YT111	24	25	24	26	**YT111**	23.255	24.128	23.453	24.871
YT12	21	19	22	19	**YT12**	20.359	18.437	21.553	18.446
YT29	17	19	17	21	**YT29**	16.871	18.289	16.581	21.000
YT92	13	11	8	12	**YT92**	12.282	10.582	7.890	11.300
YT121	23	22	25	24	**YT121**	22.436	21.271	24.460	23.318
TOT	17.625	17.625	17.875	18.375	**TOT**	17.141	17.071	17.457	17.896

The observed (Ho) and expected (He) heterozygosity per locus and overall in each population samples are reported in Tab. 5. The mean observed heterozygosity ranged from

0.7355 (71-2-Y) to 0.8219 (34-2-Y) instead the lower Ho value 0.3590 was detected at locus YT12 in sample 71-2-Y, while the highest at locus YT4 in sample 34-2-Y.

Deviations from HWE were initially detected at loci YT4, YT84, YT111, YT12 and YT29, but after Bonferroni correction only loci YT12 (in all samples) and YT84 (only in sample 41-1-Y) were still significant. This significance is likely, related to high positive F_{IS} values, suggesting deficiency of heterozygous genotypes as already highlighted with the MICROCHECKER assessment.

Tab. 5_Genetic diversity estimates: Single-locus and mean values are given. N= number of samples analysed; Na= number of alleles; As= allelic size range; Ar= allelic richness; He (n.b)= expected non biased heterozygosity; Ho= observed heterozygosity; Fis= inbreeding coefficient. Bold and underlined values indicated a significant HW disequilibrium (P<0.05); Significance after sequential Bonferroni's correction is described as follows: *P < 0.05; **P < 0.01.

		71-2-Y	77-2-Y	34-2-Y	41-1-Y
YT4	N.	40	40	40	40
	Na	16	18	16	18
	As	210-250	202-238	202-246	200-242
	Ar	15.663	17.580	15.681	17.570
	He (n.b)	0.8959	0.9168	0.9190	0.8959
	Ho	0.8250	0.9000	0.9750	0.8500
	P value	0.7600	**0.0115**	0.9480	**0.0099**
	Fis	0.0801	0.0185	-0.0618	0.0518
YT84	N.	40	40	40	38
	Na	17	16	17	15
	As	224-312	228-312	224-312	232-288
	Ar	16.300	15.590	16.373	14.886
	He (n.b)	0.9146	0.9209	0.9025	0.9049
	Ho	0.7500	0.8000	0.7500	0.6316
	P value	0.1162	**0.0304**	**0.0382**	**0.0000****
	Fis	0.1818	0.1327	0.1708	0.3049
YT87	N.	40	40	40	40
	Na	10	11	14	12
	As	269-321	269-317	269-329	269-317
	Ar	9.963	10.690	13.663	11.773
	He (n.b)	0.5535	0.7009	0.6978	0.7560
	Ho	0.5750	0.6750	0.7000	0.8000
	P value	0.7457	0.6271	0.4554	0.8542
	Fis	-0.0275	0.0237	-0.0216	-0.0020
YT111	N.	40	40	40	40
	Na	24	25	24	26
	As	144-260	158-226	158-262	156-224
	Ar	23.255	24.128	23.453	24.871
	He (n.b)	0.9491	0.9430	0.9478	0.9380
	Ho	0.8750	0.9500	0.9500	0.9000

	P value	0.3126	0.8055	0.7636	**0.0427**
	Fis	0.0789	-0.0075	-0.0024	0.0410
YT12	N.	39	40	40	40
	Na	21	19	22	19
	As	313-375	313-371	315-377	317-375
	Ar	20.359	18.437	21.553	18.446
	He (n.b)	0.9048	0.9022	0.9389	0.8959
	Ho	0.3590	0.6500	0.6500	0.4750
	P value	**0.0000****	**0.0000****	**0.0000****	**0.0000****
	Fis	0.6064	0.2821	0.3104	0.4730
YT29	N.	40	40	40	36
	Na	17	19	17	21
	As	171-215	165-223	177-219	161-213
	Ar	16.871	18.289	16.581	21.000
	He (n.b)	0.9307	0.9222	0.9038	0.9045
	Ho	0.8000	0.9500	0.9000	0.7778
	P value	**0.0051**	0.9389	0.2822	**0.0144**
	Fis	0.1420	-0.0306	0.0043	0.1419
YT92	N.	40	40	40	36
	Na	13	11	8	12
	As	204-232	214-238	214-234	206-238
	Ar	12.282	10.582	7.890	11.300
	He (n.b)	0.7323	0.7263	0.7633	0.7062
	Ho	0.7750	0.6750	0.7250	0.9000
	P value	0.3286	0.3279	0.5826	0.5497
	Fis	-0.0591	0.0714	0.0508	-0.1323
YT121	N.	40	40	40	36
	Na	23	22	25	24
	As	148-214	154-222	154-210	154-216
	Ar	22.436	21.271	24.460	23.318
	He (n.b)	0.9399	0.9370	0.9604	0.9364
	Ho	0.9250	0.9500	0.9250	0.8500
	P value	0.1271	0.2114	0.2311	0.1594
	Fis	0.0160	-0.0140	0.0374	0.0933
MEAN	He (n.b)	0.8526	0.8712	0.8792	0.8785
	Ho	0.7355	0.8187	0.8219	0.7730
	P value	0.2139	0.3691	0.4126	0.2038
	Fis	0.1258	0.0613	0.0633	0.1143

Although these loci (YT12 and YT84) do not respect the HW equilibrium and possible null alleles may be present, the Jackknife test shows that they do not give different signals respect to the other loci for F statistics values, demonstrating that all loci analysed can be used for this study (Tab. 6).

Tab. 6_Jackknife statistic analysis used for variance and bias estimation: FIS (0.01769 - 0.20125); FIT (0.01891 - 0.20462); FST (-0.00025 - 0.00431).

Jackknife	FIS	FIT	FST
YT4	0.10708	0.10875	0.00188
YT84	0.08089	0.08319	0.00250
YT87	0.10796	0.10911	0.00129
YT111	0.10668	0.10835	0.00187
YT12	0.04760	0.04901	0.00149
YT29	0.10101	0.10254	0.00170
YT92	0.10992	0.11191	0.00224
YT121	0.10579	0.10821	0.00271
Mean	0.09662	0.9838	0.00194

Data showed the presence of problematic loci that do not respect the HW equilibrium. These loci may affect final results, for this reason we performed several tests to check out how they would influence the genetic differentiation analyses.

Three tests were conducted: the first one excluding locus YT12 from the dataset, the second excluding locus YT84 and the last with the exclusion of both these two loci. In general results obtained showed very similar Fst patterns and values (Appendix 2). For this reason, we will show here only the analysis carried out with all available loci.

Pairwise Fst analysis did not reveal any significant divergence among population samples (Tab. 7), except one comparison, retaining significance also after Bonferroni correction, between samples 71-2-Y (WCPO) and 41-1-Y (WCAO) (Fst = 0.00927, P= 0.00218).

Tab. 7_Pairwise Fst values (below the diagonal) and associated significance pValue (above the diagonal). Significance after sequential Bonferroni's correction is described as follows: (*P < 0.05; **P < 0.01).

Fst	71-2-Y	77-2-Y	34-2-Y	41-1-Y
71-2-Y	-	0.40600	0.07207	**0.00218***
77-2-Y	0.00162	-	0.60469	0.36957
34-2-Y	0.00454	0.00011	-	0.78913
41-1-Y	**0.00927***	0.00163	0.00068	-

Principal coordinates analysis (PCoA) based on the pairwise Fst matrix were performed to assess differentiation among samples (Fig. 14). The scatter plot obtained showed on the first axis differentiations between Atlantic Ocean (41-1-Y and 34-2-Y) and West Pacific Ocean (71-2-Y) samples, while the East Pacific Ocean one (77-2-Y) is differentiated from the others on the second axis. However, first axis and second axis do not explain most of the variation, representing respectively 51.23 % and 25.23 %.

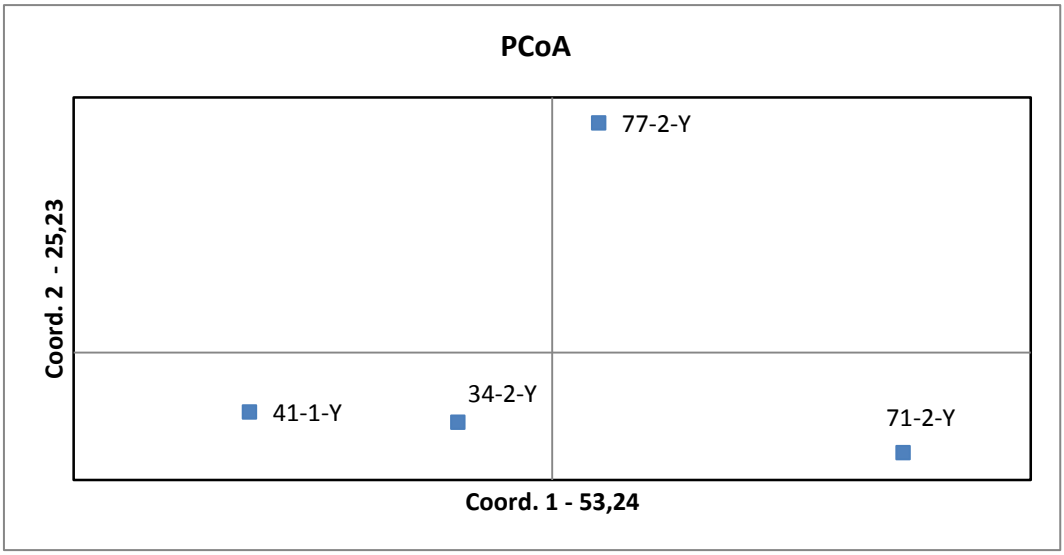

Fig. 14_Principal Coordinates Analysis (PCoA) of YFT population samples. Scatter plots built on the first two principal coordinates (coordinate 1, x axis; coordinate 2, y axis) based on the pairwise Fst values.

AMOVA analyses were carried out using different samples grouping (Tab. 8): 1) grouping samples per Ocean (Pacific Ocean vs Atlantic Ocean) (AMOVA 1) and 2) following PCoA result (Atlantic Ocean and West Pacific Ocean vs East Pacific Ocean) (AMOVA 2).

Results showed highest percentage of molecular variation within samples (AMOVA 1= 99.61% and AMOVA 2= 99.46%) and low values among group and among samples within group. The subdivision of samples in two groups (Atlantic Ocean and Pacific Ocean) was not statistically significant (AMOVA 1: Fct = 0.0034; P = 0.11144) as well the subdivision based on PCoA results (Atlantic Ocean and West Pacific Ocean vs East Pacific Ocean) (AMOVA 2: Fct = 0.031; P = 0.12512).

Tab. 8_AMOVA statistical analysis.

AMOVA 1			
PACIFIC OCEAN and ATLANTIC OCEAN			
	Variation (%)	F - Statistic	pValue
Among groups	0.34	0.0034	0.11144
Among samples within groups	0.05	0.0005	0.67155
Within samples	99.61	0.0034	0.31672

AMOVA 2			
ATLANTIC / W. PACIFIC and E. PACIFIC			
	Variation (%)	F - Statistic	pValue
Among groups	-0.31	0.031	0.12512
Among samples within groups	0.32	0.00315	0.78006
Within samples	99.46	0.0970	0.34000

Yellowfin tuna population structure was further investigated with STRUCTURE individual-based analyses Bayesian clustering. Data output obtained were displayed with STRUCTURE HARVESTER (Web version 0.6.94 July 2014) (Tab. 9).

Tab. 9_ STRUCTURE HARVESTER summary

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	ΔK
1	5	-6702,02	0,55	-	-	-
2	5	-6834,54	10,97	-132,52	46,54	4,24
3	5	-7013,60	82,27	-179,06	151,98	1,85
4	5	-7344,64	381,93	-331,04	197,08	0,52
5	5	-7478,60	277,34	-133,96	138,16	0,50
6	5	-7474,40	162,63	4,20	396,22	2,44
7	5	-7866,42	325,83	-392,02	11,12	0,03
8	5	-8269,56	297,80	-403,74	712,88	2,39
9	5	-7959,82	119,09	309,74	656,90	5,52
10	5	-8306,98	238,72	-347,16	-	-

The most likely cluster number (K) identified by the program was inferred with Pritchard method (Pritchard *et al.*, 2000). This theory analyse LnP(K) (the logarithm of the probability of the data given K) trends identifying possible K value as the nearest value to "plateau" (Fig. 14). In fact, STRUCTURE usually run for several values of K, lnP(K) is computed for each of them and plotted against K. Thus, if several values of K give similar estimates of LnP(K), the smallest seems to be the most real. No "plateau" was detected in our analysis, suggesting the lack of samples differentiation (Fig. 15).

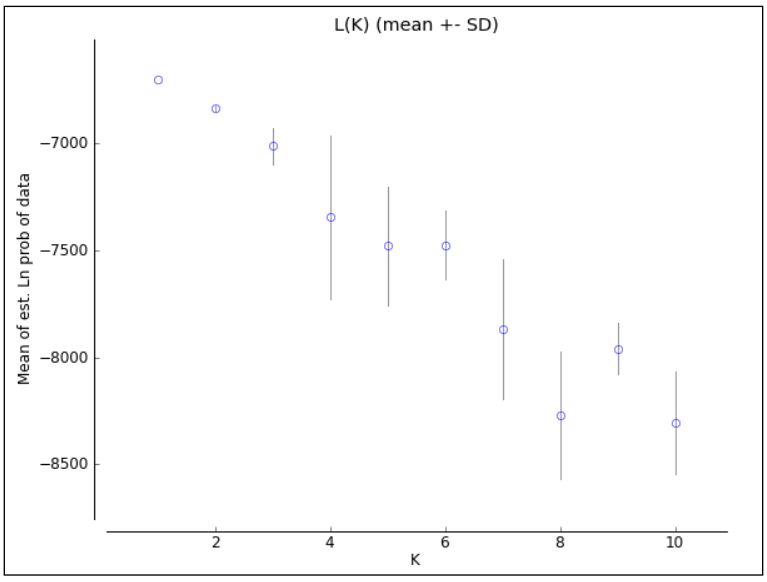

Fig. 15_ LnP(K) graph.

According with Evanno *et al.*, (2005) the likely number of K populations can also be inferred by the correlation between the second rates of change of LnP”(K) with respect to K (ΔK). This method seems to show a clear peak at the true value of K.

In fact authors found the modal value of the distribution of ΔK that represent the real K, and used the height of this value as an indicator of the strength of the signal detected by structure.

In Fig. 16 is showed the ΔK value respect to K obtained for this study.

Results are discordant to the previous LnP(K) assessment, in fact the function ΔK presents the maximum values at K=2, K=6 and K=9.

Considering both methods and the different K values obtained, we can presume that K=6 and K=9 are not realistic to correctly represents yellowfin tuna population.

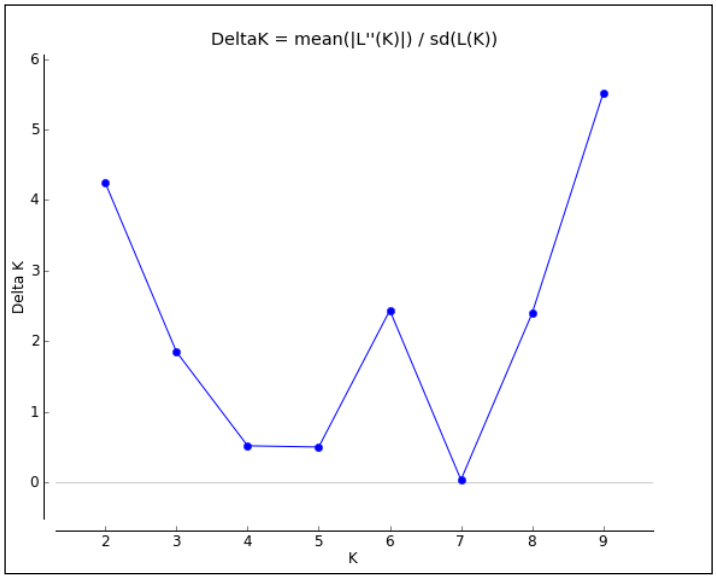

Fig. 16_Evanno’s absolut ΔK graph.

For K=2 barplot obtained showed clearly the absence of any structuring between Atlantic and Pacific YFT samples (Fig. 17). In fact, all samples showed a genetic composition whose percentage values of individual membership revealed a total admixture between them.

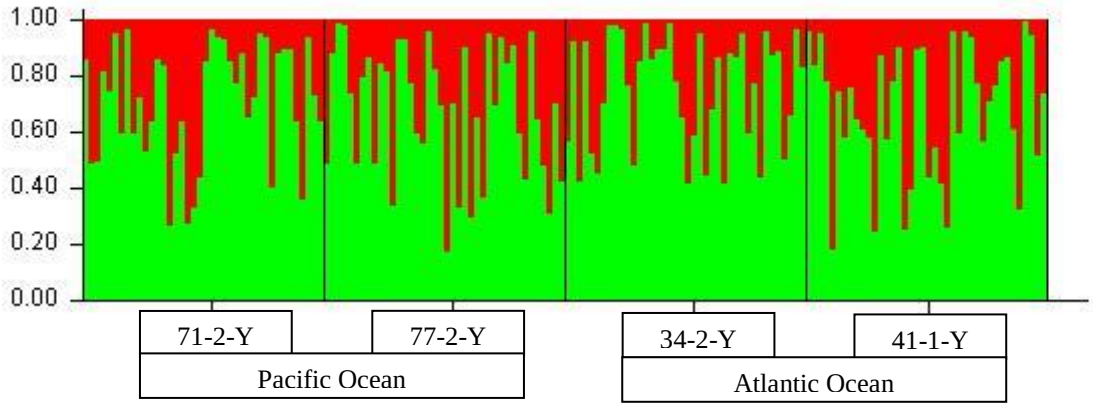

Fig. 17_ Estimated membership fraction of individuals from each sampling area : (1)= 71-2-Y; (2)= 77-2-Y; (3)=34-2-Y; (4)=41-1-Y

6. Discussions and conclusions

6.1 Species identification for YFT juveniles

Species identification of tuna juveniles is essential to increase the information on the real by-catch data, contributing decisively to more tangible and functional management plans. Unfortunately, it is quite difficult when morphological features are ambiguous or missing, such as with frozen specimens.

The immature tuna by-catch is abundantly underestimated and unreported in the fishermen's logbook worldwide (Amandè *et al.*, 2010). In fact fishermen usually do not report the real amount of little tunas by-catch because of their discard at the sea or their parallel importance in the local markets, as for instance the local market of Abidjan (Central-West Africa) (Romagny *et al.*, 2000).

However, since 2001 the European Union (EU) has developed a mandatory sampling program for the collection of data in the fisheries sector under the EU Data Collection Regulation (DCR) directive in support of its Common Fishery Policy, with the specific objective to evaluate the real amount of by-catch and discards in these fisheries (Amandè et al., 2010).

By contrast, in population structure studies, the collection of juveniles (i.e. post-larval and early-stage juvenile), according to their limited capacity to swim than the older ones, would increase the likelihood to work with individuals caught close to their nursery areas, detecting the genetic composition of the spawning populations (Carlsson *et al.*, 2007). However, post-larval and early-stage juveniles of many tuna and tuna-like species are morphologically similar between them, especially if they belong to the same genus (Robertson *et al.*, 2007) and their identification requires high-quality taxonomic skills.

Furthermore, species misidentification at early juvenile's stage could be caused by geographical variation in their morphological features (Chow and Inoue 1993). Also, the use of morphological characters (such as body shape, pigments, characteristics of the fins, etc.) is deceptive when for instance individuals are frozen or exposed to other processes (e.g. canning, filleting) which make the species identification almost impossible.

These problems could affect their real mortality information and make stock assessment more difficult.

The molecular approach is one of the most powerful and concrete among the different methodologies developed to overcome species misidentification problems (Teletchea *et al.*, 2009).

Many different DNA sequences have been used for helping with tuna and tuna-like species identification. Some tuna and tuna-like species are very close genetically and hybridization among species would create taxonomic uncertainty (Ward *et al.,* 2005) with mitochondrial introgression patterns that have been described for some of them (Alvarado Bremer *et al.*, 2005).

Among the different species of the genus *Thunnus*, species identification is particularly challenging between BET and YFT that are usually caught together with FAD-sets, due to the genetic nearby. Thus, a specific gene marker has to be selected appropriately and detailed knowledge of the DNA sequences from target species are required before setting-up the methodology and validate the final assignation. Several attempts have been done, employing different markers, to discriminate between YFT and BET, and their results were not always satisfying. In this study the use of mtDNA fragment ATCO to identify YFT specimens and to avoid any possible misidentification with BET juveniles, was a fitting choice. Our results showed the absence of variable sites between our YFT sequences and those taken from Genbank (www.ncbi.nlm.nih.gov/genbank/) confirming their high reliability. In the alignment produced 109 variable sites (V) and 15 informative sites (Pi) were detected. The Neighbour-Joining tree obtained, using Tamura-Nei distance method separated the 66 samples collected in the western part of the Pacific ocean and identified as YFT by our collaborator from 5 BET that were personally sampled in the Gulf of Guinea (with bootstrap values of 95% and 99% respectively). Our results confirmed the results of Chow and Inoue (1993), underlying the suitability of ATCO as molecular marker to discriminate between the two widely harvested species. However further tests are requested with a higher number of specimens, sampled in other fishing areas, to really assess its reliability for management purposes.

Nevertheless, previous studies carried out with RFLP analysis do not showed any concreteness in discriminating between these species. Instead the triplex-PCR approach devised by Michelini et al., (2007) through ctyb analysis, seems to be a very practical reliable and economical technique. In addition the results are simply obtained during the course of a PCR reaction, without the need of expensive sequencing.

We have not used this technique to our analysis of molecular recognition, because the method used by Michelini et al., (2007) is strongly restricted to some geographical samples and the diagnostic site for the species-specific primer annealing are not conserved when evaluate the sequence polymorphisms considering different geographical samples and broader geographical range.

The universal gene sequence capable to differentiate a vast spectrum of animal species is the portion of the mitochondrial DNA gene cytochrome oxidase subunit I (COI) (DNA barcoding), as proposed by Hebert *et al.,* (2003). But Vinas and Tudela (2009) have underlined as the COI is not the appropriate genetic marker for the identification of these species, which instead can be performed using the mtDNA control region (CR). These results were corroborated by Pedrosa-Gerasmio *et al.*, (2012), validating the mtDNA CR as a robust marker, in association to the liver morphology test. In contrast with these recent results, previous studies (Alvarado-Bremer *et al.,* 1997; Alvarado-Bremer *et al.,* 2005) indicated the mtDNA CR as not so efficient in discriminating within the genus *Thunnus*. Further attempts have been made using the nuclear genetic marker (ITS1). This marker showed to have good capacity for discernment especially among Scombridae family but not between tuna species (Chow *et al.,* 2006). However Vinas and Tudela, (2009) obtained promising results, employing this nuclear marker combined with the mtDNA CR.

In such context, it is clear the need to adopt an efficient marker able to discriminate between these two species properly, in order to avoid any possible misidentification issue.

However, the technique used in this study is very functional, but is time consuming and expensive, as most of the genetic analysis. For this reason we think that there is a need for a more immediate and less expensive method than the sequencing that can be applied in the field (i.e. fisherman or on-board observers) and that considers the large amount of tuna fishery.

6.2 Yellowfin tuna population structure and possible implications for its management

Understanding the processes involved in the origin and maintenance of genetic diversity is particularly difficult in the ocean realm because barriers to gene flow are far less evident in marine compared to continental species (Patarnello et al., 2007). However

many environmental factors in association with the species-specific life history traits play a central role in modelling their structure and the different degree of differentiation. Moreover climatic changes and paleo-oceanographic conditions have promoted vicariance, population size fluctuations, and secondary contact of previously allopatric populations, influencing the differentiation and geographic distribution of these species (Alvarado Bremer et al., 2005). Therefore even low levels of stock structure can have significant implications for their stock management and conservation's goals (Graves and McDowell, 2003), highlighting the need of incorporating genetic data of population structure into the fishery management of these species across the five RFMOs.

The Cape of Good Hope represents a potential genetic point break for tropical tuna and tuna-like species' gene flow between the Atlantic and Indo-Pacific oceans. In fact it has been indicated as a cause for the significant genetic drift in many pelagic species as for instance the bigeye tuna (Alvarado-Bremer *et al.,* 1998, Chow *et al.,* 2000; Martinez *et al.,* 2006,; Chiang *et al.,* 2008) as well as for other tuna and tuna like species (i.e. blue marlin: Finnerty and Block 1992; Graves and McDowell 1995; swordfish: Alvarado Bremer *et al.,* 1996; Chow *et al.,* 1997; Chow and Takeyama 2000; sailfish: Graves and McDowell 1995; albacore: Chow and Ushiyama 1995).

This potential geographic break in association with vicariant isolation events (e.g. Pleistocene glacial maxima, Isthmus of Panama) support the hypothesis of the presence of two clades for bigeye tuna populations: Clade I exclusive in Atlantic and Clade II that occurs in all oceans (Durand *et al.,* 2005).

A different situation was revealed for yellowfin tuna, for which the Cape of Good Hope does not seem to be a geographical barrier for gene flow, suggesting a global panmitic population with very low degree of genetic differentiation among each basin (Ely *et al.,* 2005). However recent studies have suggested that distinct stocks may be present within each ocean (Dammannagoda *et al.,* 2008, Swaraj *et al.,* 2013) raising many questions about the current management plan adopted by the RFMOs. Thus, ignoring their population structure might lead to overexploit some stocks with potential dramatic effects on their biology and ecology.

For population structure studies, the statistical power of genetic markers for detecting genetic differentiation is chiefly related to the total number of independent alleles rather than the number of loci (Kalinowski *et al.,* 2002). For this reason, microsatellites are

widely used for population structure studies according to their high allelic richness, lower ascertainment bias, and higher analytical power (Guichoux *et al.,* 2011), as well as their capacity to detect recent population expansions and their limited cost of analysis and genotyping (Morin *et al.,* 2004). The microsatellites employed in this study were developed by Antoni *et al.,* (2014), assessed by genotyping yellowfin tuna specimens caught off Ghana.

Our results showed very low and not significant levels of genetic differentiation at both intra- and inter-oceanic level. The only significant value (Fst=0.00927; P=0.00218*, even after Bonferroni corrections) was that measured between the two most distant samples Central-Western Pacific Ocean (71-2-Y) and Central-Western Atlantic Ocean (41-1-Y). These results could indicate a significant low rate of differentiation between the Atlantic and Pacific basins, but too low to support the presence of distinct stocks.

Indeed, our microsatellite dataset assessment indicated the presence of two possible problematic loci (YT84 and YT12) that do not respected HW equilibrium. Thus, three tests, not including these loci in the dataset, were made to check out how they would influence the genetic differentiation indices, reporting similar Fst values.

The samples were gathered in groups to evaluate their genetic differentiation by applying the analysis of molecular variance (AMOVA). Two groups were considered in the first analysis:

1) Samples of the Atlantic (34-2-Y & 41-1-Y) and 2) samples of Pacific Ocean (71-2-Y & 77-2-Y). AMOVA reported low rates of differentiation among groups and within groups. Anyway the 99.6% of the variation was elucidated by the natural variation (within each geographical sample). But even in this case the data were not statistically significant.

Similar results were carried out made two different groups following the results of the PCoA (Atlantic (34-2-Y & 41-1-Y) and Central-Western Pacific (71-2-Y) vs Central-Eastern Pacific (77-2-Y)).

Our results corroborated those indicated in previous studies, with very low and not statistically significant signs of genetic differentiation between and within oceans. For instance, Appleyard *et al.,* (2001) found out very low evidences of structure in the Western Pacific Ocean, as well as Diaz-Jaimes *et al.,* (2006) in Eastern Pacific Ocean employing 5 and 7 loci respectively. Moreover, the lack of differentiation between oceans indicated in

this study confirm the results obtained by Ely *et al.*, (2005), at the global, using mtDNA CR and RFLP of the ATCO segment.

This lack of structure in YFT populations might be linked to its large effective population size (Ne) and to the high rates of migration that could determine a high gene flow, preventing any possible separation of its population. The time since YFT population expansion began, does not provide an alternative explanation for the lack of inter-oceanic differentiation in this species. In fact, Ely *et al.*, (2005) estimated (through the mutation rate of mtDNA(CR)) that YFT expansion occurred about 522,000 years ago as well as for swordfish and bluefin tuna (450,000-470,000 years) which instead show evidence of inter-oceanic structuring.

Further investigations are required to really understand the YFT population structure within and between these oceans, especially more geographical samples and loci are needed to overcome the limits of the present study.

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Appendix A -

Allelic frequencies of eight microsatellites analyzed

		71-2-Y	77-2-Y	34-2-Y	41-1-Y
YT4	N.	40	40	40	40
	An	16	18	16	18
	200				0,025
	202		0,05	0,0125	0,025
	206		0,0125		0,0125
	208		0.0125		
	210	0.2	0.1625	0.15	0.2375
	212	0.0875	0.1	0.05	0.025
	214	0.05	0.0125		
	216	0.1125	0.05	0.0625	0.0625
	218	0.0375	0.0375	0.1125	0.0625
	220	0.1875	0.175	0.1125	0.1625
	222	0.0875	0.25	0.0375	0.05
	224	0.025	0.5	0.0125	0.0375
	226	0.0625	0.5	0.125	0.0125
	228		0.625	0.025	0.0375
	230	0.0125	0.125	0.05	0.0375
	232	0.025	0.375	0.075	0.0375
	234	0.0375	0.875	0.1	0.1125
	236		0.375		0.0375
	238	0.025	0.025	0.0375	0.0125
	242				0.0125
	244	0.025		0.125	
	246			0.25	
	248	0.0125			
	250	0.0125			
YT87	N	40	40	40	40
	An	10	11	14	12
	269	0.6625	0.525	0.5375	0.4625
	273		0.0875	0.0375	0.075
	277	0.0375	0.0625	0.075	0.0625
	281	0.0625	0.0375	0.025	0.0125
	285	0.0625	0.05	0.0375	0.0625
	289	0.0375	0.075	0.075	0.1375
	293	0.025	0.0125	0.0125	0.025
	297	0.025	0.1	0.05	0.025

		71-2-Y	77-2-Y	34-2-Y	41-1-Y
YT84	N.	40	40	40	38
	An	17	16	17	15
	224	0,0125		0,025	
	228	0,0125	0,0375	0,025	
	232		0,05	0,0125	0,0132
	236	0.0625		0.025	0.0395
	240	0.0625	0.05	0.0625	0.0395
	244	0.125	0.1	0.075	0.1053
	248	0.1125	0.075	0.1625	0.1579
	252	0.075	0.15	0.1875	0.1316
	256	0.175	0.0875	0.1125	0.1447
	260	0.0625	0.0875	0.1125	0.1447
	264	0.0625	0.0125	0.075	0.0526
	268	0.1	0.1	0.625	0.0263
	272	0.075	0.0625	0.0125	0.0526
	276	0.0125	0.0125	0.0125	0.0263
	280	0.0125	0.025		0.0263
	284		0.0125		0.0132
	288			0.0125	0.0263
	292	0.0125			
	300	0.0125	0.0125		
	308			0.0125	
	312	0.0125	0.0125	0.0125	
YT92	N.	40	40	40	40
	An	13	11	8	12
	204	0.0125			
	206				0.0125
	208	0.0125			0.0125
	210	0.0125			0.0125
	212	0.025			
	214	0.025	0.0625	0.0875	0.01125
	216	0.0125	0.0125	0.0375	0.0125
	218	0.45	0.425	0.4	0.3
	220	0.2375	0.3	0.2375	0.3
	222	0.075	0.0625	0.1	0.1
	224	0.1	0.05	0.1	0.1

	301	0.0375		0.05	0.05
	305	0.025	0.025	0.025	0.05
	309			0.0125	0.0125
	313		0.0125	0.025	
	317		0.0125		0.025
	321	0.025			
	325			0.025	
	329			0.125	
YT111	N	40	40	40	40
	An	24	25	24	26
	144	0.025			
	156				0.0125
	158	0.0125	0.025	0.0375	
	160	0.025	0.025		
	162	0.05	0.0125	0.0125	0.0375
	164			0.025	0.0125
	166	0.0125	0.0375	0.0125	0.0375
	168	0.0375	0.025	0.0375	0.0125
	170	0.075	0.1125	0.0625	0.05
	172		0.05	0.0625	0.0375
	174	0.0625	0.075	0.025	0.05
	176	0.075	0.125	0.1625	0.15
	178	0.1	0.0875	0.05	0.15
	180	0.05	0.025	0.05	0.0125
	182	0.025	0.025	0.025	0.0125
	184	0.0125	0.025	0.05	
	186	0.0625	0.0125	0.025	0.05
	188	0.05	0.1	0.0375	0.05
	190	0.05	0.0125		0.0125
	192	0.0125	0.025	0.0625	0.075
	194	0.125	0.0125	0.0625	0.0625
	196		0.075	0.0375	0.0375
	198	0.0125		0.05	0.0125
	200	0.05	0.025	0.05	0.025
	202	0.025	0.0375	0.025	0.025
	204				0.025
	206	0.0125	0.0125		0.0125
	212			0.0125	
	214		0.0125		0.0125
	216		0.0125		0.0125
	224				0.0125
	226	0.025	0.0125		
	238			0.0125	

	226	0.0125	0.025		
	228	0.0125	0.025	0.0125	0.0125
	230		0.0125		
	232	0.0125	0.0125		0.0125
	234			0.025	
	238		0.0125		0.0125
YT121	N.	40	40	40	40
	An	23	22	25	24
	148	0.025			
	154	0.0125	0.0375	0.05	0.025
	156	0.0625	0.125	0.0625	0.05
	158	0.0125	0.0375	0.0375	0.025
	160	0.0875	0.1125	0.0625	0.1125
	162	0.15	0.125	0.0875	0.15
	164	0.0625	0.75	0.0875	0.075
	166	0.05	0.0375	0.0125	0.025
	168	0.125	0.0875	0.0625	0.125
	170	0.025	0.05	0.0375	0.025
	172	0.025		0.0375	0.025
	174		0.0375		0.0375
	176	0.025		0.025	0.0125
	178	0.025		0.0375	0.025
	180	0.05	0.05	0.0375	0.0125
	182		0.025	0.0375	0.0125
	184	0.0625	0.025	0.0625	0.025
	186	0.0375	0.0625	0.0375	0.075
	188	0.0375	0.0125	0.05	0.0375
	190			0.025	
	192			0.05	0.025
	194		0.0125		0.0125
	196			0.0125	
	198	0.0375			
	200	0.025	0.025		0.0125
	202	0.025		0.0125	0.025
	204			0.025	
	206	0.0125	0.0125	0.0125	0.0375
	208	0.0125		0.025	
	210			0.0125	
	212		0.0125		
	214	0.0125	0.0125		
	216		0.0125		0.0125
	222		0.0125		
YT29	N.	40	40	40	36

	260	0.0125			
	262			0.0125	
YT12	N.	39	40	40	40
	N.a	21	19	22	19
	313	0.0128	0.0125		
	315			0.0125	
	317				0.0125
	319	0.0256	0.0125		
	321	0.0128			
	323	0.0128	0.0125		
	325	0.0128		0.05	0.0375
	327	0.0513	0.0875	0.05	0.1625
	329		0.025	0.025	
	331		0.05	0.0875	0.0875
	333	0.1154	0.1125	0.0625	0.025
	335	0.1154	0.2125	0.175	0.2375
	337	0.2179	0.1375	0.0875	0.05
	339	0.141	0.125	0.05	0.1
	341	0.0256	0.0375	0.05	0.025
	343	0.0385	0.025	0.05	0.0625
	345			0.0375	
	347		0.025	0.25	0.025
	349	0.128	0.0125	0.25	0.05
	351	0.0256			0.0125
	353		0.025	0.0375	
	355	0.0513	0.025	0.0125	
	357	0.0385	0.025	0.25	0.0125
	359	0.0128		0.05	0.025
	361	0.0256	0.025		0.0125
	365	0.0128		0.0125	
	367	0.0128			
	369			0.0375	
	371		0.0125	0.0125	0.025
	373				0.0125
	375	0.0256			0.025
	377			0.25	

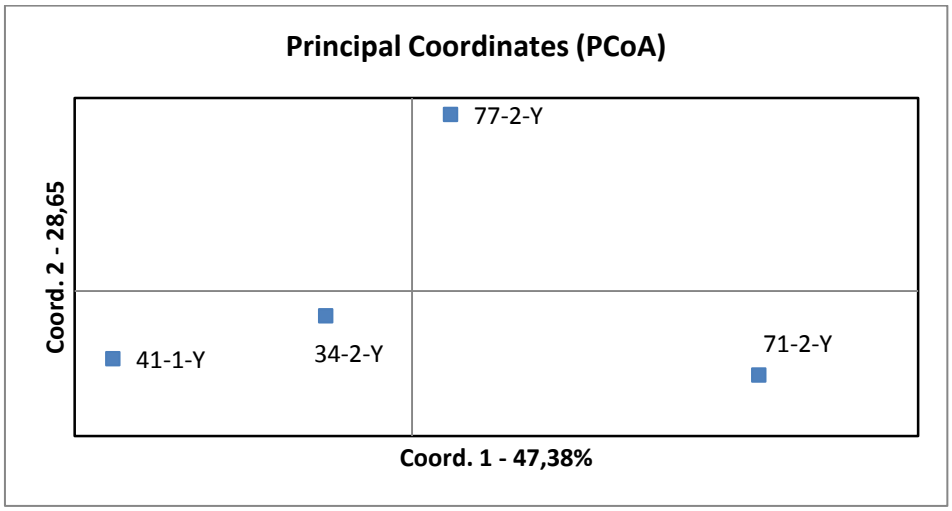
An	17	19	17	21
161				0.0139
163				0.0694
165		0.0125		
167		0.0125		0.0278
169				0.0139
171	0.25			
173				0.0139
177	0.05	0.0375	0.025	0.0278
179	0.1	0.1	0.225	0.25
181				0.0139
183	0.1375	0.125	0.1125	0.0278
187				0.0139
189	0.075	0.05	0.075	0.0694
191	0.025	0.05	0.0125	
193	0.05	0.025	0.0375	0.0139
195	0.025			0.0139
197		0.0125	0.0125	0.0139
199	0.05	0.375	0.05	0.0417
201	0.0375	0.1	0.05	0.0694
203	0.075	0.0375	0.05	0.0417
205	0.125	0.15	0.1375	0.1111
207	0.1	0.1125	0.05	0.0972
209	0.0375	0.0875	0.05	
211	0.0375	0.0125	0.0625	0.0417
213	0.0375		0.025	0.0139
215	0.0125	0.0125	0.0125	
217		0.0125		
219			0.0125	
223		0.0125		

Appendix B –

Genetic differentiation analyses without problematic loci.

Pairwise Fst values without locus YT12 (below the diagonal) and associated significance pValue (above the diagonal). Significance after sequential Bonferroni's correction is described as follows: (*P < 0.05; **P < 0.01).

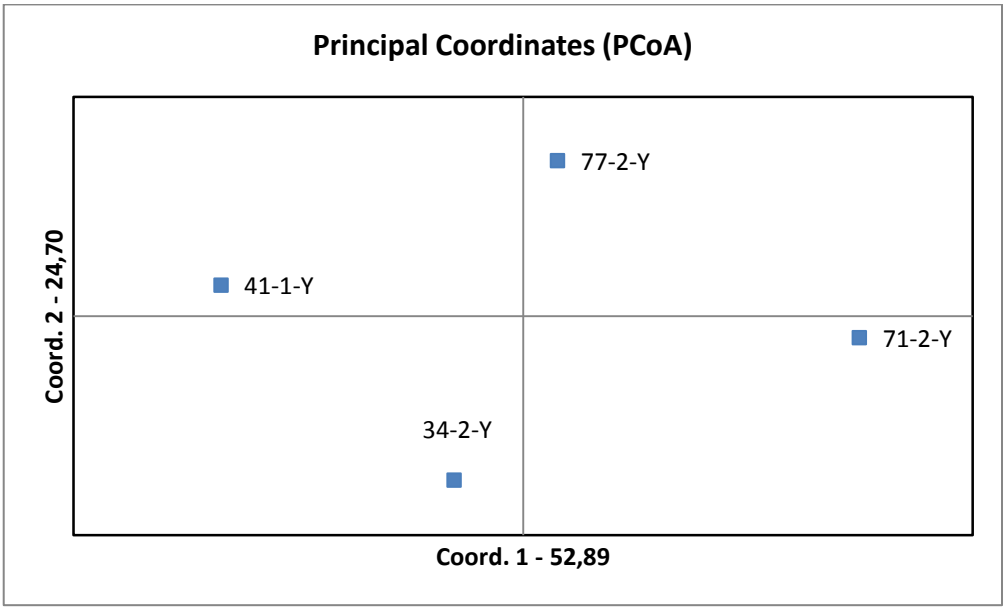
Fst	71-2-Y	77-2-Y	34-2-Y	41-1-Y
71-2-Y	-	0.81626	0.37367	0.02158
77-2-Y	0.00166	-	0.5541	0.15147
34-2-Y	0.00282	-0.00011	-	0.29205
41-1-Y	0.00579	0.00103	-0.00151	-



Principal Coordinates Analysis (PCoA) without YT12. Scatter plots built on the first two principal coordinates (coordinate 1. x axis; coordinate 2. y axis) based on the pairwise Fst values.

Pairwise Fst values without YT84 (below the diagonal) and associated significance pValue (above the diagonal). Significance after sequential Bonferroni's correction is described as follows: (*P < 0.05; **P < 0.01).

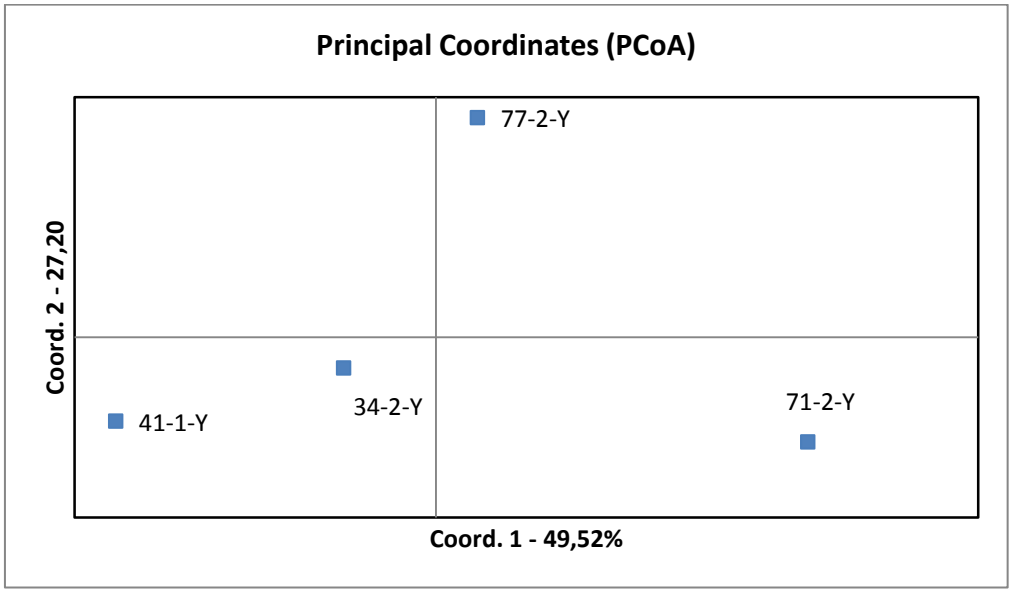
Fst	71-2-Y	77-2-Y	34-2-Y	41-1-Y
71-2-Y	-	0.40768	0.07613	**0.00059****
77-2-Y	0.00146	-	0.5326	0.35086
34-2-Y	0.00444	0.00023	-	0.59667
41-1-Y	**0.01085****	0.00154	0.00015	-



Principal Coordinates Analysis (PCoA) without YT84. Scatter plots built on the first two principal coordinates (coordinate 1. x axis; coordinate 2. y axis) based on the pairwise Fst values.

Pairwise Fst values without YT12 and YT84 (below the diagonal) and associated significance pValue (above the diagonal). Significance after sequential Bonferroni's correction is described as follows: (*P < 0.05; **P < 0.01).

Fst	71-2-Y	77-2-Y	34-2-Y	41-1-Y
71-2-Y	-	0.28423	0.18414	0.00931
77-2-Y	0.00148	-	0.46589	0.35383
34-2-Y	0.00239	0.00002	-	0.59945
41-1-Y	0.00706	0.00083	-0.00067	-



Principal Coordinates Analysis (PCoA) without YT12 and YT84. Scatter plots built on the first two principal coordinates (coordinate 1. x axis; coordinate 2. y axis) based on the pairwise Fst values.